

Original Article

Overexpression of miR-145 promotes the hypoxia induced apoptosis of vascular endothelial cells

Qingzan Kong¹, Qing Zhu², Bin Li¹, Liqi Wang¹

¹Department of Cardiology, Jinan Central Hospital Affiliated to Shandong University, No. 105, Jiefang Road, Jinan 250013, Shandong Province, P. R. China; ²Department of Pathology, Blood Center of Shandong Province, Jinan 250013, Shandong Province, P. R. China

Received December 7, 2017; Accepted October 11, 2018; Epub January 15, 2019; Published January 30, 2019

Abstract: Purpose: Cardiovascular disease (CVD) are often associated with ischemic events, leading to a reduction in oxygen and nutrient delivered to the tissues. Under hypoxic conditions, gene expression patterns in the heart are significantly altered. Therefore, exploring the effects and underlying mechanisms of hypoxia on endothelial cells would be helpful to understand the pathobiology of CVD and provide potential molecular therapies. The aim of this study was to construct hypoxic environment on human umbilical vein endothelial cells (HUVECs) and observe the functional significance of hypoxia-regulated miR-145. Methods: The expression of miR-145 was detected in HUVECs using quantitative real-time polymerase chain reaction (qRT-PCR). And Cell Counting Kit-8 (CCK-8) assay, transwell assay, and flow cytometry were used to assess cell proliferation, migration, and apoptosis, respectively. Besides, we detected the expression of apoptosis-related protein and PI3K-related protein using western blotting. Results: Hypoxia induced high expression of miR-145 in HUVECs. Then we found miR-145 overexpression inhibited cell viability and migration, while promoted the apoptosis of HUVECs, which were similar with the phenotypes in hypoxia conditions. In addition, the PI3K signaling pathway was also inhibited by miR-145 overexpression. Conclusions: We identified that hypoxia induced high expression of miR-145, which could inhibit HUVECs viability and migration, indicating that miR-145 was an important factor mediating hypoxia-induced cardiovascular disease and might be used as a target of drug treatment in the future.

Keywords: Hypoxia, miR-145, HUVEC, proliferation, migration, apoptosis

Introduction

Hypoxia is commonly associated with the pathobiology of many diseases, e.g., CVD, such as myocardial ischemia, myocardial infarction (MI), and stroke, as well as chronic lung diseases, cancer, and inflammation [1-3]. These diseases are either caused by or a consequence of hypoxia. Cardiovascular disease (CVD) is a major worldwide health burden [4]. Cardiomyocyte apoptosis, induced by the overproduction of reactive oxygen species (ROS) under ischemia or ischemia/reperfusion (I/R) or ischemic preconditioning (IPC), is an important pathological phenomenon in heart failure [5]. Oxygen not only is required for normal oxidative metabolism but also is a critical participant in the generation of many small-molecule signaling intermediates, such as nitric oxide (NO) and ROS, and in many other cellular redox processes [6]. These oxygen-associated reactions can

be either beneficial or contribute to cardiac dysfunction and death [7]. Under hypoxic conditions, gene expression patterns in the heart are significantly altered [8]. Previous studies have also shown that the oxygen sensitive master transcription factors, the hypoxia-inducible factors (HIFs), play a protective role in the pathophysiology of myocardial ischemia and pressure-overload heart failure, and contribute to the pathogenesis of pulmonary arterial hypertension [5, 9, 10]. Therefore, exploring the effects and underlying mechanisms of hypoxia on endothelial cells would be helpful to understand the pathobiology of CVD and provide potential molecular therapies.

MicroRNAs (miRNAs) are highly conserved small RNA molecules (22 nucleotides) which mostly regulate gene expression and participate in almost every cellular process [11]. Mounting literatures have proved that miRNAs

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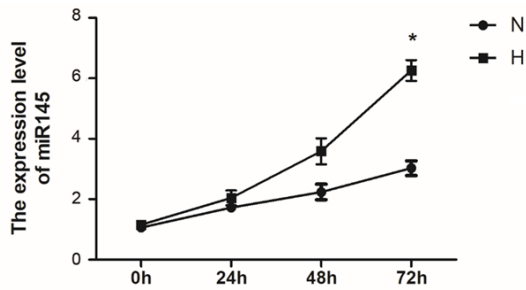


Figure 1. Effects of hypoxia on expression of miR-145 in HUVECs. It showed the expression level of miR-145 in HUVECs under hypoxic and normal conditions. Data were obtained 0, 24, 48 and 72 h using qRT-PCR. * $P < 0.05$. Data were expressed as mean \pm sd. N, under normal condition. H, under hypoxic condition (2% O_2).

are involved in multiple diseases including myocardial infarction (MI) [8, 12, 13]. A previous study has reported miRNA (miR)-99a alleviates heart remodeling and improves cardiac performance after MI [14]. Another study illustrated circulating miR-1 acts as a novel biomarker for MI [15]. A growing body of experimental evidence suggests that under hypoxic, dysregulation of miR-210 directly modulates changes in mRNA transcription, that was associated with altered control of the cell cycle, inadequate production of energy, cell death, and aberrant regulation of cell morphology [7, 16, 17]. In addition, miR-145 is reported as a marker for estimation of infarct size during MI [18]. More recently, miR-145 is also proved to be a novel biomarker for prognosticating cardiac function as well as the risk of heart failure [19]. These suggest that miR145 is associated with hypoxia-induced cardiovascular disease, but its specific role remains unclear.

In this paper, under hypoxia, the expression and role of miR-145 in HUVECs were systematically studied. Moreover, the possible target genes of miR-145 and the underlying mechanism of the modulation were also investigated.

Materials and methods

Cell culture

HUVECs were purchased from Chinese Academy of Sciences Shanghai Branch cell bank. The cells were incubated in dulbecco's modified eagle medium (DMEM) supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Until the cells grew to logarithmic phase, they were

washed 3 times by PBS, digested with trypsin and then repeatedly beat to single cell suspension. The cells were then grown into a six-well plate for subsequent experiments. When density reached about 80%, the cells were transfected. For hypoxic induction, cells were exposed to hypoxic environment (2% O_2) in a modulator chamber and grown at the same duration as cells in normal conditions.

Cell transfection

HUVECs were transfected in 6-well plates using the liposome Lipofectamine 2000 (Invitrogen, Beijing ComWin Biotech Co., Ltd) according to the manufacturer's protocol. 5 μ g of recombinant plasmid of pCMV-MIR-miR145 and 10 μ l Lipofectamine 2000 were added to 250 μ l of serum-free medium, respectively. Then they were mixed and allowed to incubate 10 mins at room temperature. 500 μ l of this mixture was then added to the cells in 1.8 mL of RPMI 1640 with 10% FCS and incubated 6 h. The transfected cells were cultured for 24 h in complete medium prior to further analysis.

RNA extraction, cDNA synthesis and qRT-PCR

Total RNAs of cells were extracted by using 1.0 ml Trizol (Invitrogen, Carlsbad, CA). Synthesis of cDNA was carried out with reverse transcriptase M-MLV (Takara). Quantitative real-time polymerase chain reaction (qRT-PCR) system (Applied Biosystems, Foster City, CA, USA) was used to detect the expression level of miR-145, and the parameters were as follows: according to the manufacturer's instructions, hot start at 95°C for 5 min; followed by 40 cycles of 95°C for 30 sec, 60°C for 45 sec and 72°C for 30 sec. The primer sequence was as follow: miR-145, F: 5'-GTCCAGTTTTCCAGGAATCCCT-3', R: 5'-CATGTCCACGGTTCTAGTTTCTTG-3'. U6 (negative control), F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'. For each miRNA, each sample was assayed in triplicate. The relative quantification of the value was determined using the $2^{-\Delta\Delta Ct}$ method.

Cell counting kit-8 (CCK-8) assay

Cell digestion was counted after transfection for 24 h and cell suspensions were prepared. Cell suspension (1000 cells/well) were seeded in 96-well plates, then the cells were incubated 1.5 h at 37°C in a CO_2 incubator. The cell viability was measured every 24 h after 10 μ l of

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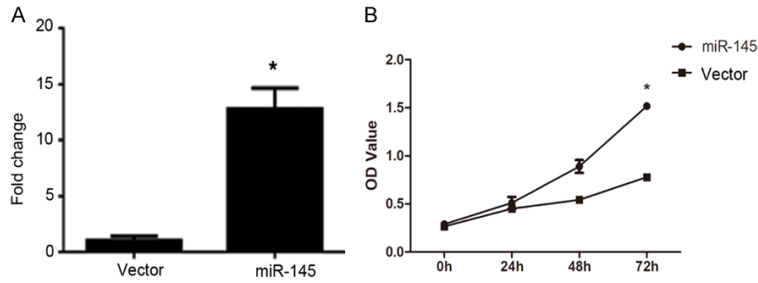


Figure 2. Expression of miR-145 in HUVECs and the effect of it on cell viability. We transfected miR-144 mimics and control into HUVECs respectively. A. Bar graphs showed the expression level of miR-145 in the two groups using qRT-PCR. B. It showed OD value of HUVECs in the two groups. Data were obtained 0, 24, 48 and 72 h following transfection by CCK-8 detection. Data were presented as mean \pm sd of three independent experiments. * $P < 0.05$. miR, microRNA. OD, optical density.

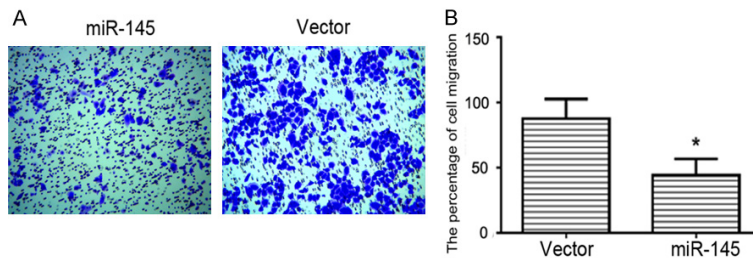


Figure 3. Effects of miR-145 overexpression on migration of HUVECs A. Representative micrographs of cell migration assay using transwell chamber. B. Bar graphs showed the percentage of cell migration in Vector group (87.13 \pm 0.36) and miR-145 group (46.54 \pm 1.07). * $P < 0.05$. Data are expressed as mean \pm sd.

CCK-8 (Beijing solarbio science & technology co., ltd.) was given to per well and optical density (OD) value was measured by 450 nm excitation to plot the proliferation curve using a microplate reader.

Cell migration assay

Cell migration was determined by using 24-well transwell chamber (Millipore). Cell suspension (1×10^5 cells/100 μ l serum-free MEM media) were added to the upper chambers and complete culture solution (500 μ l) were added to the lower chambers. To stay overnight, cells were washed in PBS, fixed for 30 min in 4% paraformaldehyde, stained for 20 min with 0.1% crystal violet. After the indicated treatments, five fields were randomly selected under the microscope and representative photographs of stained cells were taken to observation and count.

Flow cytometry analysis of cell apoptosis

After the cells were transfected for 24 h, cells were trypsinized without EDTA and collected in a centrifuge tube, then the cells were centrifuged at 1000 rpm for 5 min. Afterwards cells were resuspended in PBS pre-cooled at 4°C, centrifuged again and carefully aspirated the supernatant. After that we added 1X binding buffer to resuspend the cells, and regulated the cell density of $1-5 \times 10^6$ /ml. 100 μ l of the cells were gently mixed with 5 μ l Annexin V-FITC/PI (Beijing 4A Biotech Co., Ltd) and incubated in the dark for 5 min at room temperature. 10 μ l of PI dye and 400 μ l of PBS were added to the sample, and the results were analysed in a flow cytometer using Flowjo.

Western blotting analysis

After transfected for 48 h, cells were lysed in RIPA buffer, centrifuged at low temperature (12000 rpm, 20 min) and extracted from the protein supernatant. Protein concentration was detected using BCA Protein Assay Kit. The cell lysates (20 μ g protein/lane) were separated by SDS-PAGE and then transferred onto PVDF membranes. After blocked with 5% non-fat dry milk 1 h, the membranes were then incubated with primary antibodies overnight at 4°C: AKT (CST, 1:1000), p-AKT (CST, 1:1000), mTOR (CST, 1:1000), p-mTOR (CST, 1:1000), p70S6K (PTG, 1:1000), Cyclin D1 (PTG, 1:1000), Bcl-2 (PTG, 1:1000), Bax (PTG, 1:1000), Caspase3-p17 (PTG, 1:1000), Caspase9 (PTG, 1:1000), GAPDH (PTG, 1:5000). Followed by washing 3-5 min, secondary antibodies (labeled with HRP) were incubated 1 h at room temperature and detected with ECL chemiluminescence plus reagents. GAPDH was used as the internal reference. The gray value was scanned with QUANTITY ONE software, and the relative

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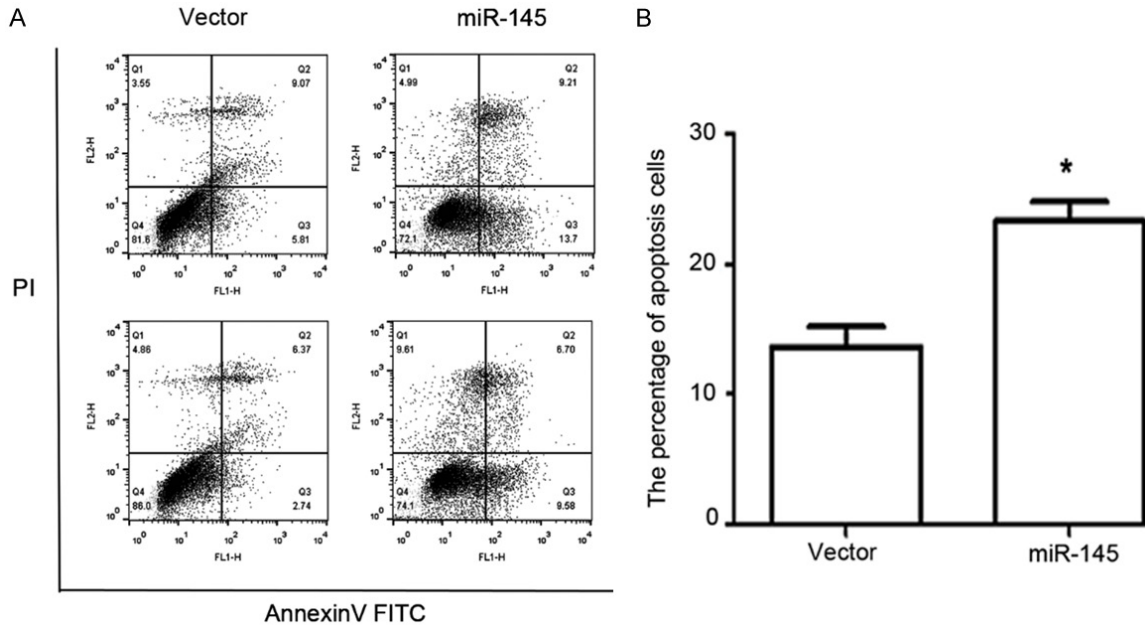


Figure 4. Effects of miR-145 overexpression on apoptosis of HUVECs. A. Flow cytometric plots showing specific cell populations in HUVEC cells. Q2 were late apoptotic cells, Q3 were live cells, and Q4 were early apoptotic cells. B. Bar graphs showed the percentage of cell apoptosis.

expression of each protein was calculated with the target protein/internal reference.

Statistical analysis

The data were presented as mean \pm standard deviation (sd). Differences between two groups were assessed using a student's t-test. Statistical differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS 18.0 software.

Results

Hypoxia induces high expression of miR-145 in HUVECs

Firstly, we detected expression level of miR-145 in HUVECs under hypoxia and normal condition by performing qRT-PCR analysis. The result showed that the expression of miR-145 was significantly increased under hypoxia ($P < 0.05$, **Figure 1**). So we confirmed hypoxia induced high expression of miR-145 in HUVECs.

miR-145 is overexpression in HUVECs and it inhibits the cell viability

The above result suggested that miR-145 might play an important role in hypoxia. For this rea-

son, we modeled the high expression of miR-145 in HUVECs under hypoxia condition, in order to observe the effect on the activity of HUVECs. First, we observed the effects of mimics on the expression of miR-145 using QRT-PCR and found that miR-145 level in the miR-145 mimic group was significantly increased compared to the control group ($P < 0.05$, **Figure 2A**), realising the method we used can effectively improve the expression of miR-145 in HUVECs. Then we performed the CCK8 assay to examine the effects of miR-145 on cell viability. And the test showed that miR-145 overexpression significantly repressed OD value since 72 h ($P < 0.05$, **Figure 2B**), that indicated that the miR-145 overexpression could inhibit the viability of HUVECs.

miR-145 overexpression inhibits the migration of HUVECs

To explore the effect of miR-145 on metastasis, transwell assay were carried out. As shown in **Figure 3**, miR-145 overexpression led to the remarkable reduction of the number of HUVECs passing through the microwells of the transwell chamber ($P < 0.05$). Collectively, the results showed that overexpression of miR-145 could inhibit HUVECs migration.

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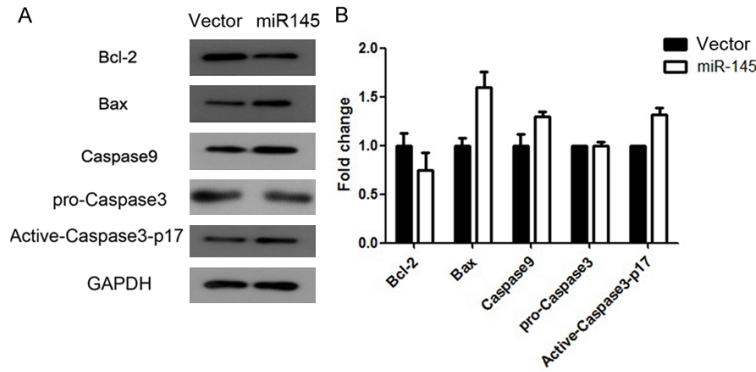


Figure 5. Effects of miR-145 on apoptosis-related proteins in HUVECs. A. Expression of Bcl-2, Bax, Caspase3-p17 and Caspase9 were determined by western blotting. B. Bar graphs showed expression levels of Bax, Bcl-2, Caspase3-p17 and Caspase9. * $P < 0.05$, compared with Vector group. Data were expressed as mean \pm sd. PI, propidium iodide.

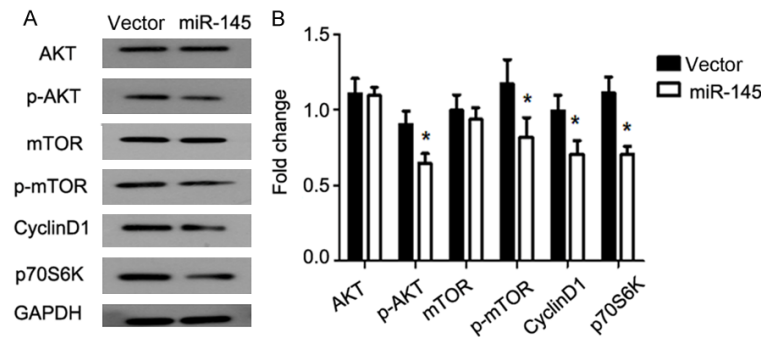


Figure 6. Effects of miR-145 overexpression on activation of PI3K signaling pathway in HUVECs. A. Expression of AKT, p-AKT, mTOR, p-mTOR, Cyclin D1 and p70S6K were determined by western blotting. B. Bar graphs showed the expression levels of AKT, p-AKT, mTOR, p-mTOR, Cyclin D1 and p70S6K. Data were expressed as mean \pm sd. * $P < 0.05$, compared with Vector group.

miR-145 overexpression promotes the apoptosis of HUVECs

To explore whether the role of miR-145 in HUVECs was correlated with the apoptosis, flow cytometry after Annexin V-FITC/PI staining and western blot assay were used. The flow cytometry analysis showed that the apoptosis of cells treated with miR-145 were increased ($P < 0.05$, **Figure 4A** and **4B**). Next, we detected the expression of apoptosis-related protein Bcl-2, Bax, Caspase3-p17 and Caspase9 after miR-145 treatment using western blotting (**Figure 5A**). As shown in **Figure 5B**, the expression levels of anti-apoptotic protein BCL-2 decreased ($P < 0.05$), and the expression of pro-apoptotic protein Bax, Caspase3-p17 and Caspase9 increased concurrently ($P < 0.05$). Therefore, we considered that miR-145 overex-

pression could promote the apoptosis of HUVECs.

miR-145 overexpression inhibits the PI3K signaling pathway in HUVECs

Phosphoinositide-3 kinase (PI-3K)/AKT/mTor signaling pathway is a well-known pathway, which is proved to participate in multiple cellular activities, including cell proliferation, apoptosis, migration and invasion [20, 21]. In order to know whether the effect of miR-145 on HUVECs via PI3K signaling pathway, we determined the expression of some key proteins in this pathway by western blot assay (**Figure 6A**). Accordingly, we found the phosphorylation of AKT and mTOR were significantly inhibited in HUVECs treated with miR-145 (**Figure 6B**, $P < 0.05$). Besides, miR-145 attenuated activity of the downstream proteins such as p70ribosomal protein S6 kinase (p70S6K) and Cyclin D1 related to cell growth (**Figure 6B**, $P < 0.05$). These results showed miR-145 overexpression inhibits the PI3K signaling pathway in HUVECs.

Discussion

Even though the treatments for CVD have been developed, CVD could cause acute death and long-term complications, for example, heart failure [22, 23]. There is a growing interest on exploration of novel therapeutic targets for CVD. In this study, we confirmed that hypoxia in HUVECs was associated with increased miR-145 expression. In in-vitro model based on HUVECs, we also observed miR-145 transfection resulted in inhibition of cell viability and migration and promoted cell apoptosis via inhibiting activation of PI3K signaling pathway.

Hypoxia is the important and original reason of cardiovascular diseases. Cellular hypoxia causes large numbers of cellular events, resulting in loss of myocardium [1]. Then, ventricular

begins to be remodeled gradually, accompanied by fibrosis, chamber enlargement and heart failure [24, 25]. Besides, hypoxia is thought to be an important factor to promote angiogenesis and can facilitate gene transcription through the role of the hypoxia inducible factor. Through this mechanism, hypoxia can modulate expression of multiple genes and therefore affect their downstream regulations [6, 10, 26]. Previous studies reported that under the hypoxic environment, miR-200a expression was induced significantly higher in ectopic endometriotic tissue, which could enhance dual specificity phosphatase-2 controlled angiogenesis [17]. The hypoxia-inducible miRNA, miR-210, augments the metastatic potential of hepatocellular carcinoma by targeting vacuole membrane protein 1 [13, 16]. In this paper, it is the first attempt to illuminate the dynamic change of miR-145 under hypoxia in HUVECs.

Hypoxia may increase the production of intracellular reactive oxygen species (ROS) that have been demonstrated to inhibit cell growth and induce cell apoptosis and necrosis [27]. The recent evidence has implicated miRNAs in the regulation of a wide variety of biological processes, such as oxidative stress and apoptosis. Our results showed the inhibition role of miR-103 in cell proliferation and migration of HUVECs [7]. Apoptosis plays a critical role in maintaining cellular homeostasis in both normal conditions or to overcome stress-induced conditions. One recent study reported that miR-145 was upregulated in H9c2 cells under hypoxic conditions resulting in increased ROS-induced apoptosis [2]. In addition, miR-145 and the hypoxia adaptations that result in cardiomyocyte death via apoptosis [28]. Using the flow cytometry, we demonstrated overexpressing miR-145 promoted cell apoptosis in HUVECs. Then, we performed western blotting to investigate the downstream regulator involved. The expression level of anti-apoptotic protein BCL-2 decreased, and the pro-apoptotic proteins (Bax, Caspase3-p17 and Caspase9) increased concurrently. The above results implied that miR-145 might be a novel hypoxia related markers in HUVECs. More recently, the PI3K/AKT pathway is reported to protect myocardial cells from ischemic injury [29]. To reveal the underlying mechanism of the miR-145-as-

sociated modulation in HUVECs under hypoxia, the phosphorylation levels of key kinases in the PI3K/AKT pathway was investigated. The results stated that the pathway was inactivated by hypoxia.

In conclusion, our results indicated that under hypoxia, the expression level of miR-145 in HUVECs was increased. And overexpression miR-145 could inhibit cell viability and migration, promote cell apoptosis via inhibiting activation of PI3K signaling pathway. The hypoxia-induced higher miR-145 expression might contribute to pathological development of HUVECs through reducing cell survival and promoting apoptosis via PI3K. And we could confirm that miR-145 was a pro-inflammatory factor and its targeting regulation might be a new treatment direction for CVD.

Acknowledgements

This study was funded by Beijing Lisheng Cardiovascular Health Foundation Linghang Funds (grant number LHJJ20159227).

Disclosure of conflict of interest

None.

Address correspondence to: Liqi Wang, Department of Cardiology, Jinan Central Hospital Affiliated to Shandong University, No. 105, Jiefang Road, Jinan 250013, Shandong Province, P. R. China. Tel: 13370582880; Fax: 0531-68623311; E-mail: wangliqi2017@163.com

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