Downregulation of microRNA-1 attenuates glucose-induced apoptosis by regulating the liver X receptor α in cardiomyocytes

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Abstract. Diabetic cardiomyopathy (DCM) is characterized by abnormal myocardial structure or performance. It has been suggested that microRNA-1 (miR-1) may be abnormally expressed in the hearts of patients with diabetes. In the present study, the role of miR-1 in glucose-induced apoptosis and its underlying mechanism of action was investigated in rat cardiomyocyte H9C2 cells. Cells were transfected with anti-miR-1 or miR-1-overexpression plasmids and the expression of miR-1 and liver X receptor α (LXRα) were determined by reverse transcription-quantitative polymerase chain reaction analysis. The proportion of apoptotic cells was determined using an Annexin-V-FITC apoptosis detection kit and the mitochondrial membrane potential (ΔΨ) was measured following staining with rhodamine 123. In addition, the expression of apoptosis-associated proteins was measured by western blot analysis. The results demonstrated that expression of miR-1 was significantly increased, whereas the expression of LXRα was significantly decreased in H9C2 cells following treatment with glucose. miR-1 knockdown significantly inhibited apoptosis, increased the ΔΨ and suppressed the cleavage of poly (adenosine diphosphate-ribose) polymerase, caspase-3 and caspase-9. It also significantly downregulated the expression of Bcl-2 and upregulated the expression of Bax. In addition, it was demonstrated that miR-1 regulates LXRα; transfection with anti-miR-1 significantly increased the expression of LXRα. Furthermore, treatment of cells with the LXR agonist GW3965 inhibited apoptosis in glucose-induced anti-miR-1 cells. These results suggest a novel function of miR-1: The regulation of cardiomyocyte apoptosis via LXRα, and provide novel insights into regarding the complex mechanisms involved in DCM.

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Introduction

Diabetes mellitus (DM) is a chronic metabolic disease characterized by elevated blood glucose levels and an estimated 415 million people are living with DM worldwide (1). The International Diabetic Federation has predicted that ~552 million people will be living with diabetes by 2030 (2). Among patients with DM, cardiovascular disease is the leading cause of disability and mortality (3); DM may affect cardiac structure and function leading to coronary artery disease and the development of diabetic cardiomyopathy (DCM). DCM is defined as 'a distinct entity characterized by abnormal myocardial structure or function in the absence of epicardial coronary artery disease, hypertension or significant valvular disease' (4). It is caused by diastolic dysfunction in patients with type 1 or type 2 DM, which is followed by systolic dysfunction induced by various structural lesions and may lead to heart failure if left untreated (5,6). Patients with DM are urged to undergo various lifestyle changes and are prescribed medical interventions to manage their condition; however, at present there are no specific treatments for DCM (7).

MicroRNAs (miRs) are endogenous small noncoding RNAs that are 20-23 nucleotides long and regulate gene expression (8). Previous studies have demonstrated the importance of miR-regulated gene expression in several different diseases, including diabetes, heart disease, neurological disorders and various types of cancer (9-11). The dysregulation of certain miRs is associated with diabetic cardiovascular dysfunction. It has been reported that miR-1 and miR-206 regulate the expression of heat shock protein 60, contributing to high levels of glucose-mediated apoptosis in cardiomyocytes (12). It has also been revealed that the expression of the insulin-sensitive glucose transporter glucose transporter type 4 is modulated by miR-133 and miR-223 in cardiomyocytes (13,14). miR-1 is specifically expressed in cardiac and skeletal muscle cells and its increased expression of miR-1 has been detected in the

hearts of patients with DM (15,16). Furthermore, it has been demonstrated that miR-1 and miR-206 attenuate lipogenesis by targeting liver X receptor α (LXRα) in hepatocytes (17).

LXRs, including LXRα and LXRβ, are key regulators of cholesterol homeostasis, and lipid and glucose metabolism, and also serve immune regulatory and anti-inflammatory functions. It has been reported that synthetic LXR activation reduces hyperglycaemia in insulin-resistant diabetes (18,19). Cannon *et al* (20) confirmed that LXRα improves myocardial glucose tolerance and reduces cardiac hypertrophy in obesity-induced type 2 diabetes. However, the signaling events that occur upstream of LXRα in cardiomyocytes and the regulatory effect of miR-1 on LXRα remain unclear. Therefore, the present study aimed to investigate the potential role of miR-1 in glucose-induced mitochondrial dysfunction and apoptosis in the rat cardiomyocyte cell line H9C2, as well as identify the downstream signaling targets of miR-1 in DCM. The present study also investigated the underlying mechanisms of miR-1 during the pathogenesis of DCM.

Materials and methods

Cell culture. The rat cardiomyocyte cell line H9C2 was obtained from ScienCell Research Laboratories, Inc. (San Diego, CA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% heat-inactivated fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and 100 U/ml penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. GW3965, an LXR agonist, was purchased from Selleck Chemicals (Houston, TX, USA) and glucose was purchased from Sigma-Aldrich (Merck KGaA Darmstadt, Germany).

Experimental groups. All H9C2 cells were initially treated for 48 h with 10% FBS in 5% CO_2 at 37˚C in 6- and 12-well plates, and then divided into 5 groups as follows: i) Control H9C2 cells, treated with PBS for 24 h and then treated with 33 mmol/l mannitol for a further 24 h; ii) glucose cells, treated with PBS for 24 h and then treated with 33 mmol/l glucose for a further 24 h; iii) NC+Glucose cells, miR-1 negative control (NC) plasmids were transfected into the cells and following 24 h, cells were treated with 33 mmol/l glucose for a further 24 h; iv) anti-miR-1+Glucose cells, anti-miR-1 plasmids were transfected into the cells and following 24 h the cells were treated with 33 mmol/l glucose for a further 24 h; v) anti-miR-1+Glucose+GW3965 cells, cells were first transfected with anti-miR-1 plasmids and following 24 h, cells were then treated with 33 mmol/l glucose and 1 μM GW3965 for a further 24 h. The volumes of PBS, and anti-miR and NC plasmids were 200 μl and 100 μl/well in 6- and 12-well plates, respectively. The volumes of mannitol and glucose, were 20 μl and 10 μl/well in 6- and 12-well plates, respectively.

Plasmid construction and cell transfection. The oligonucleotide sequences of miR-1 knockdown [an anti-miR-1 short hairpin (sh)RNA] and miR-1 overexpression were listed as follows: Anti-miR-1 forward, 5'-GATCCCCATACACACTTCTTTACATTCCATTCAAGAGATGGAATAAAGAAGTGTGTATTTTTT-3' and reverse, 5'-AGCTAAAAAATACACACTTCTTTACATTCCATCTCTTGAATGGAATAAAGAAGTGTGTATGGG-3'; miR-1, forward, 5'-GATCCCCTGGAATGTAAAGAAGTGTGTATTTCAAGAGAATACACACTTCTTTATTCCATTTTT-3'; and reverse, 5'-AGCTAAAATGGAATGTAAAGAAGTGTGTATTCTCTTGAAATACACACTTCTTTATTCCAGGG-3'. For the negative control, the sequences were as follows: Forward, 5'-GATCCCCTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTT-3' and reverse, 5'-AGCTAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAAGGG-3'. To construct the anti-miR-1 and miR-1 overexpression plasmids, these paired oligonucleotides were annealed and 2 μg double-stranded products were subsequently inserted into pRNA-H1.1 vectors (GenScript, Nanjing, China) between the *Bam*HI and *Hind*III sites. Anti-miR-1 or miR-1 plasmids were transfected into H9C2 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Lipofectamine® 2000 was added to the diluted miR-1 shRNA or miR-1 and NC plasmids and incubated for 20 min at room temperature. The complex was added to the H9C2 cells and incubated for 4 h at 37˚C. Cells were subsequently plated in 6-well plates with DMEM containing 10% FBS for 24 h at 37˚C. Following this incubation the cells were treated with 33 mmol/l glucose or 1 μmol/l GW3965 for a further 24 h depending on which group the cells were in. The cells were then collected for gene and protein examination.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using an RNApure High-purity Total RNA Rapid Extraction kit (BioTeke Corporation, Beijing, China) according to the manufacturer's protocol. cDNAs were synthesized using a Super M-MLV reverse transcriptase kit (BioTeke Corporation). For reverse transcription, the primer of miR-1 was as follows: 5'-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACATACAC-3'. For other genes, cDNA was directly obtained using oligo $(dT)_{15}$ and random primers. The reaction solution contained 2 μl RNA, 2 μl reverse transcription primers [1 μl oligo $(dT)_{15}$ and 1 μl random], 4 μl 5X reaction buffer, 0.75 μl dNTP (2.5 mM each), 0.25 μl ribonuclease inhibitor and 0.2 μl reverse transcriptase M-MLV (200 U). The reverse transcription reactions were performed at 16˚C for 10 min, 42˚C for 50 min, and 95˚C for 5 min.

The reverse transcription products were subsequently amplified by qPCR using SYBR® Green 1 (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) on an Exicycler 96 RTPCR machine (Bioneer Corporation, Daejeon, Korea). The primers used for qPCR were as follows: miR-1, forward, 5'-CGCGGTGGAATGTAAAGAAG-3' and reverse, 5'-GTGCAGGGTCCGAGGTATTC-3'; U6, forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; LXRα, forward, 5'-CTGAAGCGTCAAGAAGAGGA-3' and reverse, 5'-CCTGTTACACTGTTGCTGGG-3'; sterol regulatory element-binding transcription factor 1c (SREBP-1c), forward, 5'-TCCTGGAGCGAGCATTGAA-3' and reverse, 5'-CCGACAGCGTCAGAACAGC-3'; β-actin, forward, 5'-GGAGATTACTGCCCTGGCTCCTAGC-3' and reverse, 5'-GGC

CGGACTCATCGTACTCCTGCTT-3'. The qPCR thermocycling conditions were as follows: 94˚C for 10 min, 40 cycles of 94˚C for 10 sec, 60˚C for 20 sec and 72˚C for 30 sec, followed by 72˚C for 2 min 30 sec, 40˚C for 5 min 30 sec, melting from 60˚C to 94˚C with a 1˚C rise every 1 sec and 25˚C for 1 min. qPCR results were verified by varying the number of qPCR cycles for each cDNA and set of primers. The relative expression values of miR-1 and LXRα and SREBP-1c mRNA were calculated using the $2^{-\Delta\Delta Cq}$ method (21) with the housekeeping genes U6 and β-actin used as the internal controls. RT-qPCR was performed ≥3 times for each individual experiment.

Apoptosis detection. Apoptosis was determined using an Annexin-fluorescein isothiocyanate (FITC) apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) by fluorescence-activated cell sorting flow cytometry. All of the reagents were part of this kit. Cells were harvested, collected and washed twice in PBS. Cells were then resuspended in 500 μl binding buffer and stained with 5 μl Annexin V-FITC and 5 μl propidium iodide at room temperature for 15 min in the dark. Following this the cells were transferred into flow cytometry tubes for detection using a BD Accuri™ C6 flow cytometer and BD Accuri C6 Plus software (version C6; BD Biosciences, Franklin Lakes, NJ, USA) for 10,000 events.

Measurement of the mitochondrial membrane potential ($\Delta\Psi$). To determine the $\Delta\Psi$, cells were collected and washed in PBS and subsequently incubated with rhodamine 123 (Molecular Probes; Thermo Fisher Scientific, Inc.) at 37˚C for 30 min. Cells were then washed twice with PBS and resuspended in fresh DMEM medium for incubation for 60 min at 37˚C. Then the cells were detected with a BD Accuri™ C6 flow cytometer and BD Accuri C6 Plus software for 10,000 events.

Western blot analysis. The cells were harvested and lysed using radioimmunoprecipitation assay buffer and phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology, Haimen, China) for 30 min on ice. Protein content was determined using a BCA Protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) following the manufacturer's protocol. Proteins (40 μg/lane) were separated by 10-15% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were incubated with primary antibodies overnight at 4˚C and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit/mouse immunoglobulin G secondary antibodies (1:5,000; Beyotime Institute of Biotechnology) at room temperature for 45 min. Anti-cleaved-poly (adenosine diphosphate-ribose) polymerase (PARP; cat. no. ab32561; 1:1,000), anti-cleaved-caspase-3 (cat. no. ab2302; 1:1,000), anti-cleaved-caspase-9 (cat. no. ab25758; 1:1,000) and anti-LXRα (cat. no. ab3585; 1:300) primary antibodies were obtained from Abcam (Cambridge, MA, USA). Anti-PARP (cat. no. 9532; 1:5,000), anti-caspase-3 (cat. no. 9662; 1:5,000) and anti-caspase-9 (cat. no. 9508; 1:5,000) primary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-B-cell lymphoma-2 (Bcl-2; cat. no. BA0412) and anti-Bax (cat. no. BA0315) primary antibodies were obtained from Wuhan Boster Biological Technology, Ltd. (Wuhan, China) and used at a 1:400 dilution. Anti-SREBP-1c (cat. no. 14088-1-AP) primary antibodies were obtained from ProteinTech Group, Inc. (Chicago, IL, USA) and used at a 1:1,000 dilution. Anti-β-actin (cat. no. sc-47778) primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and used at a 1:1,000 dilution. Goat anti-rabbit (cat. no. A0208) and goat anti-mouse horseradish peroxidase-conjugated immunoglobulin G (cat. no. A0216) secondary antibodies were obtained from Beyotime Institute of Biotechnology and used at a 1:5,000 dilution. β-actin was utilized as the loading control for total protein expression. The amounts of transferred protein were quantified by the Gel Imaging System (WD-9413B; Beijing Liuyi Biotechnology, Co., Ltd., Beijing, China). Western blot quantitative analysis was performed using the Gel-Pro Analyzer software (version 4.0; Media Cybernetics, Inc., Rockville, MD, USA), and the experiments were repeated a minimum of three times.

Statistical analysis. Data are presented as the mean + standard deviation of 3 independent experiments. Multiple comparisons were performed by one-way analysis of variance and Bonferroni's post-hoc test. Data analysis and plotting were conducted using GraphPad Prism 4.0 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Silencing miR-1 expression suppresses apoptosis in glucose-induced H9C2 cells. To examine the expression of miR-1 in glucose-induced cardiomyocytes, the expression of miR-1 in H9C2 cells treated with or without glucose was determined by RT-qPCR analysis when the cells were in the logarithmic growth phase. It was revealed that the expression of miR-1 was significantly increased in glucose-treated H9C2 cells compared with non-glucose-treated control cells ($P<0.01$; Fig. 1A). LXRα is involved in the pathogenesis of DCM and serves a key role in diabetic heart disease (20). The results of RT-qPCR revealed that the expression of LXRα mRNA was significantly decreased in glucose-induced H9C2 cells compared with non-glucose-treated control cells ($P<0.01$; Fig. 1B). The expression of LXRα protein was also significantly downregulated in cells treated with glucose compared with the control cells ($P<0.01$; Fig. 1C). These results indicate that miR-1 expression is significantly increased in H9C2 cells following treatment with glucose, whereas the expression of LXRα is significantly decreased.

miR-1 expression was knocked down by transfecting anti-miR-1 plasmids into H9C2 cells (Fig. 1D) and RT-qPCR revealed that miR-1 expression was significantly inhibited in the anti-miR-1 group compared with the NC group ($P<0.01$). Fluorescence-activated cell sorting analysis was performed to determine the rate of apoptosis of cells within the different treatment groups. Transfection with anti-miR-1 alone did not significantly affect the rate of apoptosis in the cells (Fig. 1E). Treatment of untransfected cells with glucose significantly increased the rate of apoptosis compared with the control group ($P<0.01$); however miR-1 silencing significantly inhibited glucose-induced apoptosis compared with the NC+Glucose group ($P<0.01$). These results indicate that silencing miR-1 inhibits apoptosis in glucose-induced H9C2 cells.

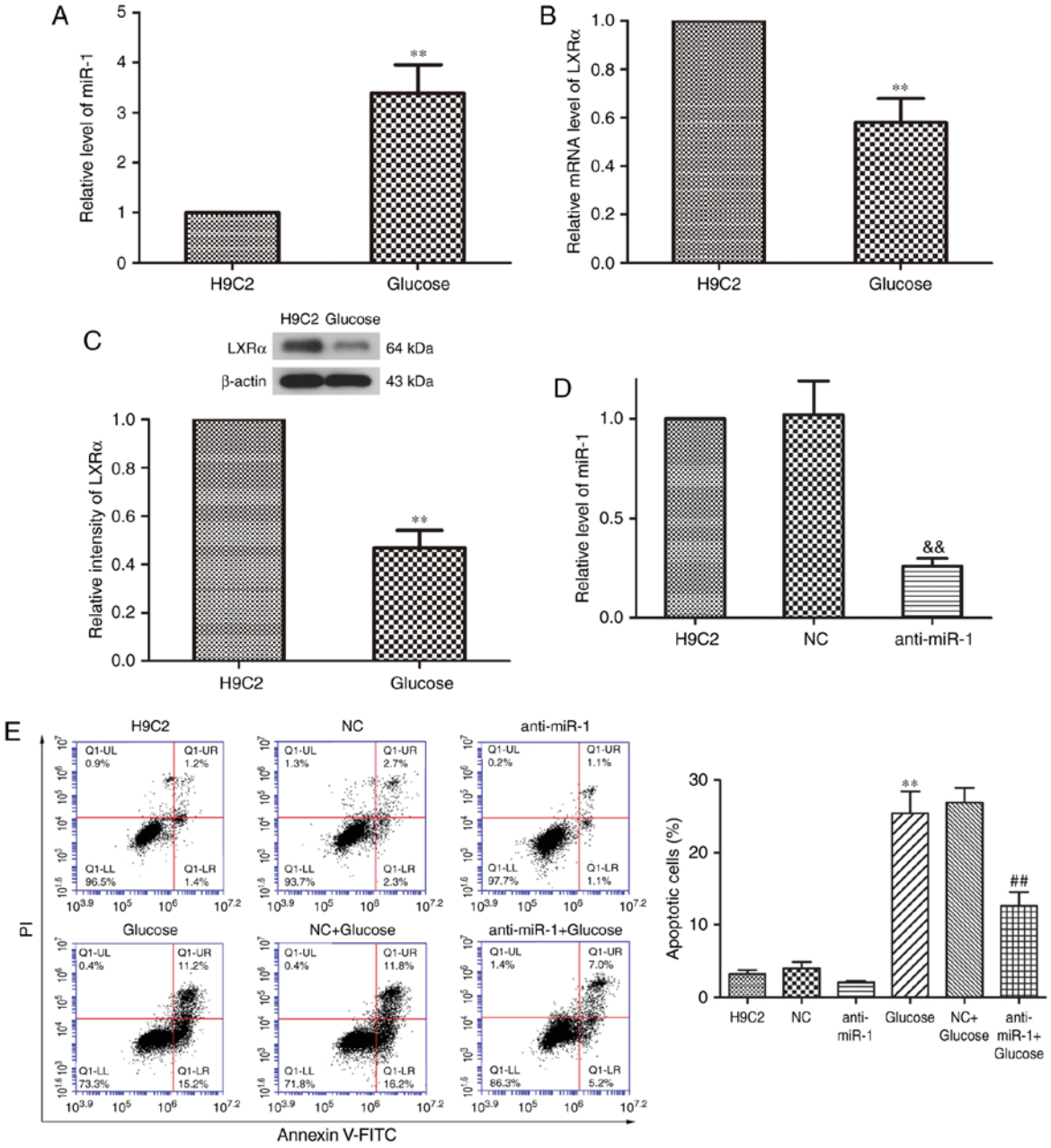

Figure 1. Expression of miR-1 and LXRα in glucose-induced H9C2 cells. The expression of (A) miR-1 and (B) LXRa mRNA in glucose-induced H9C2 cells. (C) The expression of LXRα protein in glucose-induced H9C2 cells. (D) The expression of miR-1 in H9C2 cells following transfection with anti-miR-1 and NC plasmids. (E) The proportion of apoptotic cells in groups transfected with NC, anti-miR-1 and subsequently treated with or without glucose. **P<0.01 vs. H9C2; $^{\&\&}$P<0.01 vs. NC; $^{\#\#}$P<0.01 vs. NC+Glucose. NC, negative control; miR, microRNA; LXRα, liver X receptor α; FITC, fluorescein isothiocyanate; PI, propidium iodide.

miR-1 silencing attenuates the glucose-induced decrease of the ΔΨ in H9C2 cells. Mitochondrial dysfunction is a key characteristic of apoptosis (22,23). To elucidate the potential mechanism underlying the silencing of miR-1 in glucose-induced H9C2 cells, the ΔΨ was measured to determine mitochondrial function in H9C2 cells (Fig. 2A). Treatment with glucose significantly reduced the fluorescence intensity of the ΔΨ compared with the control cells (P<0.01; Fig. 2B), however, miR-1 knockdown significantly reversed the glucose-induced ΔΨ decrease in H9C2 cells (P<0.05; Fig. 2B). These results reveal that silencing miR-1 attenuates glucose-induced apoptosis via the mitochondrial pathway.

The expression of apoptosis-associated proteins was measured by western blot analysis (Fig. 2C). There was a significant increase in the cleavage of PARP and caspase-3 in glucose treated cells (P<0.01; Fig. 2D); however inhibition of miR-1 significantly suppressed the cleavage of PARP and caspase-3 in the anti-miR-1+Glucose cells (P<0.01). This suggests that anti-miR-1-inhibited apoptosis is mediated via a caspase-associated signaling pathway. In addition, the level of cleaved caspase-9 was significantly increased by glucose (P<0.01) and also significantly inhibited in the anti-miR-1+Glucose cells (P<0.01), suggesting that the endogenous apoptotic signaling pathway is also involved in anti-miR-1-inhibited apoptosis.

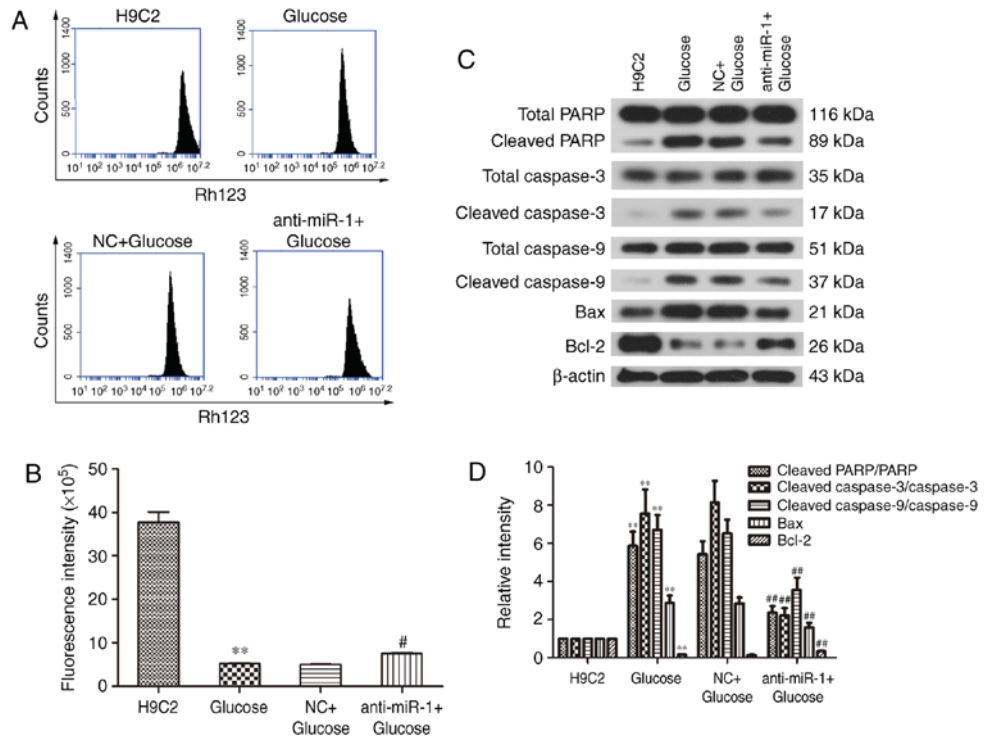

Figure 2. Effect of miR-1 silencing on glucose-induced apoptosis. (A) Effect of miR-1 silencing on glucose-induced mitochondrial dysfunction in H9C2 cells. (B) Quantification of fluorescence intensity. (C) The expression of apoptosis-associated proteins, including Bcl-2, Bax, total/cleaved caspase-3, total/cleaved caspase-9 and total/cleaved PARP in H9C2 cells. (D) Quantitative analysis of western blot analysis results. **P<0.01 vs. H9C2; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. NC+Glucose. NC, negative control; Bcl-2, B cell lymphoma-2; PARP, poly (adenosine diphosphate-ribose) polymerase; miR, microRNA.

The endogenous apoptotic signaling pathway is regulated by the balance between pro-apoptotic proteins, such as Bax and anti-apoptotic proteins, such as Bcl-2 (24). Therefore, the expression of Bcl-2 and Bax in the different groups of cells were measured. The significant downregulation of anti-apoptotic protein Bcl-2 induced by glucose (P<0.01), was significantly reversed in the anti-miR-1+Glucose cells (P<0.01) and the upregulation of pro-apoptotic protein Bax was significantly decreased following miR-1 silencing (P<0.01). There were no significant differences between the expression of proteins in the Glucose and NC+Glucose groups. These results suggest that the downregulation of Bcl-2 and the upregulation of Bax are responsible for the activation of the endogenous apoptotic signaling pathway in glucose-induced apoptosis and that this is significantly suppressed by miR-1 knockdown in H9C2 cells.

miR-1 regulates the expression of LXRα in glucose-induced H9C2 cells. It has been reported that miR-1 functions via the miR-1/LXRα signaling pathway in lipogenesis-associated diseases (17). LXRα serves a key role in the normal and DCM hearts of *db/db* mice with type 2 diabetes and serves a role in the development of DCM (25). Western blot analysis was performed to investigate whether miR-1 silencing alters the expression of LXRa in H9C2 cells (Fig. 3). The results demonstrated that miR-1 silencing significantly increased the expression of LXRα compared with the NC group (P<0.01; Fig. 3A and B). Following treatment with glucose, LXRα expression was significantly decreased (P<0.01; Fig. 3C and D). However, transfection of H9C2 cells with anti-miR-1 and treatment with glucose caused a significant increase in the relative intensity of LXRa compared with the NC+Glucose group (P<0.01; Fig. 3C). By contrast, the relative intensity of LXRa significantly decreased further following the transfection of cells with miR-1 and treatment with glucose compared with the NC+Glucose group (P<0.01; Fig. 3D). RT-qPCR was performed to determine the expression of LXRα mRNA following transfection with anti-miR-1 or miR-1 plasmids in glucose-induced H9C2 cells. miR-1 knockdown significantly increased the expression of LXRα mRNA compared with the NC+Glucose group (P<0.01; Fig. 3E), whereas the overexpression of miR-1 significantly amplified the decrease in LXRα mRNA expression (P<0.05; Fig. 3F). These results indicate that the inhibition of miR-1 suppresses glucose-induced apoptosis, potentially by regulating the expression of LXRα in H9C2 cells.

GW3965 regulates apoptosis in anti-miR-1 treated cells. LXRα may be involved in the pathogenesis of various cardiovascular and metabolic diseases and serve a key role in miR-1 knockdown in H9C2 cells. Therefore, the synthetic LXRα agonist GW3965 was used to investigate whether LXRα was associated with the inhibition of apoptosis, which is regulated by miR-1 knockdown in glucose-induced H9C2 cells. The effect of GW3965 alone on glucose-induced apoptosis was determined (Fig. 4A). GW3965 significantly inhibited glucose-induced apoptosis in a dose-dependent manner (P<0.05 and P<0.01; Fig. 4B), however treatment with 1 μM GW3965 alone did not significantly inhibit the level of apoptosis induced by glucose in H9C2 cells. Therefore, 1 μM GW3965 was used to assess whether LXRa was involved in the underlying mechanism of miR-1 on glucose-induced apoptosis in H9C2 cells. Treatment with 1 μM GW3965

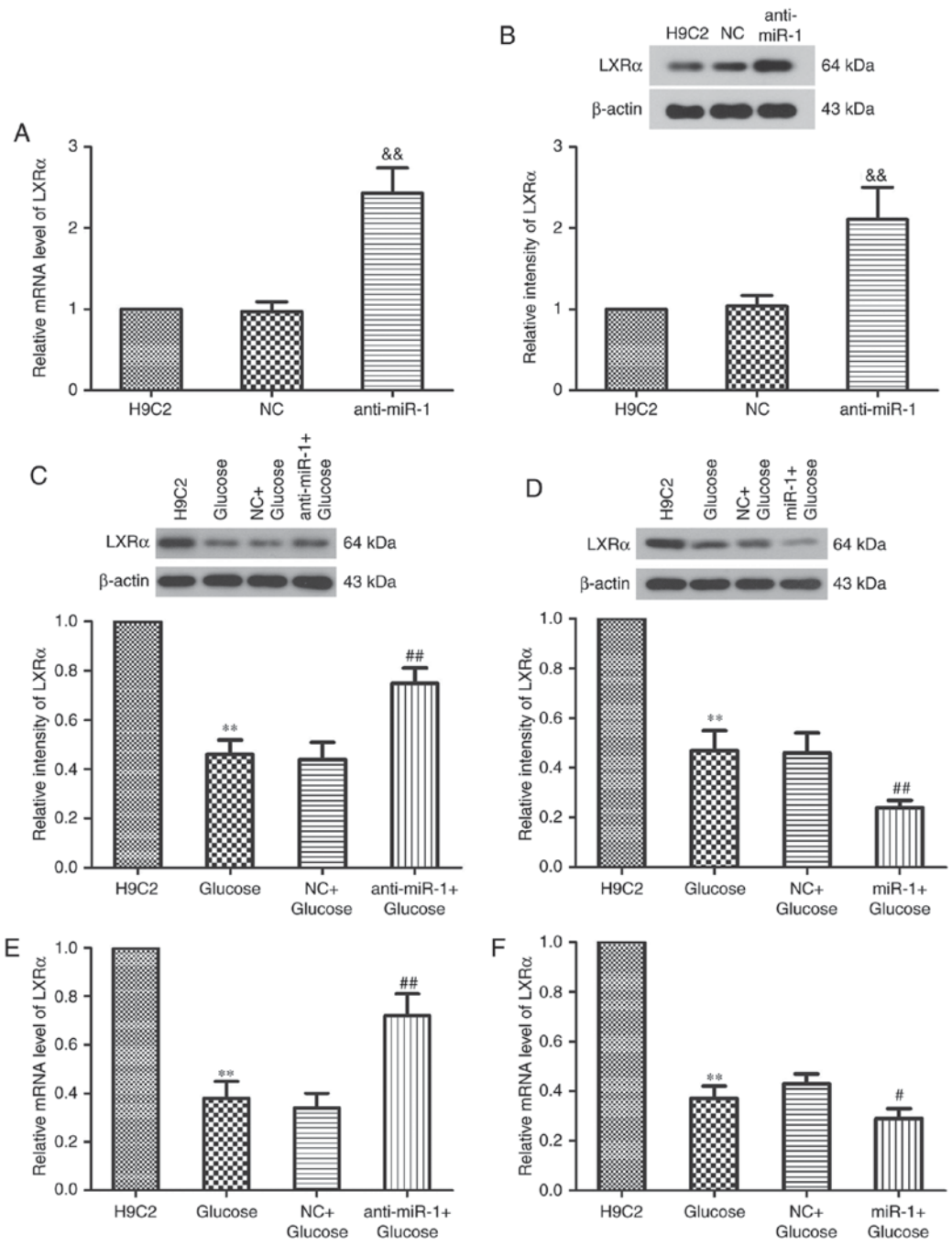

Figure 3. miR-1 regulates the expression of LXRα in glucose-induced H9C2 cells. Silencing of miR-1 upregulated the (A) mRNA and (B) protein expression of LXRα in H9C2 cells as determined by RT-qPCR and western blot analysis, respectively. The expression of LXRα protein in H9C2 cells transfected with (C) anti-miR-1 or (D) miR-1 and NC plasmids was determined by western blot analysis. The expression of LXRα mRNA in H9C2 cells transfected with (E) anti-miR-1 or (F) miR-1 was determined by RT-qPCR. **P<0.01 vs. H9C2; $^{\&\&}$P<0.01 vs. NC; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. NC+Glucose. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA; LXRα, liver X receptor α; NC, negative control.

significantly enhanced the inhibitory effect of anti-miR-1 on apoptosis compared with the anti-miR-1+Glucose cells (P<0.05; Fig. 4C and D). In addition, the increase in the ΔΨ following transfection with anti-miR-1 was significantly enhanced by treatment with 1 μM GW3965 compared with anti-miR-1+Glucose cells (P<0.05; Fig. 5A and B).

Western blot analysis indicated that the expression of cleaved PARP, cleaved caspases-3 and -9, and Bax in anti-miR-1+Glucose+GW3965 cells were significantly decreased compared with the anti-miR-1+Glucose cells (P<0.01), whereas the expression of Bcl-2 was significantly upregulated (P<0.01; Fig. 5C and D). These results indicate that the inhibition of apoptosis following miR-1 silencing on glucose-induced H9C2 cells is significantly enhanced following the activation of LXRα by GW3965 in glucose-induced H9C2 cells. Therefore, miR-1 silencing may suppress the apoptosis induced by glucose by regulating LXRα in H9C2 cells.

To investigate the regulatory effect of miR-1 on glucose-induced apoptosis, the expression of SREBP-1c, a known LXR target gene, was determined by RT-qPCR and western blot analysis. Levels of SREBP-1c mRNA and protein were significantly downregulated following

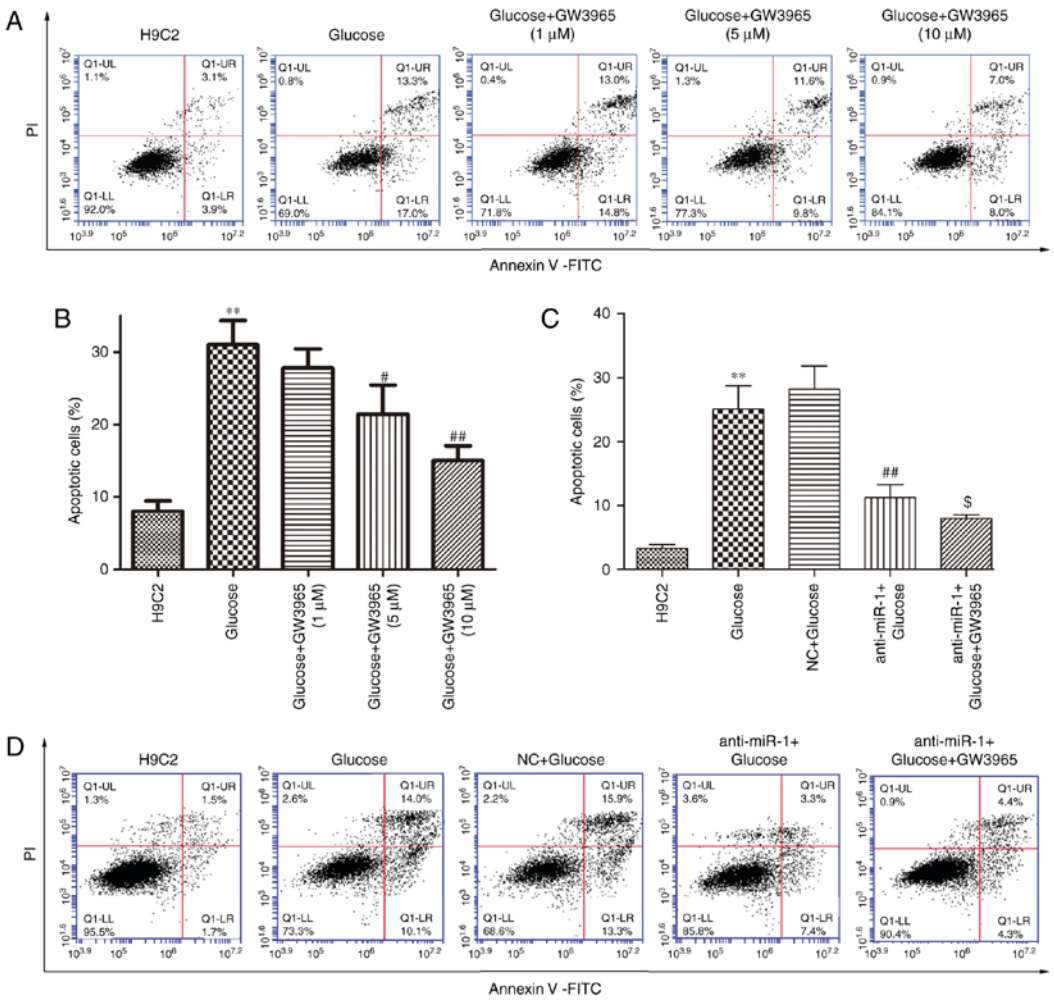

Figure 4. Effect of GW3965 on apoptosis in H9C2 cells. The apoptotic inhibitory effect of anti-miR-1 was amplified by GW3965 in glucose-induced H9C2 cells. (A) GW3965 inhibited the glucose-induced apoptosis in a dose-dependent manner in H9C2 cells, as determined by FACS analysis. (B) The results of FACS were quantified. (C) Quantification of the percentage of apoptotic cells following transfection with anti-miR-1 or NC plasmids. (D) Following transfection, and treatment with glucose or GW3965, apoptotic cells were detected by FACS analysis. **P<0.01 vs. H9C2; §§P<0.01, §P<0.05 vs. Glucose; $^{#}$P<0.05 and $^{##}$P<0.01 vs. NC+Glucose; $^{$}$P<0.05 vs. anti-miR-1+Glucose. miR, microRNA; NC, negative control; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; PI, propidium iodide.

treatment with glucose (P<0.01), however miR-1 silencing significantly reversed the decrease in SREBP-1c expression (P<0.01; Fig. 6). These results suggest that silencing miR-1 inhibits glucose-induced apoptosis via the miR-1/LXRα signaling pathway in H9C2 cells.

Discussion

DCM is characterized by left ventricular hypertrophy, diastolic dysfunction and impaired myocardial substrate metabolism (26,27). However, the effect of DM on cardiac function is not always clear, which may lead to a delayed diagnosis. The role of miRs in various diseases has attracted increased attention and previous studies have investigated the role miR-1 serves in lipid metabolic disorders as well as in diabetic complications. It has been demonstrated that circulating miR-1 is involved in the onset of myocardial infarction (28). In addition, miR-1 is a mediator of non-diabetic cardiac hypertrophy (29). However, the precise mechanism of miR-1 during the development of DCM remains unclear. In the present study, the role of miR-1 in the glucose-induced apoptosis of H9C2 cells was investigated and the association between miR-1 and LXRα was assessed. The results revealed that the expression of miR-1 was significantly increased in glucose induced H9C2 cells and silencing this miR-1 expression significantly inhibited glucose-induced apoptosis. This reduction in apoptosis occurred via the mitochondrial apoptotic pathway by upregulating Bcl-2 and downregulating Bax in H9C2 cells. The expression of LXRα mRNA was significantly decreased in glucose-treated H9C2 cells, whereas its expression was significantly increased by anti-miR-1. In addition, the inhibitory effect of anti-miR-1 on apoptosis was amplified by GW3965.

There is an association between the expression and certain regulatory functions of miR-1 and its homologue miR-206; however they are transcribed from different chromosomes and may undergo individual transcriptional activation (30). Therefore, the present study focused on the regulatory effect of miR-1 on glucose-induced H9C2 cells.

miR-1 contributes to glucose-mediated apoptosis in cardiomyocytes (12). It has been demonstrated that the levels of >125 miRs, including miR-1, are altered in glucose-induced endothelial cells and endothelin-1 has been identified as a potential

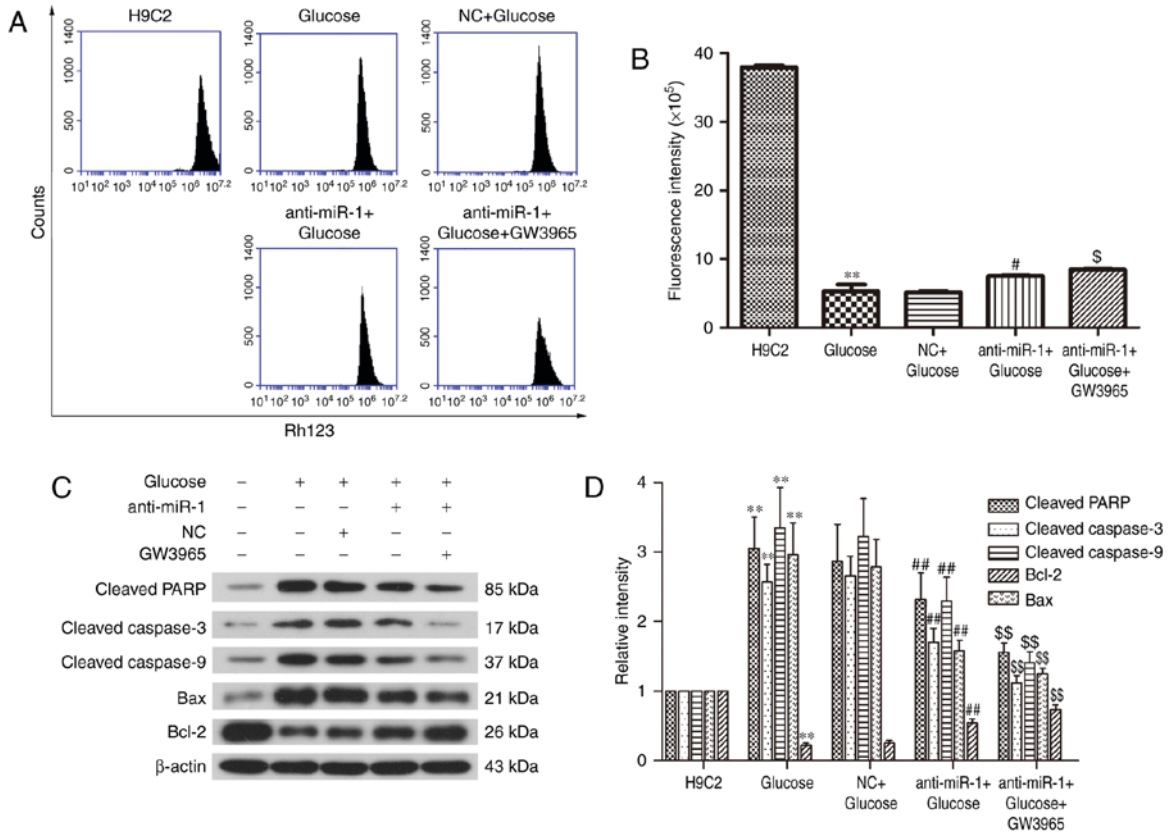

Figure 5. Effect of GW3965 on the expression of apoptosis-associated proteins. (A) The ΔΨ was detected by fluorescence-activated cell sorting analysis following staining with rhodamine 123 in H9C2 cells and (B) fluorescence intensity was quantified. (C) The expression of apoptosis-associated proteins in H9C2 cells was determined by western blot analysis and (D) the results were quantified. **P<0.01 vs. H9C2; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. NC+Glucose; $^{\$}$P<0.05 and $^{\$\$}$P<0.01 vs. anti-miR-1+Glucose. ΔΨ, mitochondrial membrane potential; miR, microRNA; NC, negative control.

target of miR-1 (31). Yu *et al* (32) reported that high glucose levels induce the apoptosis of cardiomyocytes via the miR-1 regulated insulin-like growth factor-1 signaling pathway. It has also been determined that the inhibition of miR-1 confers a protective effect via the p38/mitogen-activated protein kinase signaling pathway in high glucose induced neonatal rat cardiomyocytes (33). However, the underlying mechanisms associated with glucose and miR-1 remains unknown and further studies are required to confirm this.

Zhai *et al* (34) revealed that the inhibition of miR-1 attenuates the hypoxia/re-oxygenation-induced apoptosis of cardiomyocytes. These results suggest that miR-1 acts as a key factor during the apoptosis of cardiomyocytes. In the present study, miR-1 silencing significantly inhibited the glucose-induced apoptosis of H9C2 cells by attenuating the decrease in ΔΨ and regulating the expression of Bcl-2 and Bax. Apoptosis is mediated by the caspase cascade (35) and the interaction between anti-apoptotic proteins, such as Bcl-2 and pro-apoptotic proteins, such as Bax (36), determines whether cells undergo apoptosis. The ratio of anti-apoptotic Bcl-2 and pro-apoptotic Bax determines the response to an apoptotic signal (37). It has been reported that miR-1 participates in the H_2S protection of cardiomyocytes against ischemia/reperfusion injury-induced apoptosis by regulating Bcl-2 (34). In the present study, levels of cleaved PARP, cleaved caspase-3 and cleaved caspase-9 induced by glucose were significantly suppressed following miR-1 inhibition, suggesting that the activation of the endogenous apoptotic signaling pathway was inhibited by miR-1 knockdown. In addition, the upregulation of Bax and the downregulation of Bcl-2 induced by glucose in H9C2 cells were reversed by miR-1 silencing. Bcl-2 binds to Bax and inhibits its activation (38,39).

In the present study, treatment with glucose caused a significant reduction in Bcl-2 expression, thus releasing Bax from the Bax/Bcl-2 heterodimeric complex, leading to the upregulation of Bax. This released Bax may accelerate the formation of outer-mitochondrial membrane spanning pores and decrease the ΔΨ, triggering the activation of the caspase cascade by caspase-3 to initiate apoptosis. However, miR-1 silencing may have enhanced the expression of Bcl-2 and suppressed the expression of Bax, thereby inhibiting the release of Bax and increasing the ΔΨ. The inactivation of the caspase cascade was attributed to transfection with anti-miR-1 in glucose-induced H9C2 cells. The results of the present study suggest that silencing miR-1 by anti-miR-1 inhibits apoptosis via the mitochondrial signaling pathway in glucose-induced H9C2 cells.

LXRα may serve a role in regulating metabolism (40) and inflammation (41). It has been demonstrated that changes in LXRs are involved in the pathogenesis of various diseases, including cardiovascular (42) and metabolic diseases (43), Alzheimer's disease (44) and various types of cancer (45). LXRα is highly expressed in the majority of metabolically active tissues, including the adipose tissue, the kidney, liver

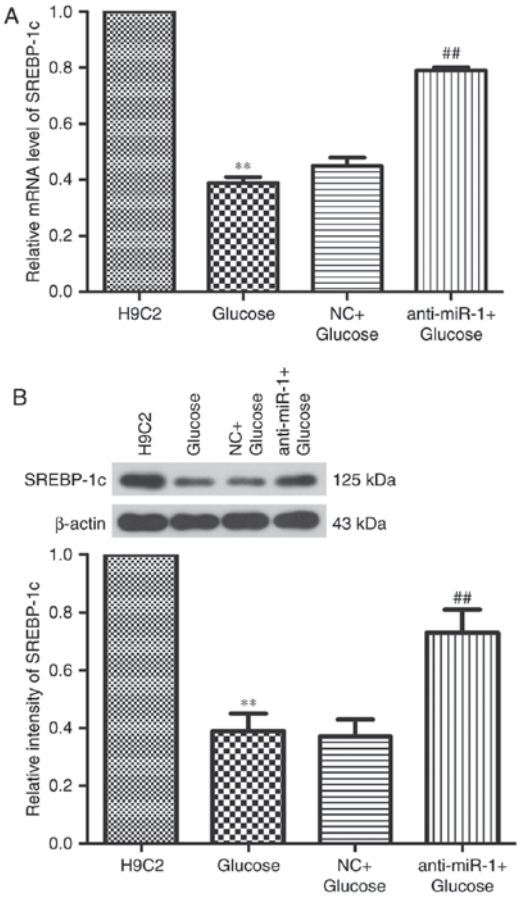

Figure 6. miR-1 silencing increases the expression of SREBP-1c in glucose-induced H9C2 cells. The (A) mRNA and (B) protein expression of SREBP-1c was determined in glucose-induced H9C2 cells transfected with or without anti-miR-1 or NC plasmids. **P<0.01 vs. H9C2; $^{\#\#}$P<0.01 vs. NC+Glucose. SREBP-1c, sterol regulatory element-binding transcription factor 1c; NC, negative control; miR, microRNA.

and intestines, where it regulates metabolic and inflammatory signaling pathways (46). LXR is therefore considered to be a potential therapeutic target in the treatment of cardiovascular and metabolic diseases (47).

In the present study, it was observed that the expression of LXRα was significantly decreased in glucose-induced H9C2 cells, which was consistent with the results of Lee *et al* (48). Furthermore, the expression of LXRα was regulated by miR-1 in glucose-induced H9C2 cells. These results indicate that miR-1 may be important in the regulation of LXRα. Zhong *et al* (17) reported that LXRα may be a target of miR-1 and miR-206. Ou *et al* (49) demonstrated that miR-613 targets the human LXR gene and mediates a feedback loop of LXR autoregulation in human hepatocytes. It has been previously reported that the activation of LXRα exhibits a cardioprotective effect against DCM by attenuating insulin resistance, reducing oxidative/nitrative stress and inhibiting the inflammatory response (25). To further explore the interaction between miR-1 and LXRα in the present study, the synthetic LXR agonist GW3965 was used. It was observed that the activation of LXRα by GW3965 significantly enhanced the inhibitory effect of anti-miR-1 on apoptosis in glucose-treated H9C2 cells. These results suggest that the activation of LXRα enhances the regulatory effect of miR-1 in H9C2 cells.

At present, more than a dozen LXR target genes have been identified. The transcription of several genes concerning the cellular cholesterol efflux, including adenosine triphosphate-binding cassette (ABC) A1, ABCG1 and apolipoprotein E are controlled by LXRs (50-52). LXRs serve key roles in the reverse cholesterol transport pathway in macrophages and other peripheral cells (53-55). In the liver, LXRs regulate the expression of proteins involved in cholesterol and fatty acid metabolism, including cholesterol 7α-hydroxylase (56,57) and SREBP-1c (58). Harasiuk *et al* (59) reported that administration of the LXRα agonist TO901317 increases the expression of LXR isoforms and their target gene SREBP-1c in the hearts of streptozotocin-diabetic rats. In the present study, the expression of SREBP-1c mRNA and protein were significantly downregulated following the administration of glucose, whereas the miR-1 silencing significantly inhibited this decrease. These results suggest that miR-1 silencing inhibits glucose-induced apoptosis via the miR-1/LXRα signaling pathway in H9C2 cells.

The present study had certain limitations, including the fact that experiments were only performed *in vitro*. Previous studies have reported that the activation of LXRα may attenuate cardiac dysfunction and improve glucose tolerance in diabetic *db/db* mice (25,60); therefore the regulatory effect of miR-1 on the activation of LXRα *in vivo* requires further investigation.

In conclusion, the present study demonstrated that silencing miR-1 significantly inhibits apoptosis via the mitochondrial signaling pathway by regulating LXRα, the activation of which attenuates the apoptosis of cardiac H9C2 cells. These results suggest that miR-1 serves an important role in the development of DCM and provide novel insights into understanding the complex underlying mechanisms involved in the pathogenesis of DCM.

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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

YXC designed the study and performed the experiments. WZ contributed to the western blot and reverse transcription-quantitative polymerase chain reaction analysis. XDZ performed the transfections and fluorescence-activated cell sorting flow cytometry experiments. LXS and YC were involved in the statistical analysis, and HRY critically reviewed the

manuscript and contributed to statistical analysis; YW performed the cell culture and transfections. YHC managed the experimental design and reviewed the manuscript. GBL reviewed the manuscript and provided funding support. YXC and WZ drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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