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Na^+–Ca^{2+} exchanger targeting miR-132 prevents apoptosis of cardiomyocytes under hypoxic condition by suppressing Ca^{2+} overload

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ABSTRACT

During ischemia-reperfusion (IR) injury of the heart, Ca^{2+} overload occurs, leading to cardiomyocyte dysfunction and eventual cell death by apoptosis. Since preventing Ca^{2+} overload during IR injury has been reported to protect cardiomyocytes, interrupting Ca^{2+} signaling cascades leading to Ca^{2+} overload may exert protective effect on cardiomyocytes under hypoxic condition. One of the key regulators of the intracellular Ca^{2+} level during IR injury is Na^+–Ca^{2+} exchanger 1 (NCX1), whose down-regulation during IR injury conferred protection of heart. In the present study, we examined whether down-regulation of NCX1 using exogenous microRNA ameliorates apoptosis of cardiomyocytes under hypoxic condition. Here, we identified miR-132 as a novel microRNA targeting the NCX1, whose expression increased during hypoxia. Delivery of miR-132 suppressed the increase of intracellular Ca^{2+} in cardiomyocytes under hypoxia, and the expressions of apoptotic molecules, such as Bax, cytochrome C, and caspase 3, and the number of apoptotic cells were also decreased by exogenous miR-132 treatment. These results suggest the potential of miR-132 as an effective therapeutic agent against IR damage to heart by preventing Ca^{2+} overload during hypoxic condition and warrant further studies to validate its anti-apoptotic effect *in vivo*.

1. Introduction

Severe damage to heart, such as ischemia-reperfusion (IR) injury and myocardial infarction, has been associated with apoptosis of cardiomyocyte [1–3]. Myocardial ischemia, which premises absence of oxygen, changes cell metabolism to anaerobic respiration, and this decreases intracellular pH by producing lactate [4]. To cope with the accumulation of intracellular H^+, Na^+–H^+ exchanger (NHE) is activated to extrude H^+, resulting in intracellular Na^+ overload. In turn, to compensate the intracellular Na^+ overload, Na^+–Ca^{2+} exchanger (NCX) goes into reverse mode to extrude excessive Na^+, eventually leading to intracellular Ca^{2+} overload [5]. Furthermore, a burst of oxidative stress is produced during reperfusion [6], and this stimulates Ca^{2+} release channel of the sarcoplasmic reticulum further exacerbating Ca^{2+} overload [7], which can lead to cytotoxicity and trigger eventual cell death [8]. The sustained rise of Ca^{2+} has been associated with irreversible cell injury, and interventions that reduced the rise in Ca^{2+} during IR injury attenuated cell death [9]. Thus, it may be possible to prevent IR-induced cardiomyocyte death by interrupting IR-induced signaling cascade leading to Ca^{2+} overload, and one of the candidate molecules whose suppression may prevent IR-induced Ca^{2+} overload is NCX.

NCX plays a crucial role in maintaining Ca^{2+} homeostasis in the heart under physiologic condition, but in its reverse mode during

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reperfusion, it facilitates and exacerbates Ca^{2+} overload [10,11]. Furthermore, different from NHE whose inhibition failed to prevent Ca^{2+} overload [12], cardiac specific ablation of NCX protected heart from IR injury [13], suggesting down-regulation of NCX may confer protection against IR injury to cardiomyocytes. In fact, there are previous studies demonstrated that inhibition of NCX using chemical compounds protected cardiomyocytes from IR injury [14–16]. In addition, recent studies have indicated that microRNAs can be used instead of chemical compounds for regulating expressions of NCX [17,18]. MicroRNAs (miRNAs or miR) are a class of endogenously expressed noncoding RNAs that control the stability and translation of protein-coding mRNAs [19]. A number of studies have reported that miRNAs as effective regulators in IR injured heart [20,21], suggesting miRNAs as potent therapeutic targets and tools for preventing IR damage to heart. Currently, there is a no single most effective way to treat/manage IR-induced heart damage. Thus, until an optimized therapeutic strategy is established, the efforts to find alternative means (i.e., different miRNAs) can be justified. In the present study, we examined the feasibility of ameliorating apoptosis of cardiomyocytes using exogenous miRNA targeting NCX1. We first screened miRNAs for targeting NCX1 on miRNA target prediction program, and the effect of selected miRNA on hypoxia-induced cardiomyocyte apoptosis was further examined.

2. Materials 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. Neonatal rat cardiomyocytes from 1 to 2 day-old Sprague Dawley rat pups were isolated. Detailed methods are presented in the Supplementary Materials.

2.2. Hypoxia and cell viability assay

Cardiomyocytes with serum free α-MEM were incubated in hypoxic chamber maintained 1% O_2, 5% CO_2, and 94% N_2 at 37 °C. After the incubation period, (cell counting kit-8, Dojindo) was added to each well for a final concentration of 0.5 mg/mL and the cells were incubated at 37 °C for 2 h. The absorbance of the samples was measured at 450 nm using a microplate reader.

2.3. Measurement of intracellular Ca^{2+}

The measurement of cytosolic free Ca^{2+} was performed by using Fluo-4 AM (Invitrogen) and confocal microscopy analysis. Detailed methods are presented in the Supplementary materials.

2.4. Reverse transcription polymerase chain reaction

Total RNA was prepared using the TRIzol® reagent (Sigma–Aldrich). Complementary DNA (cDNA) was synthesized from RNA by AMV reverse transcriptase in RT system kit (Promega). Detailed methods are presented in the Supplementary materials.

2.5. Western blot

Proteins were separated by SDS-PAGE gels and then transferred to PVDF membranes. The blotted membranes were probed with indicated primary antibodies and secondary antibodies, goat anti-mouse or goat anti-rabbit IgG-peroxidases. Detailed methods are presented in the Supplementary materials.

2.6. MicroRNA transfection

Transfections of miRNA mimics and anti-miRNAs were performed using siLentFect™ Lipid reagent (Life Science Research). After 4 h incubation in a CO_2 incubator at 37 °C, the medium was changed to conditioned medium. Detailed methods are presented in the Supplementary materials.

2.7. Luciferase reporter assay

Relative luciferase activity was measured by using Dual Luciferase assay kit (Promega) according to the manufacturer's instructions. Detailed methods are presented in the Supplementary materials.

2.8. Real-time PCR

Total RNA was isolated with the TRIzol® reagent (Sigma–Aldrich). In brief, 100 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. Detailed methods are presented in the Supplementary materials.

2.9. Flow cytometry for detection of apoptosis

Apoptosis was measured using an FITC Annexin V Apoptosis Detection Kit I (BD pharmingen™). Detailed methods are presented in the Supplementary materials.

2.10. Caspase 3 activity assay

Caspase 3 activity was measured using Caspase 3 colorimetric activity assay kit (Millipore). Detailed methods are presented in the Supplementary materials.

2.11. Statistical analysis

Quantitative data were expressed as the means ± SEM. For statistical analysis, one-way ANOVA with Bonferroni correction was performed using the OriginPro 8 SR4 software (ver. 8.0951, OriginLab Corporation, Northampton, MA, USA) if there were more than 3 groups. For two group comparison, student's *t*-test was used. A p value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Hypoxia-induced death of cardiomyocytes is associated with Ca^{2+} overload and increased NCX1 expression

To examine the effect of hypoxia on cardiomyocyte viability, cardiomyocytes were exposed to hypoxic condition for up to 12 h and cell viability was measured. The number of viable cells decreased as time increased (Fig. 1A), while hypoxia increased the intracellular Ca^{2+} level in cardiomyocytes in a time-dependent manner, indicating hypoxia-induced Ca^{2+} overload (Fig. 1B). To further examine whether the increase of intracellular Ca^{2+} was associated with Ca^{2+} handling proteins, we examined the expressions of key Ca^{2+} regulating proteins, namely RYR2 [22], NCX1, and PLB [23]. The mRNA expressions of these proteins were up-

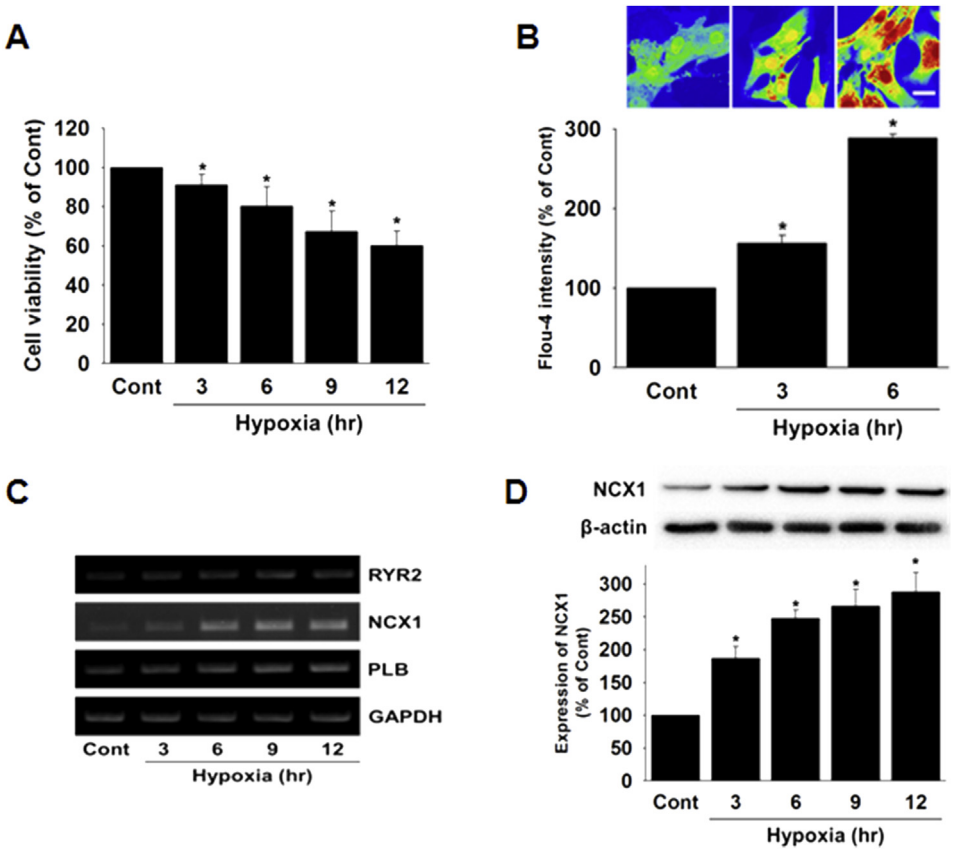

Fig. 1. Hypoxia-induced death of cardiomyocytes is associated with Ca^{2+} overload and increased Na^{+}-Ca^{2+} exchanger 1 expression. Rat cardiomyocytes were cultured under hypoxic condition for up to 12 h. A) Cell viability was evaluated using a cell counting kit-8. B) Relative amount of intracellular Ca^{2+} was determined by Fluo-4 calcium imaging. Scale bar = 20 μm. C) RNA expressions of key Ca^{2+} handling proteins. RYR2: rynodine receptor2, NCX1: Na^{+}-Ca^{2+} exchanger 1, PLB: phospholamban. D) Time-dependent changes of NCX1 protein expression under hypoxic condition. *$p < 0.05$ compared to untreated control. All the quantitative data represent the means ± SEM of three independent experiments.

regulated by hypoxia (Fig. 1C). Furthermore, the results of western blot using anti-NCX1 antibodies indicated that the expression of NCX1 also increased at protein level (Fig. 1D), suggesting the increased expression of NCX1 may have contributed to the observed Ca^{2+} overload in cardiomyocytes exposed hypoxic condition, as well as the decreased viability of cardiomyocytes under hypoxic condition.

3.2. Identification of miR-132 as a novel NCX1-targeting miRNA

Since miRNAs have been implicated in the Ca^{2+} regulation in cardiac disease [24,25] and miRNAs can regulate multiple targets [26], it was possible that miRNAs other than miR-1 and miR-214, which have been reported to target NCX1 [17,18], also down-regulate the expression of NCX1. To find such candidate miRNAs, we have screened number of miRNAs base on two criteria; 1) miRNAs predicted to target NCX1 and 2) miRNAs that have been implicated in heart failure. First, we have selected 22 miRNAs broadly conserved among vertebrates based on miRNA database (www.TargetScan.org), and candidate miRNAs were further down-sized based on possible association with heart failure by cross-checking literature [25,27–29]. With this approach, we have selected 6 candidate miRNAs for empirical verification (Fig. 2A). When cardiomyocytes were transfected with those candidate miRNAs and exposed to hypoxia, the expression of NCX1 was significantly attenuated in the miR-132 transfected group (Fig. 2B). Furthermore, miR-132 treatment significantly attenuated intracellular Ca^{2+} increase in cardiomyocytes exposed to hypoxia, while miR-212, a tandem miRNA of miR-312 belongs to a same cluster [30], did not (Fig. 2C). Also, the treatment of miR-132 or anti-miR-132 did not lead to any identifiable effects on cell viability under normal condition (Fig. 2D). The result of luciferase assay indicated that the 3′UTR of NCX1 was targeted by miR-132. Additionally, anti-miR-132 had no significant effect on the activity of luciferase (Fig. 2E).

3.3. The expression of miR-132 decrease under hypoxic condition, and hypoxia-induced NCX1 expression is attenuated by miR-132 treatment

The expression of miR-132 significantly decreased under hypoxic condition, but there was no time-dependent decrease of miR-132 was observed (Fig. 3A). However, this hypoxia-induced decrease of miR-132 suggested that the observed increase of NCX1 under hypoxic condition may be due to the decreased level of miR-132. To examine whether compensation of miR-132 using exogenous miR-132 could prevent the increase of NCX1 expression, cardiomyocytes were transfected with miR-132 for 24 h prior to hypoxic treatment. Our data indicated that miR-132 treatment attenuated the hypoxia-induced increase of NCX1 expression at both mRNA (Fig. 3B) and protein (Fig. 3C) level. Additionally, treatment with miR-132 also attenuated decrease of cell viability under hypoxic condition (Fig. 3D), suggesting miR-132 can salvage cardiomyocytes exposed to hypoxia by targeting NCX1.

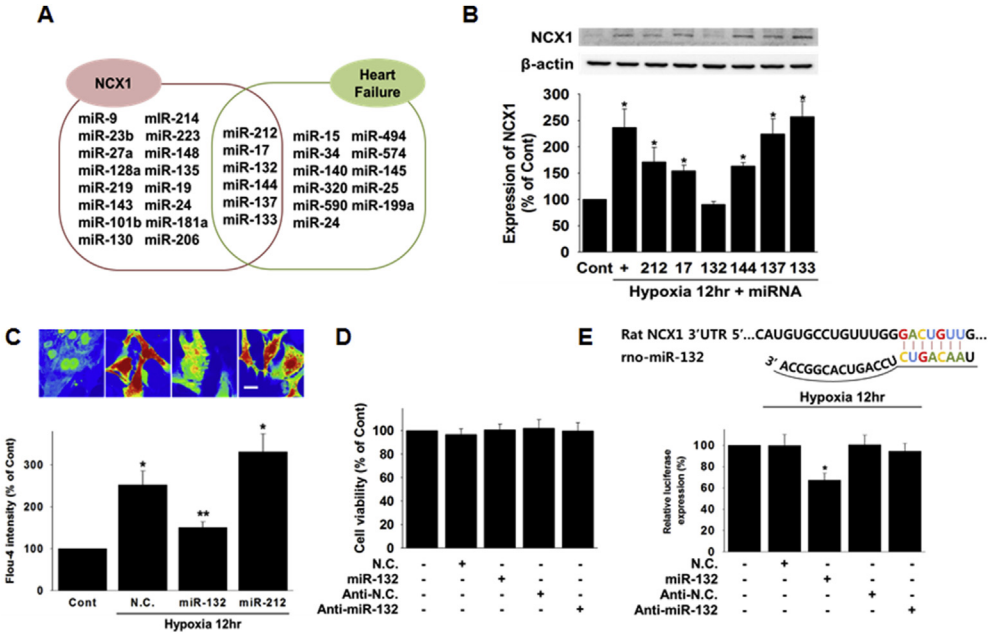

Fig. 2. Identification of miR-132 as a novel NCX1-targeting miRNA. A) Candidate miRNAs targeting NCX1 and have been associated with heart failure were selected based on miRNA target prediction database and literature search, respectively. B) Among 6 candidate miRNAs, miR-132 significantly attenuated hypoxia-induced increase of NCX1 expression. C) Treatment with miR-132 significantly attenuated hypoxia-induced increase of intracellular Ca^{2+} level. D) Overexpression of miR-132 or anti-miR-132 does not affect cell viability under normal condition. E) Relative luciferase activity using 3′UTR of NCX1 and miR-132. N.C. is a negative control. *$p < 0.05$ compared to untreated control. **$p < 0.05$ compared to hypoxia only. All the quantitative data represent the means ± SEM of three independent experiments.

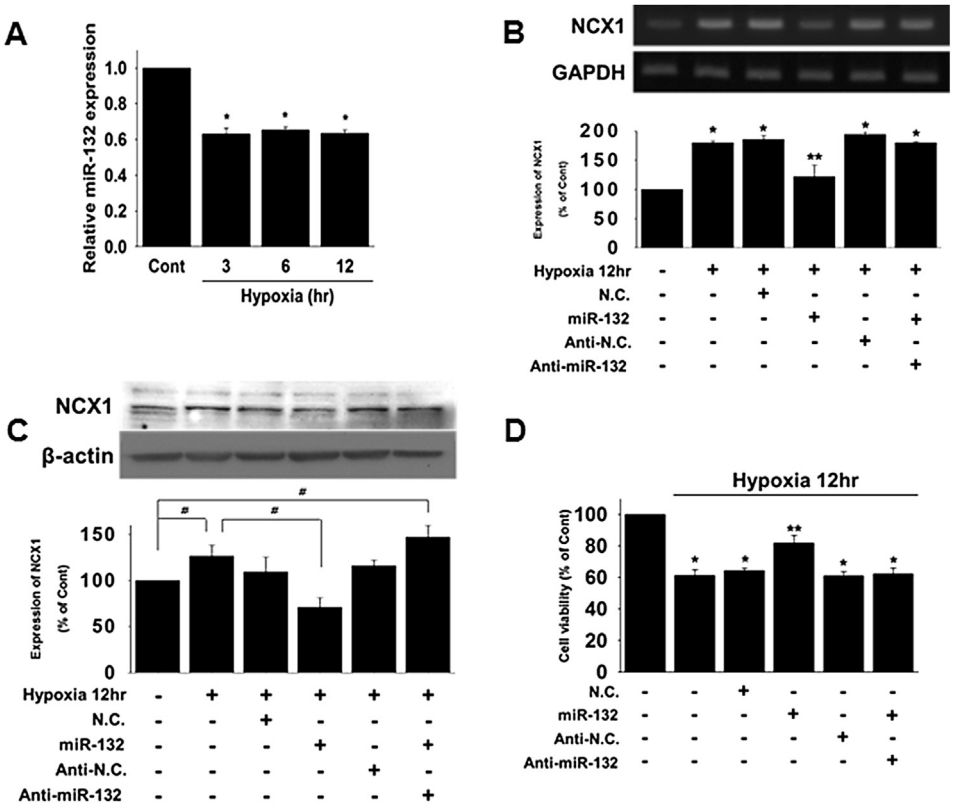

Fig. 3. The expression of miR-132 decrease under hypoxic condition, and hypoxia-induced NCX1 expression is attenuated by miR-132 treatment. A) Rat cardiomyocytes were cultured under hypoxic condition for up to 12 h, and the expression of miR-132 was measured by real-time PCR. B) Expression of Ca^{2+} handling NCX1 mRNA with miR-132 treatment prior to 12 h of hypoxic treatment. C) Expression of NCX1 with miR-132 treatment prior to hypoxia was evaluated by western blot. D) Effect of miR-132 delivery on the viability of cardiomyocytes exposed to hypoxia was assessed. N.C. is a negative control. *$p < 0.05$ compared to untreated control. **$p < 0.05$ compared to hypoxia only. #$p < 0.05$ compared to matching group. All the quantitative data represent the means ± SEM of three independent experiments.

3.4. Delivery of exogenous miR-132 attenuates hypoxia-induced apoptosis of cardiomyocytes

Hypoxia increased the number of Annexin V-positive cardiomyocytes approximately 4-folds, and miR-132 treatment significantly attenuated such increase (Fig. 4A), indicating miR-132 delivery decreased the apoptosis of cardiomyocytes under hypoxic condition. The expression Bax, a pro-apoptotic protein [31], was induced by hypoxic treatment, but was significantly attenuated by miR-132 pre-treatment prior to hypoxic treatment (Fig. 4B). Pre-treatment with miR-132 also significantly attenuated the cleavage of pro-caspase 3, one of the key events of apoptotic signaling cascade [32], in cardiomyocytes exposed to hypoxia (Fig. 4C). Furthermore, the activity of caspase 3 was significantly attenuated by miR-132 treatment (Fig. 4D), suggesting that the observed hypoxia-induced decrease of cardiomyocyte viability was due to activation of apoptotic signaling cascades and miR-132 delivery was able to significantly suppress the activation of apoptotic signaling pathway in cardiomyocytes under hypoxic condition.

4. Discussion

Ca^{2+} has been recognized as an important regulator of cardiomyocytes function in physiologic, as well as pathologic, conditions [33,34]. Accumulating evidence indicates that miRNAs are involved in the Ca^{2+}-mediated signaling in cardiomyocytes [24]. In the present study, we demonstrate that miR-132 is a novel NCX1-targeting miRNA that has potential as a therapeutic tool for preventing Ca^{2+} overload-mediated apoptosis of cardiomyocytes. The expression of NCX1 has been known to increase both at mRNA and protein level in failing heart due to ischemic cardiomyopathy [35]. As to its role during IR-injury, NCX1 has been associated with intracellular Ca^{2+} overload during IR-injury [5], and the contribution of Ca^{2+} overload to cell death in various cell types has been reported [8,36]. Thus, it is possible that NCX1-mediated Ca^{2+} overload contributes to the death of cardiomyocytes during IR-injury, making NCX1 as an effective therapeutic target for preventing IR-induced cardiomyocyte death. In fact, our group has reported that modulation of another key calcium handling protein calcium/calmodulin-dependent protein kinase type II delta (CaMKIIδ) using miR-145 suppressed Ca^{2+} overload and subsequent cardiomyocyte death [37], indicating similar miRNA-mediated approach may be applied to NCX1 and effective in preventing cardiomyocyte death during IR-injury. A recent study used miR-214, which targets NCX1, further supports such speculation by demonstrating cardioprotective effect of miRNA-mediated repression of NCX1 during IR injury [18].

Our data strongly suggest that miR-132 is a central mediator of Ca^{2+} overload-mediated cardiomyocytes apoptosis. Hypoxic condition significantly decreased the expression level of miR-132 in our experimental setting, while intracellular Ca^{2+} imaging showed a significant increase of intracellular Ca^{2+} with hypoxia. However, this hypoxia-induced Ca^{2+} increase was abrogated by miR-132 delivery prior to hypoxic treatment, strongly suggesting that the decreased level of miR-132 during hypoxia was associated with the increase of intracellular Ca^{2+}. Furthermore, since we have selected miR-132 to selectively down-regulate the expression of NCX1, these data also indicated that NCX1 is also a crucial mediator of Ca^{2+} overload under hypoxia. Our findings well agree with previous studies reported that the inhibition of NCX exerted protective effect in cerebral ischemia and in anoxia reoxygenation-injured ventricular myocytes [38–40].

As one of the members of miR-212/312 cluster [41], miR-132 has been implicated in various cellular processes such as neurological

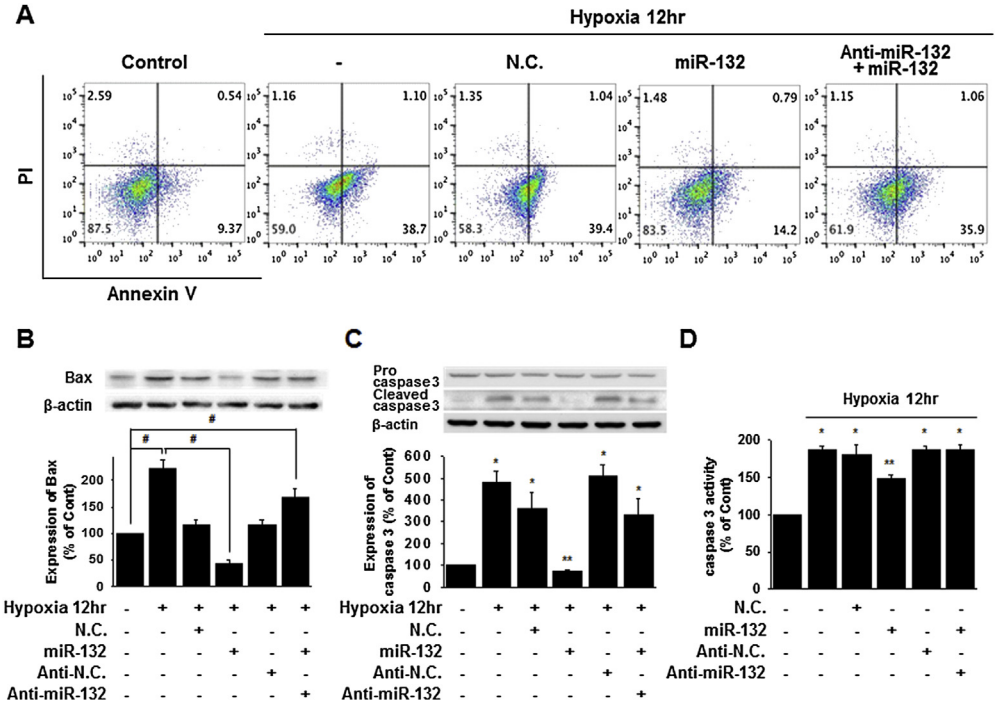

Fig. 4. Delivery of exogenous miR-132 attenuates hypoxia-induced apoptosis of cardiomyocytes. A) Apoptosis of cardiomyocytes exposed to hypoxia with or without miR-132 pre-treatment was evaluated by flow cytometry using propidium iodide (PI) and annexin V. B) Expression of Bax, a pro-apoptotic protein, in cardiomyocytes exposed to hypoxia with or without miR-132 treatment was evaluated by western blot. C) The effect of miR-132 treatment on hypoxia-induced cleavage of pro caspase 3 was assessed by western blot. D) Activity of caspase 3 in cardiomyocytes exposed to hypoxia with or without exogenous miR-132 pre-treatment was measured. N.C. is a negative control. #$p < 0.05$ compared to matching group. *$p < 0.05$ compared to untreated control. **$p < 0.05$ compared to hypoxia only. All the quantitative data represent the means ± SEM of three independent experiments.

development, inflammation, angiogenesis, and cancer [42–44]. Regarding its role in heart, it has been reported that miR-132 prevented cardiac hypertrophy by targeting calcium channel voltage-dependent beta-2 subunit (CaCn-β2) [45], and the role of miR-132/212 in angiotensin II-induced hypertension in hypertensive rat and humans also has been reported [46]. Nevertheless, to our best knowledge, there is no previous study investigated the role of miR-132 in cardiomyocytes exposed to hypoxia. In the present study, miR-132 pre-treatment prevented hypoxia-induced activation of apoptotic signaling cascades such as Bax and Caspase 3, and this study is the first *in vitro* study providing evidence that delivery of exogenous miR-132 can be an effective anti-apoptotic therapy by down-regulating NCX1 and subsequent Ca^{2+} overload. However, this anti-apoptotic effect of miR-132 has to be further validated using an *in vivo* model, and a proven, effective mean of miRNA delivery should be ready prior to conducting an *in vivo* study.

In summary, in the present study, we demonstrate that miR-132 suppresses apoptosis of cardiomyocytes exposed to hypoxia through repression of NCX1 expression and subsequent down-regulation of Ca^{2+} overload, recapitulating the importance of Ca^{2+} signaling in cardiomyocyte physiology. With further *in vivo* validation and optimization of delivery system, exogenous miR-132 can be a potent therapeutic agent for the prevention of cardiomyocyte death in pathologic conditions such as IR-injury or myocardial infarction, and the result of present study warrants further studies to elucidate more detailed underlying mechanisms.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.129.

Transparency document

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