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ARTICLE

MiR-124 regulates apoptosis in hypoxia-induced human brain microvessel endothelial cells through targeting Bim

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Abstract Human brain microvessel endothelial cells (HBMECs) are crucial for brain vascular repair and maintenance. The high-expressed expressions of microRNA-124 (miR-124) in brain have been investigated and revealed in many researches. In this work, we aimed to investigate the role of miR-124 in apoptosis of HBMECs and the underlying mechanism. Here, we found the low-expressed miR-124 in hypoxia-induced HBMECs using qRT-PCR analysis. MiR-124 targeting 3′-untranslated region (3′-UTR) of Bim mRNA was predicted by Targetscan database. Importantly, the decreased miR-124 expression and increased Bim expression, an opposite trend, were obtained in hypoxia-induced HBMECs. The further confirmation of the correlation between miR-124 and Bim was conducted by miR-124 overexpression and dual luciferase reporter assays. The inhibitory role of miR-124 in Bim expression was evidenced by results obtained from miR-124 overexpression analysis. Luciferase reporter assay further proved that miR-124 directly targeted the two conserved seed sites in the Bim 3′-UTR. The inhibited apoptosis of HBMECs was observed under both miR-124 overexpression and Bim knockdown condition in flow cytometry analysis. Collectively, these findings outline that miR-124 regulates apoptosis in hypoxia-induced HBMECs through targeting Bim, providing a better understanding of the role of miR-124 in apoptosis of HBMECs.

Keywords Apoptosis · Bim · MiR-124 · Stroke

Introduction

Characterized by a complex, multifactorial, polygenic disease, stroke pertains to a leading cause of death and disability worldwide in countries at all income levels [1, 2]. Stroke-associated ischemic damage generally involves microvascular injury, excitotoxicity, oxidative stress, blood–brain barrier dysfunction, and, ultimately, the death of neurons, glia, and endothelial cells [3, 4]. Commonly, the endothelial cell damage leads to a significant microvascular injury that directly results in cerebral tissue damage [5]. Clinically, the main treatments comprise of symptomatic therapies involved in dehydration, intracranial pressure reduction, and hematoma removal [6]. But the therapeutic efficacy remains unsatisfactory with the loss of truly effective treatment measures and drugs. Therefore, in-depth investigation of regulatory mechanism and treatment strategy for stroke is imperative and even decisive for the diagnosis and treatment of this disease.

MicroRNAs (miRNAs) are a class of small noncoding RNAs, which modulate gene expression via posttranscriptional mechanisms, regulating the main cellular processes,

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such as cell development, differentiation, apoptosis, and metabolism [7, 8]. Extensive research generally outlines that many miRNAs play a vital role in various diseases, such as tumors [9, 10], stroke [2, 11–13], Parkinson's disease [14, 15], and Alzheimer's disease [16]. Specially, microRNA-124 (miR-124) has been reported to be highly expressed in the brain [17] and exert the neuroprotective effect on stroke [18]. Also, discussions regarding the regulatory mechanism of miR-124 involved in stroke have dominated research in recent years. MiR-124 could regulate apoptosis-stimulating protein of p53 family in stroke from experimental perspective [19]. MiR-124 also affects the apoptosis of brain vascular endothelial cells and ROS production through regulating PI3K/AKT signaling pathway [20]. However, more underlying and possible regulated mechanism of miR-124 still remains to be explored and disclosed.

Therefore, this work aimed to investigate the regulatory mechanism of miR-124 in apoptosis of human brain microvessel endothelial cells (HBMECs), which is supported by the following work. Under hypoxia condition, the expression level of miR-124 was detected by quantitative real-time polymerase chain reaction (qRT-PCR) analysis. We predicted Bim as target gene of miR-124 by means of Targetscan database and then analyzed the levels of miR-124 expression and Bim expression in hypoxia-induced HBMECs via qRT-PCR and western blot assays. Subsequently, we confirmed Bim as a direct target of miR-124 through qRT-PCR, western blot, and dual luciferase reporter assays. Finally, we investigated the potential mechanism of miR-124 on hypoxia-induced cell apoptosis determined by flow cytometry assay.

Materials and methods

Cell culture and transfection

HBMECs and HEK 293T cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HBMECs and HEK 293T cells were separately cultured in endothelial cell medium (ECM, ScienCell, San Diego, CA, USA) and Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) both containing 10% fetal bovine serum (Hyclone, Logan, UT, USA) in a humidified incubator at 37 °C with 5% CO_2. Plasmid DNA, miRNA mimic, siRNA, and Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) were separately diluted in serum-free medium according to manufacturer's instructions. After gently mixed, the solution was kept for 20 min at room temperature. Next, 400 μL of serum-free medium and the plasmid–transfection reagent mixture was gently added to 24-well plates, incubating for 4–6 h. Finally, cells were collected for subsequent experiments after another culture for 48 h in the exchange of the ECM medium containing 10% fetal bovine serum. The details for transfection were described as follows: 500 ng plasmid per well, miR-124 mimic (25 nmol/L) (5′-UAAGGCACGCGGUGAAUGCC-3′), Bim siRNA (20 nmol/L) [5′-UGACAAAUAAACACAAACCCCTT-3′ (sense)].

RNA extraction and qRT-PCR analysis

Total RNA was extracted from culture cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc., USA) according to the manufacturer's protocol. One microliter of extracted RNA was used for the concentration and $D_{260\ nm}$/$D_{280\ nm}$ detection on a Nanodrop 2000 (Nanodrop, Thermo Fisher Scientific, Inc., USA). RNA integrity was determined by 1% agarose gel electrophoresis. As for mRNA reverse transcription, a total of 11 μL of reaction mix (1 μL of oligo dT (500 ng/μL), 1 μL of total RNA, and a remainder of DEPC water) was denatured at 70 °C for 5 min. Next, a total of 20 μL of reaction mix was obtained by adding other reagents including 4 μL of 5 × M-MLV reverse buffer, 2 μL of dNTP (25 mM), 1 μL of M-MLV reverse, and 1 μL of RNase inhibitor. After kept at 42 °C for 90 min, the reaction mix was treated for the inactivation of reverse transcriptase at 95 °C for 10 min and then stored in − 20 °C before use. As for miRNA, 1 μL of total RNA was treated for tailing. A total of 25 μL reaction mix (1 μL of QmiR-RT (sequence: GCGAGCACAGAATTAATACGACTCA CTATAGGT TTTTTTTTTTTTTTTTTVN, 500 ng/μL), 2.5 μL of 10 × *E. coli* poly (A) polymerase buffer, 2.5 μL of $MnCl_2$ (2.5 mM), 2.5 μL of dNTP (25 mM), 2.5 μL of ATP (100 mM), 1 μL of M M-MLV reverse, 1 μL of E. coli poly (A) polymerase, and 0.5 μL of RNase inhibitor) was treated at 37 °C for 90 min, followed by the inactivation of reverse transcriptase at 95 °C for 10 min, and finally stored in − 20 °C before use.

qRT-PCR analysis was performed to analyze the expression levels of miR-9, miR-124, and Bim. For these analyses, SYBR Green PCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) was employed to conduct qPCR in a AriaMx Real-time PCR system (Agilent Technologies, Santa Clara, CA, USA). For miR-124 and miR-9, U6 was used as the internal control. For Bim, β-actin was used as the internal control. The data were normalized to internal controls. AriaMx software was used to calculate the fold changes of miR-9, miR-124, and Bim. The sequences used for qRT-PCR are shown in Table 1.

Western blot analysis

After 48-h transfection, cells collected were lysed for 30 min using RIPA buffer (Beyotime Biotechnology,

Table 1 Primer sequences were used in this work

Gene		Sequence (5′–3′)
Primers for qRT-PCR analysis		
miR-124	Forward	TACTATAAGGCACGCGGTGAATGC
	Reverse	GCGAGCACAGAATTAATACGAC
U6	Forward	CGCTTCGGCAGCACATATACTA
	Reverse	CGCTTCACGAATTTGCGTGTCA
Bim	Forward	TCATCGCGGTATTCGGTTC
	Reverse	GAAGGTTGCTTTGCCATTTG
β-Actin	Forward	GGCATCCTCACCCTGAAGTA
	Reverse	GGGTGTTGAAGGTCTAAA
Primers for luciferase activity analysis		
Bim-WT	Forward	CGGGGTACCGCGGTTCTCTTGTGGAGGGG
	Reverse	CCCAAGCTTAGAAGGGGAAACGGCAGACA
Bim-mut	Forward 1	CGGGGTACCGCGGTTCTCTTGTGGAGGGG
	Reverse 1	GTAATCTCTAAAAATAAAGCTTTTTAAGTTAGCTGGC
	Forward 2	CGGGGTACCGCCAGCTAACTTAAAAAGCTTTATTTTTAGAGATTAC
	Reverse 2	CCCAAGCTTCTCTTTCTTGGGTCTCCCTGGGC
	Forward 3	CGGGGTACCGCCCAGGGAGACCCAAGAAAGAG
	Reverse 3	CACATAGGGAGAGGTTGTCAAAACCTGCAGTAATTTCC
	Forward 4	CGGGGTACCGGAAACTTACTGCAGGTTTTGACAACCTCTCCCTATGTG
	Reverse 4	CCCAAGCTTAGAAGGGGAAACGGCAGACA

miR-124, microRNA-124; WT, wild type; mut, mutant

Shanghai, China) with the corresponding protease inhibitors (Roche, Basel, Switzerland). The protein concentration of the lysates was detected using BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Inc., USA). Proteins were electrophoresed in 12% SDS-PAGE gel and then transferred onto polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). Membrane was blocked in 1 × tris-buffered saline containing 0.1% Tween-20 (TBST) with 5% nonfat milk for 1 h. Next, membrane was incubated with primary antibody at 4 °C for 1 h. Membrane was washed with TBST and then incubated with secondary antibody labeled with horseradish peroxidase conjugated (HRP, 1:10,000, Millipore, MA, USA) at room temperature for 1 h. β-Actin served as a control. Finally, blots were developed using enhanced chemiluminescence reagents to detect proteins by ECL detection system (Millipore, MA, USA) according to the manufacturer's instruction.

Luciferase activity analysis

Then, DNA fragments with 2525 bp, 1344 bp, 1187 bp, and 2505 bp were obtained by PCR amplifying 3′-UTR sequences of Bim mRNA based on the template of BMEC cell genome. After electrophoresing on 1% agarose, the gel was recovered and purified. Next, the recovered DNA fragments were constructed into the pGL3-control vector by using the Kpn I and Hind III double enzymes, which is thus termed pGL3-Bim-WT, pGL3-Bim-mut 1, pGL3-Bim-mut 2, and pGL3-Bim-mut, respectively. The primers used are listed in Table 1. HEK 293T cells were planted into 24-well plates at the density of 5×10^5 per well and cultured in a humidified incubator at 37 °C with 5% CO_2. Subsequently, firefly luciferase reporter plasmid and miR-124 mimic were co-transfected into HEK 293T cells according to the manufacture's protocol. At 48 h post-transfection, relative luciferase activity was examined by normalizing the firefly luminescence to the Renilla luminescence using the GloMax 20/20 (Promega, WI, USA).

Flow cytometry analysis

HBMECs were planted into 24-well plates for 24 h. Subsequently, miR-124 mimic, negative control (NC) mimic, Bim siRNA, and control siRNA were separately transfected into HBMECs for 24 h, followed by another 6-h treatment under oxygen-free condition. The cell apoptosis was determined using Annexin/V-FITC Apoptosis Kit (Invitrogen, MA, USA) by flow cytometry under the manufacturer's protocol.

Statistical analysis

SPSS version 19.0 (SPSS, Chicago, IL, USA) was applied for statistical analysis. All experiments were repeated three times, and all data were presented as mean ± SD. One-way ANOVA was utilized for comparison between groups. $P < 0.05$ was considered statistically significant.

Results

The low-expressed miR-124 in hypoxia-induced HBMECs

We selected miR-9 and miR-124, two brain-specific miRNAs, that are highly expressed in brain tissue. Notably, miR-9 and miR-124 are wholly homologous in human, mice and rats, and associated with a broad spectrum of biological functions in stroke [21, 22]. HBMECs are crucial for brain vascular repair and maintenance, but their physiological function may be impaired during stroke [23]. The expression levels of miR-9 and miR-124 were detected in hypoxia-induced HBMECs through qRT-PCR analysis. Among two miRNAs, miR-124 was significantly down-regulated (> about 0.5 fold change) in HBMECs with the hypoxia treatment, while no significant change of miR-9 expression was obtained, as shown in Fig. 1.

The predicted miR-124 binding sites locate in the 3′-UTR of Bim mRNA

We used Targetscan7.2 bioinformatics database (http://www.targetscan.org/) to predict numbers of conserved genes that were miR-124 targets in the human genes. Among predicted miR-124 target genes, we chose a target gene related to cell apoptosis. According to Targetscan7.2 database analysis, the Bim 3′-UTR contains two conserved miR-124 target sites, separately located at position 465-471 and 2394-2400 (Fig. 2).

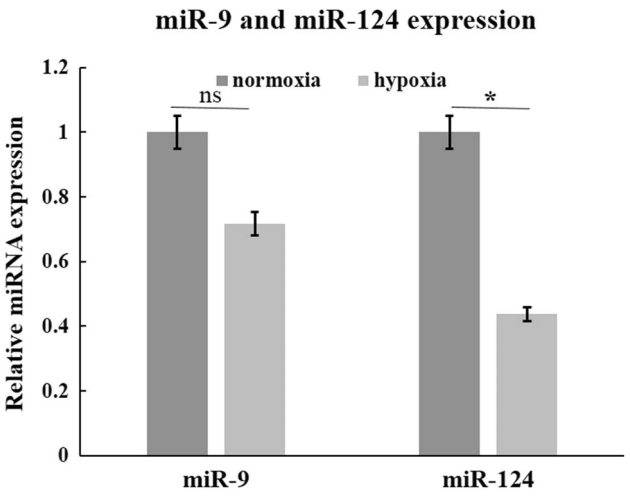

Fig. 1 Down-regulated miR-124 in HBMECs. Compared with normoxia group, miR-124 was evidently decreased in hypoxia group. However, no significant difference of miR-9 expression between these two groups was observed. ns, no significance. *$P < 0.05$

Expressions of miR-124 and Bim show an opposite trend in hypoxia-induced HBMECs

qRT-PCR and western blot assays were conducted to examine the miR-124 and Bim expression in HBMECs treated with hypoxia. qRT-PCR analysis results revealed that hypoxia constrained expression of miR-124 (Fig. 3A, $P < 0.05$), but promoted expression of Bim mRNA (Fig. 3B, $P < 0.05$). Additionally, Bim protein levels in hypoxia-induced HBMECs were assessed by western blot assay. The results showed the increased Bim protein expression (Fig. 3C), which was in correspondence with Bim mRNA expression levels. The above results indicated the possible connection between the down-regulation of miR-124 and up-regulation of Bim in hypoxia-induced HBMECs.

MiR-124 targets Bim in HBMECs

MiRNA mimics, small chemically modified double-stranded RNAs, could simulate endogenous miRNAs and enable the functional analysis of miRNA by up-regulating miRNA activity. As shown in Fig. 4A ($P < 0.05$), transfection of the miR-124 mimic into HBMECs led to an increased miR-124 expression. Bim was previously predicted as miR-124 targets [24]. As measured by qRT-PCR assay, miR-124 overexpression significantly down-regulated Bim mRNA expression levels in HBMECs (Fig. 4B, $P < 0.05$). We analyzed the levels of Bim proteins in HBMECs transfected with miR-124 mimic, and the results showed a correlation with mRNA levels, whereby Bim protein expression was significantly reduced (Fig. 4C). Collectively, the reductions in Bim expression at mRNA and protein levels in HBMECs were caused by miR-124 specifically targeting Bim.

According to bioinformatics analysis, the predicted miR-124 targeting binding sites located in the 3′-UTR of Bim mRNA (Fig. 4D). To further confirm that miR-124 directly targets the Bim 3′-UTR, two conserved target seed sites were separately cloned into the 3′-UTR of luciferase reporter plasmids to construct plasmids site 1 (465-471) and site 2 (2394–2400). Similar plasmids containing the corresponding mutant target sites, Bim-mut 1 and Bim-mut 2, were also constructed. Almost the full length of Bim 3′-UTR was cloned into the sites of pGL3 control vector and named it as Bim-WT. Luciferase reporter plasmids and

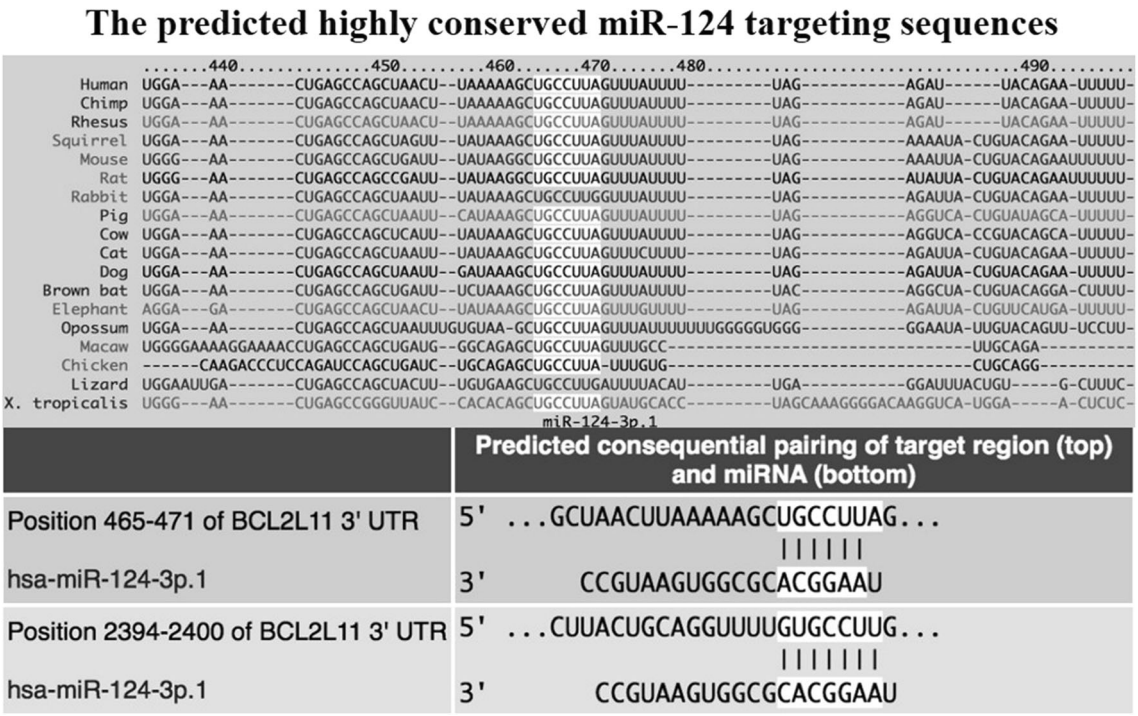

Fig. 2 Predicted highly conserved miR-124 targeting sequence located in the 3′-UTR of Bim mRNA. Target gene prediction using Targetscan database illustrated that there were two complementary binding sites in the midst of miR-124 and the 3′-UTR of Bim mRNA, separately located in 465-471 (site 1) and 2394-2400 (site 2)

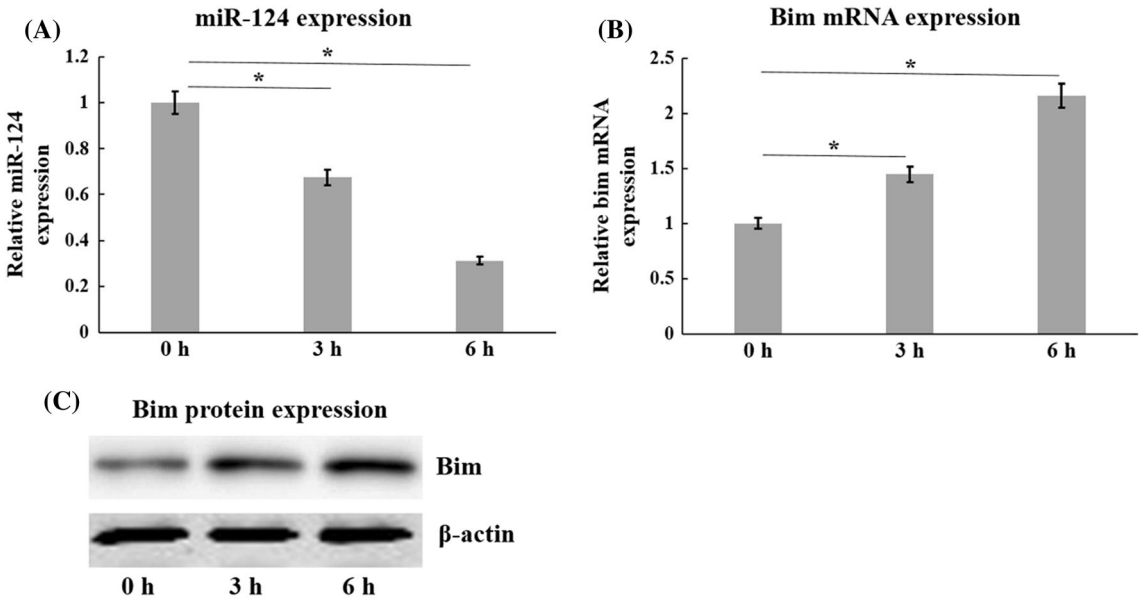

Fig. 3 Expression levels of miR-124 and Bim were detected in HBMECs under hypoxic condition. (**A**) Under hypoxic condition, qRT-PCR was conducted to evaluate the miR-124 expression in HBMECs. As time increasing, the evidently decrement of miR-124 expression was observed. *$P < 0.05$. (**B**, **C**) Under hypoxic condition, qRT-PCR and western blot were employed to assess the mRNA and protein levels in HBMECs, respectively. Both Bim mRNA level and protein level were significantly increased. *$P < 0.05$

miR-124 mimic were co-transfected into the HEK 293T cells. After 48-h treatment, luciferase activity in cells co-transfected with miR-124 mimic was significantly lower than that in cells co-transfected with NC mimic. However, the luciferase activity of cells co-transfected with miR-124 and Bim mutant target sites was not suppressed compared with NC group (Fig. 4E, $P < 0.05$). These results

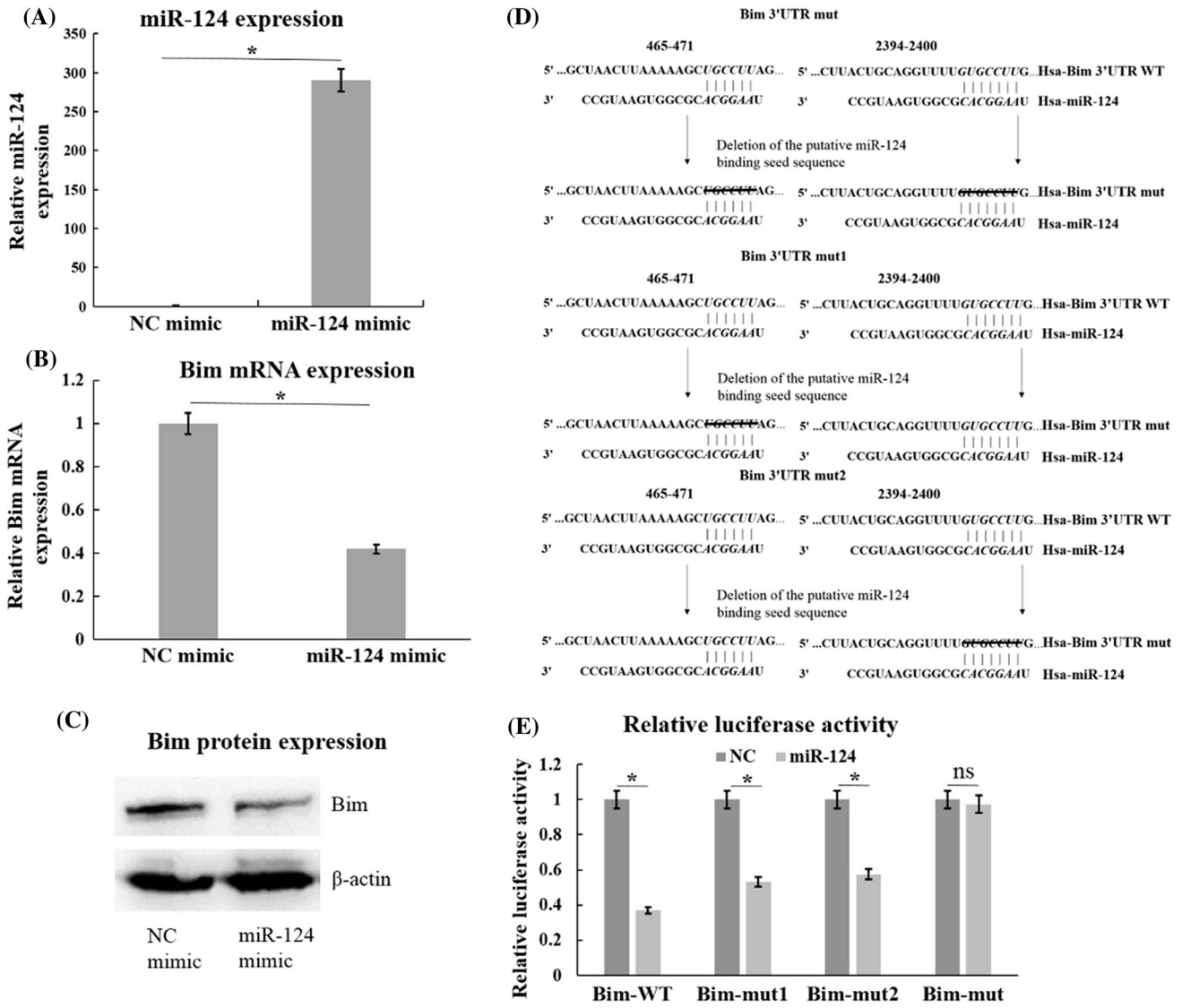

Fig. 4 Correlations between miR-124 and Bim were explored. (**A**) HBMECs were transfected with miR-124 mimic, and the increased miR-124 level was confirmed by qRT-PCR assay. NC, negative control. $*P < 0.05$. (**B**) HBMECs were transfected with miR-124 mimic, and the inhibited Bim mRNA level was confirmed by qRT-PCR assay. NC, negative control. $*P < 0.05$. (**C**) The impact of miR-124 on Bim was identified by western blot assay. Compared with control group, the overexpression of miR-124 down-regulated Bim protein level. NC, negative control. (**D**) The predicted 3′-UTR of Bim contains two miR-124 seed sequence (465-471 and 2394-2400) and deletion of the putative miR-124 binding seed sequence. (**E**) MiR-124 target gene was validated by flow cytometry analysis. A significant reduction in luminescence was displayed in HEK 293T cells transfected by miR-124 mimic and the Bim 3′-UTR wild-type reporters, whereas no significant difference was obtained in the mutant version of both sites (Bim-mut). Furthermore, HEK 293T cells transfected with miR-124 mimic and Bim-mut 1 or Bim-mut 2 exhibited an evident shrinkage. ns, no significance. $*P < 0.05$

suggested that miR-124 directly targeted the two conserved seed sites in the Bim 3′-UTR.

MiR-124 constrains hypoxia-induced HBMECs apoptosis

HBMECs apoptosis is a major occurrence in ischemic penumbra. In order to maintain the integrity of the blood–brain barrier and neurological function, it is significant to confer neuroprotection by targeting apoptotic alleviation [23]. In this study, flow cytometric analysis was aimed to detect the hypoxia-induced apoptosis in HBMECs. Compared with NC mimic group, miR-124 mimic group decreased the percentage of the apoptotic cells from 34.64 to 20.59% (Fig. 5A). Similarly, compared with control siRNA group, Bim siRNA group decreased the percentage of cell apoptosis from 28.39 to 15.69% (Fig. 5B). Taken together, these results confirmed that miR-124 inhibited HBMECs apoptosis under hypoxic condition through targeting Bim.

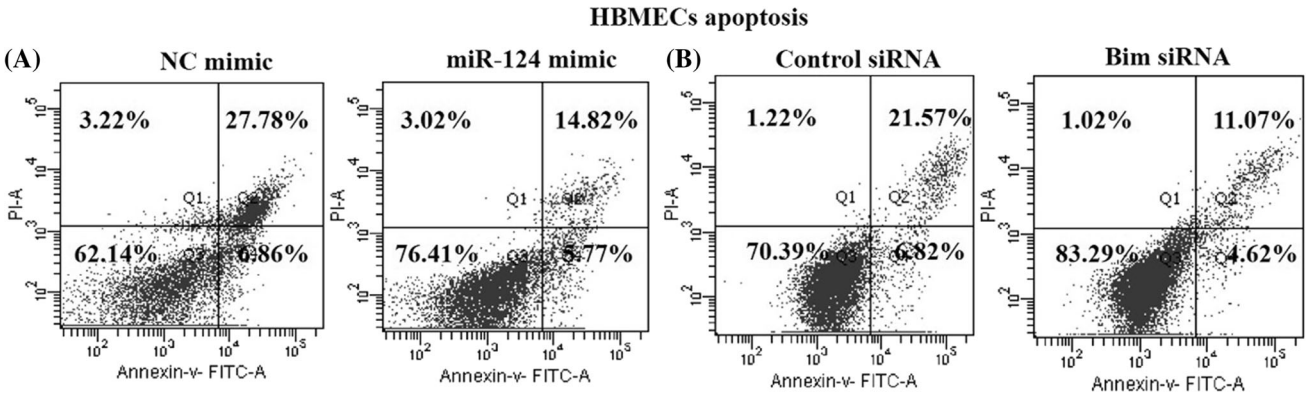

Fig. 5 Effect of miR-124 on the hypoxia-induced cell apoptosis was investigated by flow cytometry analysis. (**A**) Overexpression of miR-124 in HBMECs after treated with hypoxia 6 h resulted in inhibited apoptosis. NC, negative control. (**B**) Silencing expression of Bim in HBMECs after treated with hypoxia 6 h resulted in inhibited apoptosis

Discussion

MicroRNAs play a critical role in neuron biology, and miR-124 is bountifully expressed in the neurons [25, 26]. To date, it has been documented that miR-124 alteration is associated with a series of diseases, exemplified by stroke [2, 11–13], Parkinson's disease [14, 15], Alzheimer's disease [16], and experimental autoimmune encephalomyelitis [27]. In this present study, we investigated the role of miR-124 in HBMECs apoptosis and the preliminarily mechanism. Firstly, the down-regulated expression of miR-124 suggested its possible importance in HBMECs.

Widely accepted, apoptosis plays a vital role in the acute and chronic phases of ischemic stroke [28]. Acute ischemic stroke process profited by inhibition of apoptosis has been extensively reported [29, 30]. Two pathways, BCL2 family-regulated pathway from intrinsic aspect and death receptor-activated pathway from extrinsic aspect, jointly sense and transduce apoptotic signals. In our work, the targeting sequences of miR-124 in the Bim 3′-UTR mRNA were predicted by Targetscan database. Also, we confirmed that miR-124 targets regulation of Bim expression, consistent with the previous research [31]. In fact, plenty of evidence supports that Bim is regulated by microRNA in other diseases; by way of examples, miR-363 promotes human glioblastoma stem cell survival via direct inhibition of Bim [32], and miR-124 represses Bim and inhibits apoptosis in mouse cardiomyocytes [33].

As previously mentioned, the key role of Bim in cell apoptosis has been reported [24, 34]. We speculated the function role of miR-124 in HBMECs may be associated with Bim. The results of decrement of HBEMCs apoptosis caused by overexpression of miR-124 and low expression of Bim proved that the inhibitory role of miR-124 was related to Bim in HBMECs. Thus, the preliminary effect of miR-124 on hypoxia-induced cell apoptosis was examined, revealing the inhibitory role of miR-124 on hypoxia-induced cell apoptosis. Actually, miR-124 has been identified to exert a neuron-protective role via apoptosis-inhibiting pathway in ischemic stroke [35], evidenced by governing neuron apoptosis and death induced by oxygen and glucose deprivation and regulating antiapoptosis protein, Bcl-2 and Bcl-xl.

To conclude, our findings conveyed valuable information about the regulated mechanism of miR-124 in HBMECs and confirmed Bim as a direct target of miR-124. However, only preliminary verification was conducted regarding the functional role and regulatory mechanism of miR-124 in hypoxia-induced HBMECs and further investigation regarding the correlation of Bim with stroke remains yet to be elucidated. It would be interesting to test whether inhibition of miR-124 could result in reduction in basal autophagy and neurodegeneration or not. Besides, we also suppose that the mechanisms underlying neuroprotection of miR-124 in HBMECs may be more complicated, not just only by targeting Bim. More research design alike would be contained in our next work on the basis of this work.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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