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Exosomal MicroRNA-126 from RIPC Serum Is Involved in Hypoxia Tolerance in SH-SY5Y Cells by Downregulating DNMT3B

Junhe Cui,[1,2,3] Na Liu,[1,2,3] Zhehan Chang,[1,2,3] Yongsheng Gao,[1,2] Mulan Bao,[1,2] Yabin Xie,[1,2,3] Wenqiang Xu,[1,2] Xiaolei Liu,[1,2] Shuyuan Jiang,[1,2] You Liu,[1,2] Rui Shi,[1,2] Wei Xie,[1,2] Xiaoe Jia,[1,2] Jinghua Shi,[1,2,3] Changhong Ren,[3] Kerui Gong,[4] Chunyang Zhang,[5] Rengui Bade,[1,2] Guo Shao,[1,2,3,5] and Xunming Ji[3]

[1]Inner Mongolia Key Laboratory of Hypoxic Translational Medicine, Baotou Medical College, Inner Mongolia, PRC; [2]Biomedicine Research Center, Basic Medical College and Baotou Medical College of the Neuroscience Institute, Baotou Medical College, Inner Mongolia, PRC; [3]Beijing Key Laboratory of Hypoxic Conditioning Translational Medicine, Xuanwu Hospital, Capital Medical University, Beijing, PRC; [4]Department of Oral and Maxillofacial Surgery, University of California San Francisco, San Francisco, CA, USA; [5]Department of Neurosurgery, The First Affiliated Hospital of Baotou Medical College, Inner Mongolia, China

Ischemic tolerance in the brain can be induced by transient limb ischemia, and this phenomenon is termed remote ischemic preconditioning (RIPC). It still remains elusive how this transfer of tolerance occurs. Exosomes can cross the blood-brain barrier, and some molecules may transfer neuroprotective signals from the periphery to the brain. Serum miRNA-126 is associated with ischemic stroke, and exosomal miRNA-126 has shown protective effects against acute myocardial infarction. Therefore, this study aims to explore whether exosomal miRNA-126 from RIPC serum can play a similar neuroprotective role. Exosomes were isolated from the venous serum of four healthy young male subjects, both before and after RIPC. Exosomal miRNA-126 was measured by real-time PCR. The miRNA-126 target sequence was predicted by bioinformatics software. SH-SY5Y neuronal cells were incubated with exosomes, and the cell cycle was analyzed by flow cytometry. The expression and activity of DNA methyltransferase (DNMT) 3B, a potential target gene of miRNA-126, were examined in SH-SY5Y cells. The cell viability of SH-SY5Y cells exposed to oxygen-glucose deprivation (OGD) was also investigated. To confirm the association between miRNA-126 and DNMT3B, we overexpressed miRNA-126 in SH-SY5Y cells using lentiviral transfection. miRNA-126 expression was upregulated in RIPC exosomes, and bioinformatics prediction showed that miRNA-126 could bind with DNMT3B. DNMT levels and DNMT3B activity were downregulated in SH-SY5Y cells incubated with RIPC exosomes. After overexpression of miRNA-126 in SH-SY5Y cells, global methylation levels and DNMT3B gene expression were downregulated in these cells, consistent with the bioinformatics predictions. RIPC exosomes can affect the cell cycle and increase OGD tolerance in SH-SY5Y cells. RIPC seems to have neuroprotective effects by downregulating the expression of DNMTs in neural cells through the upregulation of serum exosomal miRNA-126.

INTRODUCTION

Stroke is a leading cause of mortality and disability worldwide, and the economic burden of treatment and poststroke care are increasing.[1,2] Remote ischemic preconditioning (RIPC), as an endogenous physiological mechanism, is a clinically feasible strategy to protect against ischemia-reperfusion injury after stroke.[3] RIPC showed neuroprotective effects against focal cerebral ischemic injury induced by transient middle cerebral artery occlusion (MCAO) in a rodent model.[4] Although the exact mechanisms involved in signal transmission from the periphery to the brain have not been fully elucidated, both humoral factors and an intact nervous system seem to have critical roles.[5] RIPC induces the local release of endogenous chemical mediators, which activate protective effects in remote organs.[6]

Exosome-mediated intercellular communication may contribute to the beneficial effects of RIPC treatment.[7] Exosomes have a particle size of 50–200 nm and are excreted by intracellular multivesicular bodies and cell membranes.[8] Exosomes secreted by neuronal and nonneuronal cells can promote the hypoxia/ischemia tolerance of neighboring or distant neurons that take up exosomes.[9–11] Exosomes can carry nucleic acids, lipids, and proteins as signaling molecules and then transfer them to recipient cells, thereby

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Correspondence: Rengui Bade, Inner Mongolia Key Laboratory of Hypoxic Translational Medicine, Baotou Medical College, Inner Mongolia, PRC.
E-mail: baard415@btmc.edu.cn
Correspondence: Xunming Ji, Beijing Key Laboratory of Hypoxic Conditioning Translational Medicine, Xuanwu Hospital, Capital Medical University, Beijing, PRC.
E-mail: jixm70@ccmu.edu.cn
Correspondence: Guo Shao, Inner Mongolia Key Laboratory of Hypoxic Translational Medicine, Baotou Medical College, Inner Mongolia, PRC.
E-mail: shao.guo.china@gmail.com

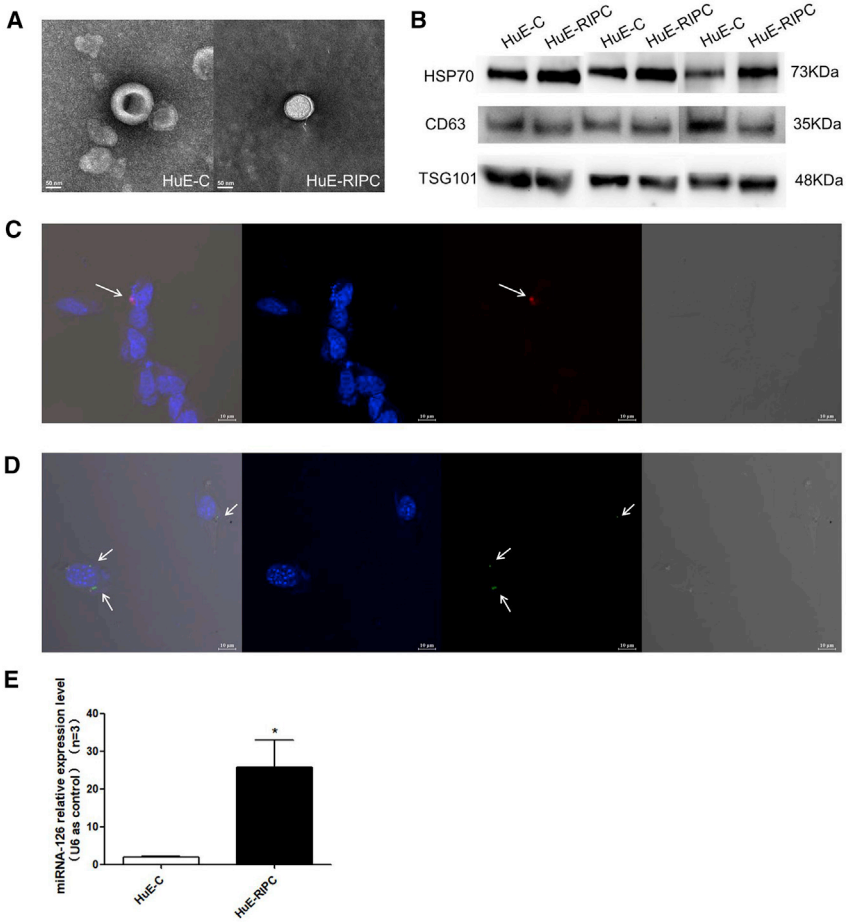

Figure 1. Exosome Characterization and Demonstration that Exosomes Derived from Serum Can Be Taken Up by SH-SY5Y Neuronal Cells

(A) Electron microscopy images of exosomes. (B) Representative western blots confirmed the expression of HSP70, TSG101, and CD63, which are markers of exosomes. (C) Control exosomes labeled with DiI emitted red fluorescence. (D) RIPC-treated exosomes labeled with DiO emitted green fluorescence. (E) RIPC-treatment increased miRNA-126 in serum exosomes. HuE-RIPC versus HuE-C, *$p < 0.05$.

modulating physiological processes in recipient cells.[12–14] The microRNA (miRNA) transferred by exosomes plays a key role in cell-cell communication in many different contexts and pathologies.[15] One report showed that miR-124 transferred by exosomes that were injected in the tail veins of mice may ameliorate brain damage after stroke.[16] Considering that miRNA is stable in exosomes, exosomal miRNA is an attractive candidate as an endogenous chemical mediator of neuroprotective signal transmission from the periphery to the brain in RIPC.

Exosomal miRNA-126 showed a rapid and significant reduction at 3 h postischemia in a rat focal ischemia model.[17] miRNA-126 in plasma may be a biomarker for ischemic stroke in humans. Exosomes enriched with miRNA-126 have stronger protective effects against acute myocardial infarction.[18] We used bioinformatics to analyze the miRNA-126 target genes and found that DNA methyltransferase (DNMT), which is involved in ischemia/hypoxia tolerance, can bind with miRNA-126. We therefore conducted this study to detect miRNA-126 changes in exosomes after RIPC and subsequently examined the role of exosomal miRNA-126 in neuroprotection. Meanwhile, DNMT levels and activity were detected. Finally, the cell cycle and the cell-cycle regulator p21 $^{WAF1/CIP1}$, which can be affected by DNMT, were measured.

RESULTS

Exosomes Isolated from RIPC Serum

To confirm that the pellet structures isolated from serum were indeed exosomes, we checked them using electron microscopy (EM) and western blotting. The EM results revealed that the pellets had a spherical/ellipsoidal/tea-tray shape with a size between 40 and 150 nm, which is consistent with the size of exosomes (Figure 1A). Three exosomal-specific protein markers, CD63, HSP70, and TSG101, were analyzed with western blotting, and the results showed that the pellets contained CD63, HSP70, and TSG101 (Figure 1B).

Exosomes Derived from Serum Can Be Taken Up by SH-SY5Y Neuronal Cells

Exosomes labeled with DiO (3,3′-dioctadecyloxacarbocyanine perchlorate) or DiI (1,1'-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) will emit red fluorescence or green fluorescence, which can be observed by laser confocal microscope. Red fluorescent spots and green fluorescent spots were observed in the SH-SY5Y cells incubated with the exosomes labeled with DiO or DiI (Figures 1C and 1D). These results demonstrated that neuronal cells have the ability to take up exosomes derived from the serum of the RIPC-treated and control groups.

RIPC Increases Exosomal miRNA-126 Levels

Next-generation sequencing (NGS) analysis showed that exosomal miRNA-126 derived from RIPC-treated individuals was increased (see Figure S1). To confirm the NGS results, we used real-time PCR analysis and we found that the expression of miRNA-126 in the exosomes derived from RIPC-treated individuals was increased compared with that in the control group (Figure 1E).

RIPC Exosomes Downregulate the Expression of DNMTs and DNMT3B Activity in SH-SY5Y Nerve Cells

To determine whether exosomal miRNA-126 from RIPC-treated individuals downregulates the expression of DNMT3B in SH-SY5Y cells, we analyzed the expression levels of three major DNMTs. The

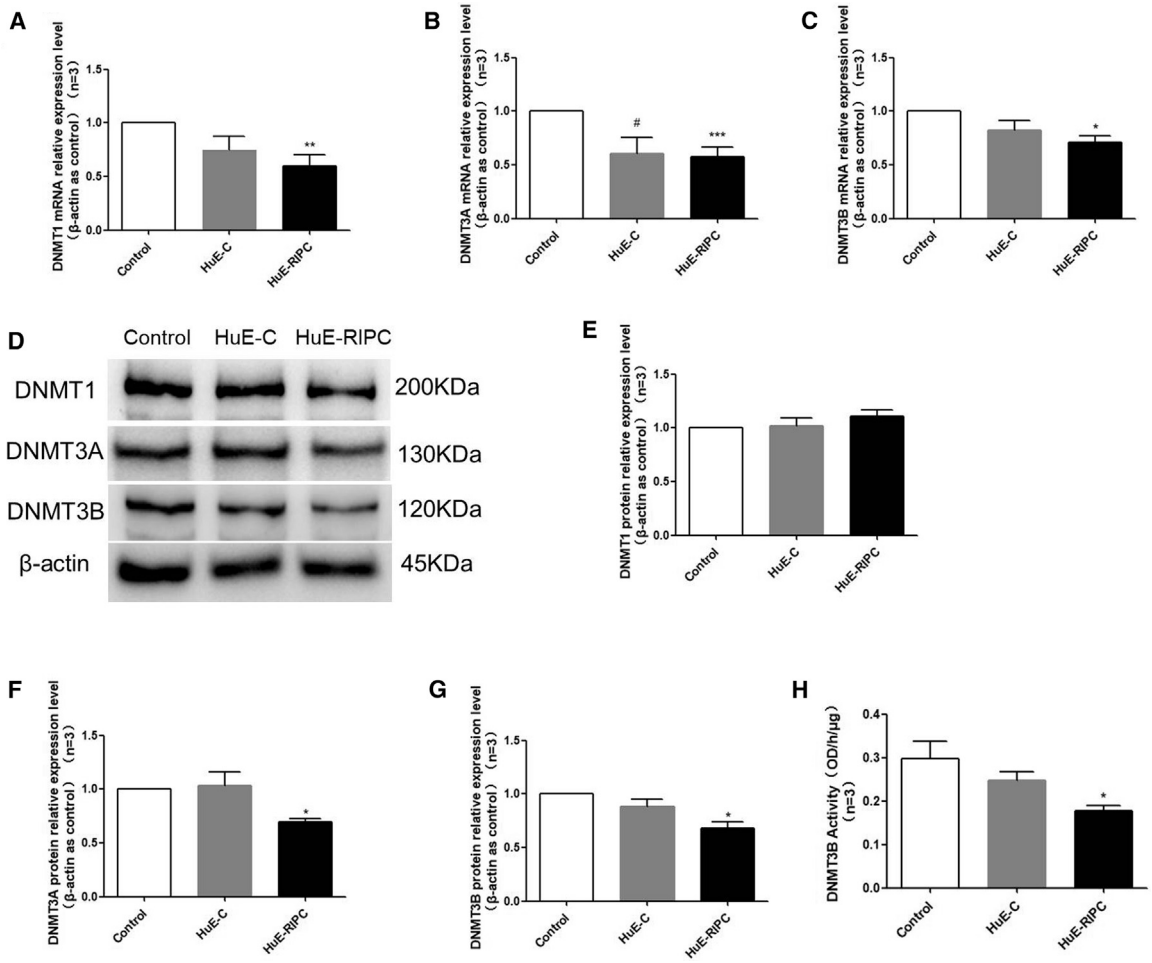

Figure 2. RIPC-Treated Exosomes Downregulated the Expression of DNMTs and Reduced the Activity of DNMT3B in SH-SY5Y Neuronal Cells

(A–C) Real-time PCR was used to quantify the mRNA expression levels of DNMT1, 3A, and 3B, respectively. (D) Typical western blot showing DNMT1, DNMT3A, and DNMT3B expression in SH-SY5Y cells. (E–G) Semiquantitative analysis indicated changes in protein levels of DNMT1, 3A, and 3B, respectivley. (H) The activity of DNMT3B in neuronal cells from both the control group and exosome group was quantified by ELISA. HuE-C versus control, # $p < 0.05$; HuE-RIPC versus control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

mRNA expression levels of DNMT1, DNMT3A, and DNMT3B were decreased in the SH-SY5Y cells incubated with human exosome control (HuE-C) and human exosome-RIPC (HuE-RIPC) exosomes, and the DNMT mRNA of the HuE-RIPC group was the lowest among the three groups (Figures 2A–2C). Compared with those in the control and HuE-C group, DNMT3A and DNMT3B protein levels were significantly downregulated in the HuE-RIPC group ($p < 0.05$) (Figures 2D–2G). There were no significant differences in DNMT1 protein levels among the three groups ($p > 0.05$).

The enzyme activity of DNMT3B in SH-SY5Y cells incubated with the exosomes of the control, HuE-C, and HuE-RIPC groups were 0.30 ± 0.04, 0.25 ± 0.02, and 0.18 ± 0.01 OD/h/μg, respectively. This result shows that the DNMT3B enzyme activity was significantly decreased in the HuE-RIPC group ($p < 0.05$) (Figure 2H).

The RIPC Exosomes Reduce the Alu and LINE-1 Methylation Levels in SH-SY5Y Cells by Targeting DNMT3B mRNA

Long interspersed element-1 (LINE-1) and Alu repetitive DNA elements are reliable indicators of global DNA methylation levels. The results showed that the relative methylation levels of the Alu sequence were 0.1272 ± 0.00258, 0.1150 ± 0.00889, and 0.1041 ± 0.00497 in the control, HuE-C, and HuE-RIPC groups, respectively. Compared with that in the normal control group (without exosomes), the methylation level of the Alu sequence in the RIPC group was significantly reduced ($p = 0.0147$, $n = 3$; Figures 3A and 3B). The methylation levels of LINE-1 were 0.4450 ± 0.01951, 0.4057 ± 0.01576, and 0.3736 ± 0.01507 and the LINE-1 unmethylation levels were 0.4970 ± 0.01387, 0.5158 ± 0.01205, and 0.5491 ± 0.00522 in three groups. Similar to Alu sequence, the DNA methylation level of LINE-1 sequence was decreased ($p = 0.0443$ in methylation/$p = 0.0246$ in unmethylation, $n = 3$; Figures 3C–3F).

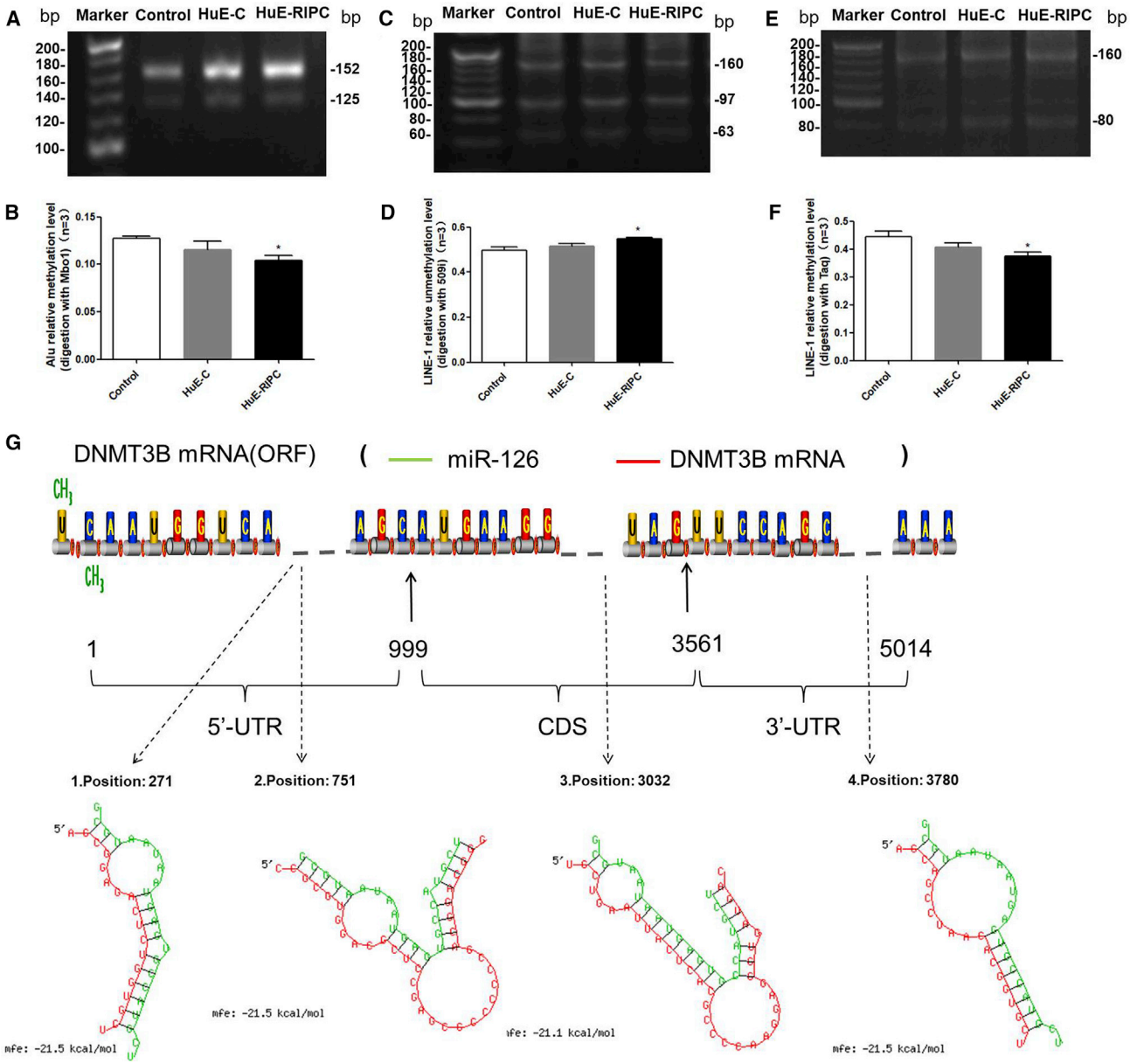

Figure 3. Upregulated miRNA-126 Expression in RIPC-Treated Exosomes May Target DNMT3B mRNA and Downregulate the Methylation of Alu and LINE-1 in SH-SY5Y Neuronal Cells

(A) Electrophoresis of COBRA products (Alu DNA sequence + MboI digestion) in agarose gel. (B) Level of DNA methylation in the Alu DNA sequence (methylation ratio = 125 bp/125 bp+152 bp). (C) Electrophoresis of COBRA products (LINE-1 DNA sequence + *Tsp*509I digestion) in agarose gel. (D) Level of unmethylated DNA in the LINE-1 DNA sequence (unmethylated ratio = 63 bp+97 bp/63 bp+97 bp+160 bp). (E) Electrophoresis of COBRA products (LINE-1 DNA sequence + TaqI digestion) in agarose gel. (F) Level of DNA methylation in the LINE-1 DNA sequence (methylation ratio = 80 bp/80 bp +160 bp). (G) Bioinformatics prediction of miRNA-126 targeting DNMT3B. Analysis by BiBiServ software showed that the seed sequence of miRNA-126 had four potential binding sites (1 in 3′ UTR, 1 in CDS and 2 in 5′ UTR) with DNMT3B mRNA, and the absolute entropy value of the site bindings was greater than 20; therefore, we speculated that miRNA-126 could bind DNMT3B mRNA and inhibit the translation process. HuE-RIPC versus control, *p < 0.05.

To understand the relationship between DNMTs and miRNA-126, we used bioinformatics tools to predict the target genes of the miRNA analyzed in this study. Bielefeld University Bioinformatics Service (BiBiServ) software analysis showed that the seed sequence (2–8 bases of the 5′ end of the miRNA) of miRNA-126 can pair with DNMT3B at the 3′ untranslated region (3′ UTR), coding sequences (CDSs) and 5′ UTR. The results of BiBiServ analysis revealed that the entropy absolute values of miRNA-126 binding with three sites are greater than 20.

Therefore, the expression of DNMT3B may be regulated by miRNA-126 through interrupting the translation process (Figure 3G).

Overexpression of miRNA-126 in SH-SY5Y Neurons by Lentiviral Transfection Can Inhibit the Expression of DNMT3B and Reduce the Methylation of Alu and LINE-1

To further demonstrate that miRNA-126 can target neural cell DNMT3B mRNA and downregulate its expression, we overexpressed miRNA-126-GFP in SH-SY5Y cells by lentiviral transfection. GFP signals were observed using a florescence inverted microscope at 48 h after transfection (Figure 4A). Moreover, the real-time PCR results showed that the expression of miRNA-126 in the miRNA-126-overexpressing group was more than double that of the control group and the scrambled group (Figure 4B). At the same time, real-time PCR and immunoblotting confirmed that miRNA-126 overexpression could effectively inhibit DNMT3B mRNA and protein expression in SH-SY5Y cells, while the expression levels of DNMT1 and DNMT3A were slightly increased ($p < 0.05$) (Figures 4C–4I).

Subsequently, the combined sodium bisulfite restriction enzyme assay (COBRA) experimental results showed that compared with the control and scrambled groups, the methylation level of the Alu gene was decreased (Figures 5A and 5B), the level of unmethylated LINE-1 was increased (Figures 5C and 5D), and the methylation of LINE-1 was decreased in the SH-SY5Y neuronal cells that overexpressed miRNA-126 (Figures 5E and 5F). This result suggests that miRNA-126 can alter genomic methylation in SH-SY5Y cells by downregulating DNMT3B.

PICR Exosomes Can Upregulate P21 Expression, Regulate the Cell Cycle, and Promote Ischemia/Hypoxia Tolerance in Neurons

The changes in DNA methylation affected the cell cycle. Both the mRNA and protein expression of P21 were significantly increased in the SH-SY5Y cells incubated with the RIPC-treated exosomes (Figures 6A–6C). At the same time, the cell cycle of SH-SY5Y cells was measured by flow cytometry. The S phase of the SH-SH-SY5Y cell from the control, HuE-C and HuE-RIPC groups were 26.73 ± 0.55%, 26.84 ± 0.07%, and 29.96 ± 0.48%, respectively (Figures 6D and 6E). These results demonstrate that the SH-SY5Y cells incubated with the RIPC-treated exosomes showed lower methylation levels and higher P21 expression and S phase fractions.

The effects of RIPC-treated exosomes on ischemia/hypoxia tolerance were measured by MTS (Figure 6F). OGD treatment can decrease cell viability. Compared with that in the normal control group and HuE-C group, the cell viability in the HuE-RIPC group was significantly restored ($p < 0.05$). This result suggested that RIPC-treated exosomes can promote the ischemia/hypoxia tolerance of SH-SY5Y neuronal cells.

DISCUSSION

Exosomes are thought to be carriers of biomarkers and intercellular biological information for specific repertoires of proteins, mRNAs, and miRNAs.[19] In the current study, we found that exosomes isolated from human serum can be taken up by cultured neuronal cells. At the same time, exosomes derived from RIPC-treated human serum can increase the cell viability of SH-SY5Y cells under hypoxic conditions. Xiao et al.[20] pointed out that endothelial cell-derived exosomes can protect SH-SY5Y neurons from ischemia/reperfusion injury. Both our and Xiao's results imply that RIPC-mediated neuroprotection may depend on exosomes, which carry information from peripheral tissues and transfers it to the central nervous system.

Exosomal miRNAs have attracted much attention in the central nervous system due to their ability to cross the blood-brain barrier and regulate gene expression. miRNAs are a specific class of noncoding single-stranded RNA (ncRNA) molecules that are 18–22 nucleotides in length and can bind with and degrade mRNAs by pairing to prevent protein synthesis.[21] Exosomal miRNA-126 isolated from the serum of RIPC-treated individuals was found to be upregulated in this study. miRNA-126, an endothelial cell-specific miRNA,[22] is critical for regulating the function of endothelial cells (ECs), controlling angiogenesis, and maintaining vascular integrity.[23] In addition to its role in ECs, increased miRNA-126 expression has been reported to be involved in cell hypoxia/ischemia tolerance in myocardial cells and nerve cells.[24,25] Further, circulating miRNA-126 may work as a biomarker for ischemic stroke in humans because it has been shown to be downregulated in ischemic stroke patients from 24 h to 48 weeks.[26] Both circulating miRNA-126 and exosomal miRNA-126 should be mostly derived from ECs, which are the main source of circulating exosomes.[17] Luo et al.[18] reported that adipose-derived stem cell (ADSC)-derived exosomes enriched with miRNA-126 have a greater protective effect against acute myocardial infarction (AMI). Similar to Luo's results, in this study, exosomes with higher miRNA-126 expression protected neuronal cells from hypoxia injury *in vitro*. Geng et al.[27] demonstrated that intravenous administration of miRNA-126^{+} exosomes post stroke improved functional recovery. Thus, exosomal miRNA-126 should play a role in hypoxia/ischemia tolerance. Therefore, miRNA-126 transferred by exosomes may be a critical biological molecule induced by RIPC and could moderate the communication between ECs and recipient cells, thereby increasing the hypoxia/ischemia tolerance of the recipient cells.

Some research has reported that miRNA-126 exerts neuroprotective effects against ischemia injury by regulating the expression of genes, such as phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2) and vascular cell adhesion molecule 1 (VCAM-1) in ECs.[28,29] PIK3R2 and VCAM-1 are associated with resistance against endothelial dysfunction and vascular inflammation, which are two important processes related to the neuronal damage of ischemia/reperfusion.[30] Therefore, decreased inflammatory pathway activation and endothelial dysfunction should also be involved in RIPC-mediated neuroprotection via exosomal miRNA-126.[31] To our knowledge, this is the first study to evaluate the neuroprotective genes regulated by exosomal miRNA-126 in neuronal cells. Our bioinformatic analysis showed that miRNA-126 can pair with DNMT3B. Some miRNAs can target DNMTs and regulate their expression.[32,33] For example,

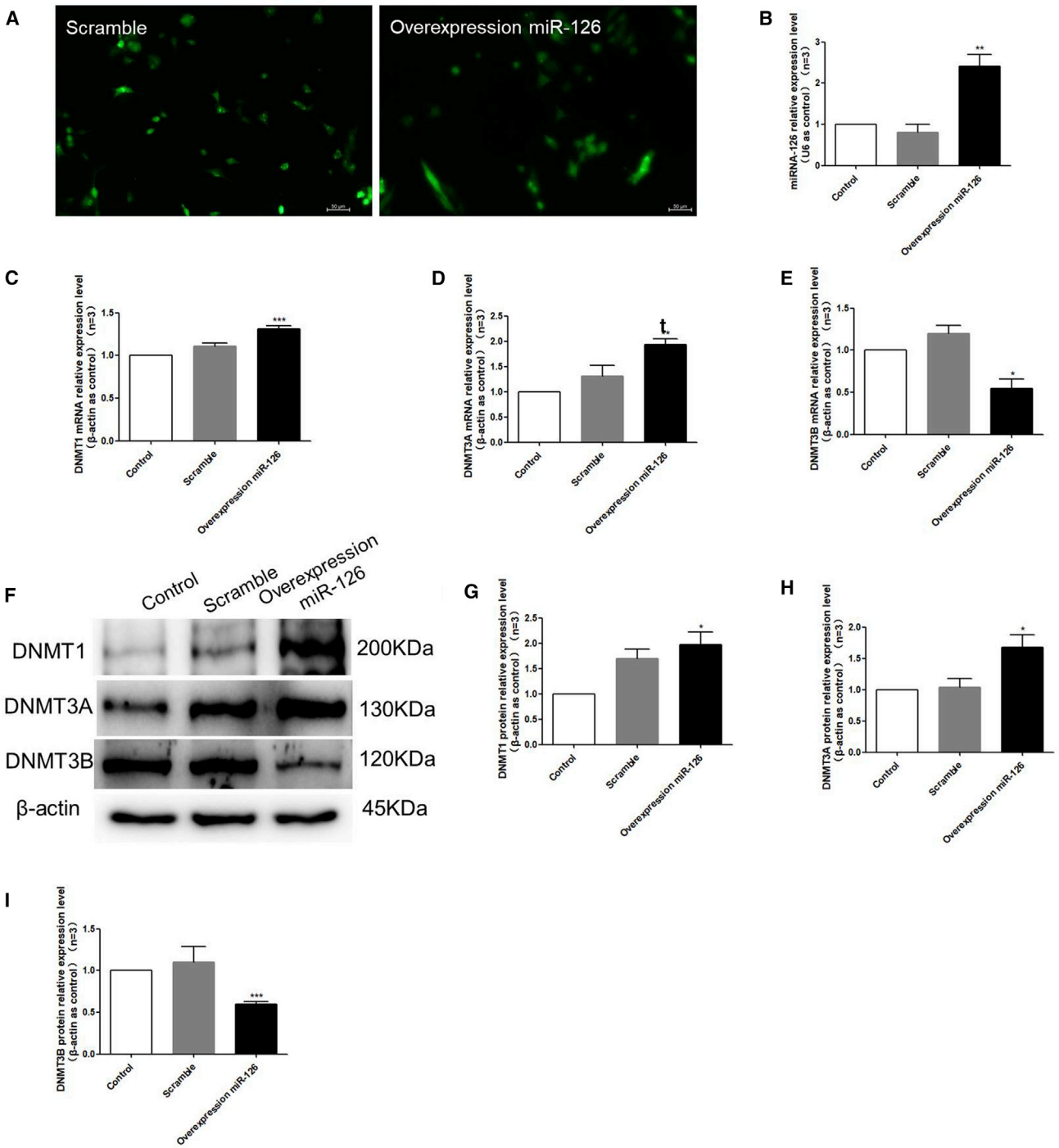

Figure 4. The Expression of the DNMT3B Gene and Global Methylation Were Downregulated in SH-SY5Y Neuronal Cells Overexpressing miRNA-126 through Lentivirus Transfection

(A) The expression of miR-126 with GFP at 48 h after transfection. (B) Significant upregulation of miRNA-126 expression in SH-SY5Y cells overexpressing miRNA-126 was observed compared with the control and scrambled group. (C–E) Real-time PCR was used to quantify the mRNA expression of DNMT1, 3A, and 3B in neuronal cells of the control group and transfected group, respectivley. (F) Typical western blot showing the expression of DNMT1, DNMT3A, and DNMT3B in SH-SY5Y cells. (G–I) Semiquantitative analysis indicated changes in protein levels of DNMT1, 3A, and 3B in SH-SY5Y cells, respectivley. miR-126-overexpressing versus control and scrambled, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

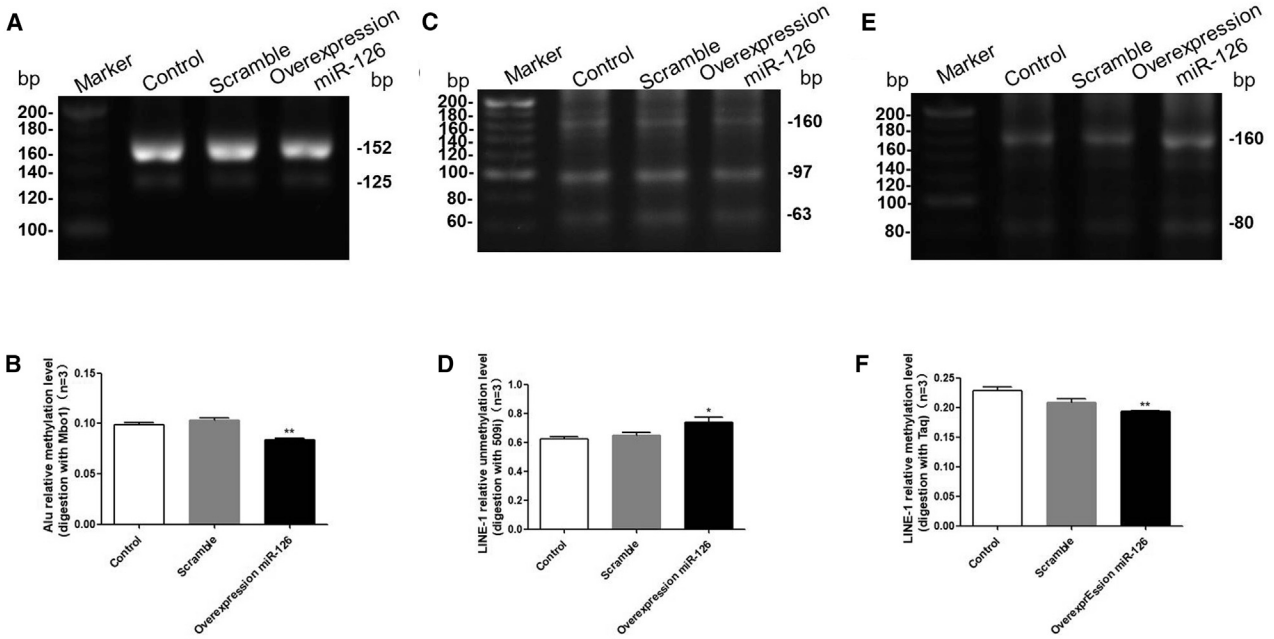

Figure 5. Overexpression of miRNA-126 in SH-SY5Y Cells Can Reduce the Degree of Genomic Methylation by Inhibiting the Expression of DNMT3B

(A) Electrophoresis of COBRA products (Alu DNA sequence + MboI digestion) in agarose gel. (B) Level of DNA methylation in the Alu DNA sequence (methylation ratio = 125 bp/125 bp+152 bp). (C) Electrophoresis of COBRA products (LINE-1 DNA sequence + *Tsp*509I digestion) in agarose gel. (D) Level of unmethylated DNA in the LINE-1 DNA sequence (unmethylated ratio = 63 bp+97 bp/63 bp+97 bp+160 bp). (E) Electrophoresis of COBRA products (LINE-1 DNA sequence + TaqI digestion) in agarose gel. (F) Level of DNA methylation in the LINE-1 DNA sequence (methylation ratio = 80 bp/80 bp +160 bp). miR-126-overexpressing versus control and scrambled, *$p < 0.05$, **$p < 0.01$.

downregulation of miRNA-152 leads to increased DNMT1 activity.[34,35] In this study, the observed decrease in DNMT3B in SH-SY5Y cells incubated with the exosomes of RIPC-treated individuals may be due to higher miRNA-126 levels. At the same time, the DNA methylation of repetitive sequences such as Alu and LINE-1 in SH-SY5Y cells was decreased after the injection of RIPC-treated exosomes, and this effect may be related to changes in the expression of DNMTs. DNMT3B expression was significantly downregulated and the gene methylation levels of Alu and LINE-1 were reduced in miRNA-126-overexpressing SH-SY5Y cells. Thus, the changes in DNMT3B expression and the DNA methylation levels of repetitive sequences such as Alu and LINE-1 in SH-SY5Y cells incubated with RIPC-treated exosomes may be due to an increase in exosomal miRNA-126 expression. Accordingly, chemical reagents such as DNMT inhibitors confer neuroprotection in rodents following mild ischemia.[36,37] Pandi et al.[38] showed that small interfering RNA (siRNA)-mediated DNMT3A knockdown decreased infarction *in vivo* and PC12 cell death *in vitro*. The mechanisms behind this neuroprotection are not yet clear. Hu et al.[39] proposed that decreased DNMT expression could induce increased expression of some genes, such as metallothionein, due to hypomethylation and that these genes protect neuronal cells from ischemia/hypoxia damage. Therefore, decreased DNMT3B expression may contribute to neuroprotection via hypomethylation and the upregulation of some beneficial genes. Interestingly, DNMT1 and DNMT3A were slightly elevated in SH-SY5Y cells overexpressing miRNA-126 in the present study. There may be some compensatory mechanisms among DNMT1, DNMT3A, and DNMT3B when one of them is lost or dramatically decreased.[40] Thus, a complex mechanism may underlie changes in DNMTs, as well as RIPC-treated exosomes, while exosomal miRNA-126 may be responsible for the changes in DNMT3B. It is well known that DNMTs catalyze DNA methylation at the 5-C position of cytosine nucleotides in CpG dinucleotides to regulate gene expression.[41] Although the molecular mechanisms underlying exosomal neuroprotection still need to be further clarified in neuronal cells, genes regulated by DNMTs should be among the possibilities.

The S-phase cell cycle and cell-cycle regulator $p21^{WAF1/CIP1}$ were increased in SH-SY5Y cells incubated with RIPC-treated exosomes in the current study. Our previous study found that upregulation of P21 expression can increase the percentage of cells in the S phase.[42] Therefore, in this study, RIPC-treated exosomes may affect $p21^{WAF1/CIP1}$ to change the cell cycle. The increased expression of $p21^{WAF1/CIP1}$ has been regarded as an adaptive response to cerebral ischemia.[43] Inhibition of the cell cycle has been shown to be neuroprotective in multiple models and leads to improved behavior/function in stroke.[44] Nikkola et al.[45] found evidence for coordinated expression and methylation changes in a small set of key genes related to the mitotic cell cycle in patient's blood after RIPC treatment. The gradual diminution of DNMT protein levels induced a corresponding

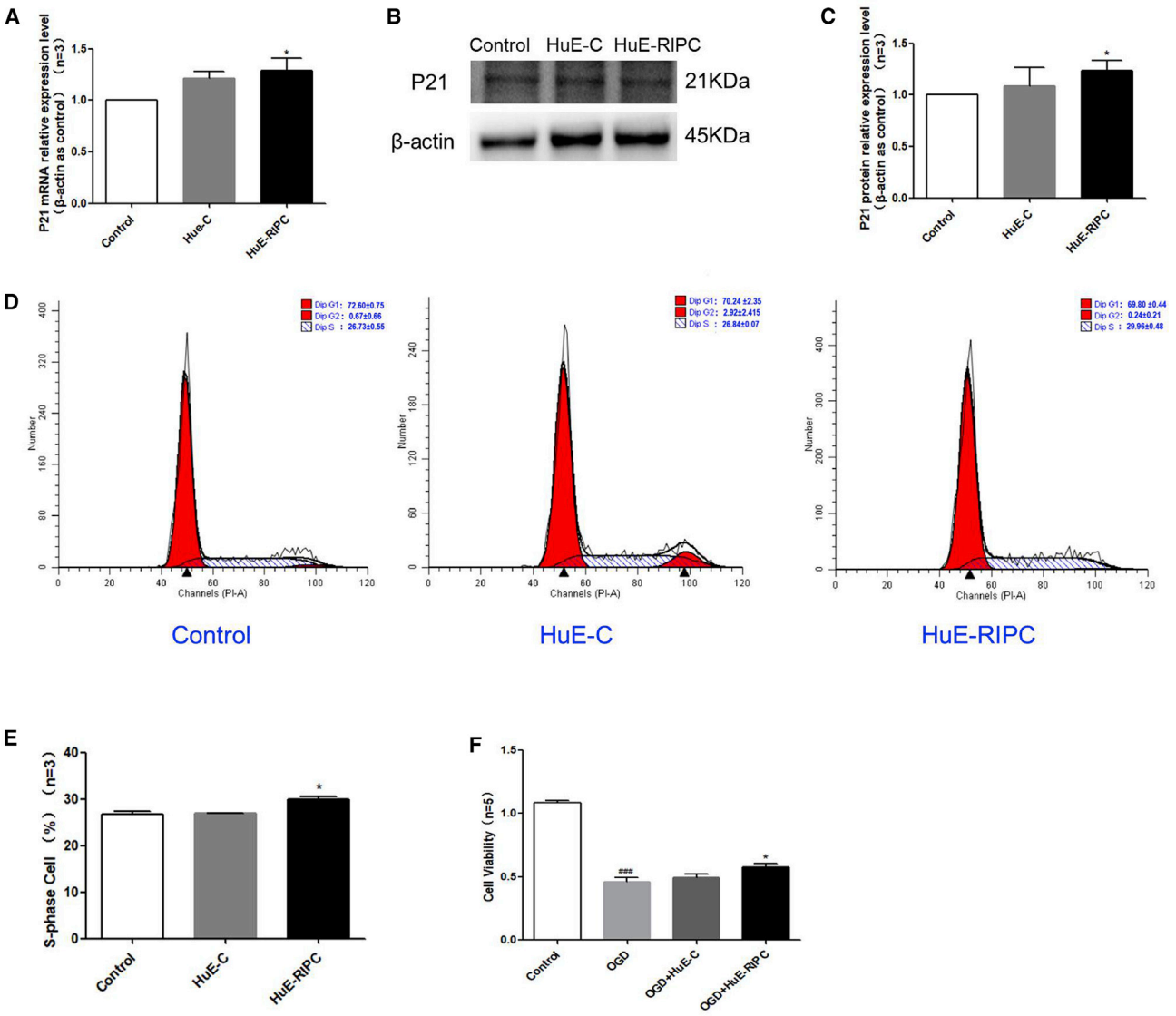

Figure 6. PIRC Serum Exosomes Can Affect the Cell Cycle and the Tolerance of the Cells to OGD

(A) Real-time PCR was used to quantitatively analyze the P21 mRNA expression levels of neuronal cells in the control group and exosome group. (B) Typical western blot showing P21 protein expression in SH-SY5Y cells. (C) Semiquantitative analysis indicated changes in the protein levels of P21 in SH-SY5Y cells. (D) The changes in the SH-SY5Y cell cycle of different groups were detected by flow cytometry. (E) Flow cytometry was used to detect the S-phase changes of neuronal cells in the control group and exosome group. (F) RIPC-treated exosomes can restore the vitality of neuronal cells in an ischemia/hypoxia state. OGD+HuE-PIRC versus OGD and control, *$p < 0.05$, **$p < 0.01$; OGD versus control, ### $p < 0.005$.

rapid increase in the $p21^{WAF1/CIP1}$ protein, demonstrating a link between DNMTs and the cell-cycle regulator $p21^{WAF1/CIP1}$.[46] RIPC exosomal treatment can increase the tolerance of SH-SY5Y cells to OGD in this study. The change of cell cycle and cell-cycle regulator $p21^{WAF1/CIP1}$ may contribute to this tolerance. Because SH-SY5Y cells are a transformed neuronal cell line that may differ from primary neurons, the neuroprotective effects of human RIPC-treated serum exosomes on neuronal cells need to be clarified further. Therefore, the neuroprotective effects of RIPC-treated exosomes may be partly due to DNMTs, which can affect the cell state and cell-cycle regulator genes.

In summary, our findings demonstrate a direct effect of exosomes from RIPC-treated human serum on hypoxia and can provide benefits to cultured nervous cells against hypoxic injury. The neuroprotective effects of RIPC-treated exosomes are likely related to increased miRNA-126 expression, which downregulates DNMTs. The limitation of this study was that the data were generated from an *in vitro*

study. We will further confirm our results in an *in vivo* RIPC treatment model. Our results suggest that exosomal miRNA-126/nervous DNMT3B pathway may be of value in the treatment of stroke.

MATERIALS AND METHODS

Subject Population and Induction of RIPC

Four healthy undergraduate college students (male, aged 20–30, height 170–180 cm, weight 60–75 kg, early morning, awake, fasting) participated in this study as volunteers. 20 milliliters of venous blood were drawn from each subject before RIPC treatment as a control group (HuE-C). Then, RIPC was induced by inflating a 12-cm-wide blood pressure cuff placed around the upper portion of the subject's nondominant arm for 5 cycles. Each cycle consisted of a 5-min period of 200 mmHg inflation with 5 min of reperfusion as previously described.[47] The same volume of venous blood was drawn using the same method as the experimental group immediately after RIPC. This study was approved by the Baotou Medical College Ethics Committee. All participants signed the informed consent before enrollment.

Exosome Isolation and Electron Microscopy Characterization

Exosomes were prepared from blood by ultracentrifugation as follows: the collected blood was allowed to stand at 4°C for 1 h, centrifuged at 1,000 × *g* for 15 min at 4°C to obtain serum, and the serum was uniformly mixed with phosphate-buffered saline (PBS) at a ratio of 4:5. After that, the samples were processed with the following procedure: mixed, centrifuged at 2,000 × *g* for 30 min at 4°C to remove the cells, centrifuged at 12,000 × *g* for 45 min to remove the cell debris, centrifuged at 110,000 × *g* for 2 h at 4°C using an ultracentrifuge, exosomal precipitation, and washed with 1 mL of PBS. After the pellet was precipitated, it was centrifuged at 110,000 × *g* for 70 min and 150 μL of PBS was added to the exosomes to prepare a serum exosomal suspension. The quantification of the exosomes was performed as previously described by Luhtala and Hunter.[48] Then, the exosomes were aliquoted and stored at −80°C.

A total of 20 μL of purified exosomes from RIPC-treated and control serum were resuspended in PBS and imaged with a JEM-1400 transmission electron microscope, as detailed by Grigor'eva et al.[49] Then, the shape and size of the exosomes were analyzed.

Cell Culture and Model Preparation

Human neuroblastoma SH-SY5Y cells were cultured in 1640 medium containing 15% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin. There are three groups in this study: SH-SY5Y cells cultured with normal medium (control), SH-SY5Y cells cultured with normal medium + normal serum exosomes (HuE-C), and SH-SY5Y cells cultured with normal medium + RIPC-treated serum exosomes (HuE-RIPC). The ratio of exosome suspension to medium was 1:100 (approximately 35 μg exosomes/mL medium). The cells were harvested for subsequent experiments after incubation for 24 h.

Exosomal Tracking Experiment

Lipophilic tracers DiI and DiO were prepared in stock solutions of dimethyl sulfoxide (DMSO) for the *in vitro* study. DiI emits orange-red fluorescence under the excitation of light with a wavelength of 549 nm. DiO emits green fluorescence under the excitation of light with a wavelength of 484 nm. Exosomes derived from the control group and RIPC-treated group were incubated with 10 μM of DiO or DiI at 37°C for 20 min. The excess dye was removed by washing with PBS 3 times. The fluorescence-labeled exosomes were incubated with SH-SY5Y neuronal cells for 24 h. Then, the cells were fixed with 4% paraformaldehyde for 30 min. The uptake of DiO or DiI by the exosomes of SH-SY5Y neuronal cells was visualized using confocal microscopy with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) stain.

Lentivirus Preparation and Transfection

miRNA-126 and scrambled control lentiviruses were prepared by a commercial biocompany (Hanbio Biotechnology, Shanghai, China). SH-SY5Y cells were seeded at a density of 5×10^4 cells per well in 24-well plates and lentiviral vectors were transferred to the cells (MOI = 20) when the SH-SY5Y cells reached 70% confluency. miRNA-126-overexpressing cells and control cells were selected with 3 mg/mL puromycin dihydrochloride hydrate (BBI Life Sciences Corporation, Shanghai, China).

Cell Ischemia Model Construction and 3-(4,5-dimethylthiazol-2-yl)-5 (3-carboxymethonyphenol)- 2-(4-sulfophenyl)-2H-tetrazolium (MTS) Assay

Cell viability was measured by MTS assay using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA). According to the manufacturer's protocol, SH-SY5Y cells were seeded into 96-well plates at a density of 2×10^3 cells per well and cultured under regular conditions (5% CO_2, 21% O_2, 37°C). At 24 h later, the cells were incubated with exosomes and were cultured in OGD conditions for 6 h, followed by reoxygenation with normal culture medium for 24 h as described by Xiao et al.[20] MTS was added and detected every half hour. Experiments were performed as previously described by Tian et al.[42] Each experiment was conducted in triplicate.

Genomic Methylation Assay

The DNA of SH-SY5Y cells incubated with HuE-RIPC and HuE-HC exosomes or control conditions (HuE-RIPC, HuE-C, and control groups) was extracted using a DNA miniprep kit (Tiangen, Beijing, China), and bisulphite treatment was conducted with an EZ DNA Methylation kit (ZYMO Research, Irvine, CA, USA). The methylation levels of the Alu and LINE-1 genes were analyzed using a COBRA according to our previous description.[41] The DNA methylation levels of Alu and LINE-1 were calculated as previously described by Sirivanichsuntorn et al.[50] Briefly, Alu and LINE-1 methylation was measured by the OD of the digestion bands/digestion bands + indigestion bands.[41] The primer sequences were as follows:

Alu F: 5′-GATCTTTTTATTAAAAATATAAAAATT-AGT-3′;

R: 5′-GATCCCAAACTAAAATACAATAA-3′;

LINE-1 F: 5′ -CCGTAAGGGGTTAGGGAGTTTTT-3′;

R: 5′-RTAAAA CCCTCCRAACCAAATATAAA-3′.

Cell-Cycle Detection

SH-SY5Y cells incubated with RIPC-treated and HC-treated exosomes or control exosomes (RIPC, HC, and C groups) were collected, washed twice with PBS, and resuspended in PBS working solution containing PI, RNase, and 1% Triton X-100. The cell suspension (1 mL) was transferred to a flow tube and stained for 15 min in the dark. Apoptosis was analyzed by FACScan flow cytometry with a concentration of 1×10^4 cells per sample, and the cell-cycle distribution was analyzed using the ModFit 3.0 program.

Real-Time PCR Detection

RNA was isolated from exosomes with the Tissue and Cell Total RNA Extraction kit (GenePharma, Shanghai, China) and reverse transcribed using GenePharma's Hairpin-it kit (GenePharma, Suzhou, China). RNA was extracted from cells with TRIzol (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized with the Revert Aid First Strand cDNA Synthesis kit (Thermo, Shanghai, China). The miRNA-126, DNMT, and P21 mRNA levels were measured by real-time PCR in the ABI 7900 real-time PCR machine; U6 snRNA was used as the internal reference for miRNA-126, and β-actin was used as the internal reference for the other genes. The primer sequences were as follows:

miRNA-126-3P: F: 5′-TCGTCTGYCGTACCGTGAGTAAT-3′.

R: 5′-CACTTCCTCAGCTCTTGTTGGTAT-3′.

U6-Hu: F: 5′-CGCTTCGGCAGCACATATACTA-3′.

R: 5′-CGCTTCACGAATTTGCGTGTCA-3′.

β-actin: F: 5′-AGGTGAAG GTCGGAGTCA-3′.

R: 5′-GGTCATTGATGGCAACAA-3′.

P21: F: 5′-TGTCCGTCAGAACCCATGC-3′.

R: 5v-AAAGTCGAAGTTCCATCGCTC-3′.

DNMT1: F: 5v-AACCTTCACCTAGCCCCAG-3′.

R: 5′-CTCATCCGATTTGGCTCTTTCA-3′.

DNMT3A: F: 5′-GACAAGAATGCCACCAAAGC-3′.

R: 5′-CGTCTCCGAACCACATGAC-3′.

DNMT3B: F: 5′-AGGGAAGACTCGATCCTCGTC-3′.

R: 5′-GTGTGTAGCTTAGCAGACTGG-3′.

All procedures were performed according to the protocols obtained from the manufacturers. The relative miRNA and mRNA expression levels were calculated using the Delta-delat ct (DD) value.[51]

Western Blotting

The exosome precipitates or cell pellets were lysed by radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Shanghai, China), and the protein concentration was determined using the bicinchoninic acid (BCA) method with the Thermo Fisher Scientific Pierce BCA Protein Assay Kit (Thermo Fisher, Rockford, IL, USA). Lysed samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Roche Diagnostics, Indianapolis, IN, USA), and then the membranes were blocked with 5% skimmed dry milk blocking buffer. Then, the membranes were incubated with primary antibodies (CD63, HSP70, TSG101, P21, DNMT1, DNMT3B, and β-actin, internal reference) at 4°C for 16 h. The membranes were then washed with Tris-buffered saline containing 0.05% tween20 (TBS-T) 3 times and incubated with the corresponding secondary antibodies (Tiangen Biological, Beijing, China) for 1 h at room temperature. After secondary antibody incubation, the membranes were washed 3 times with TBS-T and the blots were detected by a Tanon 5800 automatic chemiluminescence imaging analysis system using ECL supersensitive luminescent solution.

Bioinformatics Prediction and Enzyme-Linked Immunosorbent Assay

miRWalk software (http://mirwalk.umm.uni-heidelberg.de/) was used to find the miRNAs related to DNMTs and validate them using Bielefeld Bioinformatics Service software (https://bibiserv.cebitec.uni-bielefeld.de/). The DNMT3B and miRNA-126 binding sites were located. DNMT3B viability was measured using Epigntek's EpiQuik DNMT3B Activity/Inhibitor Screening Assay Core Kit (Epigentek, Farmingdale, NY, USA).

Statistical Analysis

The experimental data are expressed as the mean ± standard error. Statistical analyses were completed by one-way analysis of variance (ANOVA) or bilateral t test using SPSS software. $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.04.008.

AUTHOR CONTRIBUTIONS

J.C., N.L., Z.C., Y.G., M.B., Y.X., W.X., X.L., S.J., Y.L., and R.S. conducted the experiments and drafted the manuscript. W.X., X.J., J.S., C.R., K.G., and C.Z. contributed to the experimental design and statistical analyses. R.B., G.S., and X.J. designed the experiments and revised the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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