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Original Article

MiR-26a enhances hypoxia-mediated cardiomyocytes apoptosis by targeting CDK8

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Abstract: Acute myocardial infarction (AMI) is one of the major causes of death worldwide. Based on the importance of cardiomyocyte apoptosis in AMI pathogenesis, potential treatments focusing on protecting cardiomyocytes from hypoxia-induced apoptosis are needed. Therefore, we aimed to explore potent methods to inhibit hypoxia-induced apoptosis of cardiomyocytes and provide more information for the development of new treatments of AMI. Herein, we investigated the potential role of miR-26a in hypoxic rat neonatal cardiomyocytes by targeting CDK8. H9c2 cardiomyocytes were subjected to hypoxia and we found that miR-26a was significantly elevated, while CDK8 was decreased in H9c2 cells after hypoxic treatment. Downregulation of miR-26a significantly inhibited hypoxic H9c2 cells apoptosis. CDK8 was identified as a direct target of miR26a. Knockdown of CDK8 abolished the protective effect of inhibiting hypoxic H9c2 cells apoptosis owing to miR-26a inhibitor. In conclusion, miR-26a enhances hypoxia-induced cardiomyocyte apoptosis by targeting CDK8 and represents a potential target for therapies development of AMI.

Keywords: miR-26a, cardiomyocytes, CDK8

Introduction

Acute myocardial infarction (AMI) is one of the major causes of death worldwide with stubbornly high morbidity and mortality these years [1]. A large number of reports indicate that excessive apoptosis could happen in cardiomyocytes undergoing hypoxia, which then induces ischemic heart diseases including AMI [2, 3]. Based on the importance of cardiomyocyte apoptosis in AMI pathogenesis, potential treatments focusing on protecting cardiomyocytes from hypoxia-induced apoptosis are needed to be developed. However, the mechanisms underlying this physiological process are still waiting to be figured out to lock effective targets of new therapeutic approaches for AMI.

MicroRNAs (miRNAs) are an abundant class of short (8-25 nucleotides) non-coding RNAs, which could directly bind to the 3'-UTR of their target mRNAs and lead to degradation or translational inhibition [4]. Thus, miRNAs play critical roles in a variety of cellular processes, including hypoxia-induced cardiomyocyte apoptosis [5, 6]. In this way, miRNAs could be key therapeutic targets to address AMI [7, 8].

Cyclin dependent kinase 8 (CDK8), one of the CDK family members, has been proved to be abnormally expressed in several cancers and inhibit cancer cell apoptosis [9-12]. Furthermore, rapidly growing tissues like tumors always have a distinguishing feature of hypoxia [13]. Previous studies indicated that CDK8 is vital for many HIF1A target genes induction, which could be activated by hypoxic stress [14]. Taken together, CDK8 is closely associated with cell apoptosis and hypoxia response. Nevertheless, there is little information about the mechanisms underlying the dysregulation of CDK8 and its function in regulating hypoxia-induced cell apoptosis. It was suggested by the TargetScan and miRDB online software that 3'-UTR of CDK8 mRNA might be the direct target of miR-26a [15, 16].

Numerous studies showed that the overexpression of miR-26a can suppress cancer cell proliferation and promote cancer cell apoptosis, including ovarian cancer and hepatocellular carcinoma [17-19]. Moreover, it has also been reported that upregulated miR-26a could induce hypoxic rat neonatal cardiomyocytes apoptosis [20]. Thus, miR-26a plays an important role in the apoptosis process of various cells including cardiomyocytes. According to the possible interaction between miR-26a and CDK8 and their known functions, we hypothesized that miR-26 may promote the apoptosis of cardiomyocytes by targeting CDK8.

Herein, we investigated the potential role of miR-26a in hypoxic rat neonatal cardiomyocytes. We found that miR-26a in cardiomyocytes was significantly upregulated by hypoxia. Downregulation of miR-26a significantly increased viability of hypoxic cardiomyocytes and inhibited hypoxia-induced cardiomyocyte apoptosis. After proving that CDK8 could be directly targeted by miR-26a in cardiomyocytes, it was also found that reduction of miR-26a promoted CDK8 expression and reduced the activity of caspase-3 in hypoxic cardiomyocytes. Therefore, it was demonstrated that overexpression of miR-26a could enhance the hypoxia-induced apoptosis of cardiomyocytes via targeting CDK8 and vice versa, so that miR-26a may be a novel molecular target for the new therapies of AMI.

Materials and methods

Cell cultures

Rat myocardium-derived H9c2 cells and 293T cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest Europe, Nuaillé, France) and 1% penicillin-streptomycin solution (SigmaAldrich, St. Louis, MO, USA) in a humidified incubator containing 5% CO_2 at 37°C. For hypoxia models, H9c2 cells were subjected to 1% O_2, 94% N_2 and 5% CO_2 at 37°C for 12 h or 24 h in a hypoxia incubator.

Cell transfection

The miR-26a mimics with the sequence of 5'-TTCAAGTAATCCAGGATAGGCT-3', miR-26a inhibitor with the sequence of 5'-AGCCTATCCTGGATTACTTGAA-3', negative controls (NCs) (GenePharma, Shanghai, China), CDK8 siRNA (forward, 5'-GGGAAUGGUGAAGUVAVUAUUAUAUTT-3'; reverse, 5'-AUAUAAUAGUGACUUCACCAUUCCCTT-3'), and NC siRNA (forward, 5'-ACGUGACACGUUCGGAGAATT-3'; reverse, 5'-UUCUCCGAACGUGUCACGUTT-3') (Santa Cruz, CA, USA) were transfected into cells using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocols. Briefly, miR-26a mimic, miR-26a inhibitor, CDK8 siRNA, NC siRNA, and Lipofectamine 2000 were diluted with serum-free medium, respectively. The diluted Lipofectamine 2000 was then added into each solution respectively, and incubated for 20 min at room temperature before adding them into the H9c2 cell suspension. After incubating the H9c2 cells at 37°C with 5% CO_2 for 6 h, the medium in each well was replaced by DMEM with 10% FBS, and cultured for another 48 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carksbad, USA) following the manufacturer's protocol. cDNA was synthesized using Moloney Murine Leukemia Virus Reverse Transcriptase (BioTeke Co., Ltd., Beijing, China) or miScript Reverse Transcription Kit (Qiagen, Dusseldorf, Germany) for mRNA or miRNA, respectively. RT-qPCR was performed with SYBR-Green qPCR Master Mix (BioRad, Hercules, CA, USA) and appropriate primers on an ABI Prism 7700 analyzer (Applied Biosystems, Warrington, UK) under the reaction condition of 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. U6 and β-actin were used as the internal control of miR26a or CDK8, respectively. The relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in the experiments were as follows: miR-26a forward, 5'-GTTAACGTGGCCTCGTTCAAGTAATCCAGGATAGGCTGT-3' and reverse, 5'-AACTCGAGAGCCTATCCTGGATTACTTGAACGAGGCCACG-3'; U6 forward, 5'-GCGCG TCGTGAAGCGTTC-3' and reverse, 5'-GTGCAGGGTCCG AGGT-3'; CDK8 forward, 5'-ATGCACTGTTGCGAATGCTG-3' and reverse, 5'-AATGCTTGCCCCTAGCACAT-3'; β-actin forward, 5'-TCAGGTCATCACTATCGGCAAT-3' and reverse, 5'-AAAGAAAGGGTGTAAAACGCA-3'. All experiments were conducted in triplicate.

MTT assay

MTT assay was carried out to assess cell viability. Briefly, the cells were plated in 96-well plates at a density of 2×10^4 cells/well and cultured overnight. The cells were subsequently transfected with the miR-26a inhibitor or NC inhibitor for 24 h and exposed to hypoxia for 12 h or 24 h. After the hypoxia treatment, 20 μl of 5 mg/ml MTT (Sigma-Aldrich) were added into the cells following a 4 h-incubation. Ten minutes after being dissolved by 150 μl dimethylsulphoxide (DMSO), the production of formazan was detected by determining the absorbance at 490 nm using a microplate reader (BioRad Laboratories, Inc., Hercules, CA, USA).

Lactate dehydrogenase (LDH) assay

Cell injury was tested using an LDH assay kit (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's protocol. In brief, 0.2% Triton X-100 (Sigma-Aldrich) was used to lyse the cardiomyocytes and a centrifugation at 10,000 × g for 10 min at 4°C was performed subsequently to get the supernatants. A 30 min-incubation at 37°C of the supernatants was then carried out with pyruvate and nicotinamide adenine dinucleotide hydrogen (Roche Applied Science, Indianapolis, IN, USA). After adding 0.4 M NaOH, the absorbance of the solution at 530 nm was detected using a microplate reader (BioRad Laboratories, Inc.).

Annexin V/propidium iodide (PI) apoptosis assay

The percentage of apoptotic cardiomyocytes was examined using the Annexin V/PI apoptosis detection kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions. Briefly, the cells were digested with 2.5 g/l trypsin (Sigma-Aldrich) and then washed with phosphate-buffered saline (PBS). The cells were then re-suspended in binding buffer supplemented with 10 μl of Annexin V. After incubating for 30 min, 5 μl of PI solution was added and another incubation of 5 min was performed. Cells were finally analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Caspase-3 activity assay

Caspase-3 activity was determined by a caspase-3 activity assay kit (Roche Applied Science) following the manufacturer's instructions. In brief, the cells were lysed and centrifuged at 16,000 × g for 15 min at 4°C before collecting the supernatants. The supernatants were then incubated with 10 μl Ac-DEVD-pNA (2 mM) for 2 h at 37°C. The absorbance of the solution at 405 nm was then detected using a microplate reader (BioRad Laboratories, Inc.).

Western blot analysis

Proteins of H9c2 cells were extracted using RIPA (Beyotime, Nantong, China) and the concentration was tested using the bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). Equivalent amounts of protein were loaded on 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gels) and separated, then transferred to a PVDF (polyvinylidene fluoride) membrane (Millipore, Billerica, MA, USA). After blocking the membrane with 5% non-fat dry milk for 1 h at 37°C, then membranes were incubated with primary antibodies (Santa Cruz Biotechnology) at 4°C overnight. The membrane was washed with Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:1000; Santa Cruz Biotechnology) at room temperature for 1 h. After being washed with TBST, the protein bands were developed using a Pierce ECL Western Blotting kit (Pierce, Rockford, IL, USA). The protein bands were then analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). The primary antibodies used included rabbit anti-human CDK8 (1:1000; Santa Cruz Biotechnology), rabbit antihuman caspase-3 (1:1000; Santa Cruz Biotechnology), rabbit anti-human cleaved caspase-3 (1:1000; Santa Cruz Biotechnology), and rabbit anti-human GAPDH (1:1000; Santa Cruz Biotechnology).

Luciferase assays

The 3'-UTR of CDK8 harboring either the miR-26a binding site (CDK8 3'-UTRWT) or a mutant (CDK8 3'-UTR-MT) was cloned into the pmirGLO luciferase vector (Promega, Madison, WI, USA). The constructed vectors were co-transfected into 293T cells with the miR-26a inhibitor or NC inhibitor using Lipofectamine 2000 (Invitrogen Life Technologies,) and incubated for 48 h. The cells were harvested and

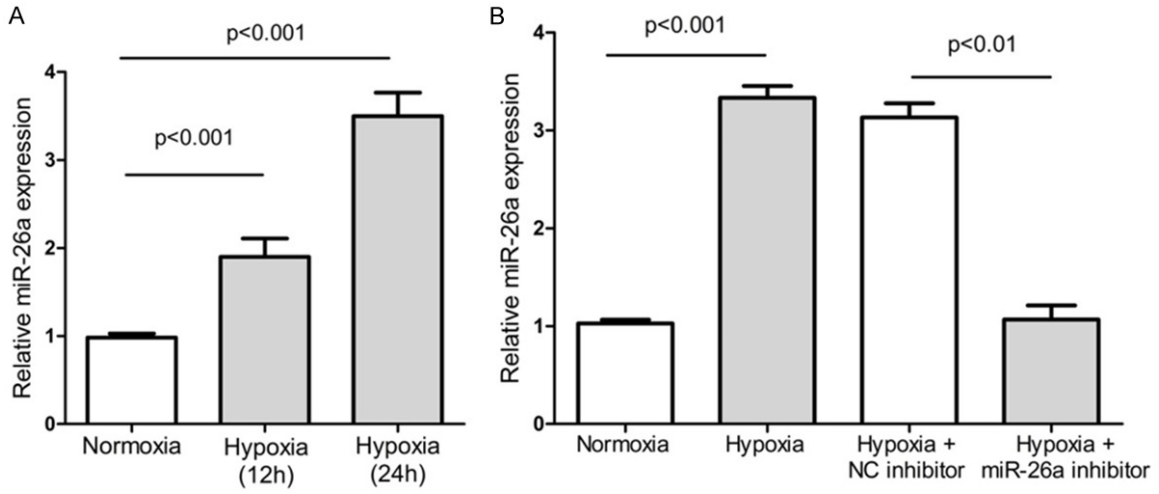

Figure 1. Expression of miR-26a in cardiomyocytes. A. The expression of miR-26a in H9c2 cells under normoxic or hypoxic conditions for 12 and 24 h. B. The expression of miR-26a in miR-26a inhibitor-transfected H9c2 cells. H9c2 cells were transfected with miR-26a inhibitor or NC inhibitor for 24 h and subjected to hypoxia for 24 h.

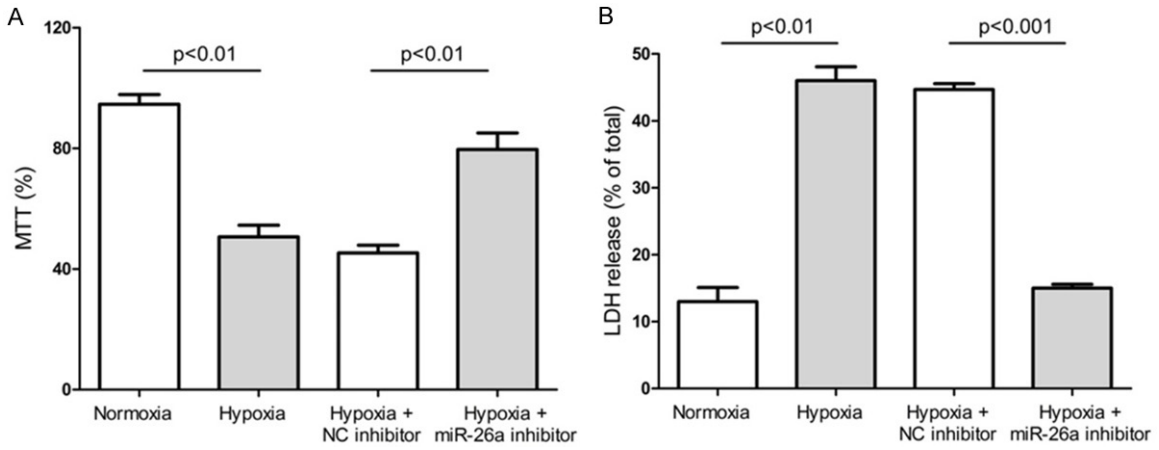

Figure 2. Downregulation of miR-26a attenuates hypoxia-induced cell injury. H9c2 cells were transfected with miR-26a inhibitor or NC inhibitor for 24 h and subjected to hypoxia for 24 h. A. Cell viability was detected by the MTT assay. B. Cell injury was detected by the lactate dehydrogenase (LDH) assay.

lysed before detecting the luciferase activities using a Dual-GLO Luciferase Assay system (Promega). All experiments were performed at least three times.

Statistical analysis

The results are presented as mean ± standard deviation (SD). Statistical analyses were conducted using SPSS software (version 20.0; IBM SPSS, Armonk, MY, USA). Statistical significance was determined by Student's ttest and one-way analysis of variance (ANOVA). Differences with a $P < 0.05$ were regarded as statistically significant.

Results

MiR-26a was upregulated by hypoxia in cardiomyocytes

To investigate the possible role of miR-26a in ischemic heart disease, we detected the expression of miR-26a in cardiomyocytes exposed to hypoxia using RT-qPCR. The results showed that miR-26a was significantly upregulated after hypoxia treatment (**Figure 1A**), indicating that miR-26a has an important role in the hypoxic-induced effects of cardiomyocytes.

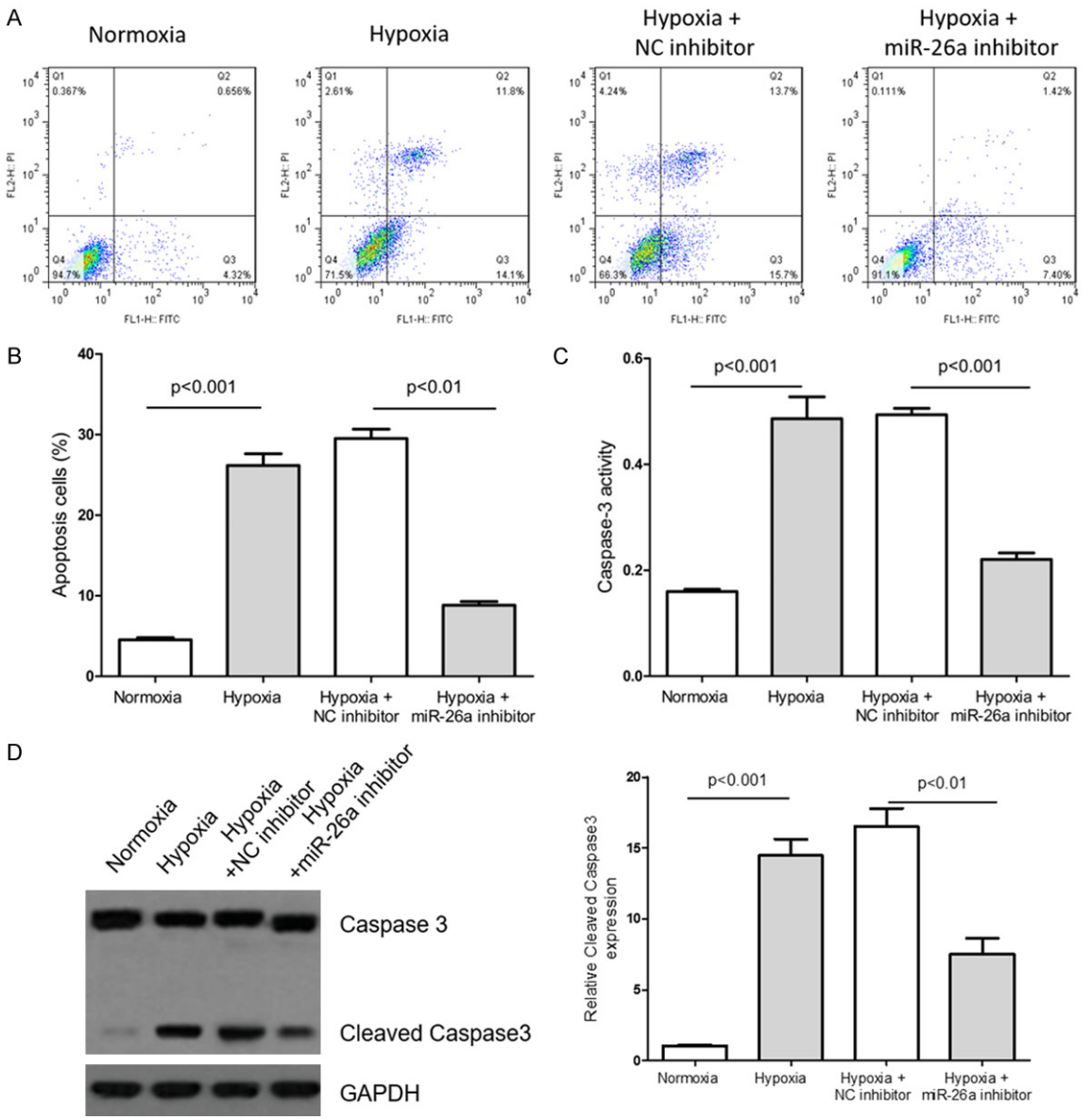

Figure 3. Downregulation of miR-26a inhibits hypoxia-induced cardiomyocyte apoptosis. H9c2 cells were transfected with miR-26a inhibitor or NC inhibitor for 24 h and subjected to hypoxia for 24 h. A. Cell apoptosis was examined with the Annexin V/PI apoptosis assay. B. Quantitative data of Annexin V/PI apoptosis assay. C. Caspase-3 activity was measured by the caspase-3 activity assay. D. Protein expression of caspase-3 was detected by western blotting.

Downregulation of miR-26a weakened hypoxia-induced cell injury

To investigate the biological effects of miR-26a on hypoxia-treated cardiomyocytes, we used miR-26a inhibitor to reduce the expression of miR-26a in H9c2 cells (**Figure 1B**). We then detected the changes of cell viability caused by downregulation of miR-26a using the MTT assay. The results showed that the cell viability sharply declined by hypoxia could be obviously increased again by inhibiting miR26a (**Figure 2A**). Moreover, the effect of miR-26a inhibition on hypoxia-induced cell injury was also evaluated with the LDH assay. As displayed in **Figure 2B**, the hypoxia-induced cell injury was also dramatically reversed by the miR-26a inhibitor (**Figure 2B**). Taken together, these data suggest that downregulation of miR-26a could help hypoxic cells to survive.

Inhibition of miR-26a attenuated hypoxia-induced cardiomyocyte apoptosis

To explore the mechanism underlying the protective effect of miR26a inhibition on hypoxic

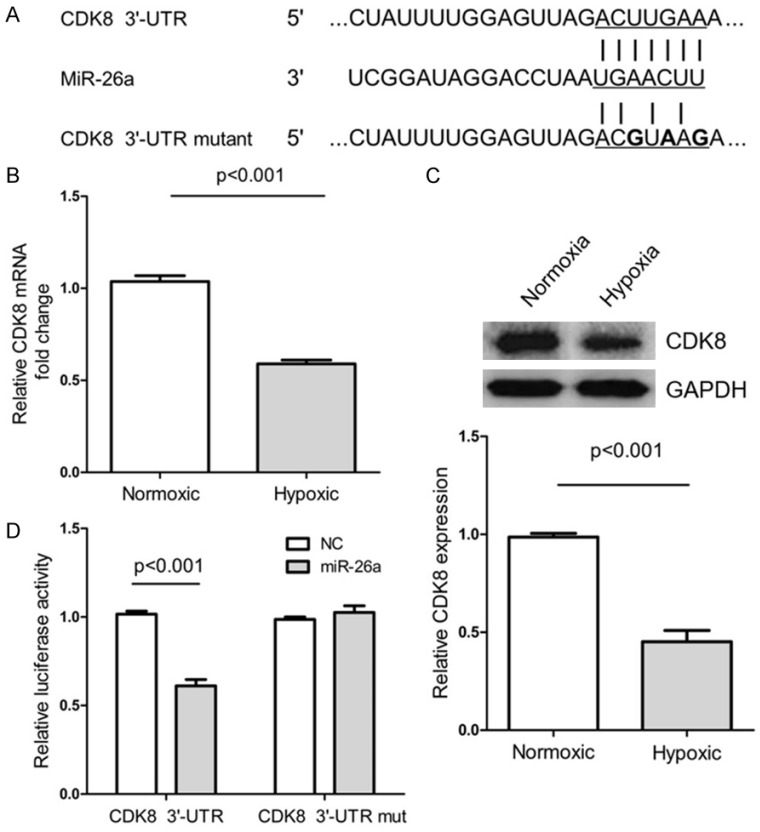

Figure 4. MiR-26a interacts directly with mRNA 3'-UTR of CDK8. A. Schematic diagram of the binding sites between miR-26a and GATA-4 3'-UTR. B. The mRNA expression of CDK8 in hypoxic H9c2 cells. C. The protein expression of CDK8 in hypoxic H9c2 cells. D. The effect of miR-26a on luciferase activity was detected by the dual-luciferase reporter assay.

cardiomyocyte, we further detected the changes of cardiomyocyte apoptosis rate caused by miR26a suppression. The Annexin V/PI apoptosis assay showed that hypoxia-induced apoptosis was significantly deadened by the downregulation of miR26a (**Figure 3A** and **3B**). At the same time, miR26a inhibition also significantly reduced the cleavage of caspase-3 (a pro-apoptotic protein), which was drastically increased after the hypoxia treatment (**Figure 3C**). And this was also confirmed by the western blotting results displayed in **Figure 3D** that the protein expression level of cleavage caspase-3 in cardiomyocytes was obviously decreased by miR-26a inhibitor compared with the hypoxia group.

CDK8 is a potential target of miR-26a

According to the results of both Targetscan and miRBD, we speculated that CDK8 was a potential target gene of miR-26a. The 3'-UTR of CDK8 has a matched site for miR26a to bind (CDK8 3'-UTR-WT), and a mutant (CDK8 3'-UTRMT) that cannot bind with miR-26a was designed (**Figure 4A**). It was also found that the expression of CDK8 at both mRNA and protein levels could be significantly decreased by hypoxia (**Figure 4B** and **4C**). To confirm the direct interaction between miR-26a and CDK8 3'-UTR, CDK8 3'-UTR-WT or CDK8 3'-UTR-MT was cloned into the pmirGLO vector to perform the luciferase reporter assay. The results showed that the luciferase activity of pmirGLO-CDK8 3'-UTR-WT was significantly decreased by miR-26a overexpression (**Figure 4D**). However, the luciferase activity of pmirGLO-CDK8 3'-UTR-MT was almost not affected by miR-26a overexpression (**Figure 4D**). These results suggest that miR-26a directly targets the 3'-UTR of CDK8. We then examined the effect of miR-26a on CDK8 protein expression by western blot analysis. The results showed that the downregulation of miR-26a significantly increased the protein expression of CDK8 (**Figure 5A**), which were decreased by hypoxia in the cardiomyocytes.

Knockdown of CDK8 abolishes the protective effect of miR-26a downregulation

To verify that CDK8 contributes to the miR-26a inhibition-mediated protective effect against hypoxia, we knocked down the expression of CDK8 with CDK8 miRNA and inhibited miR-26a at the same time. The results of the LDH assay showed that miR-26a suppression could protect H9c2 cells from hypoxia injury, while ensued knockdown of CDK8 will abolish the protection brought by miR-26a suppression (**Figure 5B**). This finding was further confirmed by detecting the activity of caspase-3 and we got the result that hypoxic H9c2 cells treated with both miR-26 inhibitor and CDK8 siRNA had

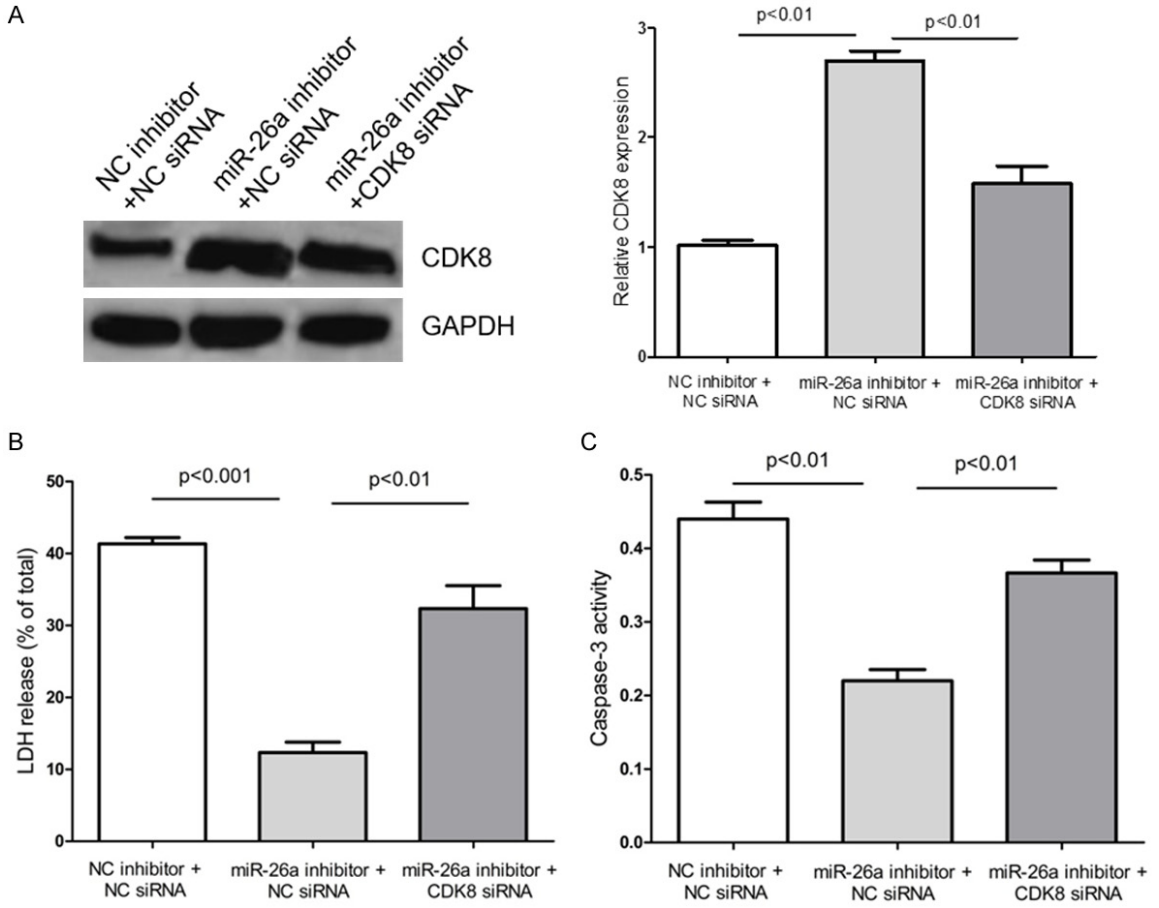

Figure 5. MiR-26a may function through downregulating CDK8. H9c2 cells were transfected with the miR-26a inhibitor or NC inhibitor, and CDK8 siRNA for 24 h and subjected to hypoxia for 24 h. A. The protein expression of CDK8 was tested by western blotting. B. Cell injury was detected by the LDH assay. C. Cell apoptosis was measured by the caspase-3 activity assay.

much more active caspase-3 than hypoxic H9c2 cells only treated with miR-26 inhibitor (**Figure 5C**). This result suggests that miR-26a inhibits H9c2 cell apoptosis through regulating CDK8.

Discussion

Hypoxia-induced cardiomyocyte apoptosis is a major cause of AMI, and inhibiting cardiomyocyte apoptosis is an important direction to develop new therapeutic approaches for AMI. In this study, it is indicated that miR-26a is a novel target to regulate hypoxia-induced cardiomyocyte apoptosis. We found that miR-26a in cardiomyocytes was significantly upregulated by hypoxia. Through directly upregulating the expression of CDK8, downregulation of miR-26a could protect cardiomyocytes from hypoxia-induced apoptosis, which could provide a potential therapeutic target for AMI.

MiRNAs are known to be direct targets for regulating the expression of genes affecting the response of cardiomyocytes to hypoxia treatment. Up to now, a lot of reports have revealed that miRNAs play essential roles in heart diseases [21, 22]. According to previous studies, dysregulation of various miRNAs could be found in cardiomyocytes undergoing sever apoptosis caused by hypoxia [23, 24]. It has been reported that miR-26a, which is highly expressed in rat and human cardiomyocytes, could promote cancer cell apoptosis [17-19]. Moreover, miR-26a has been proved to express higher in hypoxic rat cardiomyocytes and induce apoptosis [20]. However, the functions and mechanisms of miR-26a regulating cardiomyocyte apoptosis caused by hypoxia are not fully clear. In the present study, we found miR-26a was significantly overexpressed in hypoxic cardiomyocytes suffering strong apoptosis, while miR-26a inhibitor could obviously rescue this apoptosis,

confirming its function of promoting hypoxia-induced cardiomyocyte apoptosis.

MiRNAs exert the function through inhibiting their target mRNAs. Thus, in order to explore the relationship between miR-26a and hypoxia-reduced cardiomyocyte apoptosis, we identified its potential target mRNA, which should be a protector of cardiomyocytes against hypoxia. Based on the results of the online prediction softwares, we found CDK8 could be an important target gene of miR-26a. It was indicated that CDK8 could work as a coactivator of a variety of transcriptional processes, including the classic Wnt/β-catenin pathway [11, 25] and the p53 network [26, 27]. Furthermore, previous studies also demonstrated diverse functions of CDK8 related with cellular proliferation [28, 29] and cell cycle progression [30]. Noteworthily, CDK8 was found to serve as an oncogene to improve cell proliferation and inhibit apoptosis of colon cancer, laryngeal squamous cell carcinoma and gastric cancer [12, 31, 32]. Thus, based on both the predicted results online and previous reports, we speculated that CDK8 may play an important role during the process that miR-26a regulates hypoxia-induced cardiomyocyte apoptosis.

In this study, we verified that downregulation of miR-26a could increase the expression of CDK8, which could markedly protect cardiomyocytes from hypoxia-induced apoptosis. In addition, downregulation of both miR-26a and CDK8 obviously recovered the severe apoptotic effect of hypoxia, which means miR-26a affects hypoxia-induced cardiomyocyte apoptosis through targeting CDK8. These results suggest that miR-26a targeting CDK8 has potential to be a promising target for the development of new therapies for AMI.

In conclusion, our study demonstrated that miR-26a in cardiomyocytes could be upregulated by hypoxia and further promote apoptosis. Downregulation of miR-26a could protect cardiomyocytes against hypoxia-induced apoptosis by enhancing CDK8 expression. Our study suggests a potential therapeutic molecular target for the treatment of AMI.

Disclosure of conflict of interest

None.

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