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The role of miRNA-29b1 on the hypoxia-induced apoptosis in mammalian cardiomyocytes

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ABSTRACT

Cardiomyocyte apoptosis is a complex biological process involving the interaction of many factors and signaling pathways. In hypoxic environment, cardiomyocytes may trigger apoptosis due to insufficient energy supply, increased production of oxygen free radicals, and disturbance of intracellular calcium ion balance. The present research aimed to investigate the role of microRNA-29b1 (miR-29b1) in hypoxia-treated cardiomyocytes and its potential mechanism involved. We established an *in vitro* ischemia model using AC16 and H9C2 cardiomyocytes through hypoxia treatment (1% O_2, 48 h). Cell apoptosis was evaluated by flow cytometry using Annexin V-FITC-PI staining assay. Moreover, we used Western blot and immunofluorescence analysis to determine the expression of Bcl-2, Bax, caspase-3 and Cx43 proteins. We found that miR-29b1 protected AC16 and H9C2 cells from hypoxia-induced injury as evidence that miR-29b1 attenuated the effects of hypoxia treatment on AC16 and H9C2 cell apoptosis after hypoxia treatment. In conclusion, our findings suggest that miR-29b1 may have potential cardiovascular protective effects during ischemia-related myocardial injury.

Key words: hypoxia; cardiomyocyte; apoptosis; miR-29b1; Cx43.

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Introduction

Ischemic heart disease is one of the primary causes of death worldwide, and apoptosis in the ischemic myocardium is significant in the early stages of acute myocardial infarction (MI).[1-3] Hypoxia induced cardiomyocytes often exploited as simulating the condition of ischemic myocardium *in vitro* experiments. Hypoxic stress is a key factor in ischemic heart disease, triggering apoptosis by activating the caspase cascade through cytochrome c (CytC) release from mitochondria to the cytoplasm.[4,5] Myocardial cell apoptosis directly leads to severe cardiac dysfunction.[6-8] Studies have shown that cardiomyocyte autophagy can be rapidly activated in the early stages of MI and can rescue myocardial cells in the infarct margin area and limit the expansion of MI area. On the contrary, excessive autophagy will lead to the development of cardiovascular diseases; for example, prolonged hypoxia can induce the autophagic death of cardiomyocytes.[9] Therefore, the control of the hypoxia-induced apoptosis in cardiomyocytes is considered as a vital therapeutic approach in the treatment of ischemic heart disease. MicroRNAs (miRNAs) are a novel class of small non-coding RNAs that are 18 to 25 nucleotides long, which are engaged in cardiac development and the progression of cardiac hypertrophy.[10] miRNAs mediate gene expression at the translational or post-transcriptional level by suppressing translation from protein-encoding messenger RNAs (mRNAs) or by advancing degradation of their target mRNAs.[11] The miR-29 family consists of three largely homologous members, miR-29a, miR-29b, and miR-29c, and is greatly expressed in fibroblasts and vascular smooth muscle cells.[11] Van Rooij *et al*.[11] found miR-29 is dramatically downregulated in the region of the fibrotic scar after AMI. MiR-29b1 expression is varied in different areas in the early phase of AMI, most commonly in the 3′-untranslated regions (UTR). Given the importance of miR-29 in vascular homeostasis and the post-transcriptional regulation and the evidence that alienating miR-29 can release genetic deficiency situations in cells and manipulate aneurysm expansion.[12-17]

Gap junctional intercellular communication (GJIC) is made by docking of two hemichannels that are composited by the oligomerization of six proteins, the connexins, and a protein family with more than 20 different types in mammals.[18] Gap junctions (GJs) are most acknowledged for their role in the exchange of molecules of up to 1000 Da such as ions, metabolites or small signaling molecules[19,20] between the cytoplasm of neighboring cells. This provides them with not only important physiological functions but also pathological functions. GJs regulate cell fate by facilitating the transmission of pro- and anti-apoptotic molecules, influencing cell survival and death.[21] Prior research, including our own, has shown that CytC-induced apoptosis spreads from apoptotic cells to nearby healthy cells *via* GJs.[22-27] Of the known connexin (Cx) genes, Cx43 is most ubiquitous expressed in many organs, tissues and cell types, and we will focus on the role of Cx43 in the cardiovascular system.[28]

Hypoxia-inducible factor-1α (HIF-1α) was first discovered while studying the gene function of hepatoma cells.[29] HIF-1α was found regulating the transcription of many hypoxia-related genes, such as basic metabolism, survival, differentiation and apoptosis of cells.[30] HIF-1α is also involved in the downstream signal transduction process of hypoxic response.[31] HIF-1α/BNIP3 pathway can induce the autophagy and apoptosis of H9c2 during myocardial ischemia-reperfusion injury.[32] Nevertheless, the regulatory effect of miR-29b1 on hypoxia-induced myocardial injury is not clear. Therefore, in this study, we explored the role of miR-29b1 in repairing hypoxia-induced cardiac damage in AC16 and H9C2 cardiomyocytes.

Various rodent models have also indicated that the tissue levels of miR-29b1 are not protective against transverse aortic constriction (TAC)-induced cardiac hypertrophy. Increased expression of miR-29b1 in cardiac tissue is associated with TAC-induced cardiac hypertrophy whereas miR-29b1 deficient mice or those treated with miR-29a antagomirs had reduced levels of TAC-induced cardiac hypertrophy and myocardial fibrosis[33] miR-29b1 expression is also upregulated in murine heart tissues of myocardial ischemia-reperfusion injury and overexpression of miR-29b1 promotes cell apoptosis through suppression of insulin-like growth factor I in a rat myoblast cell line.[34] Moreover, suppression of miR-29b1 can reduce myocardial infarct size and IR injury-induced cell apoptosis via the upregulation of myeloid cell leukemia 1 (MCL-1), which is a target of miR-29b1.

Materials and Methods

Major materials and equipment

Human-derived AC16 cells and rat-derived H9C2 cells were kept in-house. Dulbecco's modified Eagle's medium culture was purchased from Life Technologies (Gaithersburg, MD, USA). PVDF membrane was purchased from Pall Life Sciences (Gaithersburg, MD, USA). Western blotting reagents were purchased from Bio-Rad (Hercules, CA, USA). Chemiluminescent HRP substrate (ECL) reagent was purchased from Merck Millipore (Billerica, MA, USA). Rabbit polyclonal anti-caspase3, mouse monoclonal anti-caspase-9 rabbit polyclonal anti-Bax, and rabbit polyclonal anti-TGF-β were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Rabbit polyclonal anti-Bcl-2 and rabbit polyclonal anti-Cx43 were purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated Goat anti-rabbit IgG was purchased from Abbkine (Redlands, CA, USA). And the HRP-conjugated monoclonal mouse anti-glyceralde-3-phosphate dehydrogenase (GAPDH) was purchased from KANGCHEN (Nanjing, China). Flow cytometry kit was purchased from BD Inc. (Franklin Lakes, NJ, USA).

Cell culture and treatment

Human cardiomyocyte cell line AC16 and rat cardiomyoblast H9C2 cell line were kept in-house. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, South Logan, UT, USA) with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Gibco, Rockville, MD, USA), penicillin (100 IU/mL) and streptomycin (100 IU/mL) (Gen-View, USA). Cells were incubated at 37°C with humidified atmosphere of 5% CO_2.

AC16 and H9C2 cardiomyocytes were randomly and equally assigned into the four groups with different treatments: i) Control group (normoxia): no defined specific treatment; ii) SGOD group: hypoxic incubator containing 5% CO_2 and 1% O_2 in the regular medium for 48 h; iii) MiR-NC group: transfection of scrambled miR-NC plus hypoxic culture; iv) MiR-29b1 group: transfection of miR-29b1 mimics plus hypoxic culture. The AC16 and H9C2 cells were passaged regularly and sub-cultured to 80% confluence before the experiments.

miRNA transfection

MiR-29b1 mimics (miR-29b1) and scrambled negative control (miR-NC) were synthesized by Ribobio Co., Ltd. (Guangzhou, China). Transfection of miR-29b1 and the negative control was performed using the Transfection Reagent Kit (Ribobio Co., Ltd) with concentration of 50 nM according to the manufacturer's protocol.

Apoptosis assay

Cell apoptosis was detected *via* annexin V-FITC/PI apoptosis kit. Briefly, after incubated in hypoxia incubator for 48 h, AC16 and H9C2 cardiomyocytes were collected and washed by cold phosphate-buffered saline (PBS). Then, cells were resuspended in a final density of 1×10^6/mL in staining buffer following with Annexin V-FITC/PI in the dark for 15 min. Finally, the samples were measured by flow cytometry analysis with a FACS Calibur Flow Cytometer (Beckman Coulter, Franklin Lakes, NJ, USA). A total number of cells of 10,000 were counted. The Annexin V-/PI- were considered as viable cells, the annexin V+/PI- were considered as early stage apoptotic cells and annexin V+/PI+ were considered as late apoptotic or necrotic cells. The data were analyzed by the Summit software.

Western blot analysis

The cells were in lysis buffer (130 mM Tris HCl pH6.8, 2% SDS, 1% β-MSH, 20% glycerol) and allowed to sit on ice for 20 min. The homogenate was centrifuged at 12,000 *g* for 20 min at 4°C, and the supernatants were used as whole cell lysates. The protein concentrations were determined with a BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA), and fractionated by 10% SDS-PAGE. The gels were transferred onto a PVDF membrane (GE Healthcare, Tokyo, Japan). The membrane was incubated for 1 h with PBS containing 0.1% Tween20 and 5% skim milk, and then incubated with anti-caspase-3, anti-caspase-9, anti-Bcl-2, anti-BAX, anti-TGF-β, and anti-Cx43 antibodies at 4°C overnight. After washing three times with PBS, the membrane was incubated with HRP-conjugated Goat anti-rabbit IgG. The quantification of protein expression was normalized to GAPDH using a densitometer and the reaction product was visualized using ECL regents. Expression of caspase-3, caspase-9, Bcl-2, Bax, TGF-β, and Cx43 proteins was quantified by densitometry analysis using ImageJ software.

Immunofluorescence staining

AC16 cells and H9C2 cells were seeded on coverslips and subsequently fixed using 4% paraformaldehyde. Permeabilization was carried out with 0.3% Triton X-100, followed by blocking in 3% BSA. The cells were then incubated overnight at 4°C with primary antibodies including rabbit monoclonal anti-Bcl-2 (MA5-41096, 1:50), mouse monoclonal anti-Bax (MA5-50655, 1:50), rabbit monoclonal anti-caspase-3 (MA5-32015, 1:50), and rabbit monoclonal anti-Cx43 (MA5-49139, 1:50). These antibodies were supplied by Invitrogen Trading Co., Ltd. (Shanghai, China). Following this, goat anti-rabbit Alexa Fluor 555-conjugated IgG (H+L) (A-11011, 1:100; Invitrogen Trading Co., Ltd.); goat anti-rabbit Alexa Fluor 488-conjugated IgG (H+L) (ab150077, 1:200, Abcam, Cambridge, UK) and goat anti-mouse Alexa Fluor 488-conjugated IgG (H+L) (A-11001, 1:100; Invitrogen Trading Co., Ltd.) were applied to the cells for a 1-hour incubation. DAPI staining was then performed, and the resulting fluorescence was examined using a fluorescence microscope. Quantification of the percentage of positive cells was achieved using ImageJ software.

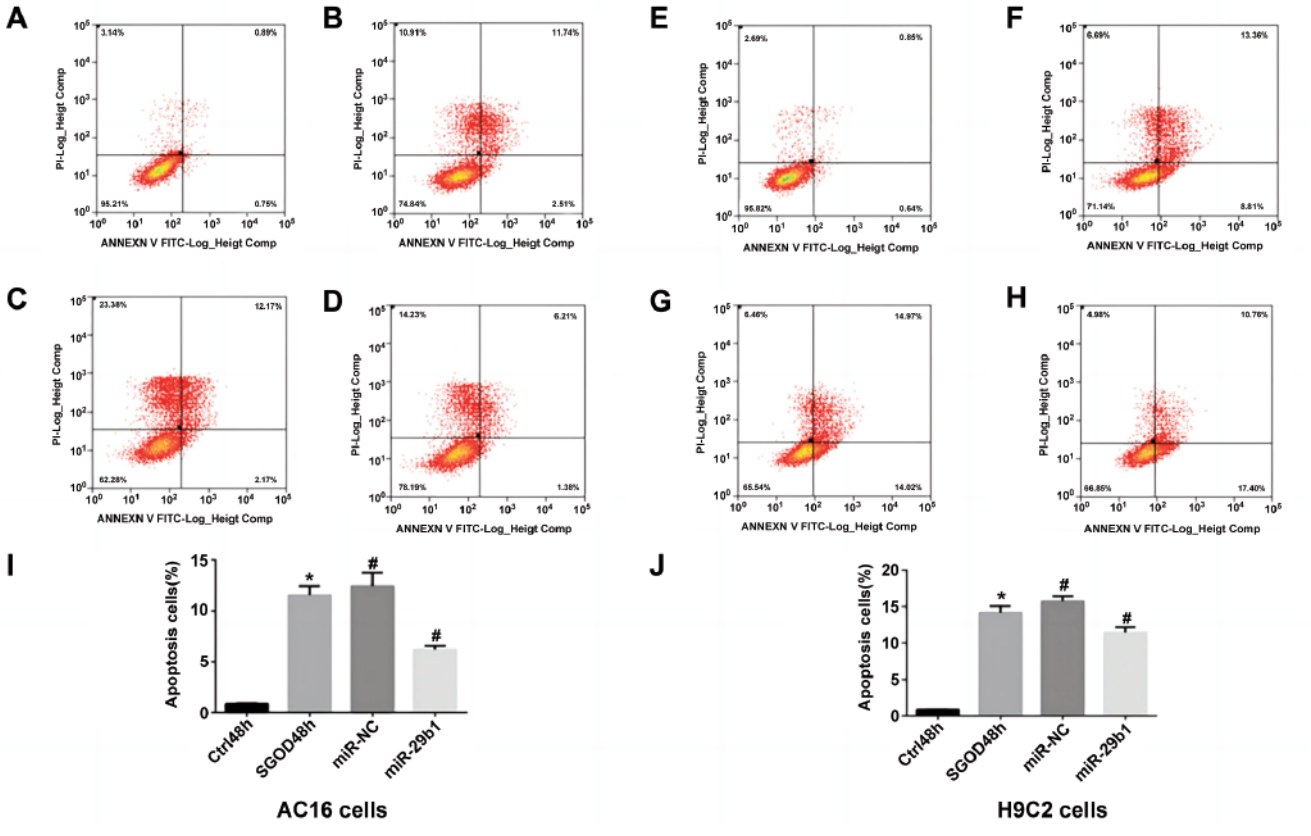

Figure 1. Effects of miR-29b1 on hypoxia-induced cardiomyocytes apoptosis. Flow cytometry analysis was carried out to determine apoptotic ratio in experimental groups: **A-D**) AC16 cells and E-H) H9C2 cells. **A,E**) Control group. **B,F**) SGOD 48h group. **C,G**) miR-NC group. **D,H**) miR-29b1 group. **I**) The relative proportion of apoptosis cells in different experimental groups in AC16 cells. **J**) The relative proportion of apoptosis cells in different experimental groups in H9C2 cells. Values presented are mean ±SD (n=5). **p*<0.05 *vs* control group; #*p*<0.05 *vs* SGOD group.

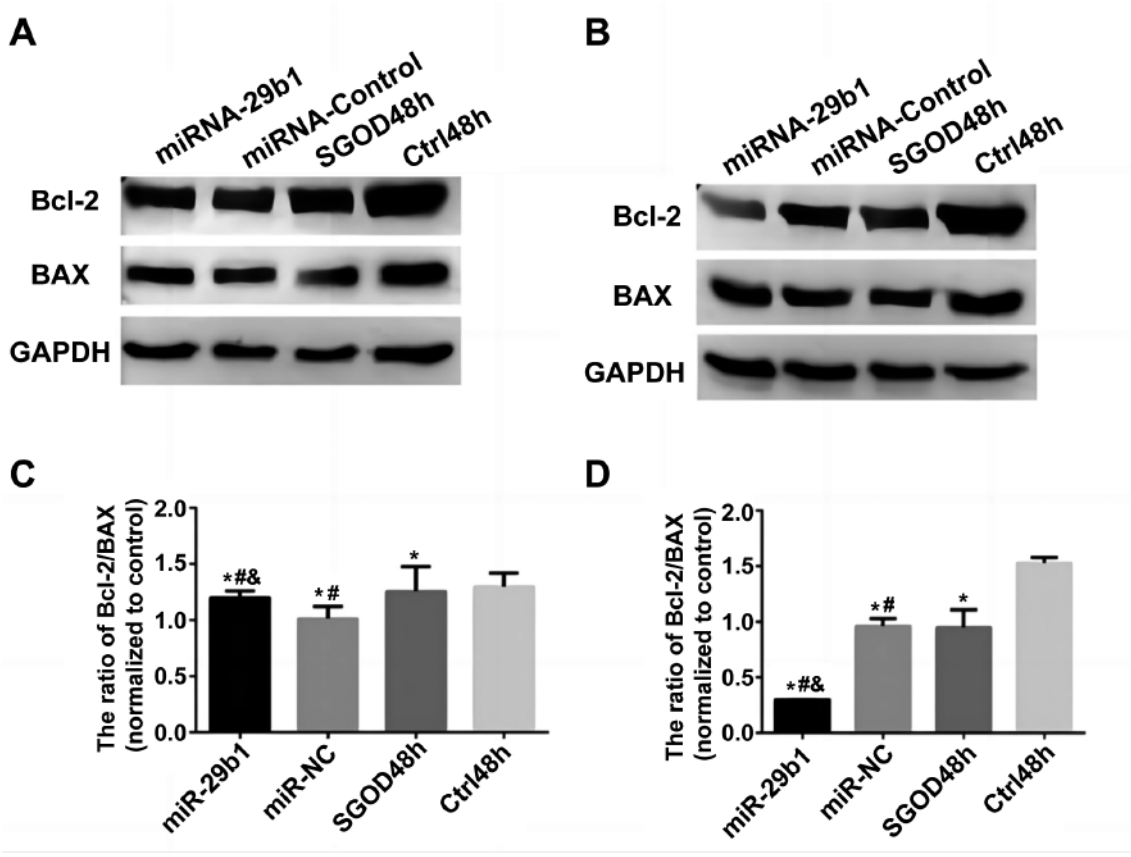

Figure 2. Expression of Bcl-2 and Bax among four groups in AC16 cells and H9C2 cells (control group, SGOD group, miR-NC and miR-29b1 group). **A**) Expression of Bcl-2 and Bax detected by Western blot analysis in AC16 cells. **B**) Expression of Bcl-2 and Bax detected by Western blot analysis in H9C2 cells. **C**) Relative expression of the ratio of Bcl-2 to BAX in AC16 cells. **D**) Relative expression of the ratio of Bcl-2 to BAX in H9C2 cells. *p<0.05 compared with the control group; $^{\#}p$<0.05 compared with the SGOD group; $^{\&}p$<0.05, compared with the miR-NC group. Bar graphs are as mean ± SD from three independent experiments.

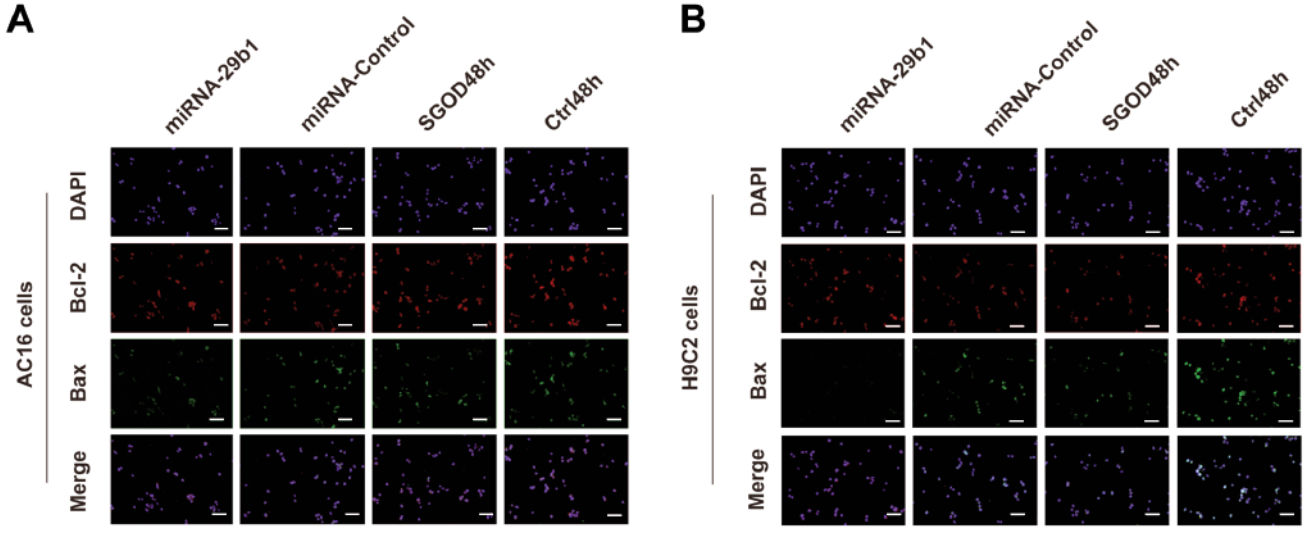

Figure 3. Expression of Bcl-2 and Bax detected by immunofluorescence in AC16 cells and H9C2 cells. **A**) Bcl-2 and Bax detected by immunofluorescence in AC16 cells. **B**) Expression of Bcl-2 and Bax detected by immunofluorescence in H9C2 cells. Scale bars: 50 μm.

Negative control samples were prepared by excluding the primary antibody and substituting it with PBS.

Statistical analysis

All data are expressed as mean ±SD values. Band densitometry was normalized by calculating the percentage ratio of each measurement relative to the highest measurement obtained. Statistical data analysis was performed using Statistic Package for Social Science (SPSS) v.19.0 (SPSS, Chicago, IL, USA) and Graphpad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) with Student's *t*-test. The significance level was set at $p<0.05$.

Results

MiR-29b1 overexpression attenuates cardiomyocytes apoptosis against hypoxia injury

Early apoptosis (annexin-V+ and PI-) and late apoptosis (annexin-V+ and PI+) were the major mechanisms of cell death caused by all the formulations. As shown in Figure 1, flow cytometry analysis results demonstrated that there was significant elevation of apoptosis in SGOD group compared to the control group in

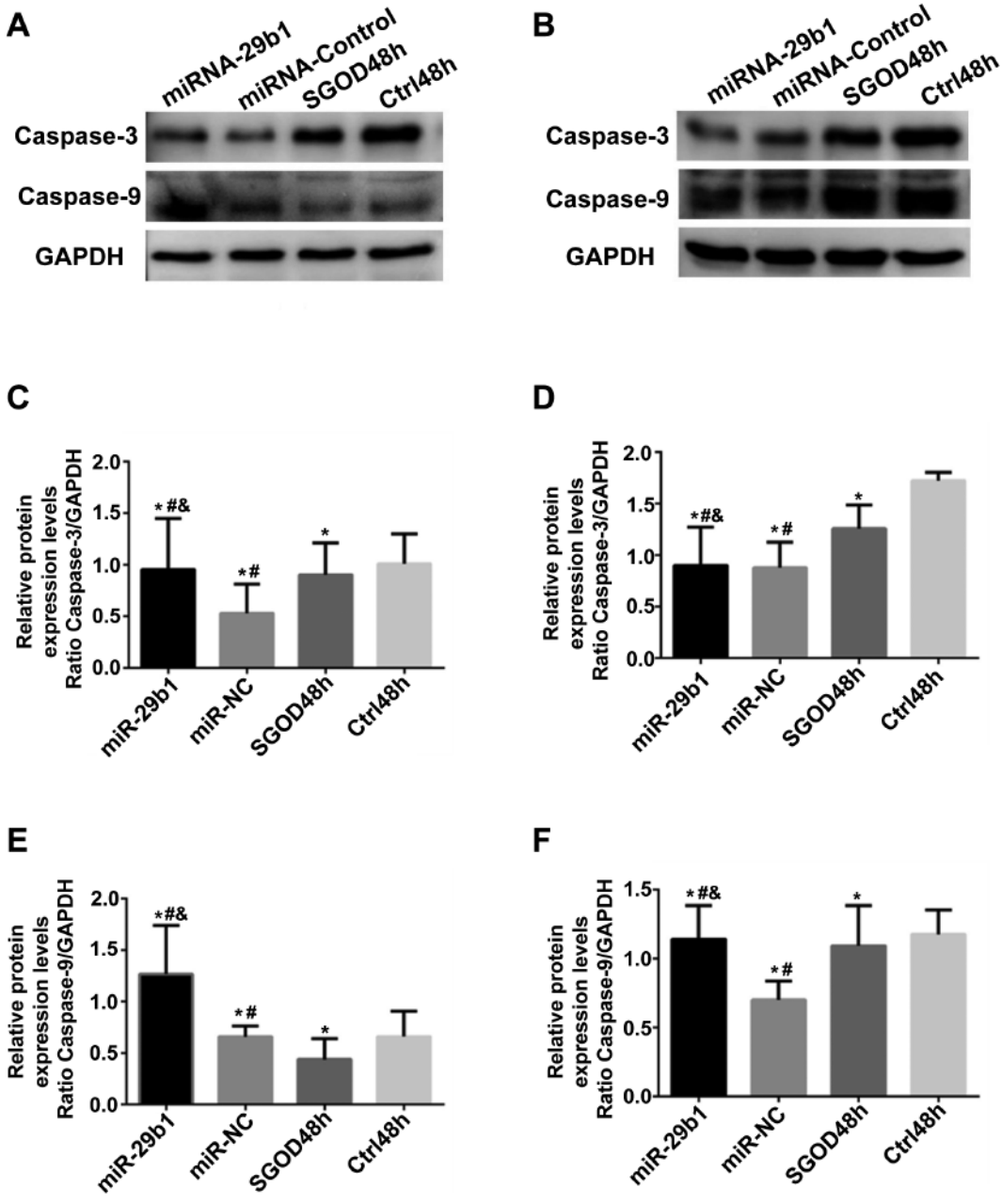

Figure 4. Expressions of caspase-3 and caspase-9 among four groups of AC16 cells and H9C2 cells (control group, SGOD group, miR-NC and miR-29b1 group). **A**) Expression of caspase-3 and caspase-9 detected by Western blotting in AC16 cells. **B**) Expression of caspase-3 and caspase-9 detected by Western blotting in H9C2 cells. **C,E**) Relative expression of caspase-3 and caspase-9 and comparison between groups in AC16 cells. **D,F**) Relative expression of caspase-3 and caspase-9 and comparison between groups in H9C2 cells. Protein levels were normalized to GAPDH. *$p<0.05$ compared with the control group; $^{\#}p<0.05$ compared with SGOD group; $^{\&}p<0.05$ compared with the miR-NC group. Bar graphs are as mean ± SD from three independent experiments.

AC16 cells (11.74% *vs* 0.89%, $p<0.05$), and (13.36% *vs* 0.85%, $p<0.05$) in H9C2 cells. To examine the effect of miR-29b1 on apoptosis, we transfected AC16 and H9C2 cells with miR-29b1 mimics or scrambled miR-NC respectively, before the cells were subjected to hypoxia. While apoptosis increased after transfection of miR-NC compared with the SGOD group, this might be due to the Transfection Reagent has some cytotoxicity that exacerbate cardiomyocytes apoptosis, miR-29b1 group decreased the apoptosis compared with SGOD group and miR-NC group. The results showed pretreatment of miR-29b1 inhibited hypoxia-induced apoptosis in AC16 and H9C2 cardiomyocytes.

MiR-29b1 overexpressing cardiomyocytes demonstrates decreased ratio of Bcl-2 to Bax levels

To understand the molecular details regarding repair from myocardial injury, we further monitored the change in the expression of pro-apoptotic marker Bax and the anti-apoptotic marker Bcl-2, which regulates the intrinsic pathway of apoptosis. Results clearly show that the miR-29b1 group remarkably decreased Bcl-2 expression and increased expression of Bax compared to the miR-NC and SGOD group in H9C2 cells. While in AC16 cells, the expression of Bal-2 of miR-29b1 group was increased compared with the miRNA-NC group and decreased with the SGOD and control groups, the expression of BAX of miRNA-29b1 group was decreased compared with the control group but increased with miRNA-NC and SGOD groups. Besides, the ratio of Bcl-2 to BAX of miR-29b1 group was slightly increased in AC16 cells while declined in H9C2 cells compared with miR-NC groups, due to the Transfection Reagent has some cytotoxicity that might exacerbate cardiomyocytes apoptosis. These results suggested that miR-29b1 protected against hypoxia-induced apoptosis by modulating the balance of Bcl-2 and Bax proteins in AC16 cells was more obvious than that in H9C2 cells (Figures 2 and 3).

MiR-29b1 overexpression show increased caspase-3 and caspase-9 levels in cardiomyocytes

Caspase-3 plays a determinant role in apoptotic progress. Compared with control groups, expression of caspases-3 and caspases-9 were decreased in SGOD and miR-NC groups of both AC16 and H9C2 cells, which means that expression of the cleaved-caspases-3 and cleaved-caspases-9 was increased in the same groups compared to the control groups. Transfection of miR-29b1 reversed these decreases significantly ($p<0.05$) in AC16 cells while slightly in H9C2 cells compared with the corresponding groups. These results showed that miR-29b1 overexpression promoted efficiently expression of relevant factors that was necessary for recovery from myocardial damage (Figures 4 and 5).

MiR-29b1 overexpression show reduced Cx43 and elevated TGF-β levels in cardiomyocytes

Western blotting data showed significantly decreased Cx43 in other three groups compared with control groups in two types of cells ($p<0.05$). What's more, in miR-29b1 groups was not increased the expression of Cx43 compared to the other groups in cardiomyocytes. The same results were observed through immunofluorescence staining. Furthermore, we found that the elevated TGF-β levels in the transfection of miR-29b1 groups compared to miR-NC groups and SGOD groups. This trend of changes in AC16 cells was coincidence with that in H9C2 cells (Figure 6).

Discussion

MicroRNAs (miRNAs) are small, non-coding RNAs which act as essential regulators for almost all aspects of intracellular signal-

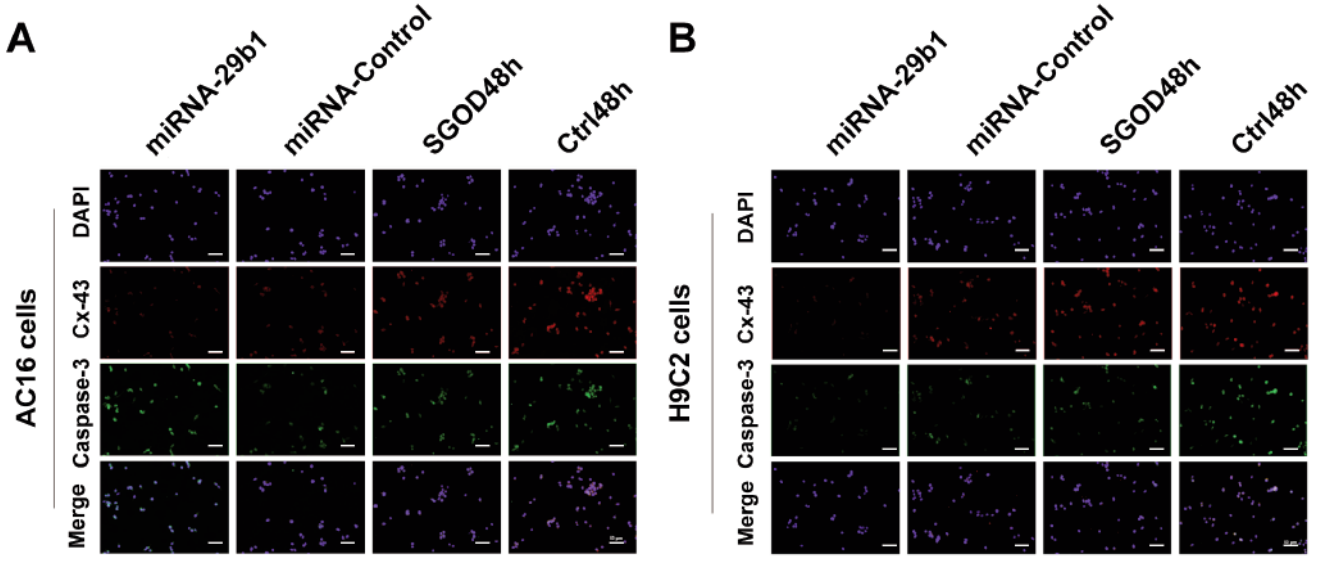

Figure 5. Expression of Cx43 and caspase 3 detected by immunofluorescence in AC16 cells and H9C2 cells. **A**) Expression of Cx43 and caspase-3 detected by immunofluorescence in AC16 cells. **B**) Expression of Cx43 and caspase-3 detected by immunofluorescence in H9C2 cells. Scale bars: 50 μm.

ing pathways in eukaryotic cells. Recently, miRNA have been identified as critical molecular targets in the course of myocardial injury and recovery.[35,36] MiRNA-29b1 involved in myocardial fibrosis after MI. Apoptosis is pivotal for development and preservation of tissues and it has been exhibited to contribute to ischemia-induced myocardial damage and cardiac dysfunction which is predominately dependent on mitochondrial function.[37] It is well known that mitochondria control the energy metabolism of the body and regulate cell apoptosis as well.[38] Bcl-2 and Bax proteins, the Bcl-2 family members, serve important characters in triggering mitochondrial mediated apoptosis through controlling the permeabilization of the mitochondrial membrane.[37] Cook *et al.* have reported that Bcl-2 regulates mitochondrial permeability transition pore opening in opposition to Bax, prevents cytochrome c leakage, suppresses caspase activity and decreases cell apoptosis.[39] Bcl-2/Bax ratio is another important parameter in mitochondria signaling pathway. Alteration of the Bcl-2/Bax ratio influences apoptotic balance.[40-42] Mounting evidence proposes that ischemia-induced cell apoptosis is intimately linked with mitochondria dysfunction.[43,44] Therefore, ameliorating the mitochondrial function is an effective method to attenuate the hypoxia-induced injury in cardiomyocytes.

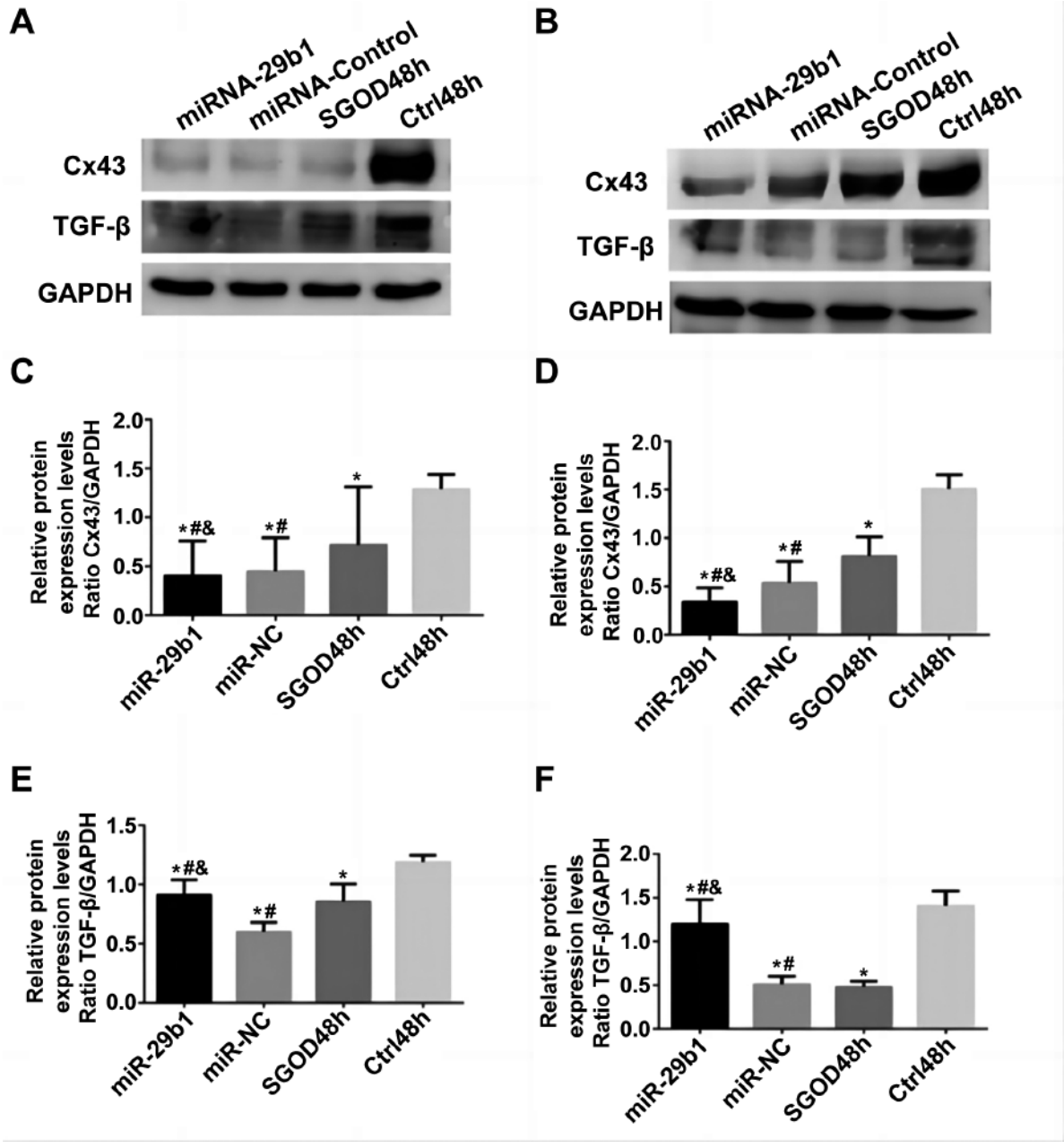

Figure 6. Expressions of Cx43 and TGF-β among four groups of AC16 cells and H9C2 cells (control group, SGOD group, miR-NC and miR-29b1 group). **A**) Expression of Cx43 and TGF-β detected by Western blotting in AC16 cells. **B**) Expression of Cx43 and TGF-β detected by Western blotting in H9C2 cells. **C,E**) Relative expression of Cx43 and TGF-β and comparison between groups in AC16 cells. **D,F**) Relative expression of Cx43 and TGF-β and comparison between groups in H9C2 cells; protein levels were normalized to GAPDH. *p<0.05 compared with control group; $^{\#}p$<0.05 compared with SGOD group; $^{\&}p$<0.05, compared with the miR-NC group. Bar graphs are as mean ± SD from three independent experiments.

Mitochondrial outer membrane permeabilization is the important process causing caspase activation in the intrinsic pathway.[45] CytC released into the cytosol from the intermembrane space binds to the apoptosis protease activating factor (Apaf-1), which then oligomerizes in the occurrence of ATP.[46] Pro-caspase-9 molecules can then bind to each of the Apaf-1 monomers *via* the caspase recruitment domain (CARD) forming a caspase-activating compound, the apoptosom.[47] Active caspase-9 participates in activation of downstream caspases-3 and -7. As with the previous report,[48] hypoxia significantly increased cleaved caspase-3 expression and caused apoptosis in H9C2 cardiomyoblasts. Hypoxia motivates the enrollment of integrins to cell membrane and produces apoptosis through the activating caspase-3 cascade. Cx43 is by far the most abundant heart connexin, expressed at high levels in atrial and ventricular myocytes, and to a lower extent in parts of the ventricular conduction system.[42] Cardiac remodeling occurs in response to a variety of cardiac disorders, and is characterized by structural and electrical alterations that decrease heart electrical stability.[49-51] Lateralization is observed in a variety of acquired and inherited arrhythmic syndromes, including ischemic heart disease, hypertrophic cardiomyopathy and arrhythmogenic right ventricular cardiomyopathy.[49-52] Therefore, it seems that the total Cx43 expression decrease during cardiac ischemia/hypoxia that can be a result of ATP depletion and/or increased activity of protein phosphatase 1A.[50,51,53] Transforming growth factor-β (TGF-β) super family members are critical regulators of cell proliferation and differentiation, developmental patterning and morphogenesis, and disease pathogenesis. We observed that increased expression of miR-29b1 contributes to attenuate in AC16 and H9C2 cells; thereby, our study demonstrated that miR-29b1-overexpressing cadiomyocytes increased the expression of TGF-β and pro-apoptotic marker Bcl-2, lightened the apoptosis process through enhanced the expression of Caspase-3 and Caspase-9 in AC16 and H9C2 cells. However, we found remained decreased expression of Cx43 compared with control group in AC16 and H9C2 cells illustrated that the Cx43 channels as crucial guarantees heart function are not fastened in modification of the heart to hypoxia-induced damage in coordination with miR-29b1. Therefore, our study illustrated that the miR-29b1 overexpressing AC16 and H9C2 cells efficiently regulated hypoxia-induced myocardial apoptosis, and it might have the ability to repair myocardial injury by ischemia. However, the intrinsic role of miR-29b1 on cardiac damage is unclear and further studies are necessary to better understand its functional role during cardiac damage in response to hypoxia.

Acknowledgements

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