CircRNA MFACR is Upregulated in Myocardial Infarction and Downregulates miR-125b to Promote Cardiomyocyte Apoptosis-induced by Hypoxia

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Research

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Abstract

Background: CircRNA MFACR promotes cardiomyocyte death, which contributes to myocardial infarction (MI). We analyzed the role of MFACR in MI.

Methods: RT-qPCR was applied to determine MFACR and miR-125b expression in plasma samples from both MI patients (n=61) and healthy controls (n=61). MFACR and miR-125b were overexpressed in AC16 cells (cardiomyocytes) to study the interactions between them. Methylation of miR-125b gene in cells with MFACR overexpression was analyzed by methylation-specific PCR. Cell apoptosis after transfections was analyzed by cell apoptosis assay.

Results: MFACR was overexpressed in MI and inversely correlated with miR-125b. In AC16 cells, hypoxia treatment increased MFACR expression and decreased miR-125b expression. In AC16 cells, MFACR overexpression decreased miR-125b expression and increased the methylation of miR-125b gene. Under hypoxia, MFACR overexpression increased AC16 cell apoptosis, and miR-125b overexpression decreased cell apoptosis. In addition, miR-125b overexpression reversed the effects of MFACR overexpression on cell apoptosis.

Conclusion: MFACR may increase the methylation of miR-125b gene to downregulate its expression, thereby promoting the apoptosis of cardiomyocytes in MI.

Background

Myocardial infarction (MI), also refers to a heart attack, is a clinical disorder caused by the reduced or even ceased blood supply to the heart [1] . MI can cause severe heart muscle damages, and the symptoms are mainly chest pain and shoulder, back, arm, neck or jaw discomforts [2,3]. It is estimated that MI causes about 30% mortality rate, and more than half of all deaths occur prior to hospitalization [4,5]. Even worse, about 10% of the survivors will die with 1 year after discharge [4,5]. MI patients are usually treated and prevented with beta blockers and aspirin, which are antiplatelet drugs [6]. However, there are no available approaches to reverse heart damages (myocardial scar) [6]. Therefore, novel therapeutic approaches are still needed.

Molecular factors play critical roles in the onset and progression of MI [7,8]. These molecular players participate in MI mainly by regulating inflammatory responses and cell apoptosis [9,10]. In effect, some molecular pathways have shown potentials in novel anti-MI therapies, such as targets therapy that suppress MI by regulating gene expression [11] . CircRNAs have no capacity of protein-coding but play theirs roles in human diseases by regulating gene expression [12,13], indicating that circRNAs are promising candidate targets for targeted therapy. CircRNA MFACR promotes cardiomyocyte death, which promotes MI [14]. Through our microarray analysis we observed an inverse correlation between MFACR and miR-125b, which plays protective roles in cardiac dysfunction by suppressing cell apoptosis. We then analyzed the crosstalk between MFACR and miR-125b in MI.

Methods

MI patients and controls

A total of 61 MI patients (acute phase) and 61 healthy controls were enrolled at the Third People's Hospital between May 2018 and June 2020. The Ethics Committee of this hospital approved this study. MI was diagnosed by ECG (pathologic Q waves and ST segment elevation) and serum biomarkers ((increased troponin T or I, and CK-MB). In view of the fact that other clinical disorders and therapies may also affect gene expression, this study excluded patient complicated with other severe clinical disorders and the ones with initiated therapy. All healthy controls were enrolled at the physiological heath center of the aforementioned hospital. Controls with a history of MI were excluded. All patients signed informed consent.

Blood extraction and plasma preparations

Prior to therapy, blood (3ml) was extracted from both healthy controls and MI patients. To prepare plasma samples, blood was mixed with citric acid (1:10), followed by centrifuging the mixture for 15 min at 1200 g to collect the supernatant (plasma). Plasma samples were kept in liquid nitrogen storage prior to the subsequent assays.

Cardiomyocytes

AC16 cardiomyocyte cells (Sigma-Aldrich) were used in this study. AC16 cells were cultivated in DMEM medium supplemented with 12% FBS and 1% penicillin and streptomycin (Sigma-Aldrich). In an incubator, AC16 cells were cultivated at 37°C, and 95% humidity, a 5% CO_2.

Transfections

MFACR and miR-125b were overexpressed in AC16 cells by transfecting AC16 cells with MFACR expression vector (Invitrogen) or mimic of miR-125b (Sigma-Aldrich) through lipofectamine 2000 (Invitrogen)-mediated transient transfections. NC experiments (NC miRNA- or empty vector-transfected cells) and C experiments (control cells with no transfections) were included in each experiment. The following experiments were performed 48h later.

RNA preparations

Extraction of total RNA from AC16 cells and plasma samples was performed using Ribozol reagent (VWR biotech). Incubation with DNase I (Invitrogen) was performed for 85 min at 37°C to completely remove genomic DNA. Total RNA samples were separated using 5% urea-PAGE gels to check RNA integrity. RNA purity was analyzed by determining the OD 260/280 ratios of the RNA samples

RT-qPCR

SS-IV-RT (Invitrogen) was used to reverse transcribe the RNA samples with satisfactory integrity and OD values close to 2.0 into cDNA samples. With cDNA samples as template, qPCRs were performed using SYBR® Green Real-Time PCR Master mix (Toyobo) to determine the expression of MFACR with 18S rRNA internal.

Expression of mature miR-125b was determined through following steps: 1) poly (A) addition; 2) miRNA reverse transcriptions; 3) qPCRs. All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia) was used to complete all the steps.

$2^{-\Delta\Delta CT}$ method was used to normalize Ct values of target genes to corresponding internal controls.

Methylation-specific PCR (MSP)

AC16 cells were used to extract genomic DNA using conventional methods. DNA samples were converted using Methylation-GoldTM kit (ZYMO RESEARCH). Taq 2X master mix (NEB) was used to perform both routine PCRs and methylation-specific PCRs.

Cell apoptosis assay

Cells were cultivated in 6-well plates with 12000 cells in 2 ml medium per well. Three replicate wells were set for each experiment. Under hypoxic conditions (1% O_2, 5% CO_2, and 94% N_2), AC16 cells were cultivated for further 48h, followed by washing with ice-cold PBS. After that, AC16 cells were resuspended in binding buffer, followed by staining with Annexin V-FITC and propidium iodide (PI) (Dojindo, Japan). After that, flow cytometry was used to analyze cell apoptosis.

Statistical analysis

MI and Control groups were compared by unpaired t test. ANOVA Tukey's test was used to analyze the differences among multiple transfection groups. <0.05 was statistically significant.

Results

MFACR was overexpressed in MI and inversely correlated with miR-125b

RNA isolations and RT-qPCRs were perfromed to analyze the expression of MFACR and miR-125b. Compared to Control group, significantly overexpressed MFACR (Fig.1A, $p<0.001$) and significantly under-expressed miR-125b (Fig.1B, $p<0.001$) were observed in MI group. Pearson's correlation coefficient analysis showed that MFACR and miR-125b were inversely and significantly correlated across MI samples (Fig.1C) and control samples (Fig.1D). Therefore, a crosstalk may exist between MFACR and miR-125b.

Hypoxia treatment altered the expression of MFACR and miR-125b in AC16 cells

AC16 cells were cultivated under hypoxic conditions for 24, 48, 72 and 96h, followed by determine the expression of MFACR and miR-125b by RT-qPCR. It was observed that, in AC16 cells, hypoxia treatment increased the expression of MFACR (Fig.2A, $p<0.05$) and decreased the expression of miR-125b (Fig.2B, $p<0.05$).

MFACR overexpression decreased miR-125b expression through methylation in AC16 cells

To study the effects of crosstalk between MFACR and miR-125b, AC16 cells were transfected with either MFACR expression vector or miR-125b mimic, followed by checking the overexpression of MFACR and miR-125b every 24h until 96h. It was observed that MFACR and miR-125b were significantly overexpressed between 24h and 96h (Fig.3A, $p<0.05$). MFACR overexpression decreased the expression of miR-125b between 24h and 96h (Fig.3B, $p<0.05$), while miR-125b overexpression failed to significantly affect the expression of MFACR (Fig.3C). To study the effects of the overexpression of MFACR overexpression on the methylation of miR-125b gene, MSP was performed at 96h post-transfection. MFACR expression vector transfection significantly increased the methylation of miR-125b (Fig.3D). Therefore, MFACR may downregulate miR-125b through methylation.

MFACR overexpression increased AC16 cell apoptosis induced by hypoxia through miR-125b

Cell apoptosis under hypoxic conditions was analyzed. MFACR overexpression increased AC16 cell apoptosis, and miR-125b overexpression decreased cell apoptosis. In addition, miR-125b overexpression reversed the effects of MFACR overexpression on cell apoptosis (Fig.4, $p<0.05$).

Discussion

In this study we explored the involvement of MFACR and miR-125b in MI. We found that MFACR was overexpressed in MI and miR-125 was downregulated in MI. Interestingly, MFACR may downregulate miR-125b through methylation to promote the apoptosis of AC16 cells induced by hypoxia.

In a recent study, Wang et al. characterized a novel circRNA with overexpression in mice model of MI. Knockdown of MFACR reduced the apoptosis of cardiomyocytes and attenuated mitochondrial fission by increasing the activity of miR-652-3p [14].However, the expression pattern and function of MFACR in MI patients are unknown. In this study we showed that MFACR was overexpressed in MI. Interestingly, hypoxia treatment significantly increased the expression of MFACR in AC16 cells. Therefore, the overexpression of MFACR in MI patients is likely induced by the hypoxic condition in patients' heart. Moreover, cell apoptosis analysis showed that overexpression of MANCR increased the apoptosis of AC16 cells induced by hypoxia. Therefore, hypoxia-inducible MFACR may promote MI progression by promoting cell apoptosis.

MiR-125b can target TRAF6 to regulate p53-mediated apoptotic signaling and the activity of nuclear factor κB to suppress the apoptosis of cardiomyocytes, thereby suppressing the development of cardiac dysfunction [15]. Consistently, our study observed the downregulation of miR-125b in MI. Interestingly,

hypoxia treatment decreased the expression of miR-125b in AC16 cells, and overexpression of miR-125b reduced cell apoptosis under hypoxic conditions. Therefore, overexpression of miR-125b may serve as a potential therapeutic target for MI. However, clinical trials are needed to verify our hypothesis.

In this study we showed that MFACR overexpressed decreased the expression of miR-125b by increasing the methylation of miR-125 gene. This finding enriched our knowledge of the interactions between circRNAs and miRNAs. However, the mechanism remains to be explored.

In conclusion, MFACR is overexpressed in MI and miR-125b is under-expressed in MI. MFACR may downregulate miR-125b through methylation to promote the apoptosis of cardiomyocytes to promote the progression of MI.

Abbreviations

Not applicable.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

MHJ designed the study. SJW carried out experiments and wrote the manuscipt, MHJ revised the paper, SJW, LL and WJD collected patient specimens and related information. SJW, LL and WJD contributed to analysing the data. All authors reviewed the results and approved the final version of the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of Third People's Hospital all the patients or parents/ guardians of patients provided written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figures

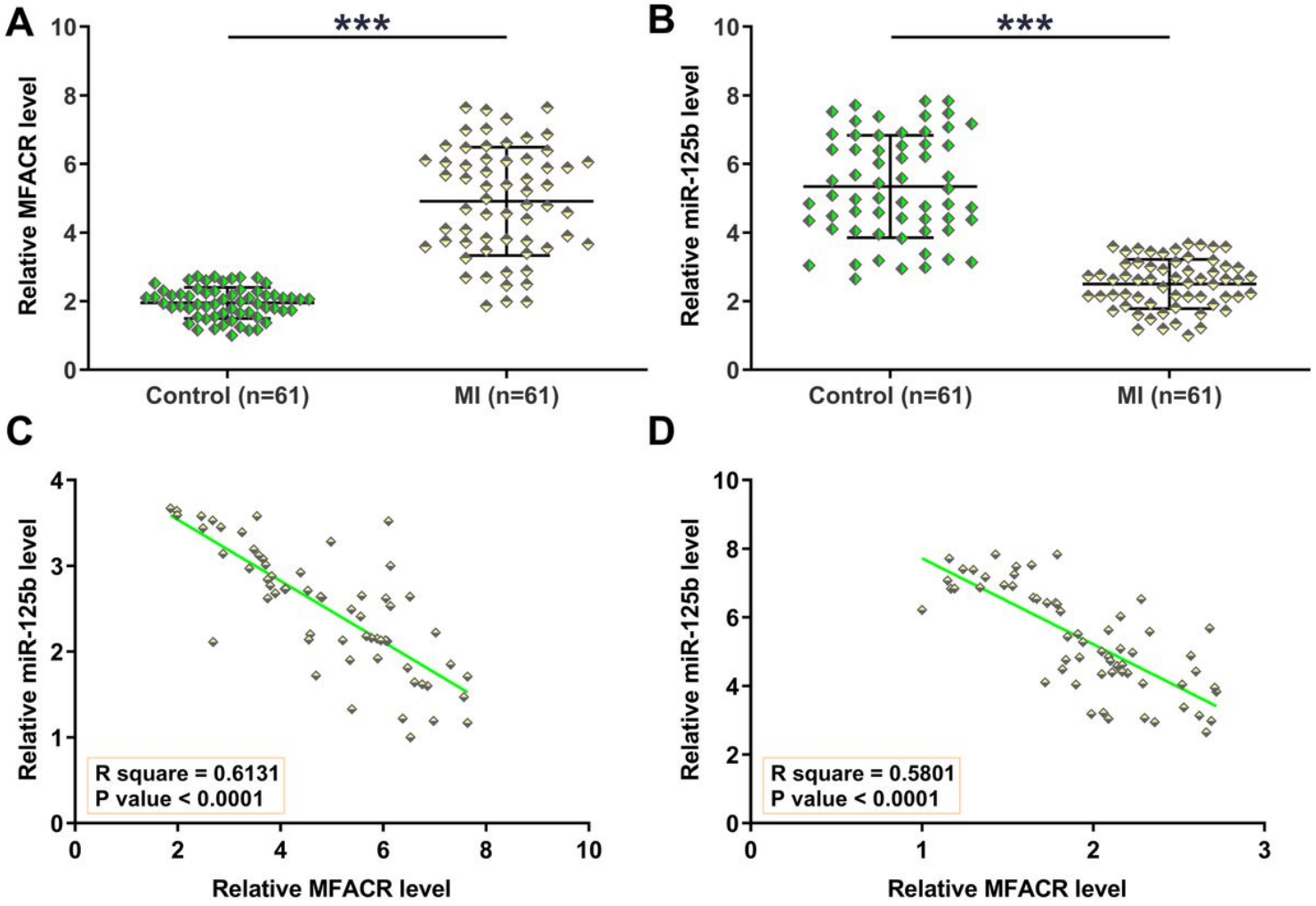

Figure 1

MFACR was overexpressed in MI and inversely correlated with miR-125b RNA isolations and RT-qPCRs were performed on plasma samples to analyze the expression of MFACR (A) and miR-125b (B). Pearson's correlation coefficient analysis was performed to analyze the correlations between MFACR and miR-125b across MI samples (C) and control samples (D). ***,p<0.001.

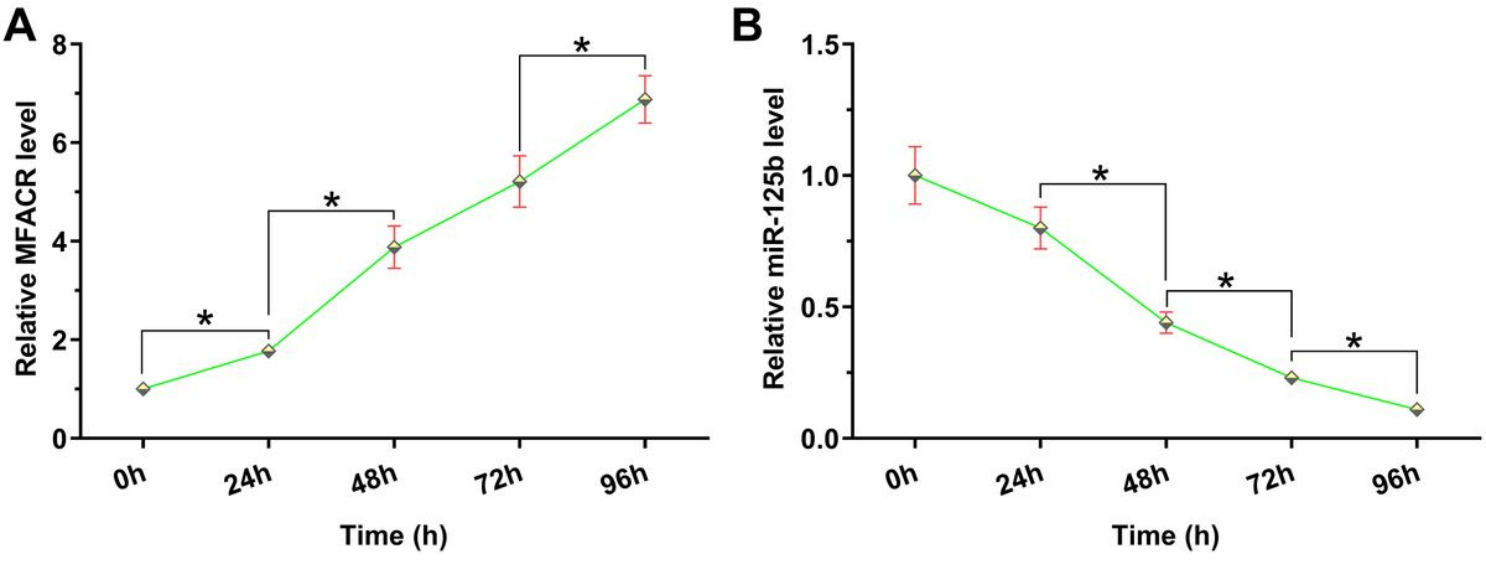

Figure 2

Hypoxia treatment altered the expression of MFACR and miR-125b in AC16 cells AC16 cells were cultivated under hypoxic conditions for 24, 48, 72 and 96h, followed by determine the expression of MFACR (A) and miR-125b (B) by RT-qPCR. *,p<0.05.

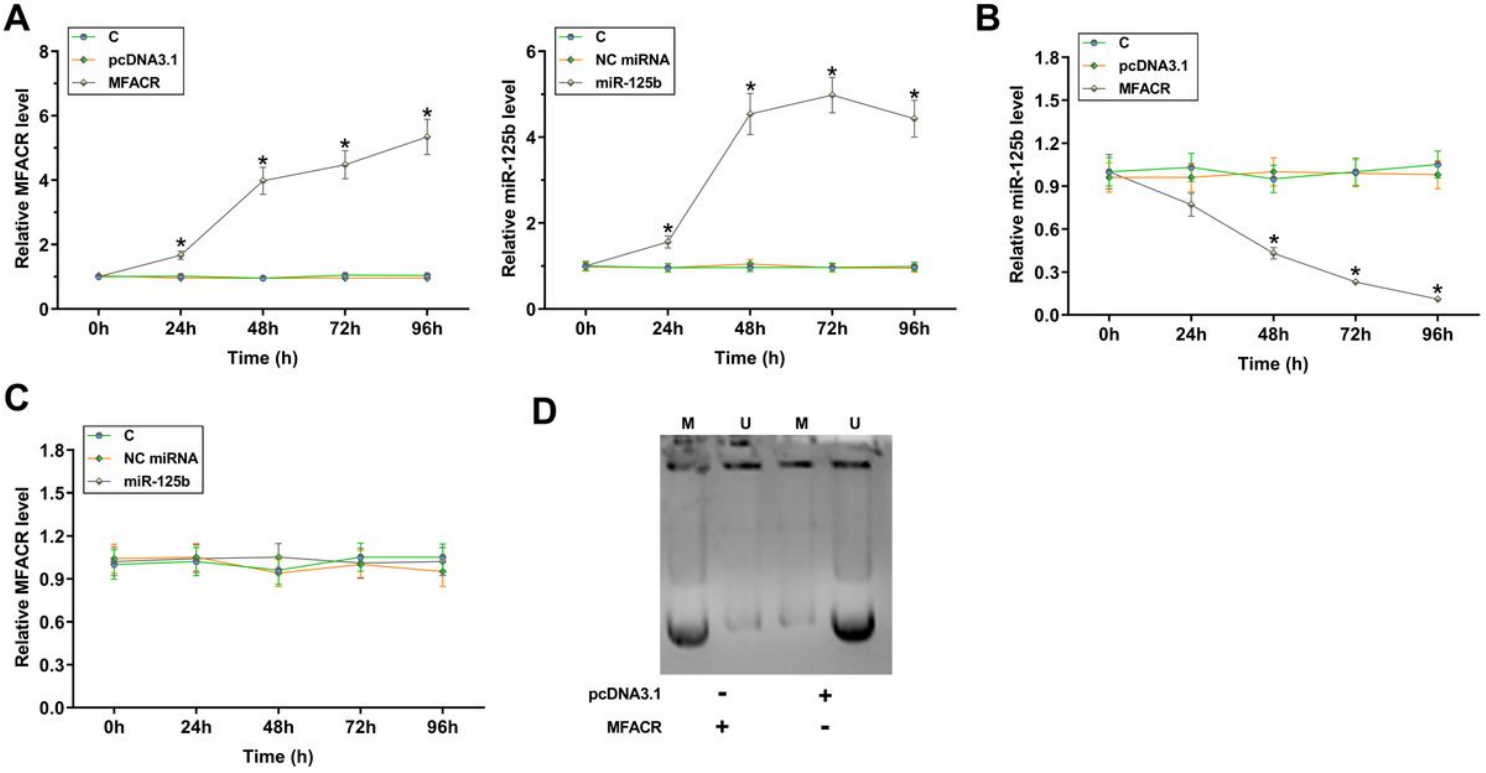

Figure 3

MFACR overexpression decreased miR-125b expression through methylation in AC16 cells To study the effects of crosstalk between MFACR and miR-125b, AC16 cells were transfected with either MFACR expression vector or miR-125b mimic, followed by checking the overexpression of MFACR and miR-125b every 24h until 96h (A). The effects of MFACR overexpression on the expression of miR-125b (B), and the effects of miR-125b overexpression on the expression of MFACR (C) were analyzed by RT-qPCR. MSP was performed at 96h post-transfection to analyze the effects of the overexpression of MFACR overexpression on the methylation of miR-125b gene (D).M, methylation; U, un-methylation; *,p<0.05.

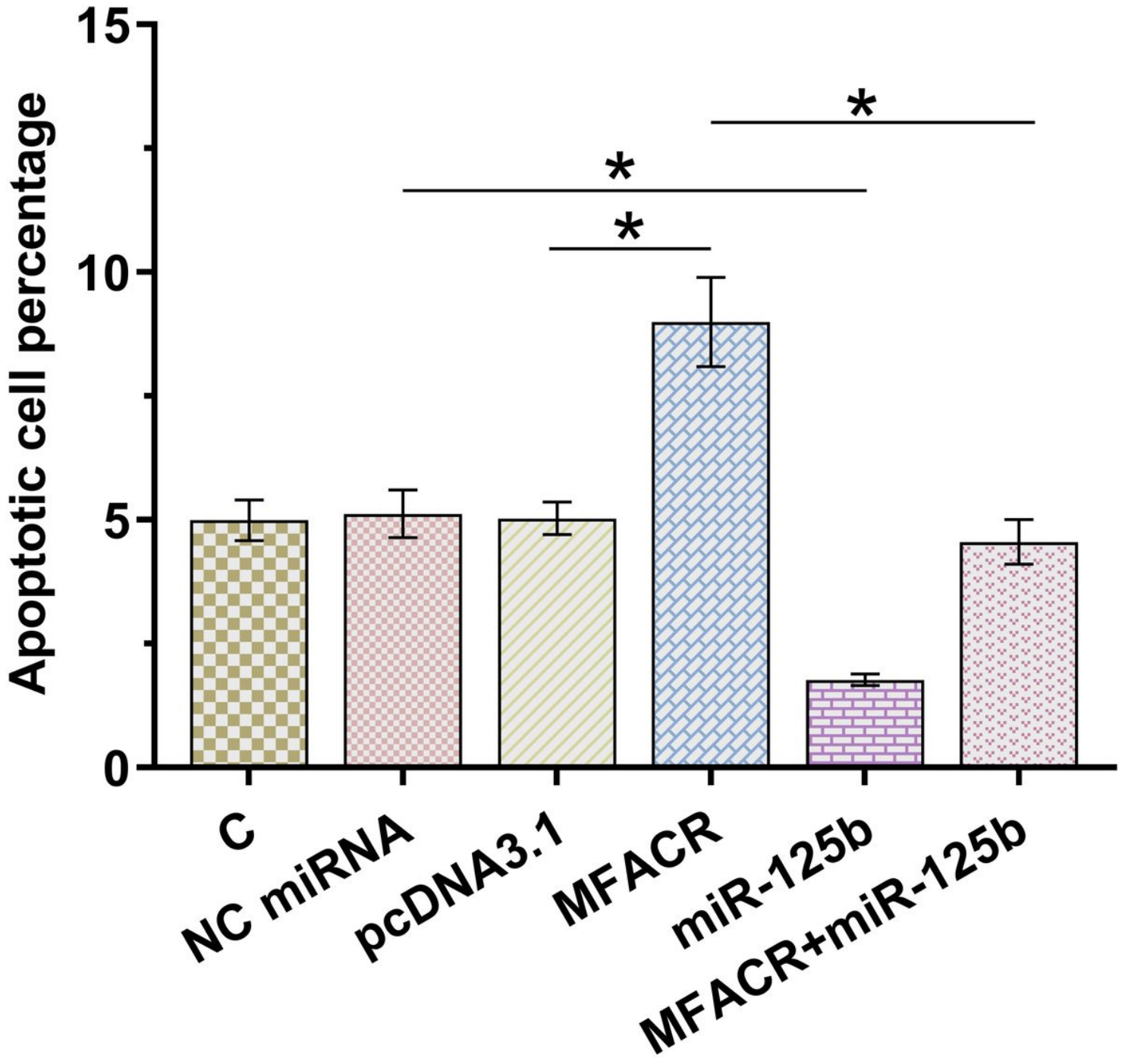

Figure 4

MFACR overexpression increased AC16 cell apoptosis induced by hypoxia through miR-125b Under hypoxic conditions, the role of MFACR and miR-125b in regulating the apoptosis of AC16 cells was analyzed by cell apoptosis assay.*,$p<0.05$.