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MiR-223 promotes cardiomyocyte apoptosis by inhibiting Foxo3a expression

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Abstract. – OBJECTIVE: MicroRNAs (miRs) are proven to possess diversified functions in the pathogenesis of cardiac diseases. The current study is designed aiming at determining the effect of miR-223 on oxidative stress induced apoptosis in cardiomyocytes.

MATERIALS AND METHODS: Mouse model of myocardial infarction (MI) was constructed, and endogenous level of miR-223 in the border zone of infarcted heart tissues was determined. Primarily cultured cardiomyocytes were exposed to H_2O_2 treatment to mimic the oxidative stress stimulation. Multiple approaches including quantitative reverse transcription polymerase chain reaction (qRT-PCR), cell viability assay, luciferase assay, Western blot assay and flow cytometry assay were employed to determine its expression, function and mechanism in apoptosis.

RESULTS: MiR-223 expression was significantly upregulated in the border zone of infarcted heart ventricular tissues and in cardiomyocytes treated with H_2O_2. Overexpression of miR-223 in cardiomyocytes promoted apoptosis, whereas inhibition of endogenous miR-223 protected cardiomyocytes from oxidative stress induced apoptosis. MiR-223 directly targets the 3'untranslated region (UTR) of Foxo3a mRNA. Overexpression of miR-223 inhibited Foxo3a protein expression, however, inhibition of miR-223 suppressed its expression. Silencing Foxo3a using small interfering RNA (siRNA) mimicked the effect of miR-223, indicating its functional significance.

CONCLUSIONS: MiR-223 is an important regulator of cardiomyocyte apoptosis under oxidative stress. Inhibition of the miR-223/Foxo3a signaling axis may be a potential therapeutic strategy for cardiac injuries.

Key Words:
miR-223, Apoptosis, Cardiomyocyte, Foxo transcriptional factor, Oxidative stress.

Introduction

Ischemic heart disease, which is commonly caused by coronary artery occlusion induced myocardial infarction (MI)[1], possesses a high morbidity and mortality worldwide. Physiologically, cardiomyocytes are vulnerable to energy and nutrition depletion, and they will experience cell death immediately after MI. Loss of these cells can cause irreversible damage to the organ, which leads to its structural and functional changes[2]. Therefore, understanding how cardiomyocytes undergo cell death is key to the prevention and treatment strategies for many heart diseases. MicroRNAs (miRs) are a class of noncoding endogenous single RNA strands that exert their biological functions by specifically binding to and inhibiting their target genes[3]. MicroRNAs have been established as important pathogenic molecules in heart diseases as evidenced by the accumulating data. These microRNAs have been broadly implicated in the cell death signaling in myocardium[4,5]. To date, numerous microRNAs have been identified to serve as regulators of apoptosis involved in the pathogenesis of acute cardiac injury. MicroRNAs may have diverse functions in various pathogenic scenarios. Recent studies have demonstrated that miR-223 is upregulated in MI, which then inhibited the expression of genes encoding the potassium channel Kv4.2[6]. As a consequence, miR-223 promotes arrhythmia in MI. Loss of miR-223 also protects heart from hypertrophic program in mice[7]. Whether and how miR-223 regulates acute cardiac injury and oxidative stress induced apoptosis needs further investigation. In the current study, we aimed at exploring the role of miR-223 and identifying new targets in cardiomyocytes. We show that in

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mouse MI model, miR-223 expression is significantly upregulated in the border zone in response to ischemic stimuli. *In vitro* H_2O_2 treatment has a similar effect on its expression. Furthermore, we demonstrate that miR-223 promoted cardiac apoptosis by suppressing the expression of the cardioprotective factor, Foxo3a. Our study will aid in understanding the pathogenesis of multiple cardiac diseases and providing potential therapeutic targets for myocardial infarction.

Materials and Methods

Mouse Model

To establish mouse model for MI, male C57BL/6 mice aged 8 weeks were purchased from HFKbio (Beijing, China). The left anterior descending coronary artery was then ligated with a 7-0 suture to introduce left ventricular myocardial infarction. For control mice, sutures crossed the left anterior descending coronary artery and were untied. Border zone cardiac tissues were determined using evans blue staining. All the procedures were approved by the Ethics Committee of Hebei University of Engineering. Heart tissues were collected at the indicated time points.

Primary Cell Culture

Primary culture of cardiomyocytes was performed as previously described [8]. In brief, neonatal mice born within 3 days were dissected, and the hearts were collected. After removing of the blood, heart tissues were cut into small pieces and subjected to series pancreatin digestion. Cell suspensions were collected in DMEM, followed by plating in flasks for 2 h at 37°C to remove cardiofibroblasts. Cardiomyocytes were then re-plated in 6-well plates or 96-well plates according to the experimental demands. Cells were cultured under 5% CO_2 37°C condition in a humidified atmosphere. To manipulate the endogenous level of miR-223, we purchased miR-223, miR-223 inhibitor (miR-223-In) and their respective negative controls (NC and NC-In) from Genepharma Biotech., (Shanghai, China). The oligonucleotides were then transfected into the isolated cells at the dose of 200 nM. The following experiments were conducted 48 hours after transfection.

Quantitative Reverse Transcription-PCR

TRIzol reagent was used to isolate total RNA from tissue and cell samples. The RNA samples were treated with DNase I (Qiagen, Hilden, Germany) to avoid residue DNA contamination. Reverse transcription was conducted using a ReverTra Ace qPCR RT Kit (Toyobo, Tokyo, Japan) according to the manufacturer's protocol. Stem-loop primers for miR-223 reverse transcription and amplification primers for miR-223 detection were purchased from Genepharma Biotech (Shanghai, China).

Cell Viability Assay

Cardiomyocytes were seeded into 96-well plates at the concentration of 5×10^3 /ml. 20 µl methyl thiazolyl tetrazolium (MTT) solution (5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) was then added to each well and normally incubated for 4 h. 100 µl dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was then added to each well to visualize viable cells. Absorbance values at 570 nm were collected using a spectrophotometer.

Western Blot

Cells were harvested with SDS lysis buffer, and centrifuged at 15000 g at 4°C for 15 min. The supernatant was collected. Then protein concentration was quantified with a BCA kit (Beyotime, China). 50 µg protein was loaded on each lane for Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Proteins were transferred onto PVDF membranes, and the membranes were then incubated with primary antibodies against cleaved-Caspase 3 (Cell Signaling Technology, Danvers, MA, USA), Foxo3a (Santa Cruz Biotech., Santa Cruz, CA, USA) and b-actin (Santa Cruz Biotech, Santa Cruz, CA, USA) at 4°C overnight. Horseradish peroxidase (HRP) conjugated goat anti rabbit secondary antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) was applied to the membrane at room temperature for 1 h to detect the protein. BeyoECL Plus kit (Beyotime, China) was used for final visualization of the protein bands.

Apoptosis Assay

Apoptosis was determined using Annexin V-fluorescein isothiocyanate (FITC)/ Sodium dodecyl sulfate (PI) double staining kit (Beyotime, China) according to the manufacturer's protocol. Cells were digested with 0.25% pancreatin, stained with Annexin V-FITC and PI, and then analyzed using flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA).

Luciferase Activity Assay

The 3'UTR of Foxo3a was subcloned into the multiple cloning site (MCS) of pmiRGLO reporter construct (Promega, Madison, WI, USA). The mutant 3'UTR was generated using TaKaRa MutanBest site-directed mutagenesis kit (TaKaRa, Otsu, Shiga, Japan). To examine the effect of miR-223 binding on Foxo3a 3'UTR, the wild type or mutant reporter was transfected into HEK293T cells along with miR-223. 36 h after transfection, luciferase activity was measured using dual-luciferase system (Promega, Madison, WI, USA) according to the instructions provided by the manufacturer.

Statistical Analysis

All data were expressed as means ±S.E.M. statistical significance of the difference between groups was analyzed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test or Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

MiR-223 Expression Is Increased in the Border Zone of MI Heart and H_2O_2 Treated Cardiomyocytes

In the first part of our study, we established mouse model of MI to study the expression of miR-223 in this pathological states. MiR-223 expression level in the isolated border zone area was measured. As shown in Figure 1A, after MI, miR-223 expression was increased over time. On the cellular level, we employed H_2O_2 treatment on cardiomyocytes, and we found that miR-223 expression was also upregulated in injured cells

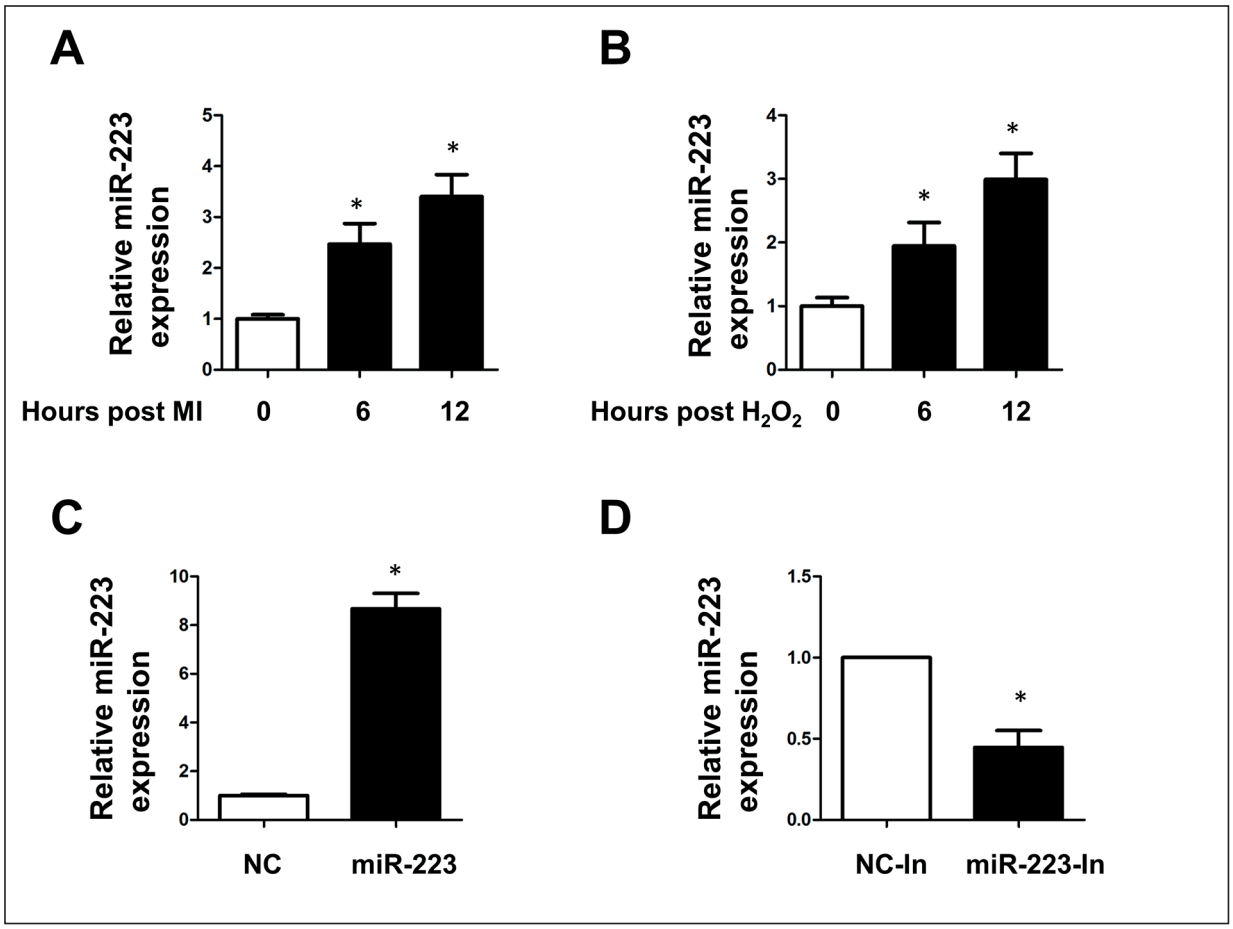

Figure 1. Foxo3a silencing promotes apoptosis. ***(A)*** The relative expression of miR-223 in the border zone ventricular tissues after myocardial infarction (MI). ***(B)*** The relative expression of miR-223 in cardiomyocytes underwent H_2O_2 treatment. ***(C)*** The relative expression of miR-223 in cardiomyocytes after transfection of miR-223. ***(D)*** The relative expression of miR-223 in cardiomyocytes after transfection of miR-223 inhibitor (miR-223-In). *$p < 0.05$ *vs.* 0 h or NC or NC-In, $n \geq 3$. NC, negative control.

(Figure 1B). To further ascertain the role of miR-223 in cardiomyocytes, we transfected miR-223 and its inhibitor (miR-223-In) into primarily cultured neonatal cardiomyocytes to manipulate its endogenous level and analyze its function (Figure 1C and D).

MiR-223 Promotes Apoptosis in Cardiomyocytes

H_2O_2 treatment significantly decreased cell viability as determined by MTT assay, overexpression of miR-223 enhanced this reduction in cell viability, whereas miR-223-In opposed this effect (Figure 2A), indicating the deleterious role of miR-223 in oxidative stress induced cardiac injury. We next sought to investigate the role of miR-223 in apoptosis, a common type of cell death in oxidative stress induced cardiac injury. Western blot analysis of the apoptosis marker Cleaved-Caspase 3 (C-Casp3) revealed that miR-223 promoted its expression under oxidative stress condition (Figure 2B). In contrast, miR-223-In attenuated its expression (Figure 2B). Flow cytometry analyses have shown that, under oxidative stress condition, miR-223 transfection leads to more cells rendered in apoptotic program. However, miR-223-In transfection had an opposite effect on its apoptosis.

Foxo3a is a Target of miR-223 in Cardiomyocytes

To study the function of miR-223 at the molecular level, we used bioinformatics tools to predict its target. We found that miR-223 is predicted to bind to the 3' untranslated region (UTR) of Foxo3a mRNA (Figure 3A). Luciferase assay showed that miR-223 inhibited luciferase reporter gene expression in construct carrying wild type 3'UTR of Foxo3a, and mutation of the binding sequence abolished the effect (Figure 3A and B). Transfection of miR-223 into cardiomyocytes inhibited Foxo3a expression, whereas miR-223 inhibitor increased its level (Figure 3C). Importantly, H_2O_2 treatment induced reduction of Foxo3a expression can be partially reversed by miR-223-In (Figure 3D).

Foxo3a Silencing Promotes Apoptosis

The above data suggest the possibility that miR-223 targets Foxo3a in cardiomyocytes during oxidative stress. To confirm the function of miR-223/Foxo3a axis in regulating apoptosis, we transfected Foxo3a small interfering RNA (siRNA) into cardiomyoctes (Figure 4A). This led to reduced cell viability under oxidative stress and increased C-Casp3 expression (Figure 4B and C). Flow cytometry analysis confirmed that inhibition of Foxo3a enhances apoptosis (Figure 4D). These data showed that Foxo3a inhibition mimicked the effect of miR-223-In, suggesting that Foxo3a serves as a functional target of miR-223 in cardiomyocytes.

Discussion

As cardiomyocytes have very limited capacity to regenerate after injury and are vulnerable to multiple stresses, one of the key issues in identifying the molecular pathogenesis of cardiac diseases is to explore critical regulators of cardiomyocyte death. In the border zone of the infarcted area, apoptosis is frequently occurred, which leads to a progressive loss of myocardium[9]. In the present study, we have provided evidence that miR-223 is significantly upregulated in this region. Functional assays using the cellular model of primary cultured cardiomyocytes revealed that overexpression of miR-223 promoted H_2O_2 induced apoptosis, whereas miR-223 inhibitor exerted an opposite effect. We have shown that Foxo3a is a novel target of miR-223 in cardiomyocytes, and that inhibition of Foxo3a mimicked the effect of miR-223. Our study thus provides pilot evidence of miR-223 in oxidative stress induced cardiomyocyte apoptosis, which indicates the therapeutic potential of miR-223 inhibitor in multiple heart dysfunctions related to cardiomyocyte apoptosis.

Over the last decades, a large number of microRNAs have been identified to participate in the regulatory programs of cardiac injury induced apoptosis. For instance, it has been shown that miR-145 directly targets Bnip3, which suppressed the mitochondria apoptotic pathway upon H_2O_2 treatment and ischemia reperfusion[10]. MiR-30 is also implicated in the regulation of β-adrenergic signaling pathway critical for cardiomyocyte apoptosis[11]. PI3k/Akt/mTOR pathway is crucial for cell survival under various pathological conditions; microRNAs such as miR-28 and miR-378 are implicated in this pathway to control a fate of cell survival against apoptosis[12, 13]. Regardless of various reports and evidence, the role of miR-223 in heart is still elusive. A previous study has shown

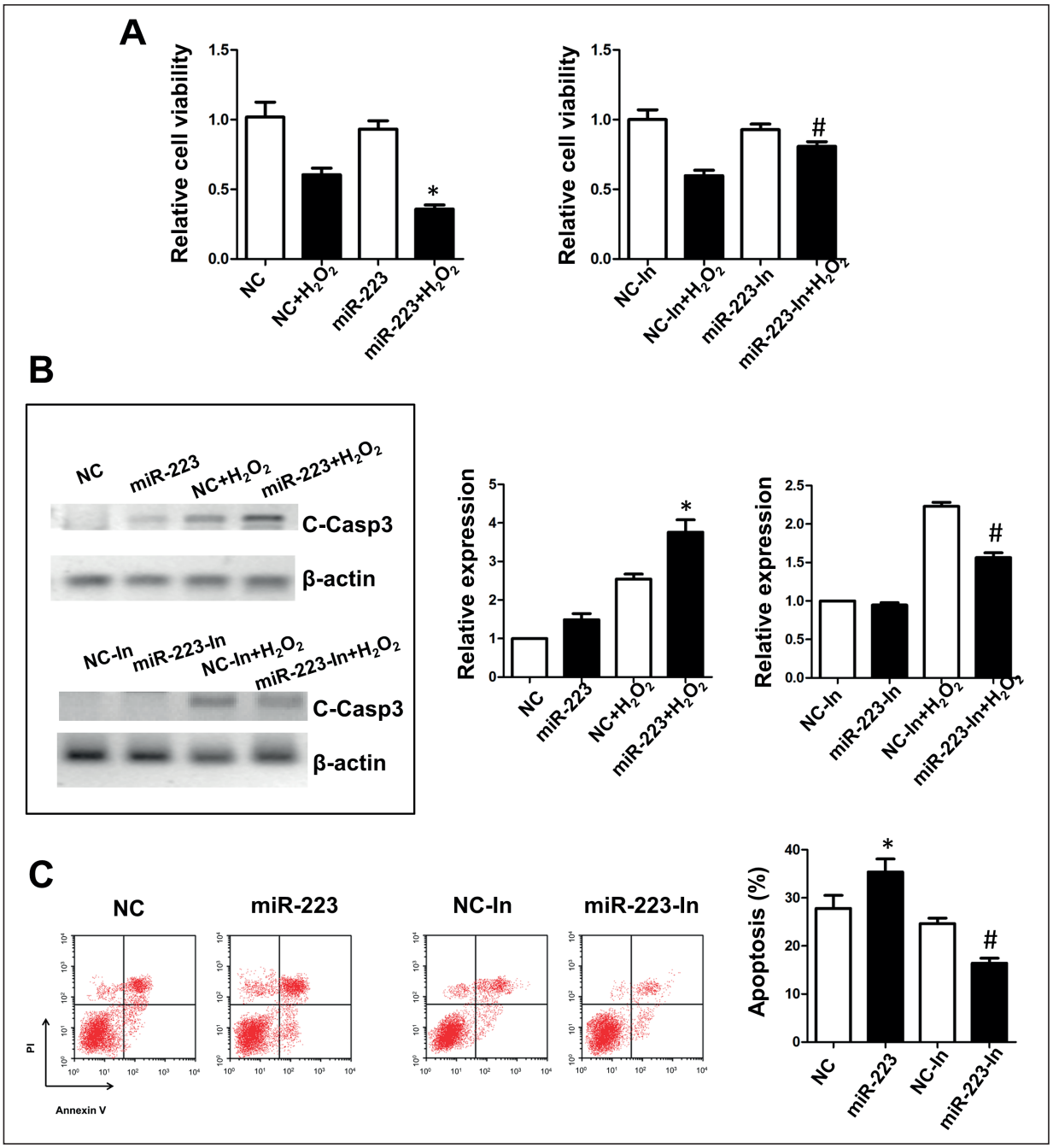

Figure 2. Foxo3a is a target of miR-223 in cardiomyocytes. ***(A)*** The relative cell viability of cardiomyoctes transfected with miR-223 (left) or miR-223-In (right) under normal and oxidative stress conditions. ***(B)*** Cleaved-Caspase3 (C-Casp3) expression in cardiomyoctes transfected with miR-223 or miR-223-In under normal and oxidative stress conditions. ***(C)*** Apoptosis in cardiomyoctes transfected with miR-223 or miR-223-In under oxidative stress condition. *$p < 0.05$ *vs.* NC+H_2O_2, #$p < 0.05$ *vs.* NC-In+H_2O_2, n ≥ 3. NC, negative control.

that the expression of miR-223 is significantly upregulated in the border zone of myocardial infarction shortly after ligation of the left anterior descending coronary artery in rodents[6]. In that study, the authors have proposed that miR-233 is an arrhythmogenic microRNA that directly represses the expression of Kv4.2 potassium channel. However, our study, by focusing on the aspect of cardiomyocyte apoptosis, demonstrates that miR-223 is also an important

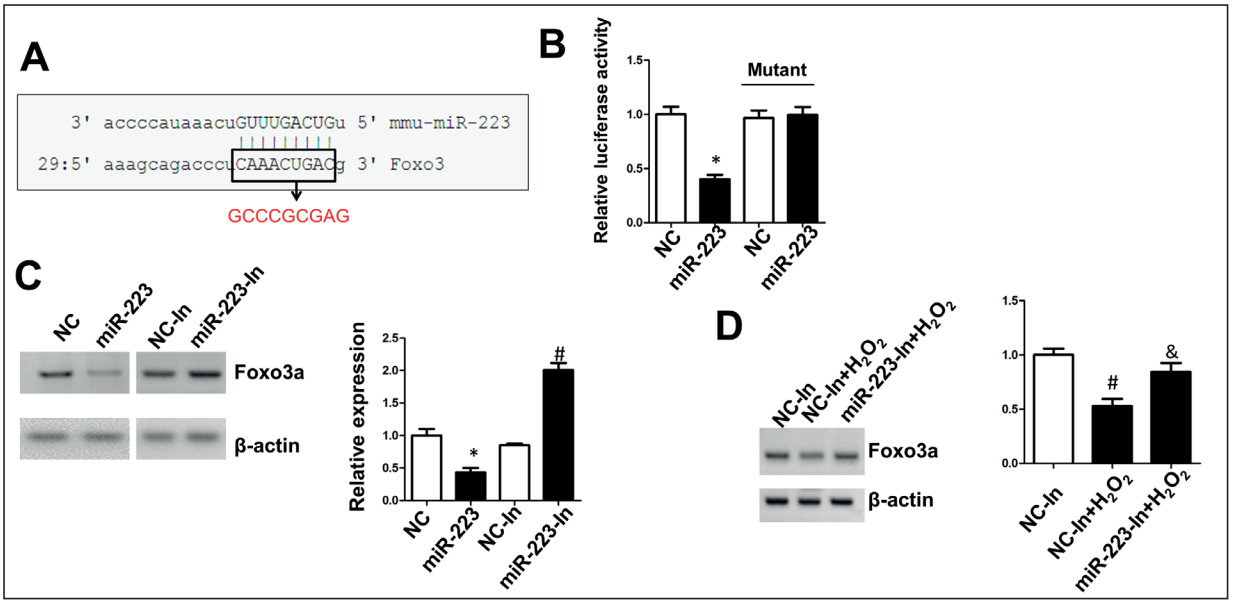

Figure 3. MiR-223 promotes apoptosis in cardiomyocytes. ***(A)*** The schematic diagram of interaction between miR-223 and the Foxo3a mRNA 3'UTR, the mutagenesis strategy is shown in red. (B) Relative luciferase activity of wild type and mutant luciferase reporter after transfection of miR-223. ***(C)*** The protein expression of Foxo3a after miR-223 or miR-223-In transfection. ***(D)*** The protein expression of Foxo3a after miR-223-In transfection under oxidative stress condition. **p*< 0.05 *vs.* NC. #*p* < 0.05 *vs.* NC-In, &*p* < 0.05 *vs.* NC-In + H_2O_2, n ≥ 3. NC, negative control.

regulator of cell survival under oxidative stress condition. Using a knockout mouse model, Wang et al[7] have shown that loss of miR-223 resulted in less hypertrophic damage to heart muscle cells, suggesting that miR-223 plays a detrimental role in cardiac hypertrophy model. In line with these studies, we demonstrate that miR-223 also exerts a detrimental effect on oxidative stress induced apoptosis, which is relevant to myocardial infarction injury. Therefore, it may be proposed that inhibition of miR-223 might be a general approach for multiple cardiac disorders. However, Qin et al[14] recently have shown that miR-223-5p and 3p cooperatively suppresses necroptosis in ischemia/reperfusion (IR) induced apoptosis in mouse. The reason for this phenotype is unknown; nevertheless, it suggests that the actual effects of miR-223 might reflect the specific responses to distinct types of stimuli. A more accurate *in vivo* model is still required to evaluate this issue. In this study, we have identified that Foxo3a is a functional target of miR-223 in regulating cardiomyocyte apoptosis. Foxo3a is a well-characterized cardiac protective factor that is implicated in regulation of multiple pathways such as PI3K/AKT and AMPK signaling pathways[15-17]. Before our study, Foxo3a has been identified to be a critical target of several other microRNAs. In diabetic cardiomyopathy, miR-30d suppresses Foxo3a to induce pyroptosis[18]. In cardiac hypertrophy, Foxo3a is negatively regulated by miR-23a, and inhibition of Foxo3a leads to hypertrophic program[19]. We have identified that miR-223 can target Foxo3a in cardiomyocytes to promote oxidative stress induced apoptosis, which reinforces the previously proposed regulatory signaling network where Foxo3a plays central role. It is intriguing to note that miR-223 directly suppresses ARC in cardiac hypertrophy[7], an anti-apoptotic molecule that acts downstream of Foxo3a. Therefore, part of the pro-apoptotic function of miR-223 may be mediated directly by suppressing ARC in our cellular model. However, this hypothesis needs further experimental validation.

Another unanswered question is how the level of miR-223 is regulated in pathological conditions. Wang et al[7] recently have shown that circRNA HRCR acts as an endogenous miR-223 sponge to lower its level, thus inhibiting cardiac hypertrophy and heart failure. Whether other mechanisms, such as transcriptional regulation and epigenetic regulation, contribute to its up-regulation during MI or oxidative stress needs further demonstration.

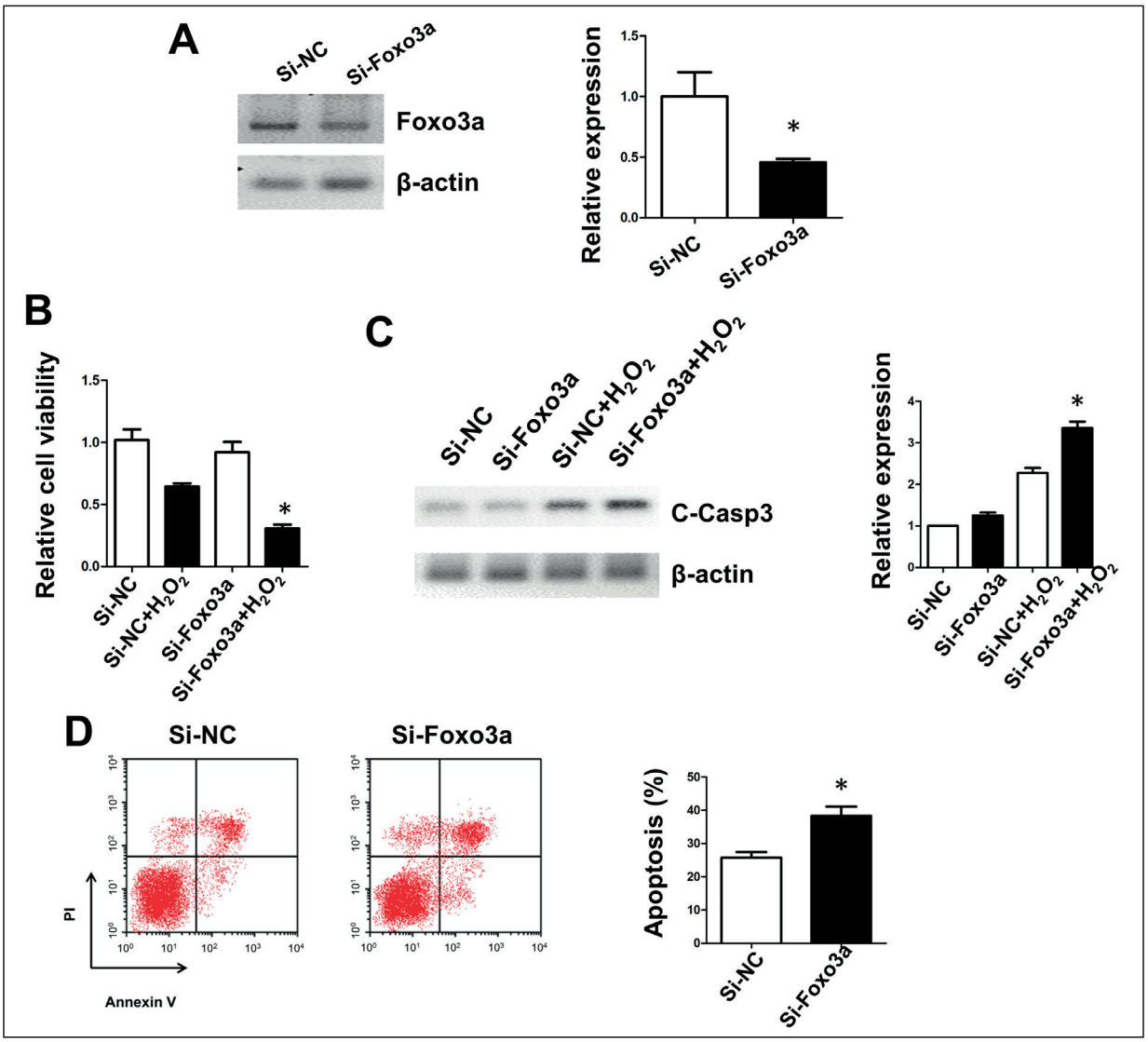

Figure 4. MiR-223 expression is increased in the border zone of MI heart and H_2O_2 treated cardiomyocytes. ***(A)*** Foxo3a expression after transfection of small interfering RNA for Foxo3a (Si-Foxo3a). ***(B)*** The relative cell viability of cardiomyoctes transfected with Si-Foxo3a under normal and oxidative stress conditions. ***(C)*** C-Casp3 expression in cardiomyoctes transfected with Si-Foxo3a under normal and oxidative stress conditions. ***(D)*** Apoptosis in cardiomyoctes transfected with Si-Foxo3a under oxidative stress condition. *$p < 0.05$ *vs.* Si-NC or Si-NC+H_2O_2, n ≥ 3. NC, negative control.

Conclusions

We identify a novel miR-223/Foxo3a signaling pathway that functions critically in regulating cardiomyocyte apoptosis. Inhibition of miR-223 can upregulate Foxo3a, which protects cardiomyocyte from oxidative stress induced apoptosis. Therefore, miR-223 inhibitor may represent a novel therapeutic agent for multiple heart diseases.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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