# Inhibition of microRNA-101 attenuates hypoxia/reoxygenation-induced apoptosis through induction of autophagy in H9c2 cardiomyocytes

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**Abstract.** Autophagy is a cellular self-catabolic process responsible for the degradation of proteins and organelles. Autophagy is able to promote cell survival in response to stress, and increased autophagy amongst cardiomyocytes has been identified in conditions of heart failure, starvation and ischemia/reperfusion. However, the detailed regulatory mechanisms underlying autophagy in heart disease have remained elusive. MicroRNAs (miRNAs) have been implicated in the regulation of autophagy in cells under stress. In the present study, the protective effect of miRNA (miR)-101 on hypoxia/reoxygenation (H/R)-induced cardiomyocyte apoptosis was investigated. It was revealed that H/R induced apoptosis in H9c2 cardiomyocytes, accompanied by a downregulation of miR-101 expression. Further investigation identified Ras-related protein Rab-5A (RAB5A) as a direct target of miR-101. RAB5A was previously reported to be involved in autophagy; therefore, the present study further focused on the role of miR-101 in the regulation of autophagy under H/R and found that the inhibition of miR-101 attenuated H/R-induced apoptosis, at least partially, via the induction of autophagy. In conclusion, the results of the present study revealed a beneficial effect of miR-101 inhibition on H/R-induced apoptosis in cardiomyocytes, indicating that miR-101 inhibition may present a potential therapeutic agent in the treatment or prevention of heart diseases.

## Introduction

Autophagy is a cellular self-catabolic process responsible for the degradation of long-lived proteins and organelles. During autophagy, cytoplasmic constituents are sequestered into autophagosomes and degraded via the lysosomal pathway (1). Autophagy is able to promote cell survival in response to stress. However, progressive autophagy also induces cell death (2). Therefore, autophagy not only has a crucial role in the regulation of normal development, but dysregulated or defective autophagy is associated with disease.

MicroRNAs (miRNAs) are a group of endogenous, non-coding, single-strand, small RNAs of 22-25 nucleotides, which regulate gene expression at the post-transcriptional level, predominantly through base pairing with the 3'-untranslated region (3'-UTR) of target mRNAs, which results in mRNA degradation or translational repression (3). It has been revealed that miRNAs regulate >30% of genes, which are associated with almost all major cellular processes, including cell proliferation, differentiation, apoptosis and migration, as well as immune responses (4). Myocardial tissue injury induced by ischemia and hypoxia is a major factor underlying the development of fatal diseases. The main cause of myocardial ischemic injury is myocardial cell apoptosis induced by myocardial hypoxia (5). Furthermore, subsequent reoxygenation may further aggravate the damage (6). Multiple miRNAs have been shown to function as protectors against hypoxia/reoxygenation (H/R)-induced myocardial injury (7-9). However, the specific molecular mechanisms underlying this effect have remained elusive.

Accumulating evidence has indicated that certain miRNAs function in the induction or inhibition of autophagy in cells under stress, including miRNA-101 (miR-101) (10). miR-101 has been demonstrated to participate in the regulation of various cancers (11,12). For example, miR-101 was demonstrated to be a potent inhibitor of autophagy in breast cancer cells and the inhibition of miR-101 sensitized breast cancer cells to drug-mediated cell death (10). In the present study, it was demonstrated that H/R led to cellular apoptosis as well as miR-101 downregulation in H9c2 cardiomyocytes. Further investigation suggested that the inhibition of miR-101 attenuated H/R-induced apoptosis, at least in part, via the induction of autophagy. The effect of miR-101 on H/R-induced cardiomyocyte apoptosis may be via direct targeting of Ras-related protein Rab-5A (RAB5A), which has previously been demonstrated to be involved in autophagy.

## Materials and methods

*Cell culture*. H9c2 cardiomyocytes were obtained from the American Type Culture Collection (Rockville, MD, USA).

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Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Both from Invitrogen Life Technologies, Carlsbad, CA, USA) at 37˚C in a humidified incubator containing 5% $CO_2$.

*H/R treatment of H9c2 cardiomyocytes*. Cells were pre-incubated with a selective autophagy inhibitor 3-MA (10 mM; Sigma-Aldrich, St. Louis, MO, USA) prior to hypoxia/reoxygenation treatment. Following culture in serum-free DMEM at 37˚C in 5% $CO_2$ for 12 h, H9c2 cardiomyocytes were cultured at 37˚C in 1% $O_2$/94% $N_2$/5% $CO_2$ for 4 h. Subsequently, H9c2 cardiomyocytes were cultured in DMEM containing 10% FBS and incubated at 37˚C in 5% $CO_2$ for 3 h.

*Reverse transcription polymerase chain reaction (RT-PCR) assay*. Total RNA was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). For the analysis of mRNA expression, the TaqMan Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to convert RNA into complementary (c)DNA, and PCR was subsequently performed using the Power SYBR Green kit (Thermo Fisher Scientific) on a Bio-Rad MiniOption thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β-actin was used as an endogenous control. The primers for RAB5A were as follows: Forward, 5'-CATTGGGGCTGCCTTTCTA-3' and reverse, 5'-TCCTCTGGCTGAGTTTGCG-3'. The primers for stathmin 1 (STMN1) were as follows: Forward, 5'-GCGAGAGAAGGACAAGCACG-3' and reverse, 5'-TTGGATATTTAGGAAGGGGT-3'. The primers for β-actin were as follows: Forward, 5'-AGGCCCCTCTGAACCCTAAG-3' and reverse, 5'-CCAGAGGCATACAGGGACAAC-3'. The primers for U6 were as follows: Forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. For the analysis of miRNA expression, an ABI miRNA reverse transcription kit (Applied Biosystems, Life Technologies, Foster City, CA, USA) was used to convert RNA into cDNA, according to the manufacturer's instructions. Subsequently, PCR was performed using an miRNA Q-PCR Detection kit (GeneCopoeia, Rockville, MD, USA) on a Bio-Rad MiniOption thermocycler (Bio-Rad Laboratories, Inc.). The U6 gene (GeneCopopia, Rockville, MD, USA) was used as an endogenous control. PCR cycling conditions were as follows: 95˚C for 1 min, followed by 40 cycles of 95˚C for 15 sec, 58˚C for 15 sec and 72˚C for 20 sec. All experiments were performed in triplicate. For mRNA and miRNA, the relative expression was analyzed using the $2^{-\Delta\Delta Ct}$ method.

*Western blot analysis*. Cells were solubilized in cold radioimmunoprecipitation assay lysis buffer (Fermentas, Thermo Fisher Scientific, Pittsburgh, PA, USA). Proteins were separated by 12% SDS-PAGE, and transferred onto a polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA), which was then incubated with Tris-Buffered Saline and Tween 20 (Sigma-Aldrich) containing 5% skimmed milk (BD Biosciences, Franklin Lakes, NJ, USA) at 4˚C for overnight. Subsequently, the membrane was incubated with rabbit monoclonal anti-RAB5A (1:1,000; Abcam, Hong Kong, China) and mouse monoclonal anti-β-actin (1:1,000; Abzoom, Dallas, TX, USA) primary antibodies at room temperature for 4 h. Membranes were also incubated at room temperature with rabbit monoclonal anti-mouse/rat/human Beclin1 antibody (1/500; Abcam) for 4 h, rabbit polyclonal anti-mouse/rat/human IL-1β antibody (1/200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 4 h, rabbit monoclonal anti-mouse/rat/human Bcl2 antibody (1/500; Abcam) for 3 h, rabbit polyclonal anti-mouse/rat/human LC3B antibody (1/1000; Abcam) for 4 h, rabbit polyclonal anti-mouse/rat/human/quail caspase-3 antibody (1/400; Abcam) for 4 h and mouse monoclonal anti-mouse/rat/human/rabbit/pig β-actin antibody (1/2000; Abzoom, Dallas, TX, USA) for 2 h. Following washing three times with phosphate-buffered saline with Tween 20 (PBST), the membrane was incubated with the goat anti-mouse or goat anti-rabbit secondary antibodies (1:5,000; Abcam), respectively, at room temperature for 1 h. Following washing three times with PBST, an enhanced chemiluminscence kit (Pierce Chemical, Rockford, IL, USA) was used to perform chemiluminescent detection. Relative protein expression was analyzed with Image-Pro plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA), represented as the density ratio versus GAPDH.

*Transfection*. For the miR-101 functional analysis, cells were transfected with the pre-miR-101, pre-con, anti-101 or anti-con (Genecopoeia, Rockville, MD, USA) were transfected into H9c2 cardiomyocytes using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions.

*Dual luciferase reporter assay*. A mutant 3'-UTR of RAB5A was generated using the Quick-Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). For the luciferase assay, 100,000 cells were cultured to ~70% confluence in 24-well plates and co-transfected with psiCHECK™2-RAB5A-3'-UTR or psiCHECK™2-mut RAB5A-3'-UTR vectors (Promega Corp., Madison, WI, USA) plus 50 nM miR-101 or 100 mM miR-101 inhibitor. Cells were incubated with Lipofectamine 2000/DNA complex for 5 h and the medium was subsequently replaced with DMEM containing 10% FBS. Forty-eight hours after transfection, a dual luciferase reporter gene assay kit (Promega Corp.) was used to determine the luciferase activities in each group using an LD400 luminometer (Promega Corp.). *Renilla* luciferase activity was normalized to firefly luciferase activity.

*Apoptosis analysis*. Flow cytometry was used to determine cell apoptosis with an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (BD Biosciences). At 24 h post-transfection, cells were harvested and washed twice with cold PBS. Subsequently, $10^6$ cells were resuspended in 200 $\mu$l binding buffer with 10 $\mu$l Annexin-V-FITC and 5 $\mu$l propidium iodide, and incubated in the dark for 30 min. Finally, 300 $\mu$l binding buffer was added and the cells were assessed using flow cytometric analysis (Moflo XDP; Beckman Coulter, Brea, CA, USA).

*Statistical methods*. Values are expressed as the mean ± standard deviation of three independent experiments. SPSS 18 software (International Business Machines, Armonk, NY, USA) was used to perform statistical analyses. Statistical analysis of differences between values was performed by

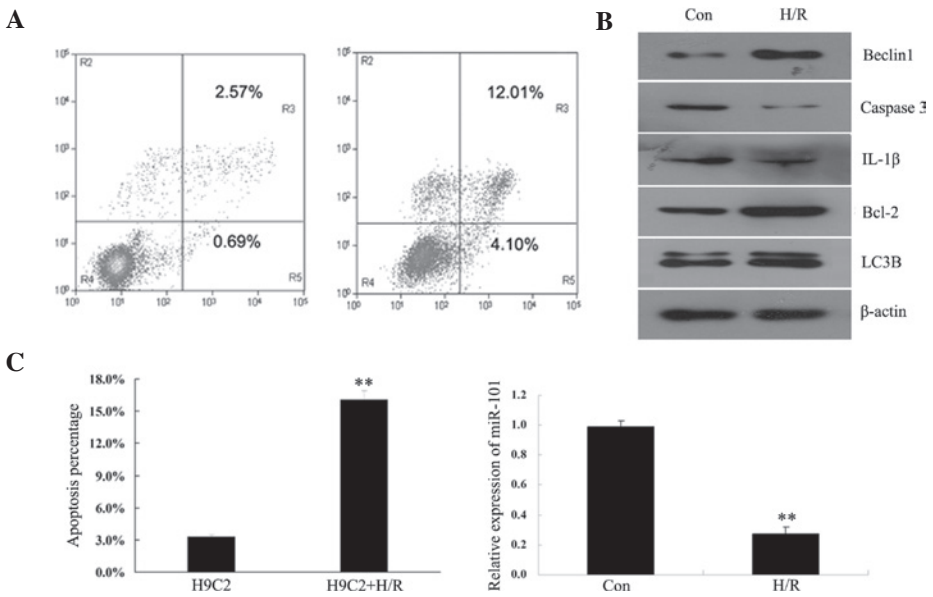


Figure 1. H/R induces apoptosis and miR-101 downregulation in H9c2 cardiomyocytes. (A) Flow cytometry was used to determine the apoptotic rate in H/R-treated H9c2 cardiomyocytes using an Annexin V-fluorescein isothiocyanate Apoptosis Detection kit. **P<0.01 vs. H9C2. (B) Western blot analysis was performed to determine the protein expression levels in H/R-treated H9c2 cardiomyocytes. (C) Polymerase chain reaction analysis was performed to determine the expression levels of miR-101 in H/R-treated H9c2 cardiomyocytes. Values are presented as the mean ±standard deviation of a representative experiment performed in triplicate. **P<0.01 vs. Con. H/R, hypoxia/reoxygenation; H9C2, H9c2 cardiomyocytes without any treatment; Con, H9c2 cardiomyocytes without any treatment; H/R, H9c2 cardiomyocytes treated with H/R; miR-101; microRNA-101; IL-1β, interleukin 1β; Bcl-2, B-cell lymphoma-2; LC3B, 1A/1B-light chain 3B.

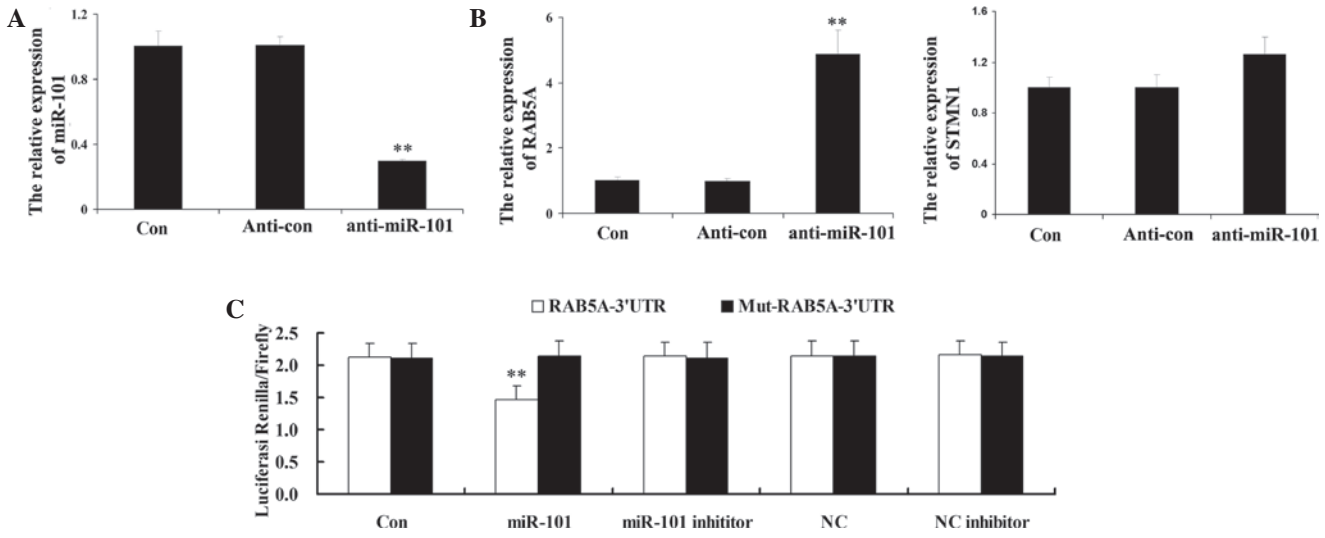


Figure 2. Evaluation of miR-101 targets in H9c2 cardiomyocytes. (A) Polymerase chain reaction analysis was performed to determine the expression levels of miR-101 in H9c2 cardiomyocytes following transfection with anti-miR-101. (B) Polymerase chain reaction analysis was performed to determine the expression levels of RAB5A and STMN1 in H9c2 cardiomyocytes following transfection with anti-miR-101. (C) Luciferase reporter assay was performed to evaluate the effect of miR-101 on luciferase activity of reporter vector with wild-type or mutant RAB5A 3'-UTR in H9c2 cardiomyocytes, respectively. Values are presented as the mean ±standard deviation of a representative experiment performed in triplicate. **P<0.01 vs. Con. miR-101, microRNA-101; Con, H9c2 cardiomyocytes without any treatment; Anti-con, H9c2 cardiomyocytes transfected with anti-control miRNAs; anti-miR-101, H9c2 cardiomyocytes transfected with anti-miR-101; NC, negative control; Mut, mutant; RAB5A, Ras-related protein Rab-5A; STMN1, stathmin 1; UTR untranslated region.

one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference between values.

## Results

*H/R induces apoptosis and miR-101 downregulation in H9c2 cardiomyocytes.* Following H/R treatment, the apoptotic rate of H9c2 cardiomyocytes was determined using flow cytometry. The results indicated that the level of apoptosis was significantly increased following H/R treatment of H9c2 cardiomyocytes (Fig. 1A), indicating that H/R induced apoptosis in H9c2 cardiomyocytes. The expression levels of certain apoptosis-associated proteins, including caspase-3, B-cell lymphoma-2 (Bcl-2), interleukin 1β (IL-1β), as well as two autophagy markers, Beclin1 and 1A/1B-light chain 3B (LC3B), were also examined. As indicated in Fig. 1B, the protein expression levels of pro-apoptotic caspase-3 and IL-1β were significantly increased, while the expression of anti-apoptotic

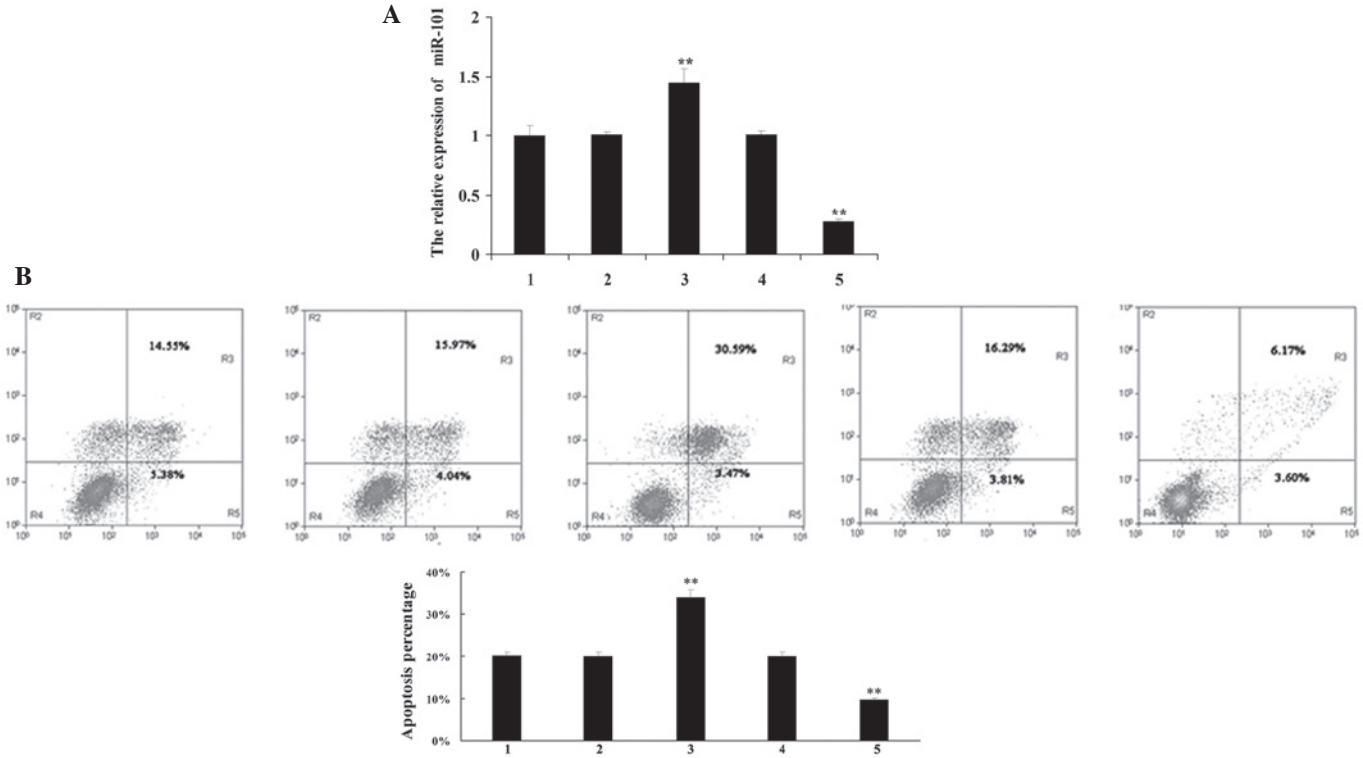


Figure 3. Effects of miR-101 on apoptosis in H/R-treated H9c2 cardiomyocytes. (A) Polymerase chain reaction analysis was performed in order to determine the expression levels of miR-101 in H9c2 cardiomyocytes following transfection with pre-miR-101 and anti-miR-101. (B) Flow cytometry was used to determine the apoptotic rates of hypoxia/reoxygenation-treated H9c2 cardiomyocytes following transfection with pre-miR-101 or anti-miR-101 using an Annexin V-fluorescein isothiocyanate Apoptosis Detection kit. 1, H9c2 cardiomyocytes without any treatment; 2, H9c2 cardiomyocytes transfected with control miRNAs; 3, H9c2 cardiomyocytes transfected with pre-miR-101; 4, H9c2 cardiomyocytes transfected with control anti-miRNAs; 5, H9c2 cardiomyocytes transfected with anti-miR-101. Values are presented as the mean ±standard deviation of a representative experiment performed in triplicate. $^{**}P<0.01$ vs. 1. miR-101; microRNA-101.

Bcl-2 was markedly decreased following H/R. Furthermore, the expression levels of autophagy-associated Beclin1 and LC3B were upregulated in H9c2 cardiomyocytes following H/R treatment. The expression of miR-101 in H/R treated H9c2 cardiomyocytes was determined by RT-PCR. The results indicated that miR-101 expression was markedly downregulated following H/R treatment of H9c2 cardiomyocytes (Fig. 1C), suggesting that H/R may have had an inhibitory effect on the expression of miR-101 in H9c2 cardiomyocytes.

*RAB5A is a direct target of miR-101 in H9c2 cardiomyocytes*. To investigate the potential targets of miR-101, H9c2 cardiomyocytes were transfected with anti-miR-101. Following transfection, the expression levels of miR-101 in H9c2 cardiomyocytes were evaluated. As shown in Fig. 2A, the expression levels of miR-101 were significantly reduced following transfection with anti-miR-101, indicating that the transfection efficiency was satisfactory. Subsequently, the expression levels of RAB5A and STMN1 were determined by RT-PCR. As indicated in Fig. 2B, the mRNA expression levels of RAB5A were significantly upregulated following the knockdown of miR-101; however, the other potential target investigated, STMN1, demonstrated no significant difference in expression to that of the control group. These results suggested that RAB5A may be a direct target of miR-101.

Based on these results, a luciferase reporter assay was performed to determine whether RAB5A was a direct target of miR-101 in H9c2 cardiomyocytes. As indicated in Fig. 2C, H9c2 cardiomyocytes co-transfected with miR-101 mimic and the wild-type 3'-UTR of RAB5A demonstrated a significant decrease in luciferase activity compared to that of the control groups. However, H9c2 cardiomyocytes co-transfected with miR-101 and mutant 3'-UTR RAB5A demonstrated no significant difference in luciferase activity. These data indicated that RAB5A may be a direct target of miR-101 in H9c2 cardiomyocytes.

*miR-101 influences the apoptotic rate of H/R-treated H9c2 cardiomyocytes*. To further investigate the role of miR-101 in H/R-treated H9c2 cardiomyocytes, H9c2 cardiomyocytes were transfected with pre-miR-101 and anti-miR-101, respectively. Once the transfection efficiency had been confirmed (Fig. 3A), H9c2 cardiomyocytes were treated with H/R and the apoptotic rate in each group was determined by flow cytometry. miR-101 upregulation significantly promoted H/R-induced apoptosis, whereas miR-101 downregulation markedly inhibited H/R-induced apoptosis in H9c2 cardiomyocytes (Fig. 3B), which suggested that miR-101 may promote H/R-induced apoptosis in H9c2 cardiomyocytes.

*miR-101 influences the expression of autophagy- and apoptosis-associated factors in H/R-treated H9c2 cardiomyocytes*. RT-PCR was performed in order to determine the expression levels of certain key autophagy- and apoptosis-associated factors, including IL-1β, caspase-3, Bcl-2 and RAB5A. The expression levels of IL-1β and caspase-3 were

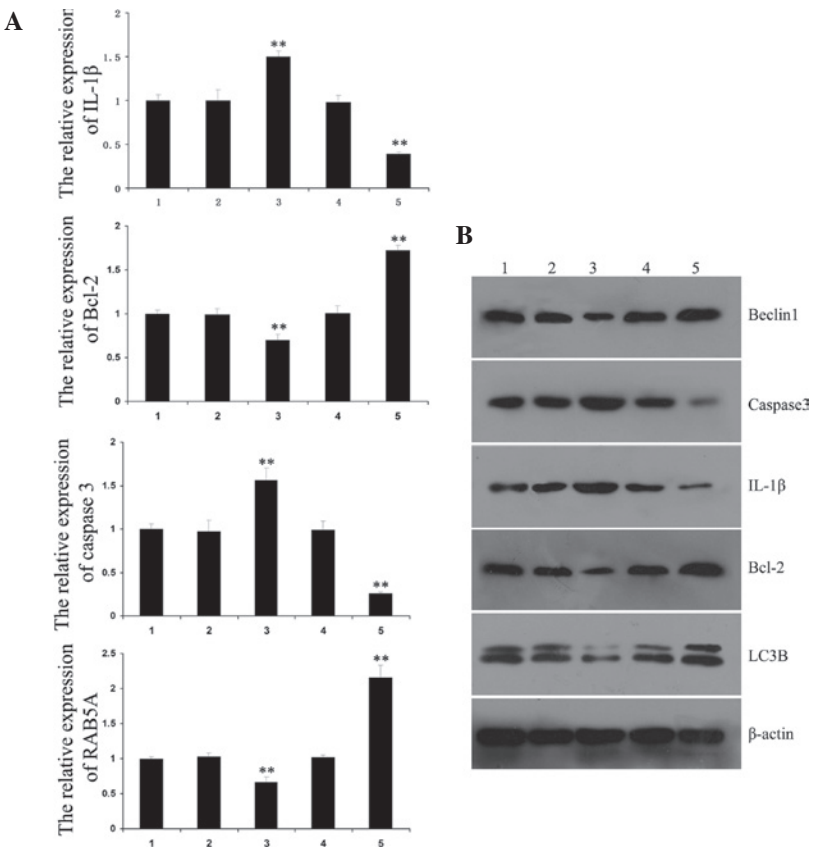


Figure 4. Effects of miR-101 on the expression of autophagy- and apoptosis-associated factors in H/R-treated H9c2 cardiomyocytes. (A) Polymerase chain reaction analysis was performed to determine the messenger RNA expression levels of autophagy- and apoptosis-associated factors in H/R-treated H9c2 cardiomyocytes following transfection with pre-miR-101 or anti-miR-101. **P<0.01 vs. 1. (B) Western blot analysis was performed to determine the protein expression levels of autophagy- and apoptosis-associated factors in H/R-treated H9c2 cardiomyocytes following transfection with pre-miR-101 or anti-miR-101. 1, H9c2 cardiomyocytes without any treatment; 2, H9c2 cardiomyocytes transfected with control miRNAs; 3, H9c2 cardiomyocytes transfected with pre-miR-101; 4, H9c2 cardiomyocytes transfected with control anti-miRNAs; 5, H9c2 cardiomyocytes transfected with anti-miR-101; Values are presented as the mean ±standard deviation of a representative experiment performed in triplicate. H/R, hypoxia/reoxygenation; miR-101, microRNA-101; IL-1β, interleukin 1β; Bcl-2, B-cell lymphoma-2; LC3B, 1A/1B-light chain 3B.

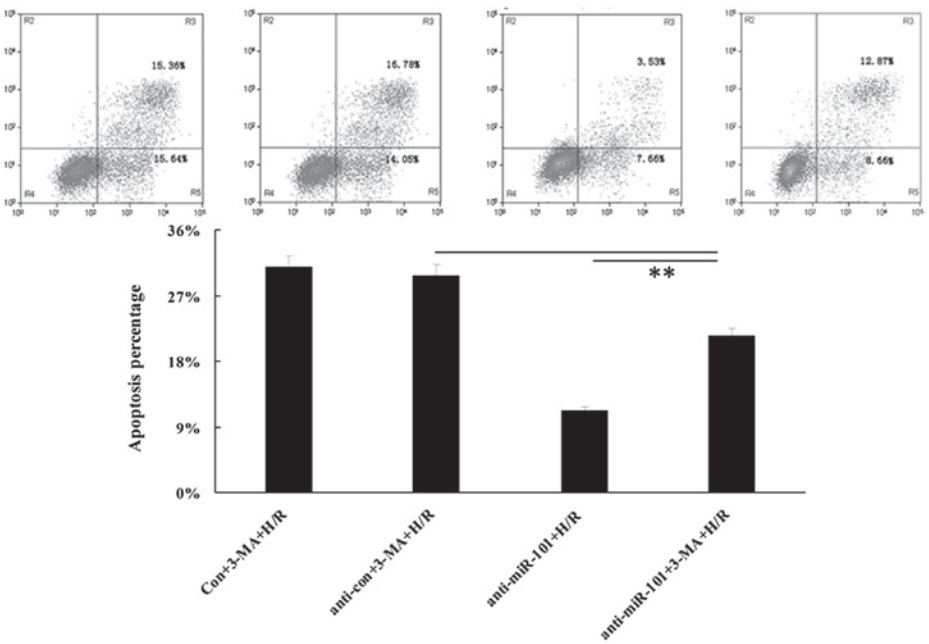


Figure 5. Flow cytometry was used to determine the apoptotic rate in H/R-treated H9c2 cardiomyocytes following transfection with autophagy inhibitor 3-MA, anti-miR-101 or both, respectively. Values are presented as the mean ±standard deviation of a representative experiment performed in triplicate. **P<0.01. Con+3-MA+H/R, H/R-treated H9c2 cardiomyocytes transfected with 3-MA; anti-con+3-MA+H/R, H/R-treated H9c2 cardiomyocytes transfected with control anti-miRNAs and 3-MA; anti-miR-101+H/R, H/R-treated H9c2 cardiomyocytes transfected with anti-miR-101; anti-miR-101+3-MA+H/R, H/R-treated H9c2 cardiomyocytes transfected with anti-miR-101 and 3-MA; H/R, hypoxia/reoxygenation; miR-101; microRNA 101; 3-MA, 3-methyladenine.

found to be increased following upregulation of miR-101, and decreased following downregulation of miR-101. However, the expression levels of Bcl-2 and RAB5A were reduced following upregulation of miR-101 and increased following downregulation of miR-101 (Fig. 4A).

Subsequently, the protein expression levels of IL-1β, caspase-3, Bcl-2, Beclin1 and LC3B were evaluated. The results indicated that the expression levels of miR-101 in each group were negatively correlated with autophagy-associated Beclin1 and LC3B, as well as anti-apoptotic Bcl-2, but positively correlated with pro-apoptotic IL-1β and caspase-3 (Fig. 4B).

*Inhibition of miR-101 attenuates H/R-induced apoptosis via an upregulation of autophagy.* As autophagy is able to protect cells from H/R-induced apoptosis to a certain extent, it was hypothesized that the promoting effect of miR-101 on H/R-induced apoptosis may be a result of its inhibitory effect on autophagy. In order to evaluate the validity of this hypothesis, autophagy inhibitor 3-methyladenine (3-MA) was added into the medium following transfection of H9c2 cardiomyocytes with anti-miR-101. Under H/R conditions, cellular apoptosis in each group was determined. As shown in Fig. 5, the apoptotic rate in the anti-miR-101+H/R group was lower than that in the anti-miR-101+3-MA+H/R group, suggesting that inhibition of miR-101 attenuated H/R-induced apoptosis, at least partially, via the upregulation of autophagy.

## Discussion

Under normal conditions, autophagy occurs at low levels; however, this process is upregulated in response to stress, including hypoxia, mitochondrial dysfunction and nutrient deprivation (13,14). It has been reported that autophagy promotes the survival of stressed cells via the recycling of proteins to generate the fatty acids and free amino acids required to maintain energy production, and the removal of damaged proteins and organelles preventing their accumulation (1,15). Accordingly, upregulation of autophagy within certain limits has protective and beneficial effects on cells subjected to stress. However, prolonged hypoxia and subsequent reperfusion may lead to excessive autophagy, which results in cardiomyocyte death by excessive self-digestion of organelles and proteins (10). Such abundant cardiomyocyte loss may result in contractile dysfunction and heart failure (16). The manipulation of autophagy may therefore become a promising therapeutic strategy for the prevention or treatment of numerous types of heart disease. In the present study, it was demonstrated that H/R induced apoptosis in H9c2 cardiomyocytes, concurrently with the downregulation of miR-101. Further investigation identified a novel target of miR-101, RAB5A, which had previously been reported to be associated with autophagy (10,16,17). Subsequently, the role of miR-101 in the regulation of autophagy under H/R was further investigated and the results suggested that inhibition of miR-101 attenuated H/R-induced apoptosis, at least partially, via the induction of autophagy.

In the present study, it was also demonstrated that alongside cellular apoptosis accompanied by the inhibition of miR-101, H/R induced the upregulation of autophagy-associated Beclin 1 and LC3B, indicating that the level of autophagy was upregulated. Enhanced autophagy was previously identified in cardiac cells following H/R, as demonstrated by an increase in the number of autophagic vesicles (18-20). These results suggested that H/R resulted in enhanced apoptosis and autophagy. As low levels of autophagy during ischemia and early reperfusion protect against cell death, it was suggested that spontaneous upregulation of autophagy may prevent excessive apoptosis of cardiomyocytes in response to H/R. Numerous studies have also demonstrated that autophagy is a beneficial response to ischemia/reperfusion (I/R) (21-24). For example, increased autophagy was demonstrated to correlate with the functional recovery of the myocardium following I/R, and cardiac myocytes exhibiting enhanced levels of autophagy were found to be negative for apoptosis (25,26).

miR-101 has been shown to participate in the regulation of autophagy. Frankel *et al* (10) for the first time, to the best of our knowledge, identified miR-101 as a potent inhibitor of basal-, etoposide- and rapamycin-induced autophagy in breast cancer cells. They also found that STMN1, RAB5A and ATG4D were three targets of miR-101 that were involved in miR-101-mediated autophagy. Furthermore, they revealed that STMN1 overexpression partially rescued cells from miR-101-mediated inhibition of autophagy, and that inhibition of miR-101 sensitized breast cancer cells to drug-mediated cell death (10). Xu *et al* (16) reported similar results, indicating that miR-101 had an inhibitory effect on autophagy, and enhanced cisplatin-induced apoptosis in hepatocellular carcinoma HepG2 cells, potentially via the inhibition of autophagy by targeting RAB5A, STMN1 and ATG4D. In the present study, it was also revealed that RAB5A expression levels were significantly upregulated following inhibition of miR-101 in H9c2 cardiomyocytes. However, no significant changes in STMN1 expression levels were detected, which suggested that the regulatory association between miR-101 and STMN1 may be cell-specific. In addition, it was confirmed that miR-101 directly targeted RAB5A via binding to the 3'-UTR of RAB5A mRNA in H9c2 cardiomyocytes. Their targeting association has also previously been revealed in breast cancer MCF7 cells and hepatocellular carcinoma HepG2 cells (10,16).

Bcl-2 has been shown to function as a negative regulator of cellular apoptosis (27) and increasing evidence has implicated Bcl-2 in the regulation of autophagy (28,29). Bcl-2 was found to bind to Beclin1 and disrupt autophagy, and overexpression of Bcl-2 in the heart inhibited autophagy in response to starvation. Furthermore, a Beclin1 mutant, which lacked the Bcl-2 binding domain, induced excessive autophagy following overexpression (30,31). These observations suggested that Bcl-2 may function as a negative regulator of autophagy via the inhibition of Beclin1. In the present study, it was demonstrated that following upregulation of miR-101, expression levels of Bcl-2 were reduced and the apoptotic rate was upregulated, indicating that miR-101 may promote H/R-induced cell apoptosis via downregulation of Bcl-2. However, despite Bcl-2 functioning as a negative regulator of Beclin1 and the reduced expression levels of Bcl-2 following overexpression of miR-101, the expression of Beclin1 was also decreased. It was therefore suggested that miR-101 may also have a suppressive effect on Beclin1 expression, through which it may inhibit H/R-induced autophagy. To further verify the hypothesis that autophagy was involved in the miR-101-mediated apoptosis in cardiomyocytes treated by H/R, autophagy inhibitor 3-MA was used.

The apoptotic rate was found to be reduced following inhibition of miR-101 in H/R-treated cardiomyocytes; however, this reduction was partially attenuated following the addition of 3-MA, indicating that inhibition of autophagy attenuated the suppressive effect of miR-101 downregulation on H/R-induced apoptosis in cardiomyocytes.

In conclusion, the present study, for the first time, to the best of our knowledge, suggested that the inhibition of miR-101 attenuated H/R-induced apoptosis, at least in part, via the induction of autophagy, and that miR-101 may function as a potential therapeutic agent for the treatment or prevention of heart disease.

## References


1. Oku M, Takano Y and Sakai Y: The emerging role of autophagy in peroxisome dynamics and lipid metabolism of phyllosphere microorganisms. Front Plant Sci 5: 81, 2014.
2. Petersen M, Hofius D and Andersen SU: Signaling unmasked: Autophagy and catalase promote programmed cell death. Autophagy 10, 2004.
3. Tessitore A, Cicciarelli G, Del Vecchio F, *et al:* MicroRNAs in the DNA Damage/Repair Network and Cancer. Int J Genomics 2014: 820248, 2014.
4. Chen LJ, Lim SH, Yeh YT, Lien SC and Chiu JJ: Roles of microRNAs in atherosclerosis and restenosis. J Biomed Sci 19: 79, 2012.
5. Xu T, Li D and Jiang D: Targeting cell signaling and apoptotic pathways by luteolin: cardioprotective role in rat cardiomyocytes following ischemia/reperfusion. Nutrients 4: 2008-2019, 2012.
6. Morita K: Surgical reoxygenation injury of the myocardium in cyanotic patients: clinical relevance and therapeutic strategies by normoxic management during cardiopulmonary bypass. Gen Thorac Cardiovasc Surg 60: 549-556, 2012.
7. Liu LF, Liang Z, Lv ZR, *et al:* MicroRNA-15a/b are up-regulated in response to myocardial ischemia/reperfusion injury. J Geriatr Cardiol 9: 28-32, 2012.
8. Qin Y, Yu Y, Dong H, Bian X, Guo X and Dong S: MicroRNA 21 inhibits left ventricular remodeling in the early phase of rat model with ischemia-reperfusion injury by suppressing cell apoptosis. Int J Med Sci 9: 413-423, 2012.
9. Liu L, Zhang G, Liang Z, *et al:* MicroRNA-15b enhances hypoxia/reoxygenation-induced apoptosis of cardiomyocytes via a mitochondrial apoptotic pathway. Apoptosis 19: 19-29, 2014.
10. Frankel LB, Wen J, Lees M, *et al:* microRNA-101 is a potent inhibitor of autophagy. EMBO J 30: 4628-4641, 2011.
11. Lin X, Guan H, Li H, *et al:* miR-101 inhibits cell proliferation by targeting Rac1 in papillary thyroid carcinoma. Biomed Rep 2: 122-126, 2014.
12. Yin J, Wang M, Jin C and Qi Q: miR-101 sensitizes A549 NSCLC cell line to CDDP by activating caspase 3-dependent apoptosis. Oncol Lett 7: 461-465, 2014.
13. Gong JS and Kim GJ: he role of autophagy in the placenta as a regulator of cell death. Clin Exp Reprod Med 41: 97-107, 2014.
14. Hou C, Zhu M, Sun M and Lin Y: MicroRNA let-7i induced autophagy to protect T cell from apoptosis by targeting IGF1R. Biochem Biophys Res Commun 453: 728-734, 2014.
15. Gustafsson AB and Gottlieb RA: Recycle or die: the role of autophagy in cardioprotection. J Mol Cell Cardiol 44: 654-661, 2008.
16. Xu Y, An Y, Wang Y, *et al:* miR-101 inhibits autophagy and enhances cisplatin-induced apoptosis in hepatocellular carcinoma cells. Oncol Rep 29: 2019-2024, 2013.
17. Li Y, Zhao Y, Hu J, *et al*: A novel ER-localized transmembrane protein, EMC6, interacts with RAB5A and regulates cell autophagy. Autophagy 9: 150-163, 2013.
18. Sybers HD, Ingwall J and DeLuca M: Autophagy in cardiac myocytes. Recent Adv Stud Cardiac Struct Metab 12: 453-463, 1976.
19. Zhang ZL, Fan Y and Liu ML: Ginsenoside Rg1 inhibits autophagy in H9c2 cardiomyocytes exposed to hypoxia/reoxygenation. Mol Cell Biochem 365: 243-250, 2012.
20. Cao X, Chen A, Yang P, *et al:* Alpha-lipoic acid protects cardiomyocytes against hypoxia/reoxygenation injury by inhibiting autophagy. Biochem Biophys Res Commun 441: 935-940, 2013.
21. Wang JH, Behrns KE, Leeuwenburgh C and Kim JS: Critical role of autophage in ischemia/reperfusion injury to aged livers. Autophagy 8: 140-141, 2012.
22. Han Z, Cao J, Song D, *et al*: Autophagy is involved in the cardioprotection effect of remote limb ischemic postconditioning on myocardial ischemia/reperfusion injury in normal mice, but not diabetic mice. PLoS One 9: e86838, 2014.
23. Kang JW, Cho H and Lee SM: Melatonin inhibits mTOR-dependent autophagy during liver ischemia/reperfusion. Cell Physiol Biochem 33: 23-36, 2014.
24. Zhang Y and Ren J: Targeting autophagy for the therapeutic application of histone deacetylase inhibitors in ischemia/reperfusion heart injury. Circulation 129: 1088-1091, 2014.
25. Zeng M, Wei X, Wu Z, *et al:* NF-κB-mediated induction of autophagy in cardiac ischemia/reperfusion injury. Biochem Biophys Res Commun 436: 180-185, 2013.
26. Hamacher-Brady A, Brady NR and Gottlieb RA: The interplay between pro-death and pro-survival signaling pathways in myocardial ischemia/reperfusion injury: apoptosis meets autophagy. Cardiovasc Drugs Ther 20: 445-462, 2006.
27. Liu G, Wang T, Song J and Zhou Z: Effects of apoptosis-related proteins caspase-3, Bax and Bcl-2 on cerebral ischemia rats. Biomed Rep 1: 861-867, 2013.
28. Ni Z, Wang B, Dai X, *et al:* HCC cells with high levels of Bcl-2 are resistant to ABT-737 via activation of the ROS-JNK-autophagy pathway. Free Radic Biol Med 70: 194-203, 2014.
29. Heath-Engel HM, Chang NC and Shore GC: The endoplasmic reticulum in apoptosis and autophagy: role of the BCL-2 protein family. Oncogene 27: 6419-6433, 2008.
30. Liang XH, Kleeman LK, Jiang HH, *et al:* Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. J Virol 72: 8586-8596, 1998.
31. Pattingre S, Tassa A, Qu X, *et al:* Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122: 927-939, 2005.