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Up-regulation of miR-26a promotes apoptosis of hypoxic rat neonatal cardiomyocytes by repressing GSK-3β protein expression

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ABSTRACT

Myocardial ischemia is the major cause of morbidity and mortality due to cardiovascular diseases. This disease is a severe stress condition that causes extensive biochemical changes which trigger cardiac cell death. Stress conditions such as deprivation of glucose and oxygen activate the endoplasmic reticulum in the cytoplasm of cells, including cardiomyocytes, to generate and propagate apoptotic signals in response to these conditions. microRNAs (miRNAs) are a class of small non-coding RNAs that mediate posttranscriptional gene silencing. The miRNAs play important roles in regulating cardiac physiological and pathological events such as hypertrophy, apoptosis, and heart failure. However, the roles of miRNAs in reactive oxygen species (ROS)-mediated injury on cardiomyocytes are uncertain. In this study, we identified at the apoptotic concentration of H_2O_2, miR-26a expression was increased. To determine the potential roles of miR-26a in H_2O_2-mediated cardiac apoptosis, miR-26a expression was regulated by a miR-26a or an anti-miR-26a. Overexpression of miR-26a increased apoptosis as determined by upregulation of Annexin V/PI positive cell population, caspase-3 activity and expression of pro-apoptotic signal molecules, whereas inhibition of miR-26a reduced apoptosis. We identified GSK3B as a direct downstream target of miR-26a. Furthermore, miR-26a attenuated viability and increased caspase-3 activity in normal cardiomyocytes. This study demonstrates that miR-26a promotes ROS-induced apoptosis in cardiomyocytes. Thus, miR-26a affects ROS-mediated gene regulation and cellular injury response.

1. Introduction

Cardiovascular disease is one of the most prevalent diseases in both developed and developing countries, with high morbidity and a high risk of death. Cardiac myocyte cell death and apoptosis by ischemia/reperfusion (I/R) are important pathological phenomena in the heart [1]. During the ischemic period, ROS including superoxide, H_2O_2 and hydroxyl radicals are found in the myocardium [2,3]. Many studies of cardiac cell types, including cardiomyocytes, vascular smooth muscle cells, endothelial cells, and fibroblasts, have shown that a common denominator, ROS, is involved in cardiovascular diseases [4,5]. I/R generates ROS, and increasing ROS is a key factor in various cardiomyocyte responses, including cell death and apoptosis [6,7]. Previously, we have reported that ROS induced apoptosis in cultured cardiomyocytes [8].

miRNAs are a class of small non-coding RNAs, derived from endogenous small hairpin precursors. MicroRNAs regulate gene expression by binding to the 3′-untranslated regions (3′-UTRs) of specific mRNAs for transcript degradation or translational repression [9,10]. Recently, the study of miRNAs renovates our understanding about gene regulation in cardiac physiology and pathology [11,12]. Many such studies have demonstrated that miRNAs can regulate cardiac apoptosis [13,14]. The first evidence for the role of miRNAs in cardiomyocyte apoptosis was found using miR-1 and miR-133, where miR-1 has a pro-apoptotic effect and miR-133 has an anti-apoptotic effect in response to oxidative stress [15]. Also, miR-1 regulates cardiomyocyte apoptosis, which is involved in post-transcriptional repression of Bcl-2 and IGF-1 [16,17]. As opposite function of miR-1 and miR-133, miR-21, miR-29, miR-30, and miR-199a are anti-apoptotic miRNAs, and miR-195 and miR-320 are pro-apoptotic miRNAs [13,14].

A previous study demonstrated that miR-26a is abundantly expressed in rat hearts and decreased in ischemic preconditioning

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(IP) [18]. Although, miR-26a initiated cell apoptosis in cancer cells [19,20], the role of miR-26a in cardiomyocytes remains unclear. Particularly, the role of miR-26a in cardiomyocyte apoptosis has not yet been studied.

Therefore, our research focused on the effect of miR-26 expression by ROS in cardiomyocytes and determined whether miR-26a regulated ROS-induced cardiomyocyte apoptosis. Our data suggests that miR-26a may be a new therapeutic target for ischemic heart diseases.

2. Material and methods

2.1. Isolation of rat ventricular cardiomyocytes

All experimental procedures for animal studies were approved by the committee for the care and use of laboratory animals, Yonsei University College of Medicine, and performed in accordance with the committee's guidelines and regulations for animal care (NIH Publication No. 85-23, revised 1996). We isolated and purified neonatal rat cardiomyocytes by previously described methods [21]. Briefly, 1–2 days old Sprague Dawley rat pups were anesthetized with ether in batches of five at a time. Using microdissecting scissors, hearts were minced to pieces of approximately 1 mm^3 and the ventricles were washed with Dulbecco's phosphate-buffered saline solution (PBS, pH 7.4, Gibco BRL) free from Ca^{2+} and Mg^{2+}. The tissues were washed with PBS, and enzymatically digested with 10 ml of collagenase II (0.8 mg/ml, 262 units/mg, Gibco BRL) for 5 min at 37 °C. The supernatant was then removed, and the tissue was treated with fresh collagenase II solution for an additional 5 min. The cells in the supernatant were transferred to a tube containing cell culture medium (α-MEM containing 10% fetal bovine serum, Gibco BRL). The tubes were centrifuged at 1200 rpm for 4 min at room temperature, and the cell pellets were resuspended in 5 ml of cell culture medium. The above procedures were repeated 7–9 times until little tissue remained. The cell suspensions were collected and incubated in 100 mm tissue culture dishes for 1–3 h to reduce fibroblast contamination. The non-adherent cells were collected and seeded to achieve a final concentration of 5×10^5 cells/ml. After incubation for 4–6 h, the cells were rinsed twice with cell culture medium and 0.1 μM 5-Bromo-2′-deoxyuridine (BrdU) was added. The supplement BrdU is recommended to culture in order to increase the purity of cardiomyocytes. The cells were then cultured with 10% (v/v) FBS in a CO_2 incubator at 37 °C.

2.2. Treatment of cells with hydrogen peroxide

After cell isolation, we incubated cardiomyocytes for one day. The cells were further incubated with α-MEM containing 0.1% FBS. And then various concentrations of H_2O_2 were added to the medium and incubated with the cells for an indicated time. For negative controls, cells were incubated with the medium alone for equivalent amounts of time.

2.3. Cell viability assay

Cell viability was measured using the CCK assay kit (Dojindo, Japan). Cardiomyocytes were seeded at the density of 5×10^3 cells per well of 96-well plates. These cells were incubated with varying concentrations of H_2O_2 for 6 h in 0.1% α-MEM. The CCK assay kit was diluted with 0.1% α-MEM and then 100 μl was added to each well and incubated for 2 h at 37 °C. The absorbance of the samples was measured at 450 nm using a microplate reader against a background control.

2.4. Measurement of caspase-3 activity

Relative caspase-3 activity was determined using the fluorimetric Homogeneous Caspases Assay according to the manufacturer's instructions (Roche, Swiss). Cells were cultured in microplates, and apoptotic stimuli were applied, causing activation of caspases. The caspase substrate, pre-diluted in incubation buffer, was added and incubated for 2 h at 37 °C for cell lysis. Free R110 was determined fluorimetrically at max = 521 nm. The amount of developed fluorochrome was proportional to the concentration of activated caspases and could be quantified by a calibration curve.

2.5. Annexin V/PI staining

Cardiomyocytes were cultured in a 4-well culture dish (1×10^4 cells/well) and pretreated with a control medium or media containing varying concentrations of H_2O_2 for 6 h. The dishes were washed with ice-cold PBS for 5 min and fixed with 4% formalin for 10 min. After being blocked with the Annexin-binding buffer at 500 μl, the cells were stained with Annexin V-FITC at room temperature in the dark for 15 min. The dishes were washed with ice-cold PBS, stained with PI and diluted with Annexin-binding buffer at room temperature in the dark for 5 min. The number of Annexin V/PI-positive cells was counted under a fluorescence microscope.

2.6. Western blot

Cardiomyocytes were washed once in PBS and lysed in lysis buffer (Cell Signaling Technology, Inc.) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM PMSF. Protein concentrations were determined using the BCA protein assay kit (Pierce). Proteins were separated in a SDS–polyacrylamide gel and transferred to the PVDF membrane (Millipore Co, Bedford). After blocking the membrane with Tris-buffered saline–Tween 20 (TBS-T, 0.1% Tween 20) containing 5% non-fat dried milk for 1 h at room temperature, the membrane was washed twice with TBS-T and incubated with primary antibodies for 1 h at room temperature or overnight at 4 °C. The membrane was washed three times with TBS-T for 10 min and then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology). The band intensities were quantified using the Photo-Image System (Molecular Dynamics, Sweden).

2.7. MicroRNA transfection

Transfections of miRNA mimics were performed using siLentFect™ Lipid reagent (Life Science Research). Mature miR-26a and miR-control (Genolution Pharmaceuticals, Inc., Korea) used a final concentration of 100 nM, and for miR-26a knockdown, anti-miR-26a and anti-miR-control (Genolution Pharmaceuticals, Inc.) was added to the transfection complexes at a final concentration of 20 nM. After 4–6 h incubation in a CO_2 incubator at 37 °C, the medium was changed to conditioned α-MEM.

2.8. Real-time PCR

Total RNA was isolated with the mirVana™ RNA Isolation Kit or Tripure isolation reagent (Roche Applied Science). In brief, 10 ng purified total RNA was used for reverse transcription (Taqman® MicroRNA Reverse Transcriptase Kit, Applied Biosystems) in combination with Taqman® MicroRNA Assays for quantification of specific miRNAs and U6 control transcripts, according to the

manufacturer's conditions. Amplification and detection of specific products were performed in a Light Cycler 480 II (Roche) at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The threshold cycle (Ct) of each target gene was automatically defined, located in the linear amplification phase of the PCR, and normalized to control U6 ($^{\Delta}$Ct value). The relative difference in expression levels of each miRNA in cardiomyocytes ($^{\Delta\Delta}$Ct) was calculated and presented as fold induction ($2^{-\Delta\Delta}$Ct).

2.9. Luciferase reporter assay

The predicted target gene of miR-26a was retrieved using a publicly available database (TargetScan, www.targetscan.org). We synthesized the 3′-UTR of GSK-3β which were 1260 nt long and contained the predicted binding sites for miR-26a. The control sequences containing several mutated bases within the binding sites were also synthesized. The corresponding genes were then cloned into the pmirGLO vector. HeLa was plated at 1×10^5 in 12-well format. After 24 h, the pmirGLO vector containing the GSK-3β binding site for miR-26a was co-transfected with the negative control using Lipofectamine 2000. The Renilla luciferase was used to normalize the cell number and the transfection efficiency. Luciferase activity was measured by the Dual Luciferase assay (Promega) according to the manufacturer's instructions after 24 h on the luminometer. Each assay was repeated three times.

2.10. Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were as performed using Student's *t*-test. Relationships were considered statistically significant when *p* values were less than 0.05.

3. Results

3.1. miR-26a expression in apoptotic cardiomyocytes

To determine which miRNA was closely related with ROS-induced cardiac apoptosis, we used real-time PCR to determine miRNA levels in normal cardiomyocytes. As shown in Fig. 1A, normal cardiomyocytes highly expressed miR-1, 26a, 133a, and 145. Also, we evaluated miRNA expression profiles of neonatal and adult heart samples and obtained similar results in heart tissue samples (data was not shown). Next, to assess the expression of miRNAs by apoptotic H_2O_2 concentration, cardiomyocytes were exposed to 100 μM H_2O_2 for a short time (6 h), and the expression of miRNAs was determined by real-time PCR. At the apoptotic concentration of H_2O_2, miR-1, miR-26a, and miR-133a expressions were increased, whereas miR-145 expression did not change (Fig. 1B). Among upregulated miRNAs, miR-26a was increased in H_2O_2-stimulated cardiomyocytes in a dose-dependent manner compared to the control (Fig. 1C).

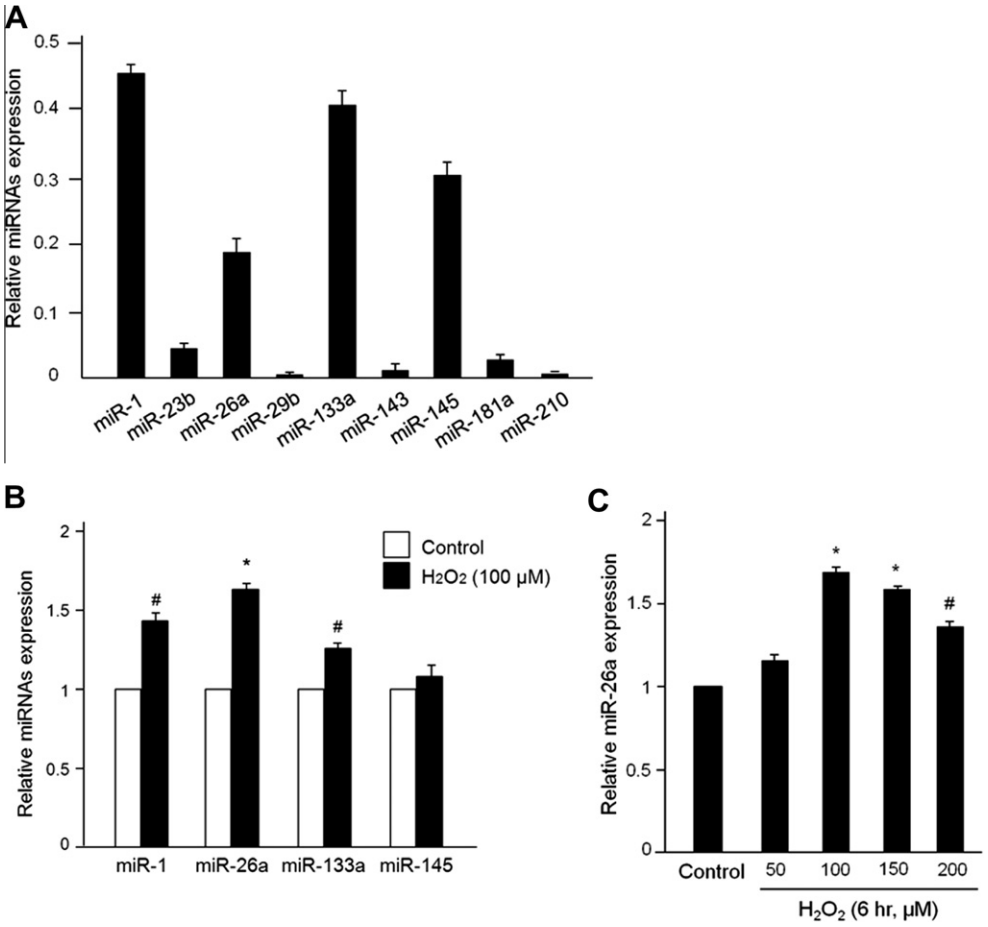

Fig. 1. miR-26a expression in apoptotic cardiomyocytes. (A) Real-time PCR analysis of miRNA expression in normal neonatal cardiomyocytes. (B) Real-time PCR analysis showed that exchanged specific miRNA expression in H_2O_2-induced cardiomyocytes compared to controls. (C) Expression of miR-26a increased cardiomyocytes with dose-dependent treatment of H_2O_2 for 6 h. U6 was used for normalization. Values are the average of triplicate determinations with the SD indicated by error bars. ($^{\#}p < 0.05$ and $^{*}p < 0.001$ compared with control.)

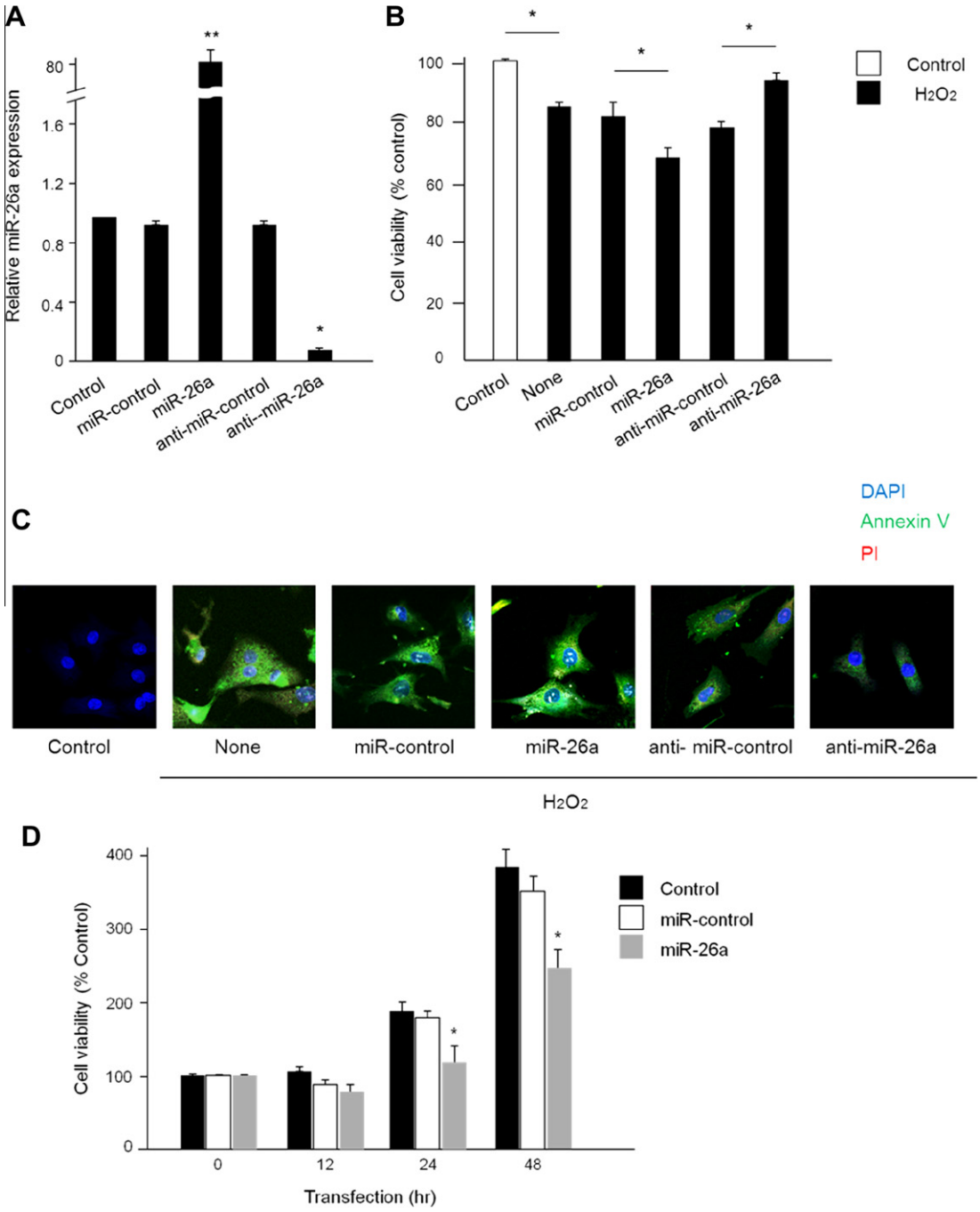

Fig. 2. The effect of miR-26a on ROS-induced cardiac apoptosis. (A) Cardiomyocytes were transfected with miR-control, miR-26a (100 nM), anti-miR-control, or anti-miR-26a (20 nM) for 4 h. miR-26a expression levels were determined 24 h later using real-time PCR. miR-control and anti-miR-control were used for control transfections. (B) Cultured cardiomyocytes pre-treated with miR-26a, or anti-miR-26a were then treated with H_2O_2 (100 μM) for 6 h. Cell viability was determinate by the CCK assay. (C) Representative AnV and PI stained cell photographs, and their merged photographs from cells treated with miR-26a, or anti-miR-26a in H_2O_2-treated cardiomyocytes. Apoptotic cardiomyocytes induced by H_2O_2 treatment stained with Annexin V (AnV)/PI and observed under a fluorescence microscope. The samples were analyzed for green fluorescence (AnV) and red fluorescence (PI): early apoptotic cells, AnV-FITC positive and PI negative; necrotic or late apoptotic cells, both AnV-FITC and PI positive; dead cells, AnV-FITC negative and PI positive. (D) Cultured cardiomyocytes treated with miR-26a, or anti-miR-26a. The effect of miR-26a on normal cardiomyocyte viability was assessed by the CCK assay. (*$p < 0.001$ compared with control.).

3.2. Modulation of miR-26a expression in cardiomyocytes

In order to determine the role of miR-26a in apoptosis, cardiomyocytes were transfected with miR-26a or anti-miR-26a. Cardiomyocytes were divided into the following 5 groups: control (untransfected cells), miR-control, miR-26a, anti-miR-control, and anti-miR-26a groups. All of these groups were subjected to the transfection procedures. As shown in Fig. 2A, miR-26a increased miR-26a expression in cardiomyocytes, and anti-miR-26a decreased miR-26a expression, while their control oligonucleotides had no effect.

3.3. The effect of miR-26a on ROS-induced cardiac apoptosis

To further demonstrate the role of miR-26a, cardiomyocytes were introduced with either miR-26a or anti-miR-26a and then

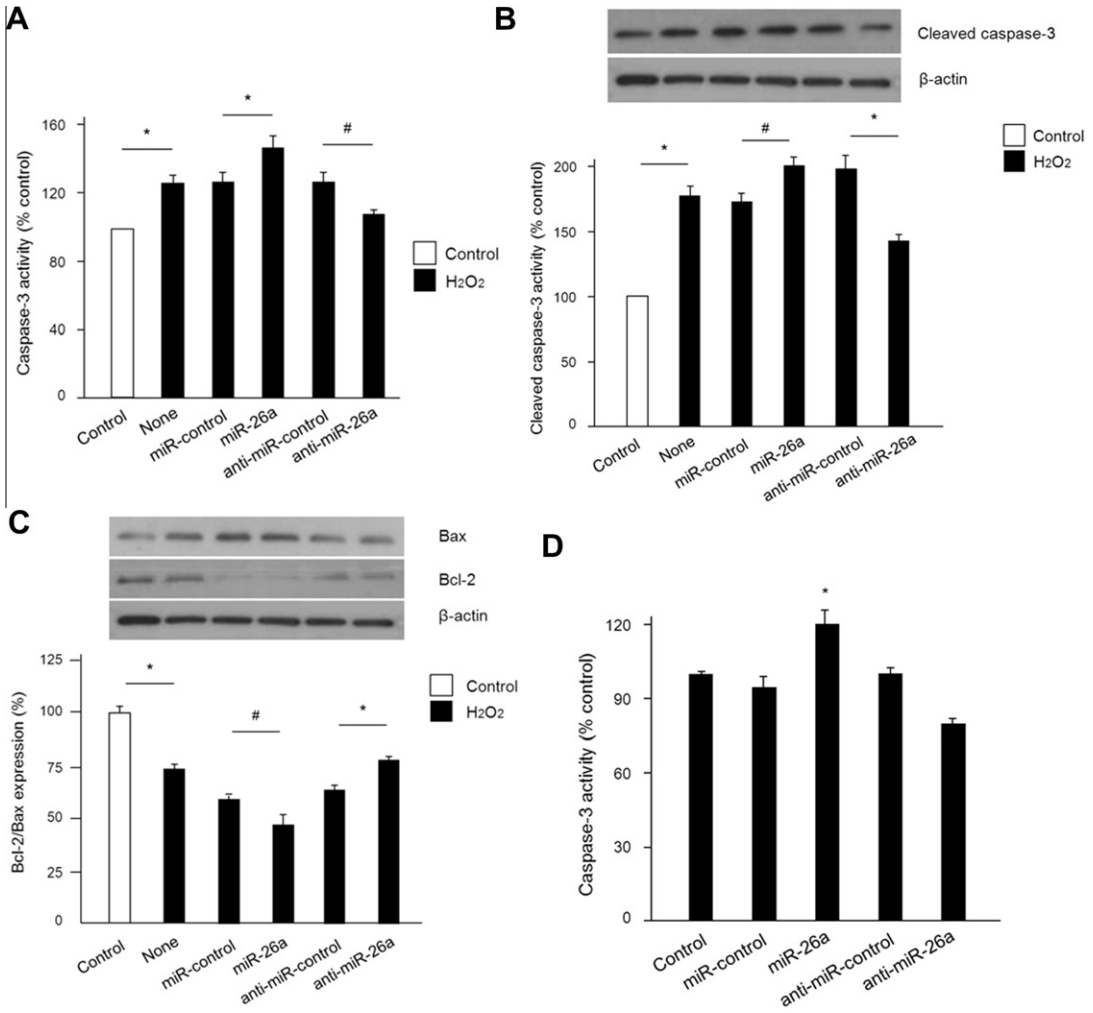

Fig. 3. The effect of miR-26a on ROS-induced apoptosis signaling. (A) Cardiomyocytes were transfected with miR-26a, or anti-miR-26a and then treated with H_2O_2 for 6 h. Caspase-3 activity was determined using the Homogeneous Caspase Assay. (B) Representative immunoblots of cleaved caspase-3 from cells treated with miR-26a, or anti-miR-26a. (C) Western blotting was performed to determine Bax and Bcl-2 protein levels in cardiomyocytes. (D) The effect of miR-26a on normal cardiomyocyte apoptosis was assessed by caspase-3 activity assay. ($^{\#}p < 0.05$ and $^{*}p < 0.001$ compared with control.)

incubated in 100 μM H_2O_2 for 6 h. Unlike miR-26a, anti-miR-26a attenuated H_2O_2-induced cardiac death in the CCK assay (Fig. 2B). The miR-26a further enhanced AnV/PI staining positive cells, whereas anti-miR-26a reduced (Fig. 2C). Next, we questioned whether miR-26a promotes cell death in normal cardiomyocytes. Fig. 2D shows that overexpression of miR-26a attenuated viability in normal cardiomyocytes. These effects of H_2O_2 on apoptosis were significantly increased by the miR-26a.

3.4. The effect of miR-26a on the apoptosis signaling pathway

While miR-26a enhanced caspase-3 activity, anti-miR-26a decreased caspase-3 activity in H_2O_2-induced cardiomyocytes (Fig. 3A). Through cleaved caspase-3 detection, the positive effect of miR-26a was confirmed (Fig. 3B). Also, pro-apoptotic Bax protein level was increased, but anti-apoptotic Bcl-2 protein level was decreased by miR-26a, whereas anti-miR-26a lead to opposite results (Fig. 3C). Furthermore, miR-26a expression increased caspase-3 activity in normal cardiomyocytes (Fig. 3D). Thus, these results indicate that miR-26a promotes apoptosis in H_2O_2-stimulated cardiomyocytes.

3.5. Prediction of target of miR-26a

Numerous genes have putative target sites of miR-26a in their 3′-UTR. Thus, we searched for predictive targets of miR-26a using the online software Targetscan (http://www.targetscan.org/), where a more negative Targetscan score indicates a higher likelihood of being a miR-26a target. Among these predicted genes, anti-apoptosis, proliferation, or growth-related genes were predicted for miR-26a targets. The GSK-3β is involved in the process of cell anti- and pro-apoptosis, then miR-26a induces smooth muscle hypertrophy by suppressing GSK-3β [22,23]. The GSK-3β 3′-UTR contains one putative miR-26a seed site which bound with imperfect complementation (Fig. 4A). To determine if miR-26a targets GSK-3β, we applied the luciferase report gene assay using the pmirGLO luciferase reporter. Firstly, we constructed a reporter vector containing a consensus miR-26a-binding site within the 3′-UTR as a positive control. After we co-transfected this reporter plasmid into HeLa with miR-26a or its control miR-control, we found that luciferase activity significantly decreased in the HeLa transfected with miR-26a, whereas it was increased with anti-miR-26a. These data indicate miR-26a can suppress expression of transcripts containing an exact miR-26a-binding site by our luciferase reporter assay (Fig. 4B). Expression level of GSK-3β protein was downregulated in cardiomyocytes treated with miR-26a, whereas it was increased in cardiomyocytes transfected with anti-miR-26a (Fig. 4C). For this reason, we suggest that miR-26a induced apoptosis in H_2O_2-stimulated cardiomyocytes by post-transcriptional regulation of GSK-3β.

4. Discussion

ROS are an important cause of heart diseases. ROS also regulates transcription factor activation, gene expression, cell growth, and apoptosis. The concentration-dependent apoptotic phenotype induced by H_2O_2 in neonatal rat cardiac myocytes is similar to previous reports, which showed the same process in adult rat ventricular myocytes and H9C2 [24,25]. Also, this study showed that a high level of ROS promotes cardiomyocyte apoptosis, caspase-3 activity, cleaved caspase-3, and Bax/Bcl_2 ratio.

In the cardiovascular field, miRNAs are known to be fundamental targets for regulating the expression of genes that govern physiological and pathological myocardial adaptation to stress. Recently, several reports have revealed important roles of miRNAs in heart failure [11,12]. During the adaptive response of the heart to stress stimuli, microarray analyses have shown upregulated, downregulated, or unchanged miRNA expression compared to that in a normal heart [26]. Moreover, differential regulation of subsets of miRNAs has been shown to characterize various etiologies. We further found that miR-1, miR-26a, miR-133a, and miR-145 were abundant in normal neonatal cardiomyocytes, which is similar to rat neonatal and adult hearts. These miRNAs were also among the enriched miRNAs in the human heart, consistent with a previous report [27].

miRNAs have been shown to regulate the cardiac apoptosis program [13,14]. The functional study reveals that overexpression of miR-320 enhances apoptosis in cardiomyocytes, whereas knockdown of miR-320 can attenuate cell death upon ischemia/reperfusion [28]. Programmed cell death4 (PDCD4) is also a direct target of miR-21 [29]. miR-199a inhibits hypoxia-inducible factor (HIF)-1α and its stabilization of p53, thereby reducing apoptosis [30]. miR-1 participates in the activation of apoptosis by reducing the expression levels of heat shock protein-60 and heat shock protein-70, whereas miR-133 antagonizes apoptosis by repressing caspase-9 expression [15]. Also, miR-1 regulated cardiomyocyte apoptosis, which is involved in post-transcriptional repression of Bcl-2 and IGF-1 [16,17]. miR-195 promotes palmitate-induced apoptosis in cardiomyocytes by targeting Sirt1, a known anti-apoptotic protein [31]. miR-1 and miR-133 is abundantly expressed in mouse, rat and human hearts and regulated in heart disease including arrhythmia and cardiac hypertrophy [32,33]. In our results, expression level of miR-1 and miR-133a increased by apoptotic stimuli. At the apoptotic concentration of H_2O_2, miR-26a expression was increased similarly to that of miR-1 and miR-133.

miR-26a is abundantly expressed in rat and human hearts [18,27] and decreased in ischemic preconditioning (IP). Also, the role of miR-26a has been studied in several cancer cells. miR-26a initiated cell apoptosis through both extrinsic and intrinsic pathways with caspase-8 and caspase-9 activation, respectively, in breast cancer cells [19]. In nasopharyngeal carcinoma (NPC) cell line C666-1 cells, ionizing radiation (IR)-induced apoptosis was dependent on ROS, and exogenous expression of miR-26a led to significant toxicity in cells [20]. However, the role of miR-26a in cardiac apoptosis is not clear. In the present study, miR-26a is abundantly expressed in cardiomyocytes and increased in an apoptotic concentration of H_2O_2. Also, specific miR-26a promotes H_2O_2-induced cardiomyocyte apoptosis.

The modulating ability of miRNAs is due to their activity in repressed translation of target mRNAs. Thus, the search for miR-26a target mRNA merits further study to determine its role in cardiac apoptosis. Its target genes may be anti-apoptotic, growth, or proliferation-related genes. It was shown previously that EZH2 and

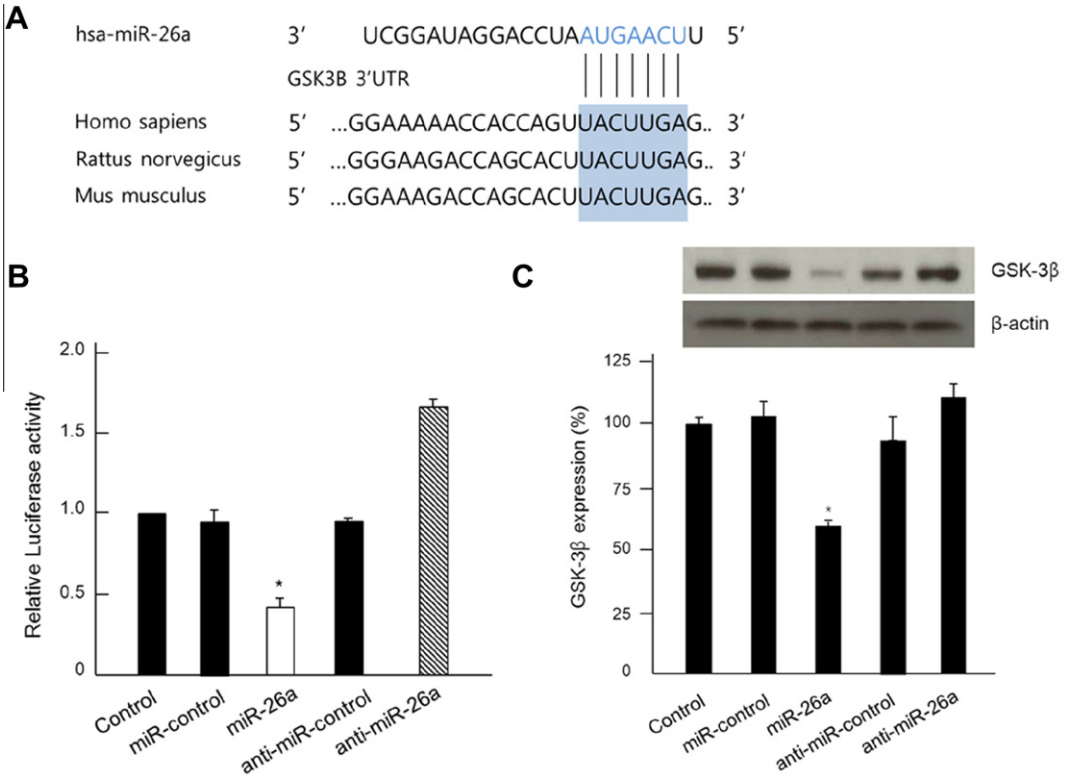

Fig. 4. GSK-3β 3′-UTR regulates target gene expression. (A) A schematic shows that the 5′ end of miR-26a contains a sequence complementary to the specific miRNA binding site within the 3′-UTR of GSK-3β mRNA. (B) miR-26a, anti-miR-26a or its control was co-transfected with the specific pmirGLO construct containing a consensus miR-26a-binding site into HeLa. All cells were harvested at 24 h after transfection, and then luciferase activities were measured and normalized to the Renilla activities. (C) Western blotting was performed to determine GSK-3β protein levels in cardiomyocytes. Three independent transfection experiments were done and data was represented as mean ± S.D. (*$p < 0.001$ compared with control)

GSK3B are targets of miR-26a, which induced apoptosis and hypertrophy in cancer cells and airway smooth muscle cells, respectively [19,20,23]. Also, miR-26b which has the same seed sequence as miR-26a, targets SLC7A11 and results in induced apoptosis in human breast cancer [34]. Previously, GSK-3β inhibits cell death caused by the extrinsic apoptotic pathway. Extrinsic apoptotic signaling, activated by cell damage, is inhibited by GSK-3β by facilitation of signals that cause disruption of mitochondria and by regulation of transcription factors that control the expression of anti- or pro-apoptotic proteins [22]. Moreover, GSK-3β regulation by miR-26a has been recently reported in human airway smooth muscle [31]. To test whether GSK-3β is a miR-26a target gene in cardiomyocytes, we have confirmed that H_2O_2 decreases GSK-3β expression in cultured cardiomyocytes [35,36]. miR-26a is able to bind to GSK-3β and regulate its expression directly using a construct in which a fragment of the 3 its exprGSK-3β mRNA with the putative miR-26a binding sequence. This result indicates that GSK-3β is indeed a functional target gene of miR-26a that is involved in miR-26a-mediated pro-apoptotic effect on cardiomyocyte injury elicited by H_2O_2.

In other studies on the apoptotic stimulus in animal models of heart diseases, the pathophysiological functions of miR-1 and miR-133 have potential clinical implication [33,34]. Our study illustrates the role of miR-26a as pro-apoptotic modulator in the regulation of GSK-3β. Future studies, especially in ischemia/reperfusion models, are necessary to validate the possible therapeutic use of miR-26a regulation.

This study has shown that miRNAs are expressed specifically in various cell types or tissues. Also, miR-26a promotes apoptosis via the caspase-3 pathway in cardiomyocytes. Thus, miR-26a may be a new apoptotic miRNA in cardiomyocytes.

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References

[1] V.P. van Empel, A.T. Bertrand, L. Hofstra, et al., Myocyte apoptosis in heart failure, Cardiovasc. Res. 67 (2005) 21–29.
[2] R.A. Kloner, K. Przyklenk, P. Whittaker, Deleterious effects of oxygen radicals in ischemia/ reperfusion. Resolved and unresolved issues, Circulation 80 (1989) 1115–1127.
[3] L.H. Opie, Reperfusion injury and its pharmacologic modification, Circulation 80 (1989) 1049–1062.
[4] D. Sorescu, K.K. Griendling, Reactive oxygen species, mitochondria, and NAD(P)H oxidases in the development and progression of heart failure, Congest. Heart Fail. 8 (2002) 132–140.
[5] H. Tsutsui, S. Kinugawa, S. Matsushima, Mitochondrial oxidative stress and dysfunction in myocardial remodelling, Cardiovasc. Res. 81 (2009) 449–456.
[6] H. Takano, Y. Zou, H. Hasegawa, et al., Oxidative stress-induced signal transduction pathways in cardiac myocytes: involvement of ROS in heart diseases, Antioxid. Redox. Signal. 5 (2003) 789–794.
[7] K.A. Kaminski, T.A. Bonda, J. Korecki, et al., Oxidative stress and neutrophil activation–the two keystones of ischemia/reperfusion injury, Int. J. Cardiol. 86 (2002) 41–59.
[8] H. Song, B.K. Kim, W. Chang, et al., Tissue transglutaminase 2 promotes apoptosis of rat neonatal cardiomyocytes under oxidative stress, J. Recept. Signal Transduct. Res. 31 (2011) 66–74.
[9] V. Ambros, The functions of animal microRNAs, Nature 431 (2004) 350–355.
[10] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, Cell 136 (2009) 215–233.
[11] M. Han, J. Toli, M. Abdellatif, MicroRNAs in the cardiovascular system, Curr. Opin. Cardiol. 26 (2011) 181–189.
[12] K. Ono, Y. Kuwabara, J. Han, MicroRNAs and cardiovascular diseases, FEBS J. 278 (2011) 1619–1633.
[13] P. Li, MicroRNAs in cardiac apoptosis, J. Cardiovasc. Transl. Res. 3 (2010) 219–224.
[14] Z. Wang, MicroRNA: A matter of life or death, World J. Biol. Chem. 1 (2010) 41–54.
[15] C. Xu, Y. Lu, Z. Pan, et al., The muscle-specific microRNAs miR-1 and miR-133 produce opposing effects on apoptosis by targeting HSP60, HSP70 and caspase-9 in cardiomyocytes, J. Cell Sci. 120 (2007) 3045–3052.
[16] Y. Tang, J. Zheng, Y. Sun, et al., MicroRNA-1 regulates cardiomyocyte apoptosis by targeting Bcl-2, Int. Heart J. 50 (2009) 377–387.
[17] X.Y. Yu, Y.H. Song, Y.J. Geng, et al., Glucose induces apoptosis of cardiomyocytes via microRNA-1 and IGF-1, Biochem. Biophys. Res. Commun. 376 (2008) 548–552.
[18] S. Dong, Y. Cheng, J. Yang, et al., MicroRNA expression signature and the role of microRNA-21 in the early phase of acute myocardial infarction, J. Biol. Chem. 284 (2009) 29514–29525.
[19] B. Zhang, X.X. Liu, J.R. He, et al., Pathologically decreased miR-26a antagonizes apoptosis and facilitates carcinogenesis by targeting MTDH and EZH2 in breast cancer, Carcinogenesis 32 (2011) 2–9.
[20] N.M. Alajez, W. Shi, A.B. Hui, et al., Enhancer of Zeste homolog 2 (EZH2) is overexpressed in recurrent nasopharyngeal carcinoma and is regulated by miR-26a, miR-101, and miR-98, Cell Death Dis. 1 (2010) e85.
[21] M.H. Cha, J.S. Choi, S.J. Bai, et al., Antiallodynic effects of acupuncture in neuropathic rats, Yonsei Med. J. 47 (2006) 359–366.
[22] E. Beurel, R.S. Jope, The paradoxical pro- and anti-apoptotic actions of GSK3 in the intrinsic and extrinsic apoptosis signaling pathways, Prog. Neurobiol. 79 (4) (2006) 173–189.
[23] J.S. Mohamed, M.A. Lopez, A.M. Boriek, Mechanical stretch up-regulates microRNA-26a and induces human airway smooth muscle hypertrophy by suppressing glycogen synthase kinase-3β, J. Biol. Chem. 285 (38) (2010) 29336–29347.
[24] Q.M. Chen, V.C. Tu, Y. Wu, et al., Hydrogen peroxide dose dependent induction of cell death or hypertrophy in cardiomyocytes, Arch. Biochem. Biophys. 373 (2000) 242–248.
[25] S.H. Kwon, D.R. Pimentel, A. Remondino, et al., H(2)O(2) regulates cardiac myocyte phenotype via concentration-dependent activation of distinct kinase pathways, J. Mol. Cell. Cardiol. 35 (2003) 615–621.
[26] Y. Cheng, R. Ji, J. Yue, et al., MicroRNAs are aberrantly expressed in hypertrophic heart: do they play a role in cardiac hypertrophy? Am. J. Pathol. 170 (2007) 1831–1840.
[27] Q. Li, X.W. Song, J. Zou, et al., Attenuation of microRNA-1 derepresses the cytoskeleton regulatory protein twinfilin-1 to provoke cardiac hypertrophy, J. Cell Sci. 123 (2010) 2444–2452.
[28] X.P. Ren, J. Wu, X. Wang, et al., MicroRNA-320 is involved in the regulation of cardiac ischemia/reperfusion injury by targeting heat-shock protein 20, Circulation 119 (2009) 2357–2366.
[29] Y. Cheng, X. Liu, S. Zhang, et al., MicroRNA-21 protects against the H(2)O(2)-induced injury on cardiac myocytes via its target gene PDCD4, J. Mol. Cell. Cardiol. 47 (2009) 5–14.
[30] S. Rane, M. He, D. Sayed, et al., Downregulation of miR-199a derepresses hypoxia-inducible factor-1alpha and Sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes, Circ. Res. 104 (2009) 879–886.
[31] H. Zhu, Y. Yang, Y. Wang, et al., MicroRNA-195 promotes palmitate-induced apoptosis in cardiomyocytes by down-regulating Sirt1, Cardiovasc. Res. 92 (2011) 75–84.
[32] A. Carè, D. Catalucci, F. Felicetti, et al., MicroRNA-133 controls cardiac hypertrophy, Nat. Med. 13 (5) (2007) 613–618.
[33] B. Yang, H. Lin, J. Xiao, et al., The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2, Nat. Med. 13 (4) (2007) 486–491.
[34] X.X. Liu, X.J. Li, B. Zhang, et al., MicroRNA-26b is underexpressed in human breast cancer and induces cell apoptosis by targeting SLC7A11, FEBS Lett. 585 (2011) 1363–1367.
[35] H.O. Jun, D.H. Kim, S.W. Lee, et al., Clusterin protects H9c2 cardiomyocytes from oxidative stress-induced apoptosis via Akt/GSK-3β signaling pathway, Exp. Mol. Med. 43 (1) (2011) 53–61.
[36] K. Ohori, T. Miura, M. Tanno, et al., Ser9 phosphorylation of mitochondrial GSK-3beta is a primary mechanism of cardiomyocyte protection by erythropoietin against oxidant-induced apoptosis, Am. J. Physiol. Heart Circ. Physiol. 295 (5) (2008) H2079–2086.