Original Article
MicroRNA-26b protects against the H$_2$O$_2$-induced injury on cardiac myocytes via its target gene HGF

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Abstract: Recent studies have revealed the critical role of microRNAs (miRNAs) in regulating myocardial ischemic injury. However, evidence for the role of miR-26b in oxidative stress injury induced by H$_2$O$_2$ is still missing. The present study attempted to determine whether MIr-26b has an influence on oxidative stress injury, if so, to investigate the mechanisms involved. We demonstrated that microRNA-26b (miR-26b) was upregulated in cardiac myocytes after treatment with hydrogen peroxide (H$_2$O$_2$). To determine the potential roles of miRNAs in H$_2$O$_2$-mediated gene regulation and cellular injury, miR-26b expression was downregulated by miR-26b inhibitor and upregulated by miR-26b mimic. H$_2$O$_2$-induced cardiac cell apoptosis and over release of creatine kinase (CK) lactate dehydrogenase (LDH) were increased by miR-26b mimic and was decreased by inhibitor. These effects of miR-26b were abrogated by over-expression of HGF, an initiation factor of the mitochondrial apoptotic pathway in cardiomyocytes. Furthermore, luciferase reporter assay and western blot analysis identified HGF as a direct target of miR-26b. Overall, this study showed an important role for miR-26b on oxidative stress injury by directly targeting HGF and may represent a potential novel treatment for ischemic heart disease.

Keywords: miR-26b, HGF, acute myocardial infarction

Introduction

Acute myocardial infarction (AMI) is the major cause of morbidity and mortality in the world which accompanied by apoptosis, cardiac fibrosis, inflammation and pathological remodeling [1]. Reperfusion is the definitive treatment for acute coronary syndromes, especially acute myocardial infarction; however, reperfusion has the potential to exacerbate myocardial injury identified as ischemia/reperfusion (I/R) injury [2]. Reactive oxygen species (ROS) that create oxidative stress acts as the key damaging factor to the heart during I/R injury, as reoxygenation during reperfusion generates a large amount of ROS which induce apoptosis [3-5]. In spite of great progress that had been made on the pathophysiologic understanding of myocardial ischemia/reperfusion injury, the complete and precise mechanism associated with myocardial ischemia is largely unknown.

MicroRNAs (miRNAs) are a kind of highly conserved endogenous non encoding small RNAs [6]. It regulates posttranscriptional gene expression by binding to the 3'-UTR region of target mRNA and participate in the pathophysiology of many diseases [7]. For instance, miRNAs was involved in the physiological and pathological processes of heart development, cardiac hypertrophy, cardiac remodeling, heart failure and arrhythmia [8-12]. Similarly, increasing evidence has illustrated miRNAs as a protective or negative regulator in myocardial ischemia reperfusion [13-15]. MiR-26 family is enriched in the heart but it is not cardiac specific. It is down-regulated in various tumor types like bladder tumor, breast cancer, oral squamous cell carcinoma and anaplastic carcinomas [16-19] and may exhibit tumor-suppressive activity during tumorigenesis in these tumors. It has been demonstrated that up-regulation of miR-26a promotes apoptosis of hypoxic rat neonatal cardiomyocytes by repressing GSK-3β protein expression.

In the present study, we investigated the changes of miR-26b expression in a cellular model of
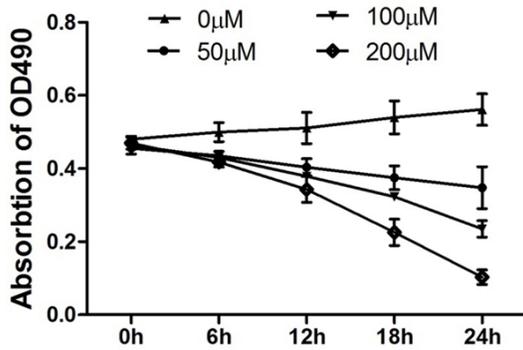
Figure 1. Effect of different concentration and time of H_2O_2 treatment on cardiac myocyte injury. After H_2O_2 of different concentration treatment, MTT assay was used to detect the cell viability. Data are expressed as mean ± SD; n = 8 *P < 0.05 vs. control, #P < 0.05 vs. H_2O_2 group.

oxidative stress induced by H_2O_2. We found the inhibition of miR-26b expression significantly reduced the cardiomyocyte apoptosis. In addition, we established HGF as a direct target gene of miR-26b.

Material and methods

Cell culture and transfection

H9c2 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, NY, USA) supplemented with 10% fetal bovine serum (GIBCO, NY, USA) , 100 U/m penicillin, 100 µg/ml streptomycin and 110 mg/ml sodium pyruvate in a humidified atmosphere containing 5% CO_2 at 37°C. MiR-26b mimics, miR-26b inhibitor and crumble control (GenePharma, Shanghai, China) were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufactures' protocols.

Cell treatment of H_2O_2

1×10^6 H9c2 cells were seeded into 6-well plate and cultured for 24 h. To induce hypoxia-like apoptosis, 100 µM H_2O_2 was added into cardiomyocyte culture for 6 h, 12 h, 24 h.

Cell viability assay

48 h after transfection, H9c2 Cardiomyocytes were plated in 96-well plates. MTT assay was used to assess the cell viability. After H_2O_2 of different concentration treatment, 10% MTT (Sigma, USA) was added to the culture medium at 37°C for 4 h. The supernatant was removed and the cell layer was diluted in DMSO and the

absorption at 490 nm was evaluated using a microplate reader (Bio-Rad, USA).

CK and LDH assay

Serum CK and LDH were measured using CK and LDH detection kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instruction.

Quantitative real-time PCR (qRT-PCR)

The H9c2 cardiomyocytes were suspended and collected. Total RNA was extracted using a Trizol (Invitrogen, USA) according to manufacturer's instructions. 2 µg RNA was reverse transcribed using a RNA PCR Kit (Takara Biotechnology, Japan) and the resulting cDNA was used as a PCR template. To detect gene expression, quantitative real-time PCR (qRT-PCR) was performed using an iCycler iQ System with the iQ SYBR Green Super Mix (Bio-Rad, USA) according to manufacturer's instructions. GAPDH and U6 snRNA were used as internal controls. The relative gene expression level was calculated using (2-ΔΔCt) method. The sequences of U6 primers were forward: 5'-GCTTCGGCAGC-ACATATACTAA-3' reverse: 5'-AACGCTTCACGAAT-TTGCGT-3'.

Western blot

The protein samples were extracted from the H9c2 cardiomyocytes using a lysis buffer (20 mM Hepes, pH7.9, 350 mM NaCl, 20% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 1 mM orthovanadate, 0.1 mM DTT, 25 mM NaF, and protease inhibitor cocktail; Sigma, USA). 30 µg protein was separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. The membranes were incubated for 2 h with 5% skimmed milk in PBS containing 0.1% Tween-20 to block non-specific binding followed by probing with primary antibodies overnight at 4°C. After washing with PBST, the membranes were incubated at room temperature for 90 min with horseradish peroxidase-conjugated goat anti-rabbit antibodies. GAPDH was used as an internal control. The blot was visualized with Pierce ECL reagents.

Flow cytometry

The cultured H9c2 cardiomyocytes were digested with trypsin, washed with cold PBS and dual
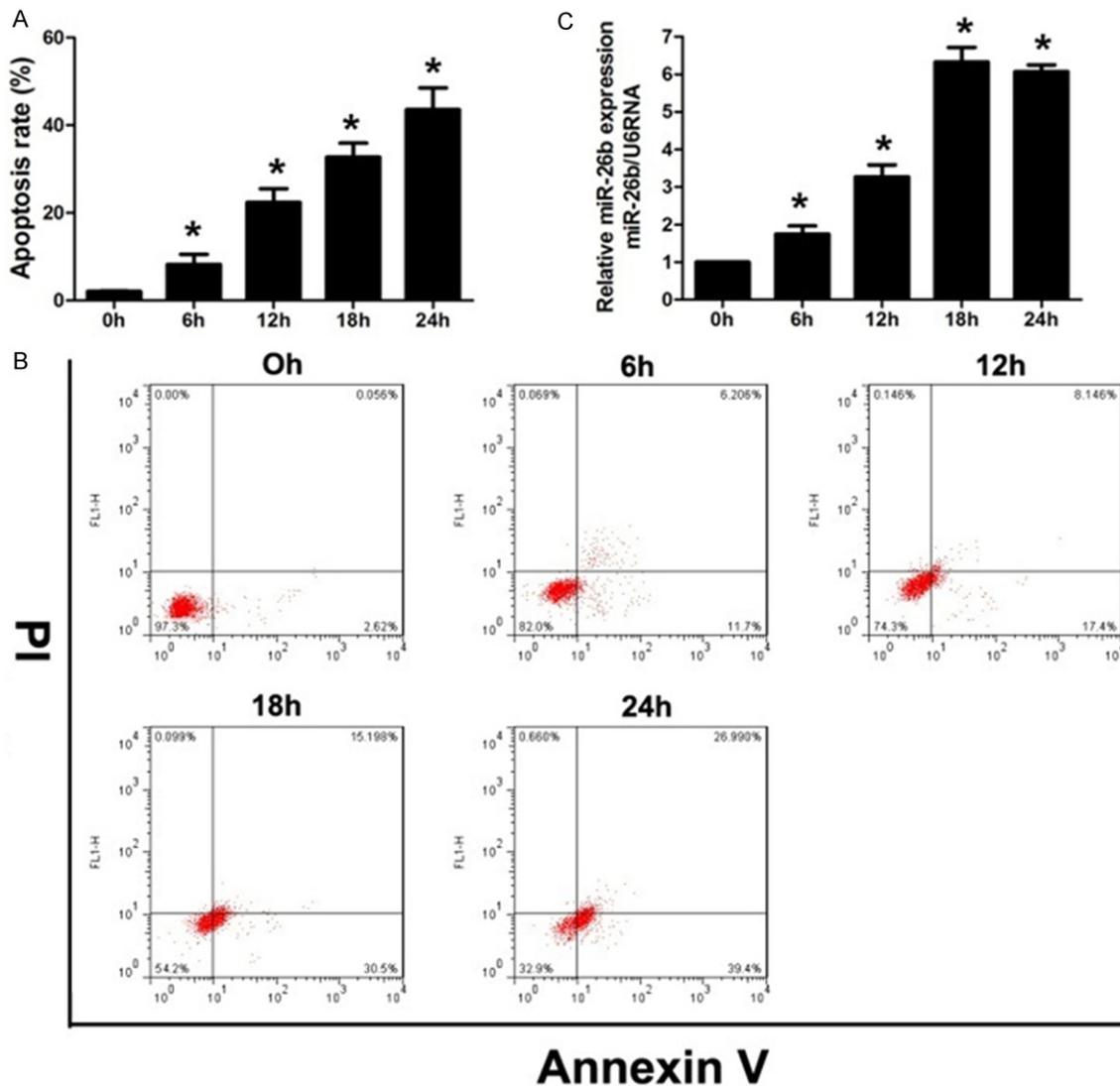
Figure 2. Effect of H_2O_2 on apoptosis and miR-26b expression in cultured cardiac myocytes. A, B. Flow cytometry was applied to investigate the apoptosis rate after different time of H_2O_2 treatment. C. Quantative real-time PCR was used to evaluated the expression of miR-26b with U6 being the internal control. Data are expressed as mean ± SD; n = 8 *P < 0.05 vs. control, #P < 0.05 vs. H_2O_2 group.

stained with nnexinV FITC/propidium iodide according to the manufacturer's instructions. Apoptosis was detected by flow cytometry on a BD FACSCalibur (Becton Dickinson, NJ, USA).

Luciferase assay

The Wild-type and mutated 3'UTRs were sub-cloned into the pGL3 vector (Promega, WI, USA) immediately downstream of the stop codon of the luciferase gene. Cells were co-transfected with the plasmid constructs of pGL3-HGF-3'UTR or pGL3-HGF-3'UTR-mut and miR-26b mimics or control using Lipofectamine 2000

(Invitrogen, CA, USA). Cells were subsequently transfected with 0.1 µg PRL-TK (TK-driven Renil-la luciferase expression vector) with Lipo-fectamine 2000 as an internal control. Luci-ferase activities were measured 48 h after transfection with a dual luciferase reporter assay kit (Promega, WI, USA).

Statistical analysis

All the data are presented as the means ± SD. Differences between multiple groups were ana-lyzed with one-way ANOVA followed by the Fisher's least significant difference (LSD) test.
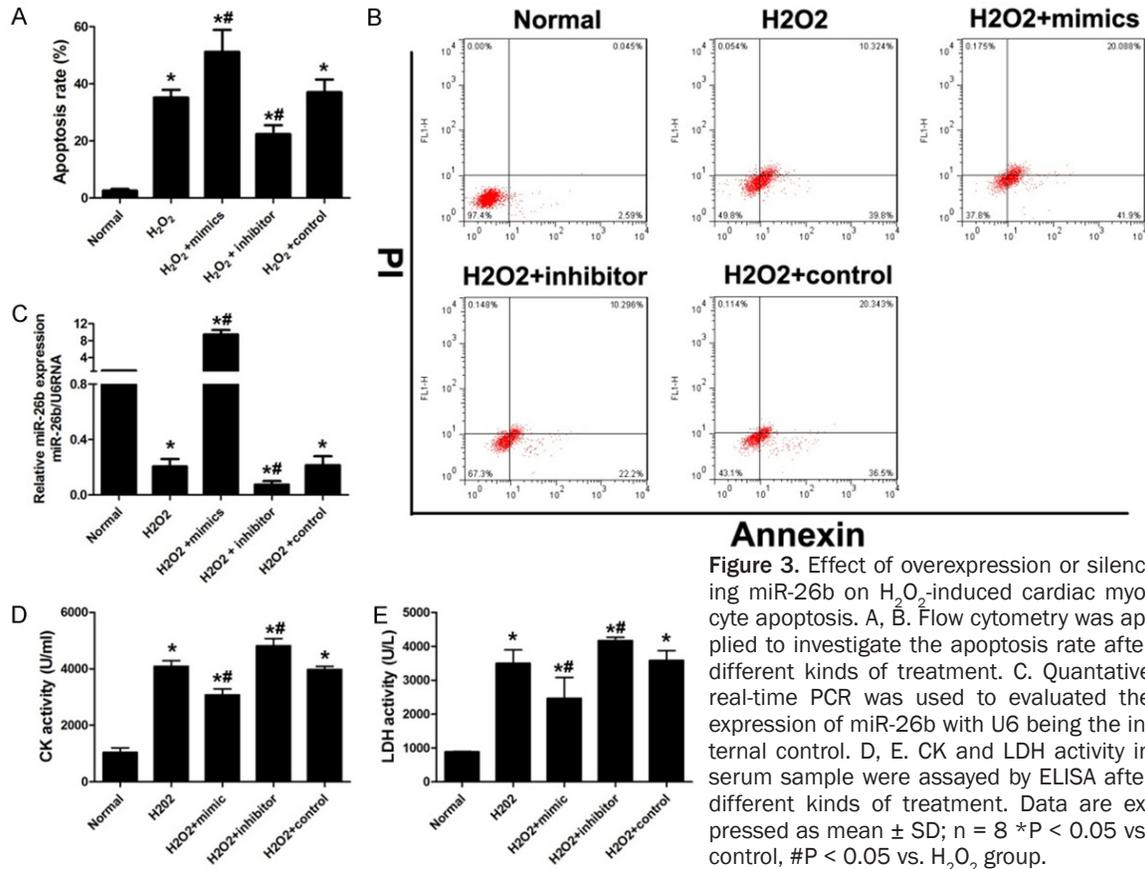
Figure 3. Effect of overexpression or silencing miR-26b on H_2O_2-induced cardiac myocyte apoptosis. A, B. Flow cytometry was applied to investigate the apoptosis rate after different kinds of treatment. C. Quantative real-time PCR was used to evaluated the expression of miR-26b with U6 being the internal control. D, E. CK and LDH activity in serum sample were assayed by ELISA after different kinds of treatment. Data are expressed as mean ± SD; n = 8 *P < 0.05 vs. control, #P < 0.05 vs. H_2O_2 group.

Differences between two groups were analyzed with Student's t-test. A P < 0.05 was considered statistically significant.

Results

Effect of H_2O_2 on cardiac myocyte injury

As **Figure 1** showed, high concentrations (50-200 μM) of H_2O_2 increased cardialmyocyte death in a dose and time dependent manner under our experimental condition. It indicated the successful establishment of cellular model of oxidative stress. The cell death was a mixture of cell apoptosis and necrosis with the majority of dead cells being apoptotic cells.

Effect of H_2O_2 on apoptosis and miR-26b expression in cultured cardiac myocytes

H_2O_2 (100 μM) promoted the apoptosis in cultured cardiac myocytes in a time dependent manner after 6 h treatment (**Figure 2A, 2B**). To investigate the relationship between miR-26b and cardiac myocytes under oxidative stress, we evaluated the expression of miR-26b (**Figure**

2C). Interestingly, exposure of cardiac myocytes to 100 μM H_2O_2 resulted in a time-dependent decrease of miR-26b expression.

Effect of overexpression or silencing miR-26b on H_2O_2-induced cardiac myocyte apoptosis

To further detect miR-26's role in regulating the cardiomyocyte response to ROS, we modulated the miR-26b expression. As **Figure 3A**, **3B** revealed, overexpression by transfection of miR-26b mimics significantly reduced the cardiac myocyte apoptosis induced by H_2O_2. On the contrary, Silencing miR-26b enhanced the apoptosis in cardiac myocyte under H_2O_2 treatment. We next evaluated the expression of miR-26b to verify the effect of miR-26b mimics and inhibitor. As expected, miR-26b mimics elevated the expression of miR-26b for more than 10 folds while miR-26b inhibitor reduced the expression of miR-26b for approximately 8 folds (**Figure 3C**). In addition, CK and LDH that are indicators of oxidative stress-induced injury were evaluated. In line with the previous findings, miR-26b overexpression elevated both CK and LDH activity (**Figure 3D**, **3E**).
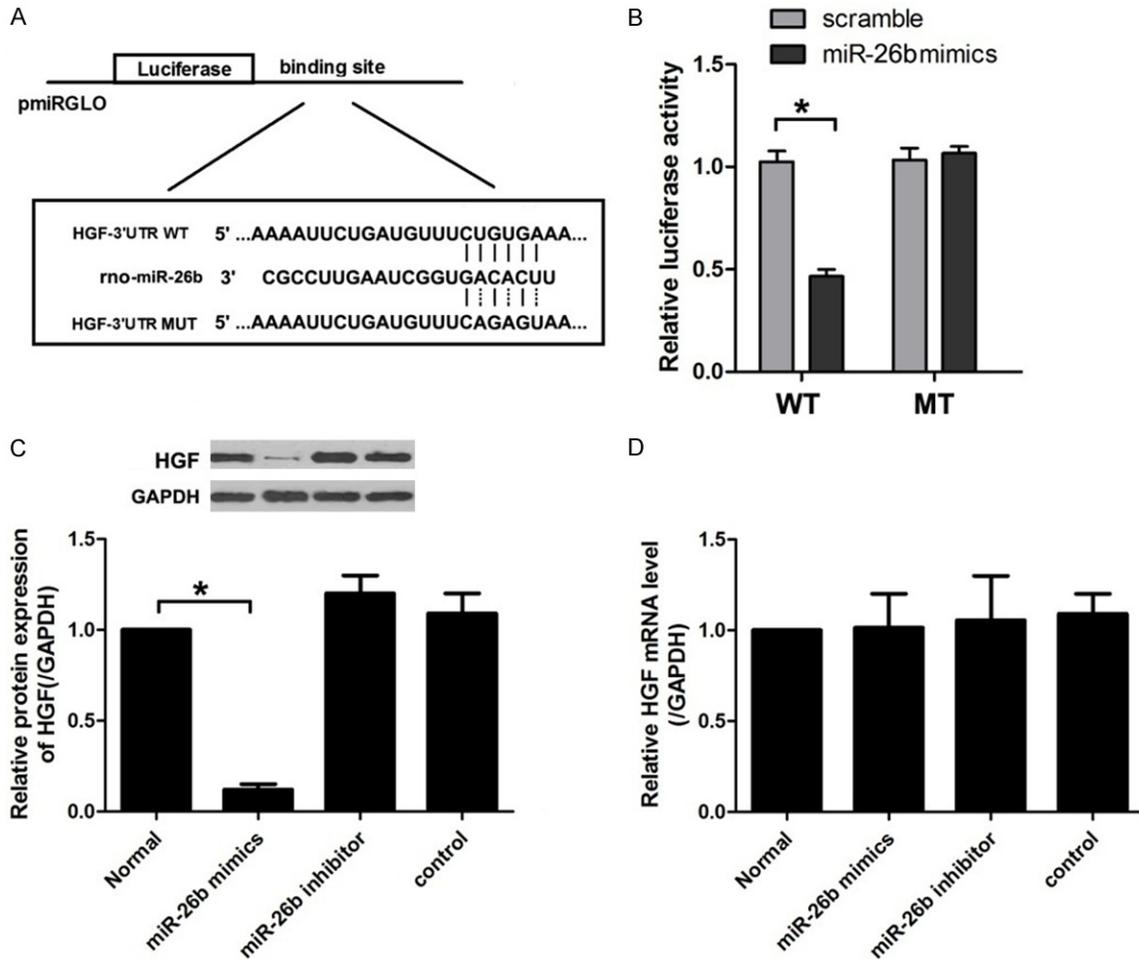
Figure 4. MiR-26b directly targets HGF. A. Sequence alignment between miR-26b and the 3'UTR of HFG of rat. B. Luciferase reporter activities of chimeric vectors carrying luciferase gene and a fragment of HFG 3'UTR containing the binding sites of miR-26b. C. Western blot analysis of protein level of HGF with miR-26b mimic or inhibitor transfected into H9c2 cells. D. Quantative real-time PCR was used to analysis the mRNA level of HGF with miR-26b mimic or inhibitor transfected into H9c2 cells. Data are expressed as mean ± SD; n = 8 *P < 0.05 vs. control, #P < 0.05 vs. H$_2$O$_2$ group.

MiR-26b directly targets HGF

To investigate the mechanism through which miR-26b inhibited ischemic injury, a potential target gene of miR-26b as predicted by bioinformatics algorithms (**Figure 4A**). To further confirm that miR-26b was able to directly bind to HGF and inhibit HGF expression, a construct in which a fragment of the 3'-UTR of HGF mRNA containing the putative miR-26b binding sequence was cloned into a firefly luciferase reporter construct and co-transfected into H9c2 cells with miR-26b mimics or scramble. As expected, we found that miR-26b decreased the luciferase activity miR-26b expression in H9c2 cells (**Figure 4B**). Quantative real-time

PCR and Western blot were carried out to verify the prediction. Unlike protein expression, the mRNA expression of HGF remained unchanged (**Figure 4C**, **4D**).

HGF overexpression reversed the effect of miR-26b on myocardiomyocyte oxidative stress injury

To test this hypothesis, we transfected cardiomyocytes with 100 nM miR-26b mimics and HGF overexpressing vector to ectopically up-regulate miR-26b and HGF (**Figure 5A**, **5B**). As expected, HGF significantly reversed the apoptosis inducing effect of miR-26b. In addition, CK and LDH activity were evaluated. Consistently,
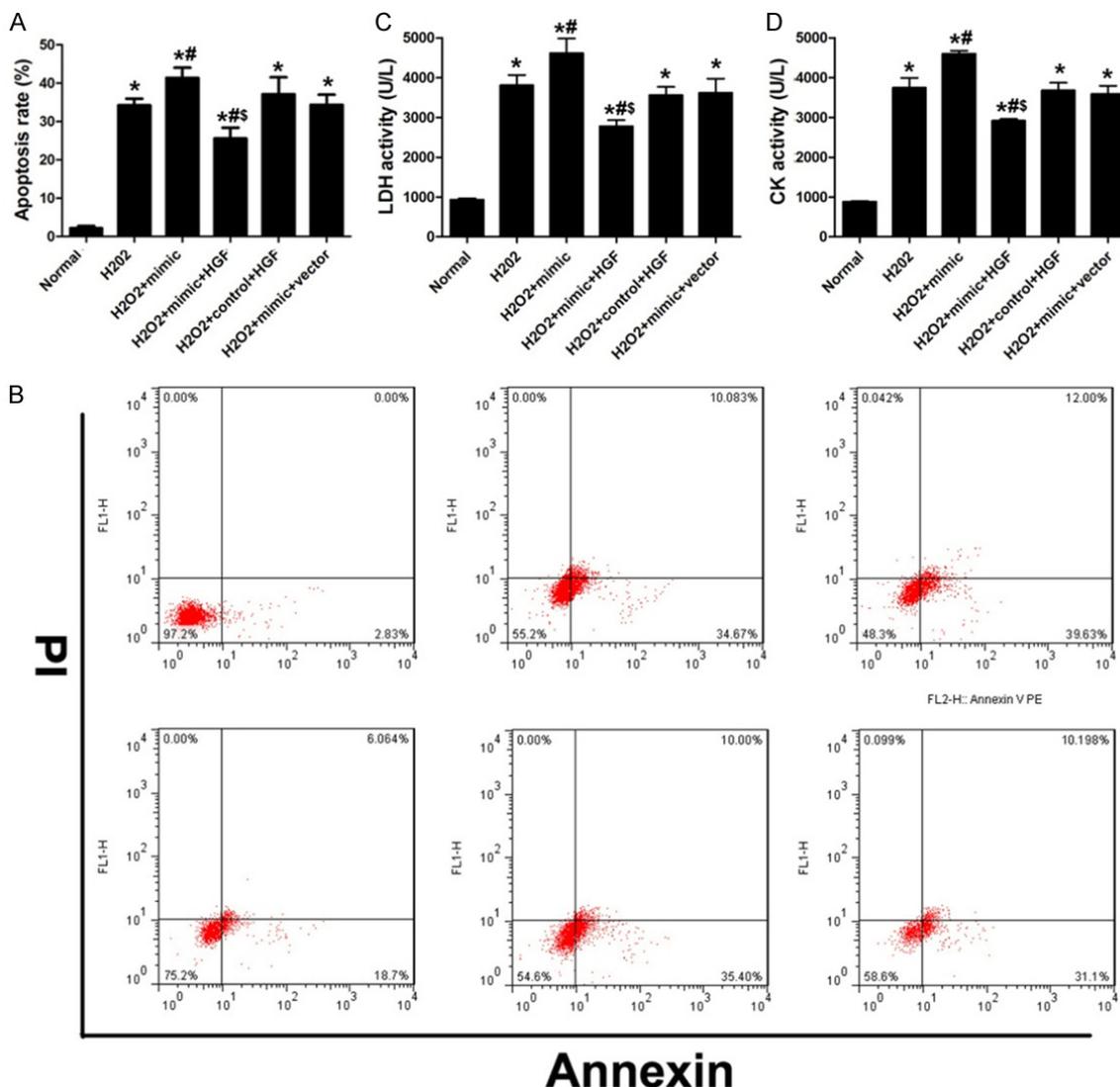
Figure 5. HGF overexpression reversed the effect of miR-26b on myocardiomyocyte oxidative stress injury. A, B. Flow cytometry was applied to investigate the apoptosis rate after different kinds of treatment. C, D. CK and LDH activity in serum sample were assayed by ELISA after different kinds of treatment. Data are expressed as mean ± SD; n = 8 *P < 0.05 vs. control, #P < 0.05 vs. H_2O_2 group, $P < 0.05 vs. H_2O_2+mimic group.

HGF overexpression down-regulated the CK and LDH activity which expression was elevated by miR-26b (**Figure 5C**, **5D**).

Discussion

ROS can be produced during the course of the cardiovascular disease, which can lead to oxidative stress damage and eventually apoptosis and necrosis of myocardial cells [20]. H_2O_2 is a kind of ROS that can induce oxidative stress injury in myocardial cells. Thus, It is most used to simulate myocardial ischemia reperfusion injury and oxidative stress damage. In this study we found that 100 μM and 200 μM H_2O_2 could both induce apoptosis of H9c2 cells significantly, we selected this concentration of to do the 100 μM experiment which is similar with the previous studies. Cultured H9c2 cardiomyocytes were significantly injured by 100 μM H_2O_2 as early as 6 h treatment. This result is consistent with previous studies indicating that hypoxia conditions generated oxygen-derived free radicals in cardiac tissues, futher played crucial role in myocardial ischemia [21].

Increasing evidences have indicated that miR-NAs are emerging as critical regulators in physi-

ological and pathogenetic processes. Physical and chemical stimuli may alter miRNA expression [22, 23]. MiR-26b is one of the most studied miRNAs [24, 25] and plays an important role in the regulation of apoptosis which is mostly studied in tumors [26, 27]. MiR-26b induces osteosarcoma and breast cancer cell apoptosis by targeting PFKFB and SLC7A11 respectively. Consistently, the miR-26b level is positively correlated with the apoptosis rate in hepatocellular carcinoma tissues [28, 29]. All these data indicate that miR-26b is a pro-apoptotic miRNA. However, the role of miR-26b in myocardial ischemic injury or oxidative stress injury has not been illustrated.

The present study examined the expression levels of miRNA-26b in response to oxidative stimulation. As expected, miR-26b expression changed dramatically. To ascertain the role of miR-26b in ROS-mediated H9c2 cells apoptosis, miR-26b expression was modulated via transfection of miR-26b inhibitor and miR-26b mimic. We found that the downregulation of miR-26b expression protected against the H_2O_2-induced injury of H9c2 cells by blocking LDH release and CK production and inhibiting cell apoptosis that are indicators of oxidative.

The major mechanism through which miRNAs inhibit target gene expression is to induce mRNA degradation or suppress mRNA translation via matching to the 3'-UTR of target mRNAs. Here, we used bioinformatics analysis to search the potential targets of miR-26b and selected HGF as a potential one. The following luciferase activity assay verified this prediction. HGF has been demonstrated to induce pro-survival Bcl-xL expression which is relevant with apoptosis 10516287. In addition, it has been determined that the expression of both HGF and its receptor Met in cardiomyocytes, as well as plasma levels of HGF rapidly increase in response to ischemia/reperfusion injury and the administration of exogenous HGF in rats with ischemia/reperfusion injury resulted in lesser degree of myocardial apoptosis, smaller size of the infarct area and better cardiac function compared with non-treated control rats [30, 31]. In our work, we firstly found that miR-26b directly target HGF which was verified by luciferase assay and blocked its protective effect on H_2O_2 induced oxidative stress injury. To further confirm what we demonstrated. MiR-26b and HGF overexpressing vector were contransfected into

H9c2 cells. Interestingly, HGF reversed the oxidative stress induction of miR-26b.

In conclusion, miR-26b owns a protective effect on H_2O_2 induced cardiamyocyte oxidative stress injury by directly targeting HFG. These novel findings may have extensive diagnostic and therapeutic implications for a variety of cardiovascular diseases related to oxidative stress.

Acknowledgements

Disclosure of conflict of interest

None.

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