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The Effects of Electroacupuncture in a Rat Model of Cerebral Ischemia-Reperfusion Injury Following Middle Cerebral Artery Occlusion Involves MicroRNA-223 and the PTEN Signaling Pathway

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: In China, electroacupuncture (EA) is used to treat the symptoms of ischemic stroke. However, the mechanisms involved in the effects of EA in cerebral ischemia remain to be investigated. This study aimed to investigate the molecular mechanism underlying the effects of EA in a rat model of cerebral ischemia-reperfusion injury (CIRI) induced by middle cerebral artery occlusion (MCAO).

Material/Methods: Seventy-five male Sprague-Dawley rats were divided into five groups: the sham group (with sham surgery), the model group (the MCAO model), the EA group (treated with EA), the EA control group, and the EA+antagomir-223-3p group. Rats in the model of CIRI underwent MCAO for 90 minutes. EA was performed on the second postoperative day and was performed at the Waiguan (TE5) and Zusanli (ST36) acupoints. The rat brains were evaluated for structural and molecular markers.

Results: EA treatment significantly upregulated the expression of microRNA-223 (miR-223), NESTIN, and NOTCH1, and downregulated the expression of PTEN in the subventricular zone (SVZ) and hippocampus. The luciferase reporter assay supported that PTEN was a direct target of miR-223, and antagomiR-223-3p reversed the effects of EA and reduced the increase in NESTIN and inhibition of PTEN expression associated with EA treatment. There was a negative correlation between PTEN expression and the number of neural stem cells (NSCs).

Conclusions: In a rat model of CIRI following MCAO, EA activated the NOTCH pathway, promoted the expression of miR-223, increased the number of NSCs, and reduced the expression of PTEN.

MeSH Keywords: **Electroacupuncture • Hypoxia-Ischemia, Brain • Neural Stem Cells • PTEN Phosphohydrolase • Receptors, Notch**

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Background

Stroke is a common acute cerebrovascular disease with a high incidence, rate of recurrence, and mortality rate [1,2]. Ischemic stroke accounts for about 80% of all cases of stroke. It is due to the local or generalized reduction in blood supply to the brain, resulting in ischemia or infarction with loss of neurological, or cerebral stroke [3]. Cerebral ischemia due to stroke results in reduced levels of ATP, accumulation of lactic acid, and activation of inflammatory cascades that result in ischemia and necrosis of brain tissue [4]. The clinical management of cerebral ischemic injury involves restoring blood flow and cerebral reperfusion, which reduces the infarct size and neurological deficit caused by the stroke. However, cerebral reperfusion induces increased damage during recovery, which termed cerebral ischemia-reperfusion injury (CIRI) [5]. Recent studies have shown that mitochondrial dysfunction, amino acid release, calcium overload, the production of reactive oxygen species (ROS), nitrogen formation, circular RNAs (circRNAs), inflammation, and apoptosis are involved in the pathogenesis of CIRI [6,7]. However, there remains a need for effective treatments for the neurological deficits that occur due to cerebral stroke and CIRI.

Acupuncture is a complementary or traditional Chinese medicinal (TCM) technique that is widely used in clinical rehabilitation. Electroacupuncture (EA) has been popularly used in treating ischemic stroke and CIRI. Waiguan (TE5) and Zusanli (ST36) are the most common acupoints in the upper and lower limbs [8]. Preliminary studies have shown that using EA at the Waiguan (TE5) and Zusanli (ST36) acupoints have neuroprotective effects on cerebral ischemia [9–11]. Recent studies have shown that EA at the Quchi and Zusanli (ST36) acupoints showed effects on CIRI through the activation of extracellular signal-regulated kinase (ERK) signaling [9]. EA at the Baihui (GV 20) and Dazhui (GV 14) acupoints resulted in ultrastructural changes in the cerebral cortex of rats with CIRI [9]. However, the underlying molecular mechanisms of the effects of EA on CIRI at the acupoints Waiguan (TE5) and Zusanli (ST36) remain to be studied in detail.

Neural stem cells (NSC) are multipotential cells of the central nervous system (CNS) that lack differentiation, have a high proliferation rate, and can differentiate into specific types of neurons or glial cells under certain conditions [12]. Nestin is a neuroepithelial stem cell protein that is encoded by the NESTIN gene, is unique to the CNS, and belongs to the family of intermediate microfilaments [13]. The expression of NESTIN is triggered by the formation of neuroblasts and ceases as neurons differentiate. The spatiotemporal pattern of NESTIN expression is consistent with the occurrence and differentiation of neural precursor cells. The Nestin protein is a specific marker for neural precursor cells and is widely used to identify neural precursor cells *in vitro* [13].

MicroRNAs (miRNAs) are 21–25 nucleotides in length [14], regulate gene expression and have significant roles in cell formation, differentiation, proliferation, and apoptosis [15]. Recent studies have indicated that microRNA-25 (miR-25) reduces apoptosis induced by CIRI [16] and promotes the development of focal cerebral ischemic NSCs [17]. EA has been shown to promote regeneration of NSCs by activating the NOTCH1 signaling pathway and facilitating the repair of CIRI [18,19]. The NOTCH1 pathway has a critical role in neuroprotection against cerebral ischemia and mediates the activation of miRNAs, including microRNA-223 (miR-223) [20,21]. The administration of miR-223 in an *in vivo* animal model of intracerebral hemorrhage has resulted in improved neurological outcomes [22]. Also, the deletion of PTEN, which is a putative target of miR-223, was previously shown to enhance the regenerative ability of neurons following spinal cord injury [23]. However, it remains to be determined whether activation of the NOTCH signaling pathway in ischemic stroke has a modulating effect on miR-223 and PTEN. Treatment with EA treatment has been reported by some studies to promote the repair of endogenous NSCs in rat models of stroke following middle cerebral artery occlusion (MCAO) [24,25]. Also, EA has been reported to trigger the proliferation and differentiation of endogenous NSCs and to stimulate the repair of injured nerves [26]. Activation of the NOTCH1 signaling pathway stimulates the regeneration and repair of nerve cells following ischemia, which can reduce or reverse neurological following cerebral ischemia [27].

Therefore, this study aimed to investigate the molecular mechanism underlying the effects of EA, including at the acupoints Waiguan and Zusanli, in a rat model of cerebral ischemia-reperfusion injury (CIRI) induced by middle cerebral artery occlusion (MCAO).

Material and Methods

Electroacupuncture (EA) and the rat model of cerebral ischemia-reperfusion injury (CIRI) induced by middle cerebral artery occlusion (MCAO)

Seventy-five specific pathogen-free (SPF) healthy male Sprague-Dawley (SD) rats (weighing 220–270 g) were obtained from the Huazhong University of Science and Technology Experimental Animal Center, Wuhan, China. The rats were randomly divided into the following five groups: the sham group (with sham surgery), the model group (the MCAO model), the EA group (treated with EA), the EA control group, and the EA+antagomir-223-3p group. The rats underwent MCAO-induced focal ischemia-reperfusion injury in the groups, except in the sham group, according to the method previously described by Longa et al. [28].

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The rats were anesthetized with 5% isoflurane by inhalation for induction and 2.5% for maintenance (RWD Life Science Co, Shenzhen, China) for the MCAO procedure. The right internal carotid artery was then occluded for 90 minutes by a nylon surgical thread. EA was performed on the second postoperative day. Acupuncture needles of 0.3 mm diameter (Hua Tuo, Suzhou Medical Appliance Company, Suzhou, China) were inserted at the acupoints Waiguan (TE5) and Zusanli (ST36) on the paralyzed limb. Continuous-wave EA of 20 Hz and 1 mA was performed for 30 min per day, for a total of 7 days using a G6805-II therapeutic EA apparatus (Shanghai Medical Electronic Apparatus, Shanghai, China). The rats were fed normal food and given free access to water and housed at 24±1°C, with a 12-hour light and dark cycle. The experimental animal protocols were approved by the Animal Experimentation Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology.

To investigate the role of miR-223-3p in MCAO-induced focal CIRI, either the miRNA antagomir-223-3p or 5 µl of scrambled-miR in 2.5 nM NaCl solution (RiboBio, Guangzhou, China) were inoculated into the right lateral cerebral ventricles at an antero-posterior depth of 0.8 mm, a mediolateral depth of 1.5 mm, as previously described [17]. The rats were stabilized and calmed by suspension in a bag during the acupuncture treatment. After seven days of treatment, the rats were euthanized by cervical dislocation following anesthesia using pentobarbital sodium.

The rat brain tissues, including those from the subventricular zone (SVZ) and the hippocampus, were collected for histology, immunohistochemistry, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays. Rat brain tissues were fixed in 4% paraformaldehyde, embedded in paraffin wax, and sectioned at 5 µm onto glass slides.

Triphenyl tetrazolium chloride (TTC) staining

Samples of rat brain tissue were immediately frozen at –20°C for 20 mins, and 2 mm thick sections were prepared, which were stored in a 2% solution of TTC (Sigma-Aldrich, St. Louis MO, USA) at 37°C for 20 mins. The stained sections were fixed with 4% paraformaldehyde. Sections were photographed using a digital camera, and areas of ischemic brain injury areas were randomly identified using the Image-Pro Plus analyzer (Media Cybernetics, Inc., Rockville, MD USA), with the infarct areas and volumes represented as percentages.

Histology using hematoxylin and eosin (H&E) staining

The paraffin sections were warmed at 60°C for 30 min before H&E staining. Tissue sections were treated with xylene for 10 min, incubated twice with 100% ethanol for 5 min, 80% ethanol for 5 min, distilled water for 5 min, hematoxylin for 15 min, and washed with water. Tissue sections were treated with 1% hydrochloric acid for 5 seconds, washed with tap water for 20 min, then stained with eosin for 3 min, followed by treating with 80% ethanol for 5 min, 95% ethanol for 5 min, 100% ethanol for 5 min, and xylene. The H&E stained tissue sections were mounted and observed by light microscopy.

Immunohistochemistry (IHC)

The paraffin sections of rat brain tissue were treated with 3% hydrogen peroxide for 20 min to block endogenous peroxidase. The rat brain tissue sections were incubated with 1% bovine serum albumin (BSA) to block nonspecific antibody binding. The tissue sections were then incubated for 2 h in primary antibodies to PTEN (1: 100) (Santa Cruz Biotechnology Inc., Dallas, TX, USA), Notch1 (1: 100) (Santa Cruz Biotechnology Inc., Dallas, TX, USA), and Nestin (1: 200) (Santa Cruz Biotechnology Inc., Dallas, TX, USA). The tissue sections were washed and incubated in horseradish peroxidase (HRP)-conjugated secondary IgG antibody for 60 min before counterstaining with the brown chromogen, 3,3'-diaminobenzidine (DAB).

TUNEL assay

Apoptosis was studied in the paraffin sections using Terminal deoxynucleotidyl transfer-mediated dUTP nick end-labeling (TUNEL) using the In-Situ Cell Death Detection Kit-POD (Roche, Basel, Switzerland). The sections were treated with TUNEL reaction mixture for 60 min following protease K treatment (10 mmol/L) for 15 min and then incubated in converter-POD for 30 min. TUNEL stained tissue sections were then photographed using light microscopy.

Luciferase reporter system

Because PTEN was proposed as the target for miR-223, the psiCHECK™-2 Vector (Promega, Madison, WI, USA) was constructed that contained the wild type (WT) or mutant type (MUT) 3' UTR sequence of PTEN. Co-transfection was performed into 293T cells with a miR-223 mimic to detect the ratio of fluorescence, according to the manufacturer's instructions.

The qRT-PCR assay

Total RNA was extracted from the SVZ and hippocampal tissues using TRIzol (Invitrogen, Carlsbad, CA, USA). Then, qRT-PCR was performed using SYBR Green Master Mix Kit (Takara, Minato-ku, Tokyo, Japan) on ABI 7500 system (ABI Biosystems, Foster City, CA, USA). The primers used for qRT-PCR are listed in Table 1. The PCR reactions were performed at 95°C for 10 min, 95°C for 10 s, 60°C for 30 s during 40 cycles. The relative gene expression levels were normalized to U6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data were calculated using the $2^{-\Delta\Delta Ct}$ method.

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Table 1. Primers used in this study (5'-3').

ID		Sequence (5'-3')
GAPDH	Forward	CCT CGT CTC ATA GAC AAG ATG GT
GAPDH	Reverse	GGG TAG AGT CAT ACT GGA ACA TG
NESTIN	Forward	TTA GAG GTC CCA GTT GCT CA
NESTIN	Reverse	TCG AGT TCC AGT CCA GTT CT
PTEN	Forward	TGT AGT ATA GAG CGT GCG GA
PTEN	Reverse	CCT CTG GAT TTG ATG GCT CC
U6	Forward	CTC GCT TCG GCA GCA CA
U6	Reverse	AAC GCT TCA CGA ATT TGC GT
rno-miR-223-3p	Forward	ACA CTC CAG CTG GGT GTC AGT TTG TCA AAT AC
rno-miR-223-3p	Reverse	CTC AAC TGG TGT CGT GGA

Western blot

Total protein was extracted from the brain tissue from the rats in each study group using RIPA buffer (Santa Cruz Biotechnology, Inc.) containing a protease inhibitor (Sigma-Aldrich, St. Louis MO, USA). A bicinchoninic acid (BCA) assay kit (Beyotime, Shanghai, China) was utilized for conducting quantitative analysis of protein concentrations. About 40 µg protein sample was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). After treating with 5% skimmed milk, primary and secondary antibodies were utilized to incubate the membranes for 1.5 h. Enhanced chemiluminescence (ECL) (Bio-Rad, Hercules, CA, USA) was used to visualize the blots. Primary antibodies used were to Notch1 (1: 1000) (ab65297; Abcam, Cambridge, MA, USA), PTEN (1: 1000) (ab31392; Abcam, Cambridge, MA, USA), Nestin (1: 1000) (ab22035; Abcam, Cambridge, MA, USA), and GAPDH (1: 2000) (ab9482; Abcam, Cambridge, MA, USA).

The Zea-Longa 5-point assessment and the modified neurological severity score (mNSS)

After MCAO and CIRI, the Zea-Longa 5-point assessment method was performed to assess neurological impairment. The mNSS test was performed at seven days following CIRI. A total score of 18 was indicated the severest neurological impairment. One point was scored for failure to perform the test or absence of a neurological reflex, with a higher score indicating a more severe degree of neurological impairment.

Statistical analysis

The results were expressed as the mean±standard deviation (SD). Data were analyzed using SPSS version 18 software (IBM, Chicago, IL, USA). Data between the study groups were compared using a t-test and one-way or two-way analysis of variance (ANOVA), followed by least significant difference (LSD) post hoc analysis. The test standard was $\alpha=0.05$. A p-value <0.05 was considered to be statistically significant.

Results

Electroacupuncture (EA) had a protective effect on cerebral ischemia-reperfusion injury (CIRI) in the rat model of middle cerebral artery occlusion (MCAO)

Preliminary experiments assessed the effects of EA treatment on non-acupoints, located at the base of the rat tail, on the neurological deficit score, assessed by the modified neurological severity score (mNSS). The results indicated that non-acupoints did not affect the neurological deficit score (Figure 1A).

The rat model of MCAO-induced focal CIRI was established. The Zea-Longa 5-point assessment method to assess neurological impairment (Figure 1B) confirmed the establishment of the model. One day after surgery, EA therapy was performed at acupoints Waiguan (TE5) and Zusanli (ST36) on the paralyzed limb for 30 min/day for seven days. Neurological deficit was assessed using the modified neurological severity score (mNSS). Rats in the acupuncture group showed significantly improved neurological function following CIRI (Figure 1C). The effect of EA treatment on the volume of cerebral infarction was assessed using triphenyl tetrazolium chloride (TTC) staining. As shown in Figure 1D, 1E, the infarct volume was significantly greater in the model group when compared with the sham group, and this effect was reduced by EA treatment. Histology of the rat brain tissue showed normal morphology in the sham group, with intact neurons with round cell bodies and clear nuclei (Figures 1F, 2A). However, in the MCAO model group, the cells in the ischemic

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cortical area were disorganized, and necrosis was present. After EA treatment, the histological changes in the rat brain tissue in the ischemic cortical region were significantly reduced when compared with the MCAO group. Apoptotic cells were reduced after EA treatment (Figures 1F, 2A). Therefore, EA at acupoints Waiguan (TE5) and Zusanli (ST36) on the paralyzed limb in the rat model had a protective effect on the subventricular zone (SVZ) and hippocampus regions.

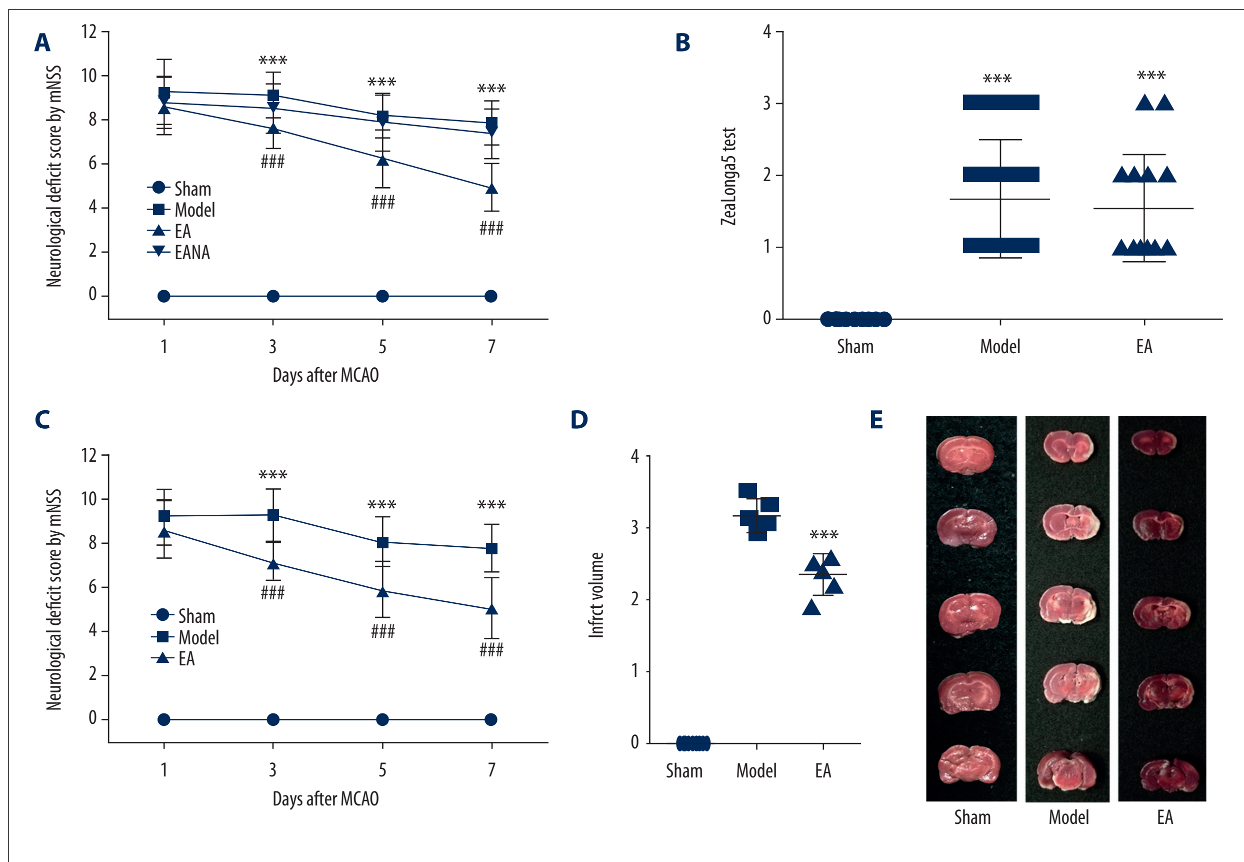
EA treatment upregulated NOTCH1 and microRNA-223 (miR-223) expression

The levels of miR-223, NESTIN, and PTEN in both SVZ and hippocampal tissues were analyzed. The levels of miR-223 were significantly downregulated (Figure 3A), and the expression of PTEN mRNA was significantly increased in the SVZ and the hippocampal tissues in the MCAO groups, compared with those of the sham group. After EA treatment, miR-223 was significantly upregulated, and PTEN mRNA was significantly downregulated (Figure 3C). NESTIN mRNA levels were significantly increased in the EA-treated group, compared with the model group (Figure 3B). There was a positive correlation between the expression levels of miR-223, NESTIN, and PTEN (Figure 3D, 3E), and between NESTIN and miR-223 in both the SVZ (Pearson's r=0.7188, p=0.0029) and the hippocampal tissues (Pearson's r=0.5954, p=0.0192). There was a negative correlation between PTEN and miR-223 levels in the SVZ tissues (Pearson's r=-0.6465, p=0.0092) and the hippocampal tissues (Pearson's r=-0.8671, p<0.001).

The protein expression levels of PTEN and Nestin in the rat hippocampal tissue (Figure 4A) and the SVZ tissue (Figure 4B) were confirmed by immunohistochemistry (IHC), which showed reduced expression of PTEN and increased expression of Nestin in the EA-treated group compared with the model group. Also, EA promoted the expression of Notch1 protein and the NOTCH1 gene. As shown in Figure 4C, EA treatment significantly upregulated the expression of NESTIN and NOTCH1 and significantly downregulated PTEN in the SVZ and hippocampal regions in the brain of the rat MCAO model. These results indicated that the effects of miR-223 during EA treatment in the rat model were mediated by the down-regulation of PTEN.

AntagomiR-223-3p reduced the effects of EA on CIRI in the rat MCAO model

The neurological deficit score was assessed by the mNSS on the seventh day after surgery. The antagomiR-223-3p reversed the effects of EA and reduced the increase in NESTIN and inhibition of PTEN expression associated with EA treatment, and



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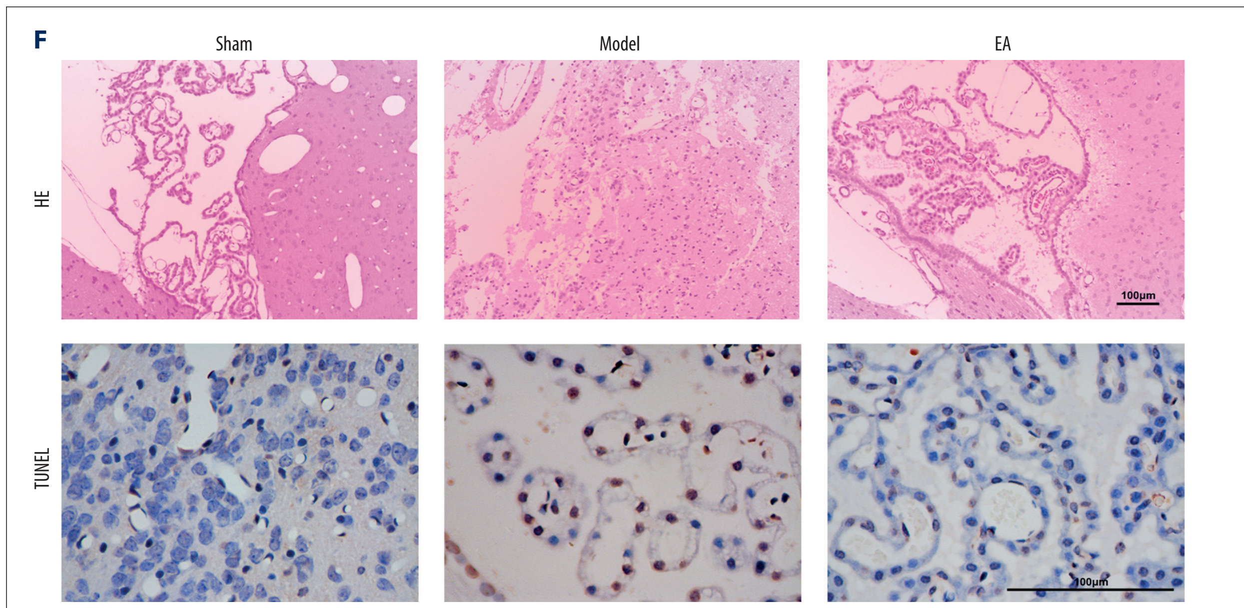
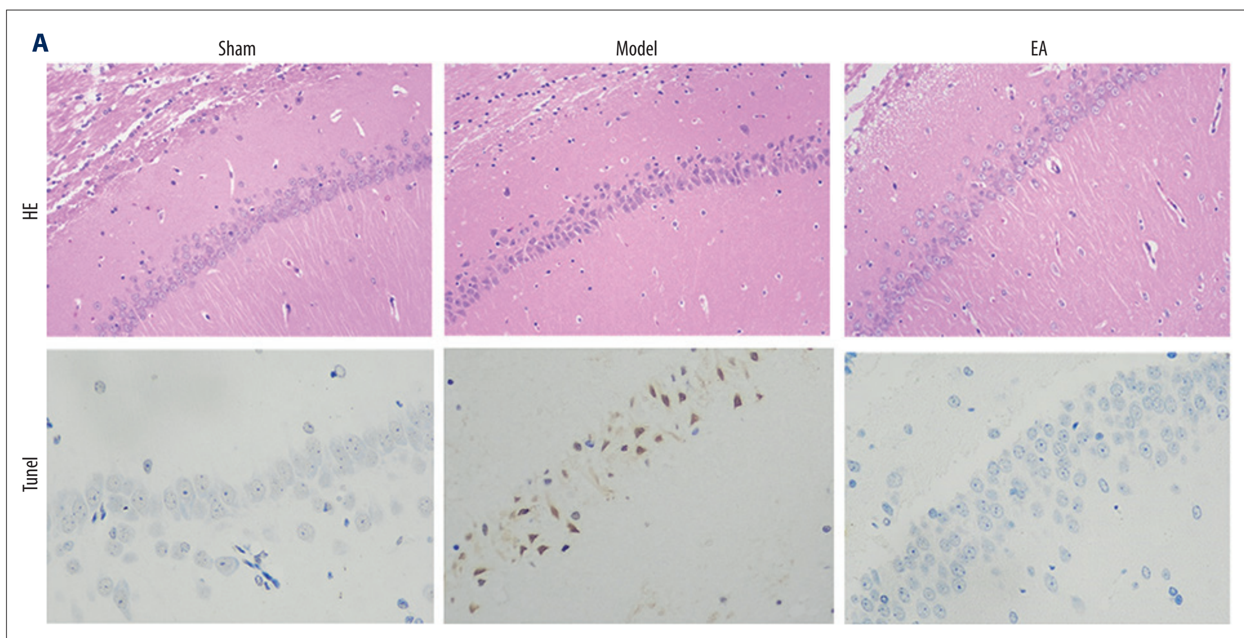


Figure 1. The protective effect of electroacupuncture (EA) on middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemia-reperfusion injury (CIRI). (**A**) Model rats were also treated with EA with or without TE5/ST36 acupoints. The neurological deficit score by the modified neurological severity score (mNSS) was assessed on the seventh day after surgery. (n=15). *** $p<0.001$ *vs.* Sham, ### $p<0.001$ *vs.* Model. The MCAO-induced focal CIRI model was established. One day after the surgery, EA treatment was performed at the Waiguan (TE5) and Zusanli (ST36) acupoints on the paralyzed limbs of the rats. Quantitative analysis of (**B**) the Zea-Longa 5-point assessment method to assess neurological impairment validated the MCAO model. (**C**) The neurological deficit score by the modified neurological severity score (mNSS) on the seventh day after surgery. (n=15). *** $p<0.001$ *vs.* Sham, ### $p<0.001$ *vs.* Model. (**D, E**) The volume of the cerebral infarction was evaluated by triphenyl tetrazolium chloride (TTC) staining in each group, and the percentage of infarct volume was calculated. (**F**) Histology using hematoxylin and eosin (H&E) staining (magnification ×100) and the TUNEL assay (magnification ×400) in the subventricular zone (SVZ).



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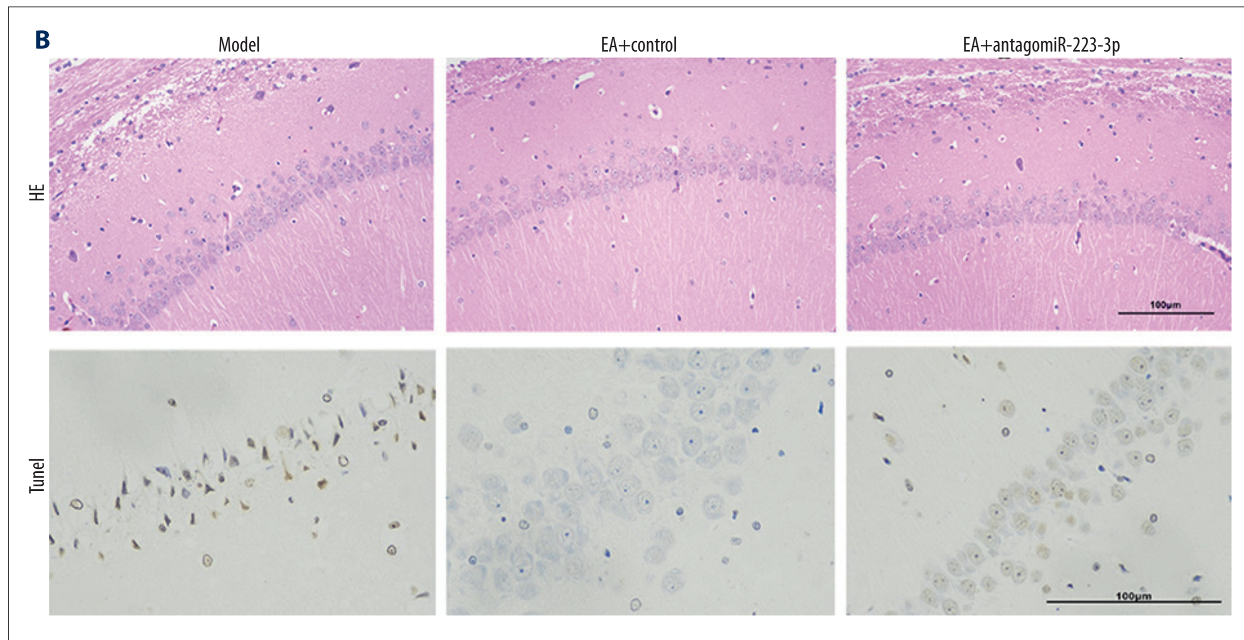

Figure 2. The effect of electroacupuncture (EA) on the hippocampal region in the rat model of middle cerebral artery occlusion (MCAO). (**A, B**) After EA treatment or the use of antagomiR-223-3p, histology using hematoxylin and eosin (H&E) staining (magnification ×200) and the TUNEL assay (magnification ×400) were performed in the rat hippocampal region.

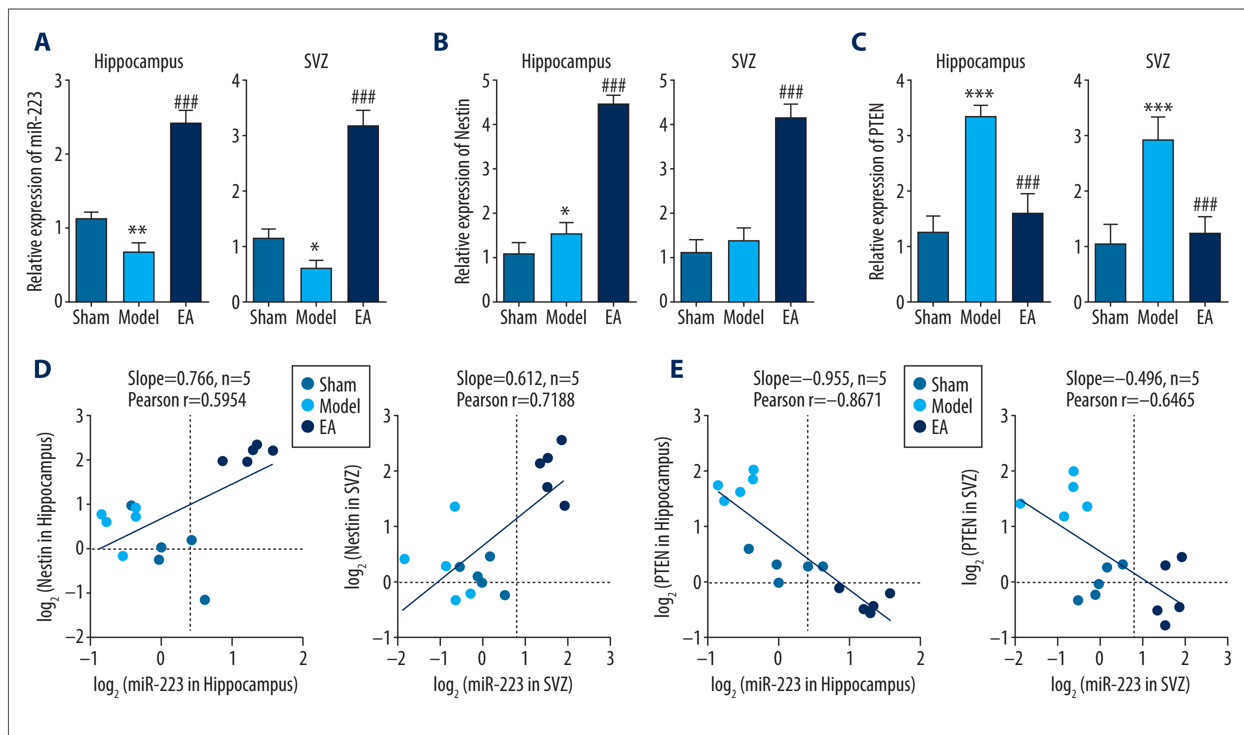

Figure 3. Expression of microRNA-223 (miR-223), NESTIN, and PTEN in the subventricular zone (SVZ) and hippocampus after electroacupuncture (EA) treatment. Levels of (**A**) miR-223, (**B**) NESTIN mRNA, (**C**) and PTEN mRNA in the hippocampus and subventricular zone (SVZ) regions. * p<0.05, ** p<0.01, *** p<0.001 *vs.* Sham; ### p<0.001 *vs.* Model. (**D**) Positive correlation between miR-223 and NESTIN in the SVZ and hippocampal regions. (**E**) Negative correlation between miR-223 and PTEN in the SVZ and hippocampal areas.

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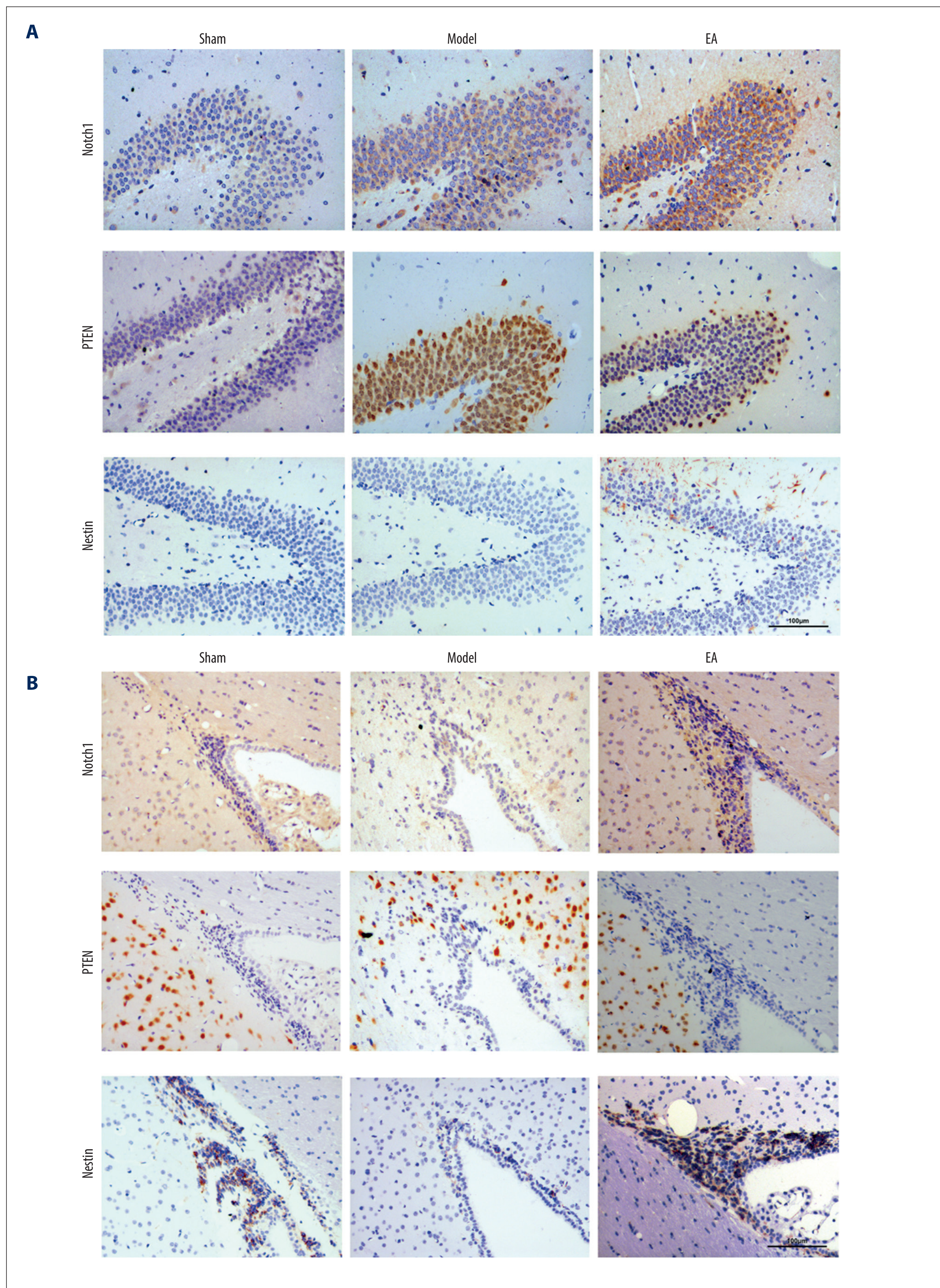

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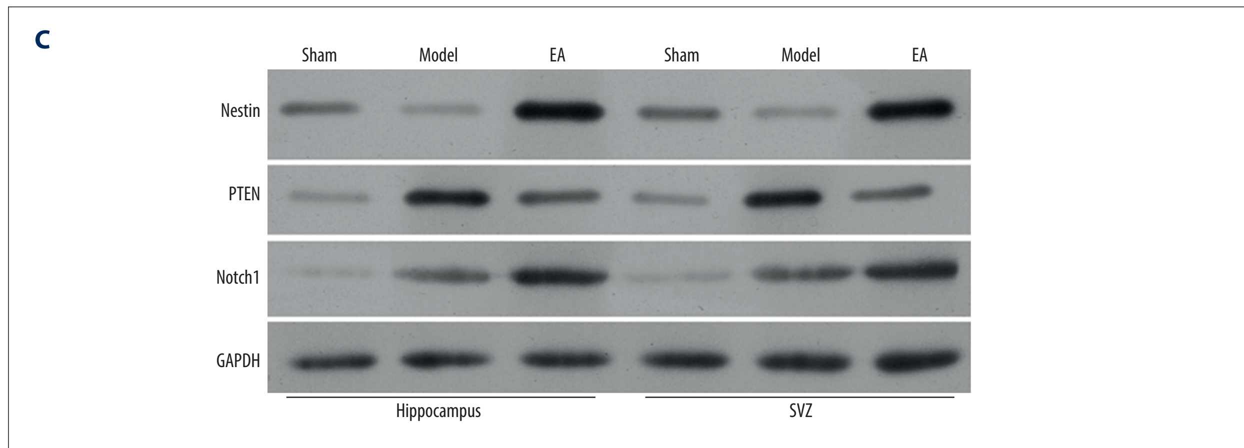

Figure 4. Expression of NOTCH1, NESTIN, and PTEN in the subventricular zone (SVZ) and hippocampus after electroacupuncture (EA) treatment. Protein levels of Notch1, PTEN, and Nestin in the hippocampus (**A**) and SVZ (**B**) detected by immunohistochemistry (IHC) (magnification ×200). (**C**) Western blot analysis of NOTCH1, PTEN, and NESTIN in the hippocampal and SVZ regions. GAPDH acted as the control.

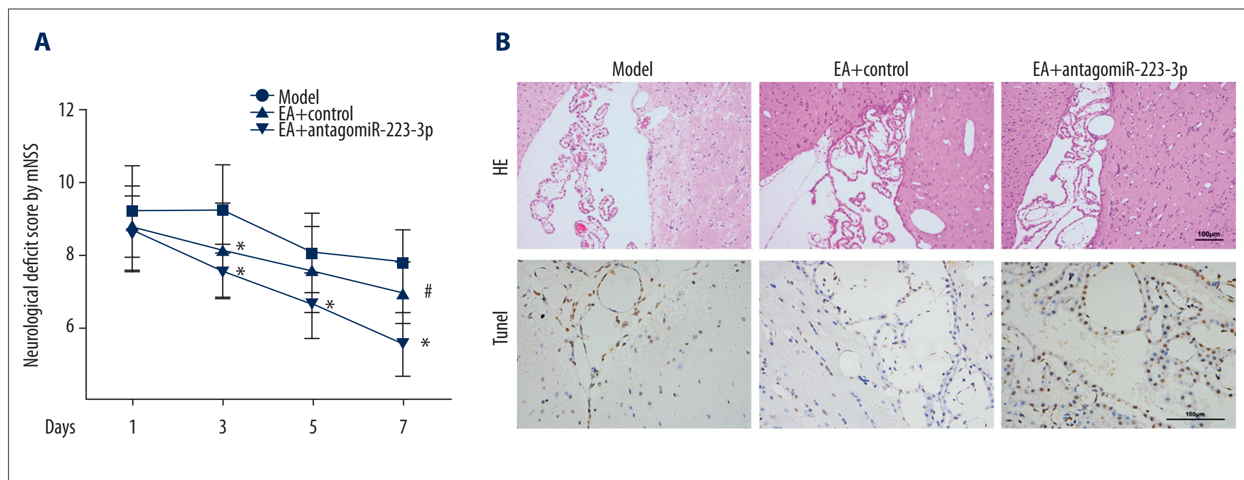

Figure 5. AntagomiR-223-3p reduced the protective effect of electroacupuncture (EA) on cerebral ischemia-reperfusion injury (CIRI) induced by middle cerebral artery occlusion (MCAO) in the rat model. (**A**) Neurological deficit was assessed using the modified neurological severity score (mNSS) at 1, 3, 5, and 7 days after surgery. (n=15). * $p<0.05$ *vs.* Model, # $p<0.05$ *vs.* EA and the Control. (**B**) After treatment with antagomiR-223-3p, the effects on the EA-treated rat MCAO model were examined by histology using hematoxylin and eosin (H&E) staining (magnification ×100) and the TUNEL assay (magnification ×400) in the subventricular zone (SVZ).

the effects were greater in the EA group than the control group (Figure 5A). Therefore, the antagomiR-223-3p reduced the protective effects of EA treatment. Histology of the rat brain tissue and the TUNEL assay for apoptosis of the SVZ and hippocampal regions of rat MCAO model showed that antagomiR-223-3p treatment significantly reduced the improvements in the of the brain tissues in ischemic cortical regions induced by EA treatment (Figures 5B, 2B). AntagomiR-223-3p treatment significantly reduced the inhibition of apoptosis induced by EA treatment in the SVZ and hippocampal regions of the rat MCAO model (Figures 5B, 2B). Therefore, antagomiR-223-3p reduced the EA-mediated protection in the MCAO rat model of CIRI.

AntagomiR-223-3p suppressed the protective role of EA treatment by regulating PTEN

Target gene prediction showed that miR-223 targeted PTEN in the MCAO rat model of CIRI. The psiCHECK™-2 Vector containing the wild type (WT) or mutant type (MUT) 3' UTR sequence of PTEN were co-transfected into 293T cells with a miR-223 mimic (Figure 6A). A significant decrease in the fluorescence ratio was found in the miR-223 mimics with WT 3' UTR PTEN, which was abolished in the MUT (Figure 6B). Therefore, PTEN was a direct target gene of miR-223. After inoculating rats with antagomiR-223-3p, the expression of EA-induced miR-223 was

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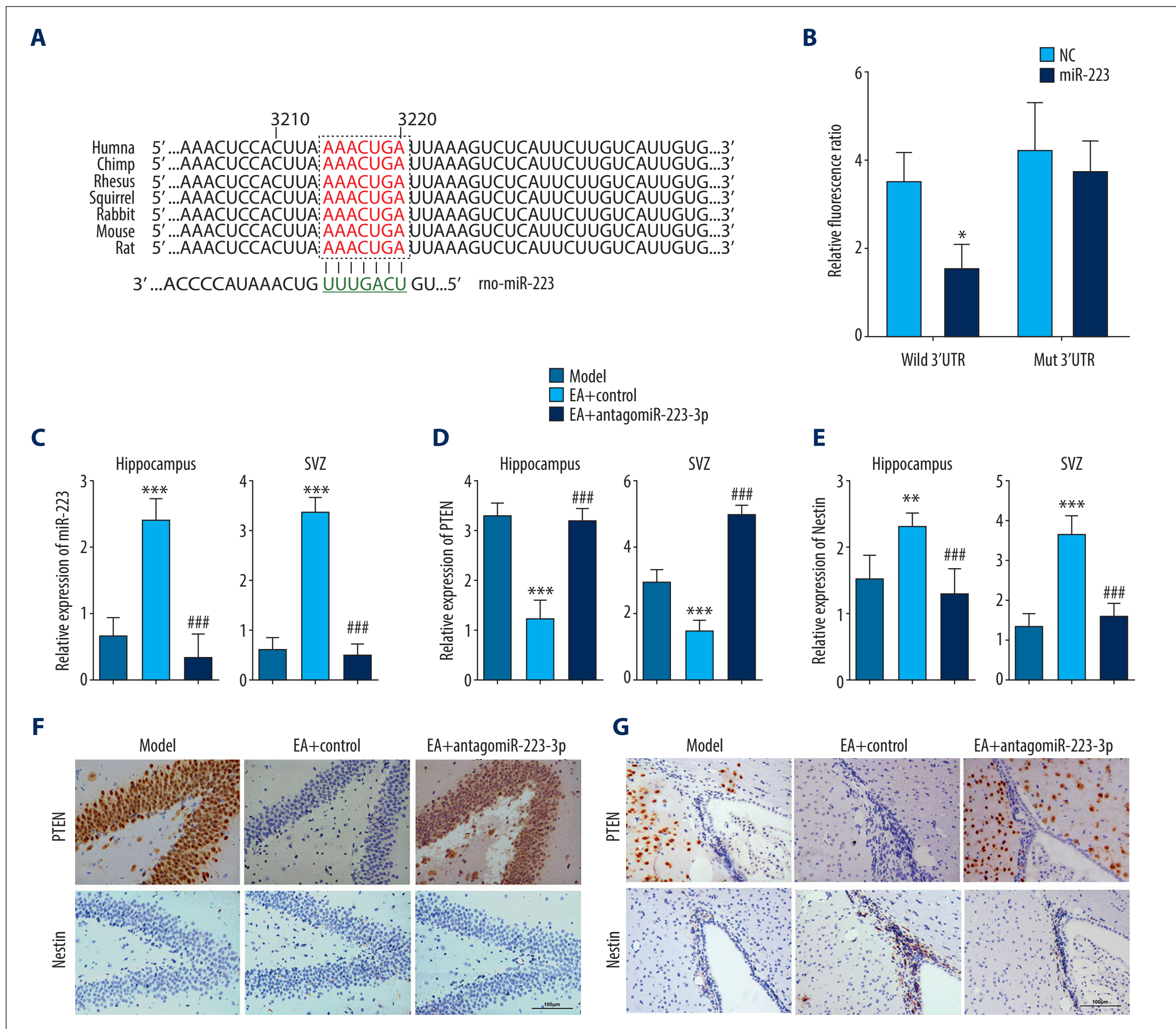

Figure 6. AntagomiR-223-3p reduced the protective effect of electroacupuncture (EA) on cerebral ischemia-reperfusion injury (CIRI) induced by middle cerebral artery occlusion (MCAO) in the rat model by regulating PTEN. (**A**) Wide type (WT) or mutant type (MUT) 3' UTR sequence of PTEN. The psiCHECK™-2 Vector containing the WT or MUT 3' UTR sequence of PTEN was constructed and co-transfected into 293T cells with a microRNA-223 (miR-223) mimic. (**B**) The fluorescence ratio was measured and compared. * p<0.05 *vs.* NC. (**C**) The miRNA antagomiR-223-3p was used in the rat model of MCAO before EA treatment. The expression of miR-223 in the hippocampal and subventricular zone (SVZ) tissues. (**D**) PTEN mRNA in the hippocampal and SVZ tissues. (**E**) NESTIN mRNA in hippocampal and SVZ tissues. (**F, G**) Protein levels of Nestin and PTEN in the hippocampus and SVZ detected by immunohistochemistry (IHC) (magnification ×200).

significantly inhibited (Figure 6C). Reduced expression levels of PTEN mRNA (Figure 6D) and increased expression levels of NESTIN mRNA (Figure 6E) were found in both the hippocampal and SVZ tissues. Consistent with the mRNA levels, immunohistochemical staining of rat brain tissue showed that the PTEN levels increased in both the hippocampus and SVZ tissues after the use of antagomiR-223-3p in the EA treatment group (Figures 6F, 6G). These findings support that, in this rat model, EA treatment on MCAO-induced focal CIRI occurred through the miR-223/PTEN signaling pathway.

Discussion

The aim of this study was to investigate the molecular mechanism underlying the effects of electroacupuncture (EA) in a rat model of cerebral ischemia-reperfusion injury (CIRI) induced by middle cerebral artery occlusion (MCAO). EA is an alternative treatment that has previously been shown to enhance functional recovery after ischemic stroke [29]. The effect of EA is primarily determined by the acupoint selection and frequency of stimulation [30]. Previous studies have shown that the stimulation

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of acupoints Waiguan (TE5) and Zusanli (ST36) had significant effects in the model of MCAO [31,32]. Previous studies have shown that the subventricular zone (SVZ) and hippocampus were regions of the brain involved in the production of neural stem cells (NSCs) [33,34]. The findings from the present study showed that the NOTCH1/miR-223/PTEN pathway was involved in the effects of EA treatment in the SVZ and hippocampus regions of the brain in the rat model of CIRI induced by MCAO.

CIRI results in necrosis or apoptosis of nerve cells, which plays a crucial role in neurological deficits caused by cerebral infarction [35]. Neuronal apoptosis occurs during cerebral ischemia and reperfusion injury, as well as glial cells and vascular endothelial cells. Recent studies have shown that the pathways that regulate apoptosis mainly include the mitochondrial, the death receptor, and the endoplasmic reticulum pathways [35,36]. In this study, in the rat model of CIRI induced by MCAO, EA had a protective role through the miR-223/PTEN pathway.

NOTCH signaling is important in neuronal repair following cerebral ischemia, and has been previously shown to be activated during cerebral ischemia in adult mice, and was shown to increase the survival of NSCs [37]. Therefore, the activation of the NOTCH signaling pathway promotes regeneration and repair of nerve cells following ischemia and reduces neurological impairment [27,38]. The NOTCH1 signaling pathway is also associated with miR-223 in several cell types. In non-small cell lung cancer (NSCLC), upregulation of miR-223 induced resistance in cells by stimulating the NOTCH signaling pathway [39]. Kumar et al. showed that miR-223 was regulated by NOTCH signaling in T-cell acute lymphoblastic leukemia (ALL) [40]. The authors further confirmed the regulatory role of NOTCH in miR-223 expression by luciferase and chromatin immunoprecipitation assays conducted on the promoter region of miR-223 [40]. In the present study, in the rat model of MCAO, NOTCH1 expression was found to increase, and EA further increased NOTCH1 expression, indicating that NOTCH1 might mediate the expression of miR-223 during EA treatment.

The findings from the present study also showed that PTEN was a target of miR-223. Previously, PTEN deletion has been shown to enhance the regenerative ability of nerve cells following adult spinal cord injury [23]. Mice with focal cerebral ischemia were also previously shown to have increased phosphorylation of PTEN [41]. In oxygen and glucose deprivation-induced injury *in vitro* and in cerebral ischemia *in vivo*, suppression of PTEN was previously shown to result in neuroprotection [42]. Also, the deletion of PTEN and mTOR resulted in significant recovery of skilled locomotion [43]. In the present study, EA suppressed PTEN expression and enhanced miR-223 expression levels. The use of antagomir-223-3p resulted in the significant down-regulation of levels of miR-223, and PTEN levels were significantly upregulated following EA treatment. Therefore, the neuroprotective effect of EA on cerebral ischemia in this rat model may have involved the activation of the NOTCH1 signaling pathway to mediate the miR-223/PTEN pathway.

Previous studies have shown that NSCs are immature cells that selectively express certain antigen markers, such as Nestin, which is a sensitive diagnostic marker, as when NSCs differentiate into mature neurons, Nestin expression is no longer present [44]. In the adult mammalian brain, regions such as the hippocampus, the SVZ, the cerebral cortex, the choroid plexus, ependyma, the striatum, and the olfactory bulb and other regions consist of NSCs. Most NSCs that persist are found in the SVZ and subgranular zones (SGZ) of the dentate gyrus (DG). The regeneration of NSCs in the SVZ is involved in the regeneration of the olfactory nerve, but NSCs in the DG are mainly involved in the regeneration of the hippocampus. EA-induced NSC proliferation and inhibition of apoptosis in both the SVZ and hippocampal tissues indicate that EA may promote the repair of injury in both the SVZ and hippocampal tissues in this rat model. However, the correlation between EA and neural cell apoptosis in ischemic areas and the proliferation of NSCs in the hippocampal and SVZ areas require further study and clarification. These results indicate a positive correlation between miR-223 and NSCs in a rat model of CIRI following MCAO. However, the role of EA in functional recovery and the underlying mechanism associated with miR-223 and NSCs should be explored in future studies.

Conclusions

This study aimed to investigate the molecular mechanism underlying the effects of electroacupuncture (EA) in a rat model of cerebral ischemia-reperfusion injury (CIRI) induced by middle cerebral artery occlusion (MCAO). The findings from this study showed that EA activated the NOTCH1 pathway, promoted the expression of microRNA-223 (miR-223), increased the number of NSCs, and reduced the expression of PTEN. These preliminary findings support the need for further studies to investigate the neuroprotective effect of EA on the ischemic rat brain and the NOTCH1/mir-223/PTEN pathways and the long-term effects of EA on in this rat model.

Conflict of interest

None.

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