Cellular Physiology
and Biochemistry

Cell Physiol Biochem 2015;37:921-932

DOI: 10.1159/000430219

Accepted: August 12, 2015

1421-9778/15/0373-0921$39.50/0

Original Paper

Combinatorial MicroRNAs Suppress Hypoxia-Induced Cardiomyocytes Apoptosis

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Key Words
MiRNA-1 • MiRNA-21 • Apoptosis • Synergy

Abstract

Background/Aims: Our previous *in silico* analysis revealed potential synergy in the activities of micro(mi)RNAs in myocardial infarction. The present study investigated whether miR-1 and -21 act synergistically to protect against cardiomyocytes apoptosis. ***Methods:*** Cell survival was analyzed with cell viability assay; apoptosis was detected by flow cytometry, terminal deoxynucleotidyl transferase dUTP nick end labeling, and the caspase-3 activity assay; and protein expression level was determined by western blotting. ***Results:*** MiR-1:miR-21 and several other miRNA pairs were evaluated for their potentially synergistic effects against myocardial hypoxia in neonatal rat ventricular cardiomyocytes. Lower combination indices suggested that miRNA pairs acted synergistically to inhibit apoptosis; miR-1 and -21 jointly blocked hypoxia-induced cardiomyocytes apoptosis. Moreover, combined application of miR-1 and -21 activated Akt and blocked hypoxia-induced upregulation of p53 in these cells. ***Conclusion:*** MiR-1 and -21 exert synergistic effects against hypoxia-induced cardiomyocytes apoptosis. These results provide a basis for the development of combined miRNA-based therapeutics to treat cardiovascular diseases.

Introduction

Ischemic heart disease is a major cause of morbidity and mortality worldwide; ischemia-induced inflammation, cardiomyocytes apoptosis, and cardiac fibrosis can lead to left ventricular dilatation and heart failure [1]. Cardiomyocytes apoptosis, which exists in all above pathological processes, is mediated by tightly regulated signaling pathways that can

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be activated by a variety of extra- or intracellular stimuli and involve downstream pro- or anti-apoptotic factors such as Akt and p53 [2–4].

Recent evidence has demonstrated key roles for micro(mi)RNAs in apoptosis [5–8]. For instance, the cardiomyocyte-specific miR-1 has anti-hypertrophic,-apoptotic, and -fibrotic effects [9], and was shown to restore cardiomyocytes function by derepressing the target gene, sodium-calcium exchanger-1 [10]. MiR-1-transfected embryonic stem cells transplanted into the heart of mice protected the host myocardium from ischemia-induced apoptosis via activation of phosphorylated (*p*-) Akt and inhibition of caspase-3, phosphorylated phosphatase and tensin homolog (*p*-PTEN), and superoxide production [11]. MiR-21 has also demonstrated anti-apoptotic effects in ischemic hearts. For example, miR-21 inhibited myocardial apoptosis by targeting programmed cell death protein (PDCD)4 in a mouse model of ischemia/reperfusion [12], and reduced ischemia-induced infarct size and suppressed apoptosis via up regulation of PTEN and Fas ligand (FasL) in mice [13]. On the other hand, the suppression of PDCD4 and PTEN activates Akt signaling, which in turn induces the up regulation of miR-21 in a positive feedback loop [14, 15].

A single miRNA can modulate the expression of multiple genes and can thus have effects similar to a multidrug cocktail. Synergistic interactions can increase the efficacy of therapeutics while reducing their side effects and slowing the development of drug resistance [16]. However, few studies have examined the therapeutic potential of using miRNA combinations for disease treatment [17, 18].

In a previous study, we explored global synergistic miRNA regulation of apoptosis by developing the topological parameter synergy score, which identifies indirect functional miRNA-miRNA interactions (MMIs) within a miRNA layer [19]. Several novel anti-apoptotic miRNA pairs were identified using this method, including miR-1: miR-21, suggesting that synergy is a key element of apoptosis regulated by miRNAs under pathophysiological conditions. The synergy score provides a quantitative measure of cooperation in miRNA-mediated gene regulation, but does not explain the molecular mechanisms underlying endogenous MMIs. In the present study, several MMIs were investigated for their synergistic regulation of apoptosis. The results provide insight into the mechanistic basis for apoptosis regulation through the coordinated activities of miRNAs.

Materials and Methods

Cell culture

Neonatal rat ventricular myocytes (NRVCs) and cardiac fibroblasts (CFs) were isolated and cultured from 1 to 3-day-old Sprague-Dawley rats, in which use of animals complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Eighth Edition, 2011) and pre-approved by the Experimental Animal Ethic Committee of the Harbin Medical University, China (Animal Experimental Ethical Inspection Protocol, No. 2009104). Briefly, hearts were quickly minced and digested with 0.25% trypsin. The cell suspensions were centrifuged at 2500 rpm for 3min, then cells were incubated for 2 h in the medium consisted of Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin. NRVCs were collected and plated in DMEM for another 48 h and CFs passaged by trypsin and used for studies at the 2nd to 4th passage. Human aortic endothelial cells (HAECs) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in Endothelial Cell Medium supplemented with endothelial cell growth factors, 5% FBS and 1% penicillin/streptomycin. The cells were maintained at 37 °C with 5% CO_2 and 95% air.

Transfection procedure and hypoxia treatment

MiR-1 (5'-UGG AAU GUA AAG AAG UGU GUAU-3'), miR-20a (5'-UAA AGU GCU UAU AGU GCA GGUAG-3'), miR-21 (5'-UAG CUU AUC AGA CUG AUG UUGA-3'), miR-146a (5'-UGA GAA CUG AAU UCC AUG GGUU-3'), miR-222 (5'-GGC UCA GUA GCC AGU GUA GAU-3'), anti-miR-1 oligonucleotide (AMO-1), negative control (NC) were synthesized by Ribobio (Guangzhou, China). After starvation in serum-free medium for 12 h. NRVCs were transfected with miR-1, -20a, -21, -146a, -222, AMO-1 and NC, or randomly matched pairs of miR-1, -20a, -21, -146a and -222 using Xtreme GENE siRNA transfection reagent (Roche, Switzerland). Transfection concentrations were in the range from 10 to 50 nM. 48 h after transfection NRVMs were treated with hypoxia by culturing cells under 1% O_2, 94% N_2, and 5% CO_2 for 24 h in a modular incubator.

Cell viability assay

Viability of cells cultured in the 96-well culture plates was assessed by measuring mitochondrial dehydrogenase activity. After miRNA transfection and hypoxia treatment, NRVCs were incubated with 10μL MTT of 0.5 mg/mL at 37 °C for 4 h. The purple formazan crystal was dissolved with 150 μL of dimethyl sulfoxide (DMSO) and added to the cells. The absorbance was measured by spectrophotometer (Tecan Group Ltd., Switzerland) at 490 nm. To calculate the combination index of synergistic cardioprotection by pair-wise miRNAs, we defined a parameter Relative Recovery Rate of cell viability (RRR) here, which could be transformed from the original results of MMT assay. The RRRs of each miRNA or miRNA pairs were calculated as described (A means absorbance value):

$$RRR = \frac{A_{miR} - \overline{A_{hypoxia}}}{\overline{A_{Ctrl}}} \times 100\%$$

TUNEL staining assay

After three times PBS washing, treated NRVCs were fixed by 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 sodium citrate buffer. Then an In Stiu cell death detection kits (Roche) were used to label apoptotic cells, and the nuclei were stained with DAPI. The numbers of total cells and TUNEL positive cells were automatically counted by Image-Pro plus version. The apoptosis rate was defined as ratio of apoptotic cells to total cells.

Caspase-3 activity assay

Caspase-3 activity was determined by using Caspase-3 Activity Assay Kit (Beyotime Institute of Biotechnology, China), which is based on the ability of caspase-3 to change acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) into the yellow formazan product p-nitroaniline (pNA). Briefly, the cells were harvested and washed with cool PBS twice, and then the cells were lysed with lysis buffer (100 μL per 2×10^6 cells) for 15 min on ice. The lysate was centrifuged (13500 r/min) for 15 min at 4°C, and then collected the supernatant and protein concentration was determined by Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, China). After incubating the mixture composed of 35 μL of cell lysate, 55 μL reaction buffer and 10 μL of 2 mM caspase-3 substrate (Ac-DEVD-pNA) in 96-well plates at 37°C overnight, the absorbance of p-nitroanilide at 405 nm was determined by using a microtiter plate reader (Bio-TEK Epoch, BioTek Instrument, VT, USA). Caspase-3 activity was calculated as a ratio of p-nitroanilide content to total protein amount. The detail analysis procedure was described in the manufacturer's protocol (Beyotime Institute of Biotechnology, China).

Detection of apoptosis by flow cytometry (FCM)

Cell apoptosis was detected by the Annexin V-FITC/propidium iodide (AV/PI) dual staining (Biosea Biotechnology, China) using commercial kits. Both staining procedures were performed in accordance with the manufacturers' instructions. Annexin V-FITC Apoptosis Detection kit was utilized to detect early apoptosis (Annexin V-FITC+/PI−, Q4), late apoptosis (AnnexinV-FITC+/PI+, Q2), and necrosis (Annexin V-FITC−/PI+, Q1). Briefly, after various treatments, the cells were digested with 0.25% trypsin and collected by centrifugation. After being washed twice with PBS, the cells were stained with Annexin V-FITC for 15 min and PI for 5 min. The apoptotic cells were identified by FCM. The number of analyzed cells was 10000 in each individual experiment.

Real-time RT-PCR

Total RNA was isolated from NRVMs using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The levels of P53, miR-1 and miR-21 were determined using SYBR Green Mix (Invitrogen, Carlsbad, CA, USA) on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The following p53 primers were used for PCR detection: Forward 5'-GCCGGCCCATCCTTACC-3'; Reverse 5'-CCGGGCAATGCTCTTCTTT-3'. Relative quantitative method was used by normalizing the amount of detected transcripts to the internal control GAPDH for target mRNAs or U6 for miRNAs. The relative value to the control sample was given by $2^{-\Delta\Delta CT}$.

Western blotting assay

Total protein was extracted from cultured cardiomyocytes and 80-μg protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane, which was blocked with 5% non-fat milk for 2 h at room temperature, then probed with primary antibody including AKT (1:1000 dilution, Cell Signaling Technology, Beverly, MA, USA), *p*-AKT (1:1000 dilution, Cell Signaling Technology, Beverly, MA, USA), P53 (1:1000 dilution, Cell Signaling Technology, Beverly, MA, USA), GAPDH (1:1000 dilution, KangchengInc, China) in PBS and incubated at 4°C overnight. The membranes were washed with PBS-T and then incubated with secondary antibody (Alexa Fluor) for 1 h at room temperature. Western blot bands were quantified by using Odyssey infrared imaging system (LI-COR) and Odyssey v3.0 software. Results are expressed as fold change after normalizing data to control values.

Statistical analyses

All data are expressed as mean ± SEM. One-way ANOVA followed by the Bonferroni multiple comparison post hoc test was carried out using the Graphpad Prism 6.0. Values of $p < 0.05$ were considered significant.

Results

MiRNAs act synergistically to protect against hypoxia-induced cardiomyocytes injury

As evidenced by the synergy score [19], some miRNA pairs acted in concert to inhibit apoptosis (Fig. 1A). Although miR-20a, -21, -146a, and -222 are anti-apoptotic miRNAs

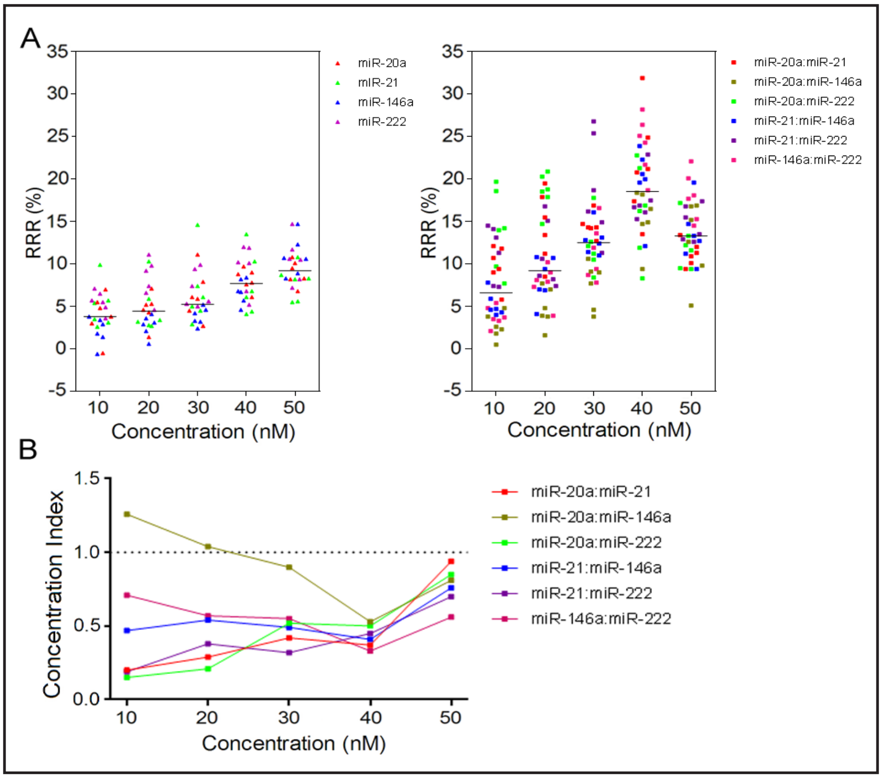

Fig. 1. MiRNA-mediated RRR under hypoxic conditions in NRVCs. A. RRRs in the presence of single miRNAs (left panel) or miRNA pairs (right panel). (B) Synergistic cardioprotection by miRNA co-transfection at concentrations ranging from 10 to 50 nM as combination index values indicated. RRR: relative recovery rate of cell viability; NRVCs: neonatal rat ventricular myocytes; n = 6 batches of cells.

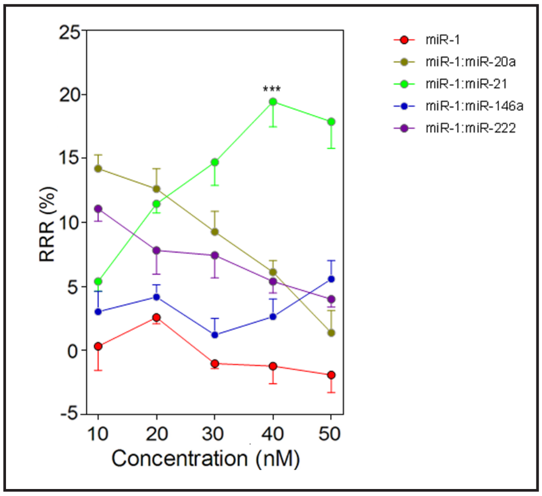

Fig. 2. Comparisons of RRRs between miR-1 alone and miRNA pairs including miR-1:miR-20a, miR-1:miR-21, miR-1:miR-146a, and miR-1:miR-22. *** $p < 0.001$, Co-transfection of miR-1 and miR-21 versus the other miRNA transfection groups at 40 nM. RRR: relative recovery rate of cell viability; n = 6 batches of cells.

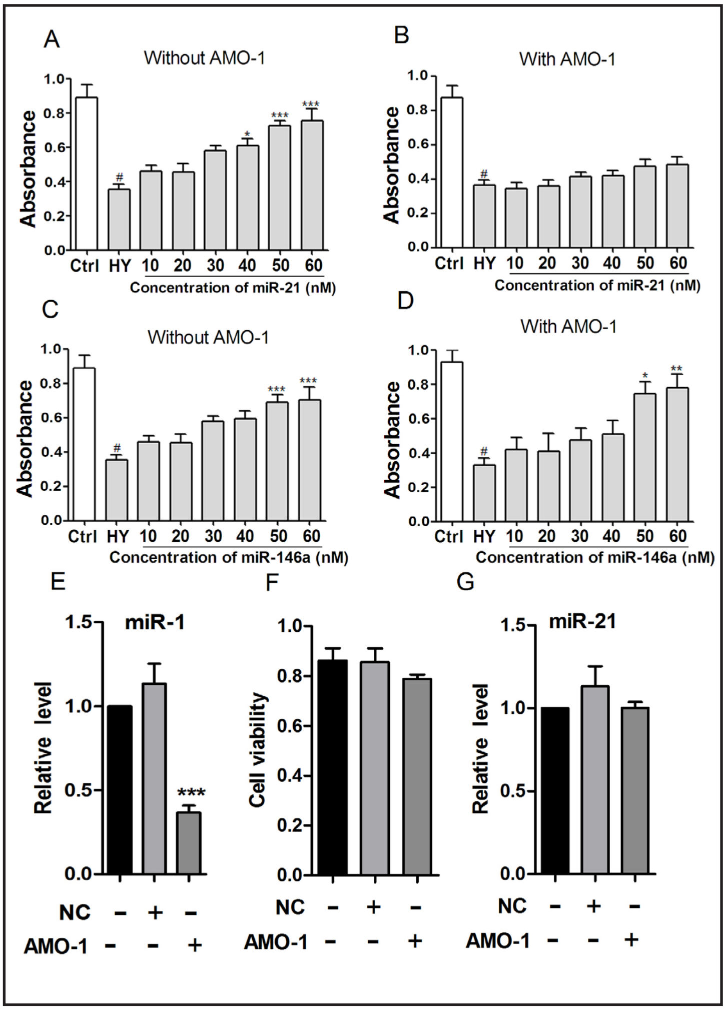

Fig. 3. Results of RRRs by miR-21 (A, B) and miR-146a (C, D) without or with co-transfection of 40 nM AMO-1 in hypoxic NRVCs. n = 6 batches of cells; miR-21 group vs. AMO-1+miR-21 group *$p < 0.05$, vs. Ctrl; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs. HY. (E) 40nM AMO-1 significantly decreased the expression of miR-1. n=4 batches of cells; ***$p < 0.001$, vs. NC. (F) NRVCs viability as detected by MTT assay showing the lack of ability of AMO-1 alone to alter cell viability. (G) 40nM AMO-1 has no obvious effect on the expression of miR-21. NRVCs: neonatal rat ventricular myocytes; Ctrl: control; HY: hypoxia; NRVCs: neonatal rat ventricular myocytes; RRR: Relative Recovery Rate of cell viability; NC indicates negative control.

[5-8], they did not significantly improve cell viability when transfected individually in the concentration range from 10-50 nM in hypoxia-treated NRVCs. In contrast, higher RRRs were obtained by transfecting miRNA pairs. For example, miR-20a and -21 (40 nM) restored cell viability by 8.2% and 7.5%, respectively, but when administered simultaneously resulted in

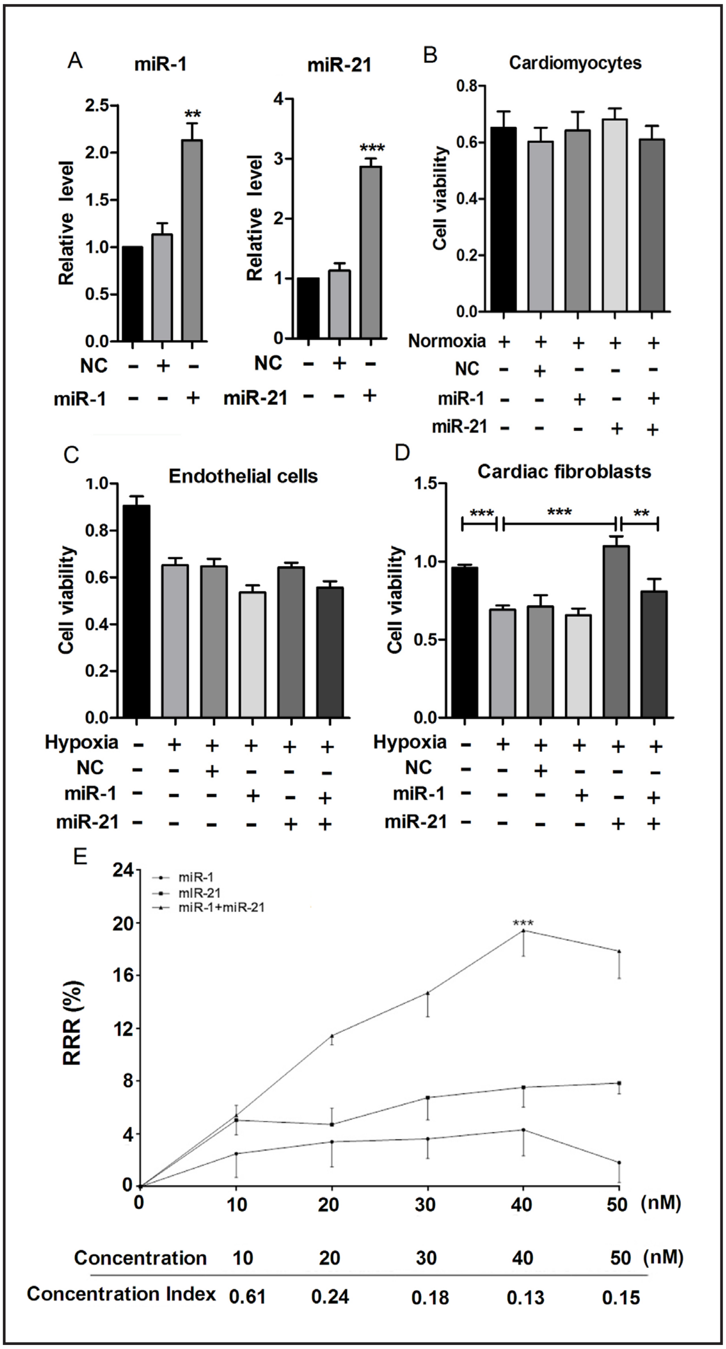

Fig. 4. Efficiency of miRNA transfection in NRVCs. (A) Transfection of miR-1/-21 (40 nM) was obviously increase the expression level of miR-1/-21 in cardiomyocytes. n=4 batches of cells; **$p < 0.01$, vs. NC; ***$p < 0.001$, vs. NC. (B) NRVCs viability was detected by MTT assay showing the lack of ability of miR-1or/and miR-21 to alter cell viability under normoxia conditions. (C) Co-transfection of miR-1 and miR-21 have no obvious effect in endothelial cells. (D) The effect of co-used of miR-1and miR-21in cardiac fibroblasts under hypoxic conditions. n = 4 batches of cells; ***$p < 0.001$, control vs. Hypoxia; ***$p < 0.001$, miR-21 vs. Hypoxia; **$p < 0.01$, miR-1+miR-21 vs. miR-21. (E) Dose-response curves of NRVCs transfected with miR-1, -21, or both (1:1 ratio) at indicated concentrations; synergy of the cardioprotective effects was determined by combination index values. NRVCs: neonatal rat ventricular myocytes; NC indicates negative control.

an average RRR of 21.6%. These findings imply a strong synergy between the two miRNAs, which was confirmed by the calculated combination index (CI) values (Fig. 1B) [16].

Specific and strong synergy is exhibited by miR-1 and -21

Synergistic effect between miR-1 and -21 was obvious, despite the fact that miR-1 alone had no significant effect on apoptosis at low concentrations (10-50 nM) (Fig. 2). The low synergy score of 1.61 observed for miR-1 and -146a suggested inefficient synergy between these two miRNAs in apoptosis (Fig. 2). Inhibition of miR-1 abolished the anti-apoptotic effect of miR-21 but not miR-146a, underscoring the importance of miR-1 interaction for the anti-apoptotic effects of miR-21 in NRVCs (Fig. 3A-D). Conversely, as predicted from the lack of synergy between miR-146a and miR-1, suppressing miR-1 expression did not affect

DOI: 10.1159/000430219

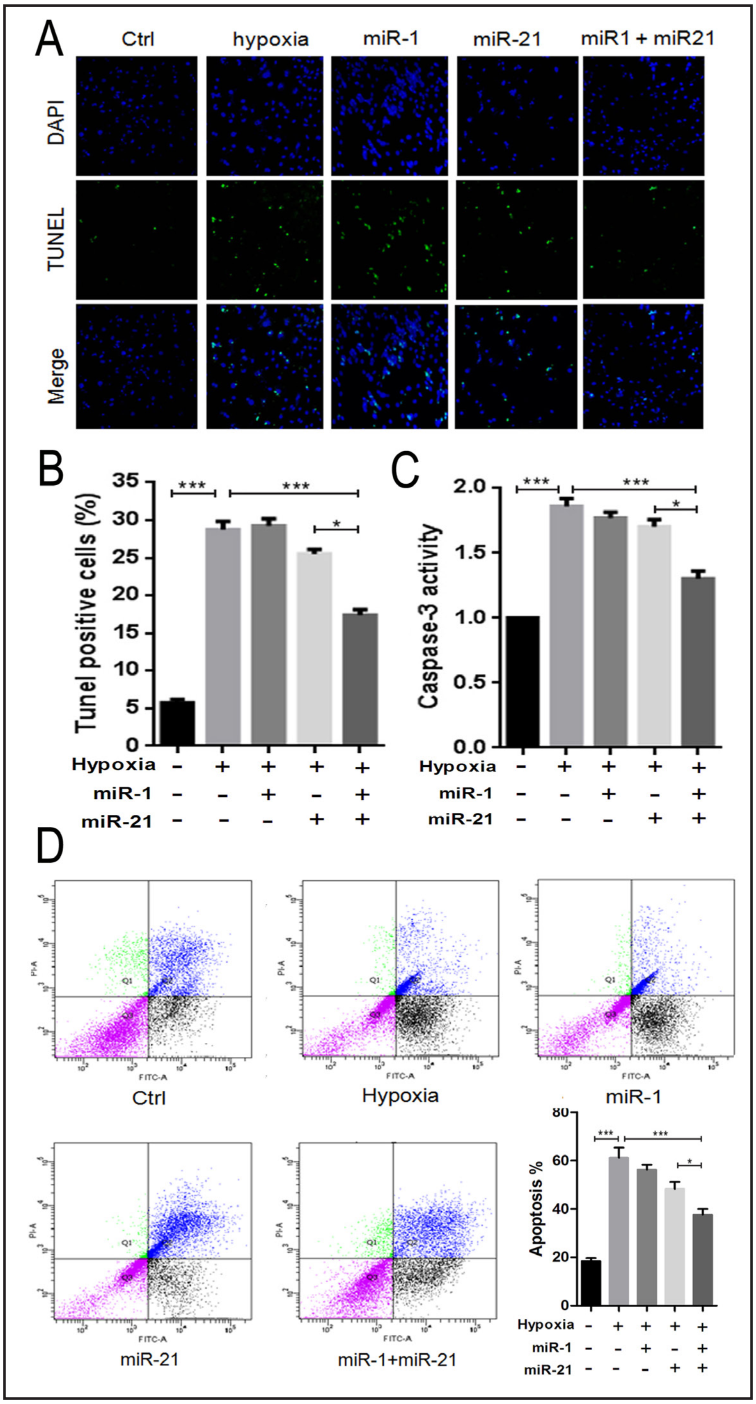

Fig. 5. Suppression of hypoxia-induced cardiomyocyte apoptosis by a combination of miR-1 and -21. (A, B) Hypoxia-induced NRVCs apoptosis was evaluated by the TUNEL assay. Apoptotic cells are green, and the nuclei are stained blue with DAPI. ***$p < 0.001$, vs. Hypoxia; *$p < 0.05$, miR-1+miR-21 vs miR-21. n = 4 batches of cells; (C) Caspase-3 activity in NRVCs after hypoxia and co-transfection of miR-1 and -21. ***$p < 0.001$, vs. Hypoxia; *$p < 0.05$, miR-1+miR-21 vs. miR-21. n = 6 batches of cells; (D) Hypoxia-induced NRVCs apoptosis detected by flow cytometry. ***$p < 0.001$, vs. hypoxia; *$p < 0.05$, miR-1+miR-21vs. miR-21. n = 3 batches of cells.

the modulation of apoptosis by miR-146a. Additionally, we examined the potential effect of AMO-1 on the expression levels of miR-1 and -21 in cardiomyocytes and the viability of the cells (Fig. 3E-G). Transfection of AMO-1 was found to remarkably inhibit the expression of miR-1 but show no obvious effect on that of miR-21 and the viability of the cardiomyocytes under normoxic conditions.

Furthermore, we evaluated the efficacy of miR-1 combined with miR-21 in NRVCs transfected with each miRNA individually or co-transfected with both miRNAs at concentrations ranging from 10-50 nM at a 1:1 ratio. Single transfection of miR-1/-21 (40 nM) was found to obviously increase the expression level of miR-1/-21 in cardiomyocytes (Fig. 4A). No obvious effect was observed on viability of cardiomyocytes by miRNA transfection

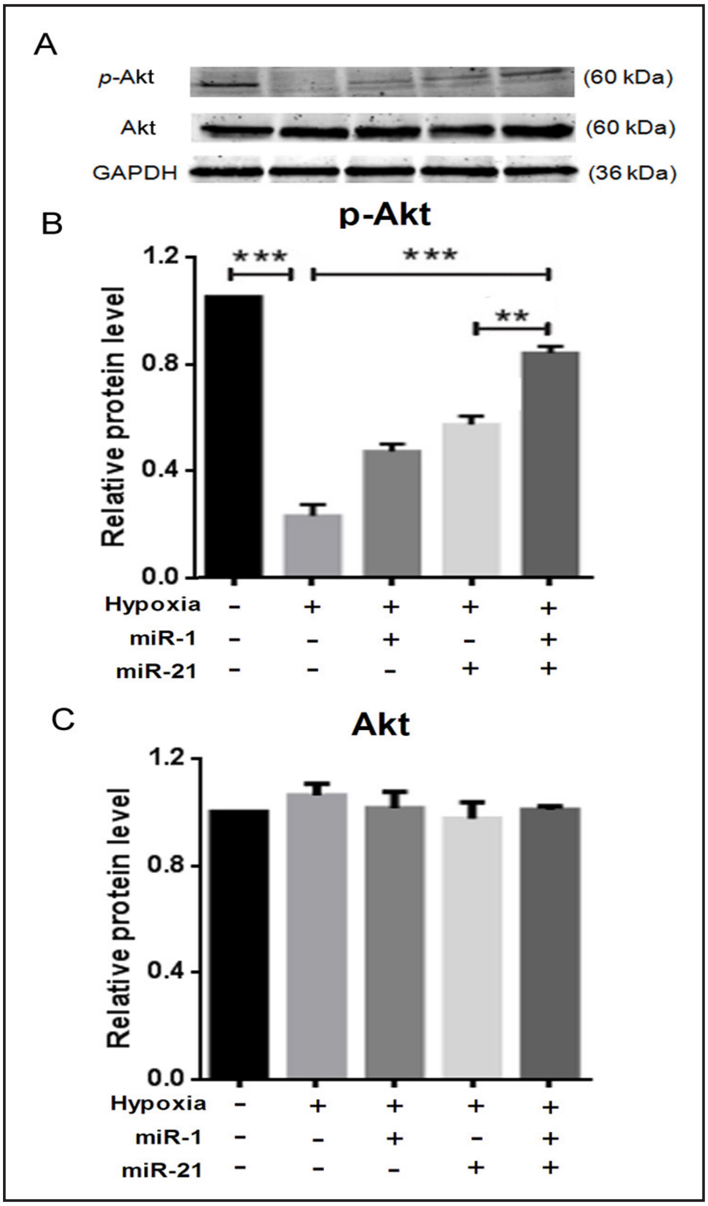

Fig. 6. Akt signaling is activated by combined treatment with miR-1 and -21. (A) Protein levels of *p*-Akt and total Akt was evaluated by western blotting; GAPDH was used as a loading control. n = 4 batches of cells; (B) Effect of miR-1, -21, or both on Akt phosphorylation level. ***$p < 0.001$, control vs. Hypoxia; ***$p < 0.001$ miR-1+miR-21 vs. Hypoxia; ***$p < 0.001$, miR-1+miR-21 vs. miR-21. n = 4 batches of cells; (C) Effect of miR-1, -21, or both on total Akt expression. n = 4 batches of cells.

under normoxic conditions (Fig. 4B). Cardiac fibroblasts and endothelial cells were also evaluated for cell survival when miR-1 or/and miR-21 were transfected (Fig. 4C and D). No obvious effect was observed in endothelial cells. In cardiac fibroblasts, single treatment of miR-21 remarkably increased the viability of the cells. However, when co-transfected with miR-1 such an effect was partially offset by miR-1. The dose-response curves for the two miRNAs revealed that at 40 nM, miR-1 and -21 jointly reduced the rate of NRVCs apoptosis (Fig. 4E), with the CI of 0.13 suggesting strong synergy between the two miRNAs [16].

MiR-1 and -21 synergize to block hypoxia-induced cardiomyocyte apoptosis

To better characterize the effects of the miRNA combination in cardiomyocytes apoptosis, we investigated the anti-apoptotic effects of miR-1 in conjunction with miR-21 in greater detail by examining NRVCs apoptosis with the TUNEL and caspase-3 activity assays and by FCM analysis. As shown in Fig. 5, the results revealed the potent synergistic activities of miR-1 and -21 against hypoxia-induced apoptosis and significantly reduced the activity of caspase-3: when miR-21was co-transfected with miR-1, the rate of apoptosis was comparable to that of control cells.

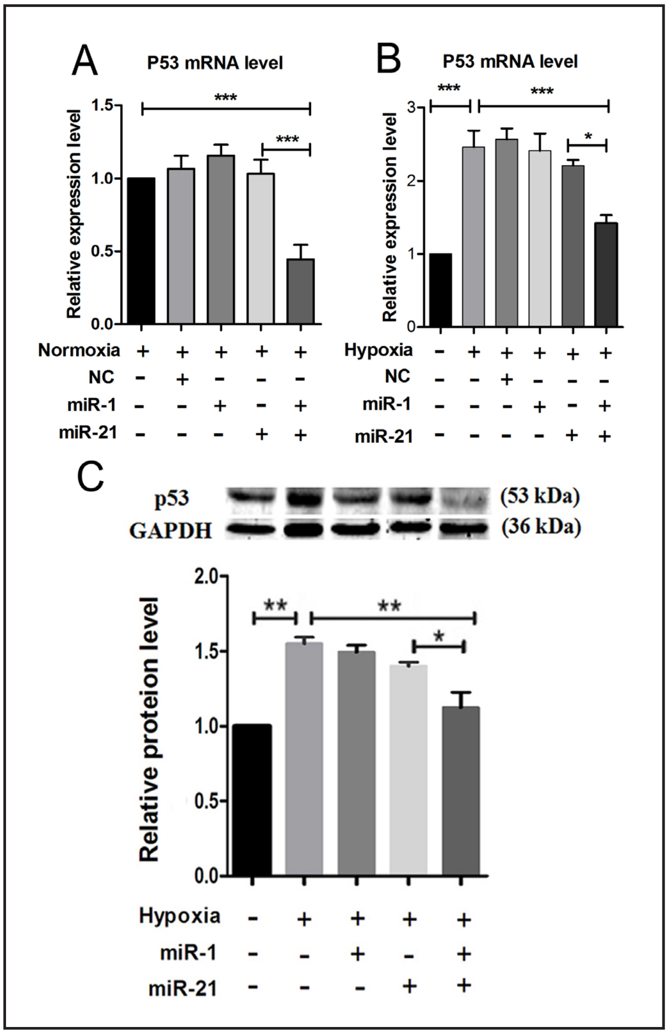

Fig. 7. Decrease in p53 expression by a combination of miR-1 and -21. (A) mRNA level of p53 was evaluated by real-time PCR after miR-1 and miR-21 co-transfection under normoxia conditions in NRVCs. n=4 batches of cells. ***$p<0.001$ control vs. miR-1+miR-21; ***$p<0.001$ miR-21 vs. miR-1+miR-21. (B) mRNA level of p53 was evaluated after miR-1 and miR-21 co-transfection under hypoxic conditions in NRVCs. ***$p < 0.001$, control vs. Hypoxia; ***$p < 0.001$, miR-1+miR-21 vs. Hypoxia; *$p < 0.05$, miR-1+miR-21 vs. miR-21. n = 4 batches of cells. (C) Protein level of p53 was detected by western blotting. **$p < 0.01$, control vs. Hypoxia; **$p < 0.01$, miR-1+miR-21 vs. Hypoxia; *$p < 0.05$, miR-1+miR-21 vs. miR-21. n = 4 batches of cells; NC indicates negative control.

MiR-1 and -21synergistically enhance Akt activation

Akt is an important component of pro-survival signaling pathways [20]; activated Akt inhibits apoptosis via direct and indirect mechanisms [21, 22].We examined the effects of combined miR-1 and -21 application on Akt activation in cultured cardiomyocytes and found that hypoxia markedly inhibited *p*-Akt expression, which was partly rescued by co-transfection of miR-1 and -21 as compared to either miRNA alone (Fig. 6), while the expression of the total AKT was unaltered.

Co-transfection of miR-1 and -21 lowers p53 expression level

Hypoxia can induce the extent of cell injury and death during acute and chronic myocardial ischemia [23, 24]. P53 is a tumor suppressor that plays a critical role in cell cycle regulation and apoptosis in response to hypoxia and ischemic stress [25-27]. Hyperactivated p53 induces apoptosis in response to ischemia; as such, inhibiting p53-mediated apoptotic signaling is a potential strategy for preventing ischemia-induced myocardial injury [28, 29]. To determine whether the anti-apoptotic effects of combined miR-1 and -21 treatments are exerted via modulation of p53, we examined the expression levels of p53 by RT-PCR and western blotting. After miR-1 and miR-21 transfection, remarkable decrease in the mRNA level of p53 was found under both normoxic and hypoxic conditions, implying strong anti-apoptotic role by miRNA combination (Fig. 7A and B). Hypoxia increased the protein expression level of p53 in cultured cardiomyocytes, but this effect was abrogated by co-expression of miR-1 and -21(Fig. 7C).

Discussion

MiRNAs play vital roles in pathological conditions involving apoptosis, including acute myocardial infarction and heart failure [30], and recent studies support combinatorial therapeutics using miRNAs as a promising approach in disease therapy [31]. However, the possibility of preventing cardiomyocytes apoptosis by the combined use of miR-1 and -21 has never been previously investigated. In the present study, we found that miR-1 and -21 act synergistically to protect against hypoxia-induced apoptosis by activating Akt and inhibiting p53 expression. These findings provide insight into the regulation of apoptosis via miRNA cross-talk.

MiRNAs are usually considered as either anti- or pro-apoptotic; however, the effect of several miRNAs working in concert may not be a linear sum of their individual activities; this is underscored by the redundancy in miRNA-mediated post-transcriptional gene regulation. In the context of apoptosis, coordinated gene regulation by miRNAs confers cells with greater flexibility in their viability status, which enables them to adapt to fluctuations in the internal and external environments. Thus, it can not be assumed that the effects of anti- and pro-apoptotic miRNAs acting coordinately cancel each other out. The lower CI values observed in our study indicate that miRNA synergy contributes to miRNA-mediated apoptosis regulation, which is consistent with the findings of previous studies [32, 33]. We propose that the regulation of gene expression by miRNAs should be viewed not only as the result of the activities of individual miRNAs but as a network of synergistic interactions.

MiR-1 and -21 were shown to act synergistically to suppress apoptosis. However, we did not observe any anti-apoptotic activity associated with miR-21 when miR-1 expression was inhibited in NRVCs under hypoxic conditions, suggesting the pro- or anti-apoptotic functions ascribed to certain miRNAs may be context-dependent. Moreover, some miRNAs such as miR-21 may function effectively only through synergistic interactions with other miRNAs [34]. These interactions are likely highly selective, since the apoptotic activity of miR-146a and others was unaffected by miR-1 inhibition.

MiR-21 has been shown to suppress the enhancement of AKT activity by PTEN and thereby inhibit apoptosis [35], while miR-1 protects against ischemia-induced apoptosis via *p*-AKT activation [11]. In the present study, we found that co-transfection of miR-1 and -21 significantly increased AKT phosphorylation and consequently, activation while suppressing p53 expression. An increase in the level of p53 is essential for ER stress-induced apoptosis [36], whereas inhibiting p53 expression attenuates myocardial ischemia-reperfusion injury [37]. Based upon the mechanisms of miRNA regulation on multiple genes, we speculated that besides AKT and p53 other biological factors and pathways might be also involved in synergetic apoptosis by miR-1 and -21. Altered global gene expression by miR-1 and miR-21 should be considered to investigate in the future for further elucidating the cooperative mechanisms underlying the cardiac protection brought by miR-1 and miR-21.

In conclusion, the present study provides novel evidence for miRNA synergy in cardiomyocytes apoptosis. Although downstream signaling by Akt and p53 was not examined in detail, our findings nonetheless indicate that functional MMIs should be considered when evaluating the contribution of miRNAs to apoptosis regulation, and also provide theoretical guidance for clinical applications such as the treatment of cardiovascular diseases. Caution should be maintained that, as a single miRNA can targets hundreds of genes, this provide an chance for the happening of potential adverse side effects when miR-1 and -21 are co-used. Thus, comprehensive *in vivo* experiments are definitely needed to investigate whether the combinatorial miRNAs constitute a substantial impact on other cells and tissues.

Acknowledgments

This work was supported by Research Fund for the Major Program of National Natural Science Foundation of China (81230081), the Doctoral Program of Higher Education (20112307130004).

Disclosure Statement

The authors declare that they have no conflict of interests.

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