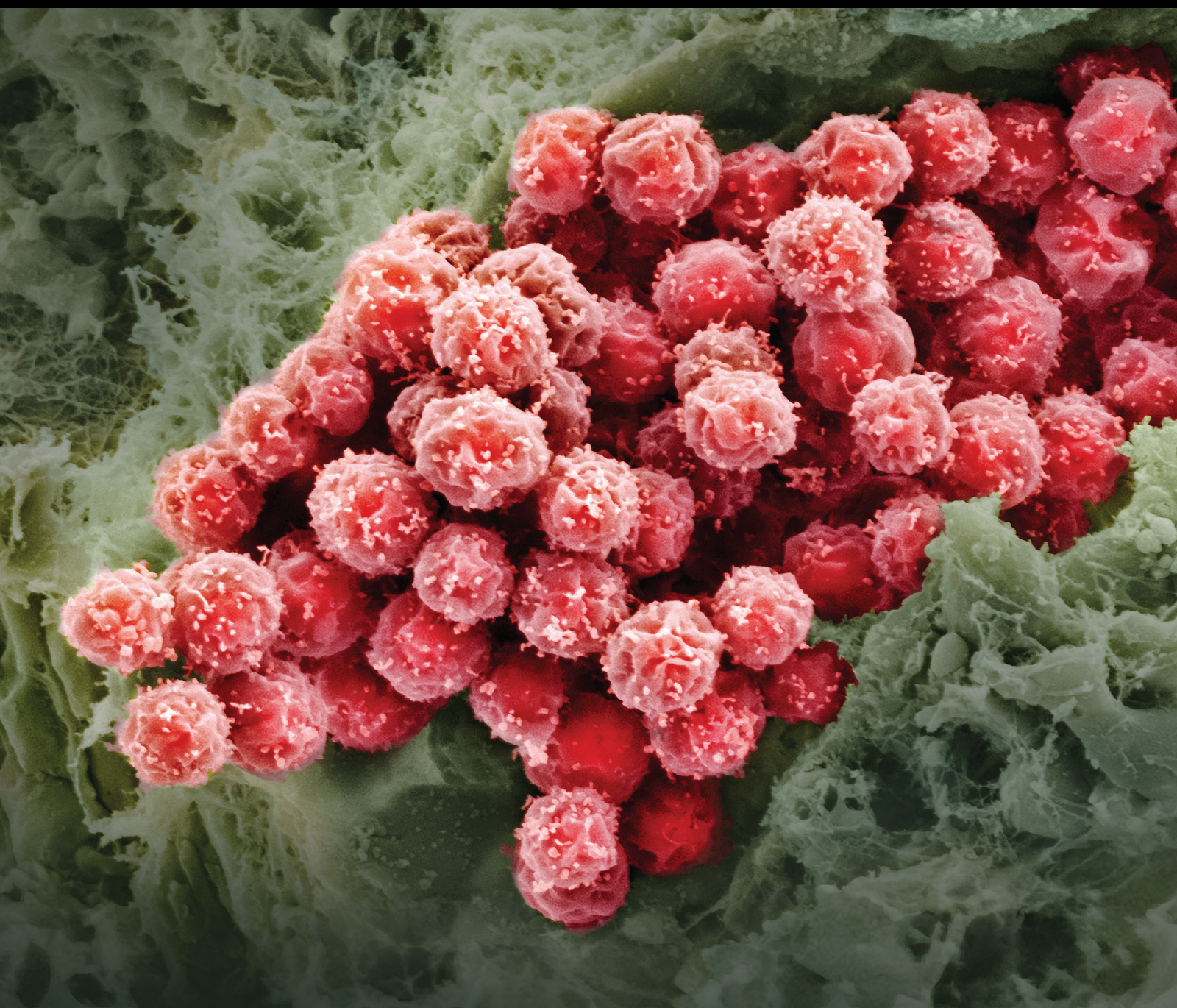
Stem Cells International

Brain Injury and Stem Cells Replacement

Lead Guest Editor: Hailiang Tang
Guest Editors: Yao Li and John Zhang



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Editorial Board

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Editorial

Brain Injury and Stem Cell Replacement

Hailiang Tang[1], **Yao Li**[2] **and John Zhang**[3]

[1]*Department of Neurosurgery, Huashan Hospital, Fudan University, Shanghai 200040, China*
[2]*Med-X Research Institute, Shanghai Jiaotong University, Shanghai 200030, China*
[3]*Center for Neuroscience Research, Loma Linda University School of Medicine, 11175 Campus St., Loma Linda, CA 92350, USA*

Correspondence should be addressed to Hailiang Tang; tangtang052105192@gmail.com

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Currently, stem cell is still the research hotspot in neuroscience due to its important role for regeneration medicine. Stem cells could aid neurogenesis and functional recovery in animals and human beings after brain injury, which was always caused by stroke or traumatic brain injury (TBI). Neural stem cell (NSC) replacement is considered a very promising therapy strategy for brain injury. Many studies had been conducted in animal experiments and clinical trials.

The purpose of this special issue is to provide readers with an overall outlook of the recent advances in stem cell replacement for brain injury. The topics cover neural stem cells and other stem cells, traumatic brain injury and ischemic stroke, overview of stem cell therapy for brain injury, mechanism of stem cell therapy, and stem cell tracking in the host brain.

This special issue published 17 excellent papers regarding the above specific topics, and the details are summarized below.

Stem cell replacement for brain injury is a very promising therapy, but how to evaluate the therapeutic effect is still difficult. Y. Zheng et al. provided the way on how to track these stem cells after treatment; they overall reviewed the advances on stem cell tracking for brain injury and summarized the current techniques applied for stem cell tracking. With regard to ischemic stroke, W. Xu et al. thoroughly reviewed the role of stem cells for this kind of stoke, and Y. Zhang and H. Yao discussed transplanted stem cells for it. Moreover, Y. Lu et al. discussed the adult neurogenesis after stroke therapy. In another group, C. Reis et al. explored the stem cell therapy options for ischemic stroke, which were very instructive. This team also reviewed the mechanism of stem cell therapy for traumatic brain injury. The two kinds of brain injury (TBI and stroke) were both covered. And what is more, M. Cui et al. interestingly described the electromagnetic regulation of neural stem cells for brain injury.

The guest editors hope this special issue provides readers with helpful information of recent advances in stem cell therapy for brain injury and may stimulate interest for further research in this area.

Acknowledgments

We would like to thank all the authors and reviewers for their excellent contributions.

Hailiang Tang
Yao Li
John Zhang

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Research Article

The Long-Term Outcome Comparison of Different Time-Delayed Kallikrein Treatments in a Mouse Cerebral Ischemic Model

Yaohui Ni,[1] Kefu Cai,[1] Yujie Hu,[2] Chuan-hui Wang,[3] Yuanyuan Zhang,[1] Hua Huang,[4] Xing Su,[5] and Jin-hua Gu[2]

[1]*Department of Neurology, Affiliated Hospital of Nantong University, Jiangsu 226001, China*
[2]*Department of Pathophysiology, Medical School of Nantong University, Jiangsu 26001, China*
[3]*Geriatric Department, Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 201900, China*
[4]*Department of Pathology, Affiliated Hospital of Nantong University, Jiangsu 226001, China*
[5]*Department of Neurosurgery, Affiliated Hospital of Nantong University, Jiangsu 226001, China*

Correspondence should be addressed to Xing Su; suxingntu@gmail.com and Jin-hua Gu; laogulaogu@hotmail.com

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Delayed administration of kallikrein after cerebral infarction can improve neurological function. However, the appropriate kallkrein treatment time after ischemic stroke has not been illuminated. In this study, we compared the long-term outcome among three kallikrein therapeutic regimens starting at different time points following mouse cerebral ischemia. Furthermore, the protective mechanisms involving neurogenesis, angiogenesis, and AKT-GSK3β-VEGF signaling pathway were analyzed. Human tissue kallikrein was injected through the tail vein daily starting at 8 h, 24 h, or 36 h after right middle cerebral artery occlusion (MCAO) until the 28th day. Three therapeutic regimens all protected against neurological dysfunction, but kallikrein treatment starting at 8 h after MCAO had the best efficacy. Additionally, kallikrein treatment at 8 h after MCAO significantly enhanced cell proliferation including neural stem cell and induced differentiation of neural stem cell into mature neuron. Kallikrein treatment starting at 8 h also promoted more angiogenesis than other two treatment regimens, which was associated with AKT-GSK3β-VEGF signaling pathway. Thus, we confirm that three delayed kallikrein treatments provide protection against cerebral infarction and furthermore suggest that kallikrein treatment starting at 8 h had a better effect than that at 24 h and 36 h. These findings provide the experimental data contributing to better clinical application of exogenous kallikrein.

1. Introduction

Stroke is the leading cause of death and disability worldwide [1]. Like other countries, ischemic stroke is the most common type of stroke in China [2]. According to the guidelines from the American Stroke Association for the early management of patients with acute cerebral ischemia, intravenous administration of rtPA remains the only recommended pharmacological therapy within 4.5 hours after acute ischemic stroke [3]. More than 4.5 hours after acute ischemic stroke, therapies are being sought to improve functional neurological recovery and reduce disability.

As an important element of the kallikrein/kinin system (KKS), tissue kallikrein can cleave low-molecule kininogen into kinins (e.g., bradykinin and kallidin). The biological function of tissue kallikrein is mainly produced by kinin binding to high-affinity kinin B1 or B2 receptors [4]. The B2 receptor is constitutively expressed, whereas the B1 receptor is expressed at very low levels under normal conditions and is triggered by inflammation or stress. Intact kinins can bind to the kinin B2 receptor, and the metabolites of kininase I, such as des-Arg9-BK and des-Arg10-kallidin, bind to the kinin B1 receptor. Additionally, kallikrein can also activate the B2 receptor directly. All components of the KKS are

expressed in the brain. After ischemic stroke, the KKS is activated and the expression of kinin, kinin B1, and kinin B2 receptors is upregulated [5–7]. Human tissue kallikrein gene transfer immediately after ischemia-reperfusion injury provides neuroprotection against cerebral ischemia injury by the kinin B2 receptor in the rat MCAO model [8]. Moreover, delayed kallikrein administration by systemic gene delivery at 8 h after MCAO was also effective in reducing neurological deficit scores and cerebral infarction without affecting blood pressure [9]. Similarly, delayed kallikrein protein administration at 24 h after focal brain infarction significantly reduced neurological deficits in hypertensive rats [10]. Therefore, compared with tPA, tissue kallikrein has a beneficial effect in experimental murine stroke model with a wide time window of several days. More importantly, in a multicenter and double-blind clinical trial, human urinary kallidinogenase (HUK) was also effective when treated within 48 h after inpatients with stroke onset [11]. The data suggest that tissue kallikrein therapy is a promising treatment for acute human ischemic stroke. Based on more than 3000 cases of phase IV clinical studies in China, kallikrein therapy efficacy reached 88%, if patients were treated with human urinary kallikrein within 2 days. Thus, HUK has been approved by the Chines FDA as a novel drug for stroke patients.

However, some detailed information about delayed kallikrein treatment has not been explored, such as, whether different time point treatments after ischemia will cause efficacy difference and if so, which time point for the delayed kallikrein treatment will produce better protection. Thus, in the present study, we compared the effects of delayed kallikrein treatment starting at 8 h, 24 h, and 36 h after stroke onset in a cerebral ischemic mouse model. In addition, from the point of view of neurogenesis and angiogenesis, the underlying mechanism of effect difference was explored. The study provided the experimental data contributing to better clinical application of exogenous kallikrein.

2. Materials and Methods

2.1. Animal Middle Cerebral Artery Occlusion (MCAO) Surgery. Animal experiments were performed in accordance with the ARRIVE guidelines. Procedure for the use of laboratory animals was approved by the Institutional Animal Care and Use Committee of Nantong University, Jiangsu, China. During the animal studies, guidelines of *the regulation for the administration of affairs concerning experimental animals of China* enacted in 1988 were followed. A total of 95 male ICR mice weighing 25–30 g were used for this study. The MCAO surgery was performed as described previously. Briefly, mice were anesthetized with intraperitoneal injection of 2.5% Avertin. Through a ventral midline incision, the right common carotid artery, internal carotid artery, and external carotid artery were surgically exposed. A 6–0 nylon suture with silicon coating (Doccol Corporation, Redlands, CA) was inserted into the internal carotid artery through the external carotid artery stump and was gently advanced to occlude the middle cerebral artery. To obtain blood reperfusion, the occluding filament was withdrawn after occlusion for 1.5 hrs. Body temperature was maintained between 37.0°C and 37.5°C with a heating pad during surgery. Cerebral blood flow was monitored by Laser-Doppler flowmetry, and only those mice with 90% of blood flow blockade during MCAO and 85–95% recovery of blood flow during reperfusion were used for further experiments. The sham-operated mice underwent identical surgery, but the suture was not inserted. Mice that died within 6 hrs after cerebral artery occlusion procedure were excluded from the study. All experiments were performed in a randomized manner. Evaluation of neurological deficits and brain loss was performed by an investigator blinded to the experimental treatments.

2.2. Experimental Groups and Human Tissue Kallikrein and BrdU Labeling. Male ICR mice ($n = 95$) weighing 25–30 g were divided into five groups: group 1, the sham-operated mice (sham, $n = 15$). In a total of 80 MCAO mice, five mice died within 6 h after reperfusion. The rest MCAO mice were randomly assigned to 4 groups at 8 h after reperfusion: group 2, the mice suffered from MCAO alone without kallikrein treatment (MACO, $n = 18$); group 3, the mice suffered from MCAO plus kallikrein treatment daily for 28 consecutive days starting at 8 h after reperfusion (KLK 8 h, $n = 18$); group 4, the mice suffered from MCAO plus kallikrein treatment daily for 27 consecutive days starting at 24 h after reperfusion (KLK 24 h, $n = 18$); group 5, the mice suffered from MCAO plus kallikrein treatment daily for 27 consecutive days starting at 36 h after reperfusion (KLK 36 h, $n = 19$). The tissue kallikrein (Techpool Bio-Pharma Co. Ltd. Guangdong, China) was dissolved with normal saline. The mice in groups 3, 4, and 5 received a bolus injection of kellikrein daily through a tail vein at the dosage of 2.4×10^{-2} PNAU/kg. BrdU, which can incorporate into the DNA of dividing cells during S-phase, was used to label proliferative cells. BrdU (75 mg/kg, Sigma-Aldrich) was injected intraperitoneally twice daily for 5 consecutive days starting 48 h after reperfusion in all mice.

2.3. Neurological Functional Assessment and Brain Area Loss Measurement. In all animals, a battery of behavioral tests was performed at 14 and 28 days after MCAO by an investigator blinded to the experimental treatments. To evaluate neurological function, modified neurological severity score (mNSS) was applied [12].The mNSS contains the motor, sensory, balance, and reflex tests. Neurological function was graded on a scale of 0 to 18(normal score, 0; maximal score, 18).A higher score indicates a more severe injury. Additionally, pole test was used for evaluating the mouse movement disorder. The pole test was adapted to Matsuura et al. with minor modifications [13]. In brief, the mouse was placed head upward near the top of the pole, which was covered with a tape to create a rough surface. The time taken to turn completely downwards (T/turn) and the total time to reach the floor with all four paws (T/floor) were recorded. If the animal was unable to turn completely, the time to reach the floor was also attributed to T/turn. Each animal was tested on 5 trials, and the average score was taken as the final pole test score.

After removing and photographing the intact brains at 28 days after MCAO, brain area loss was determined using cresyl violet staining to assess the remaining area by four 20 μm

coronal sections cut on a cryostat. Sections were mounted on gelatin-coated microscope slides, incubated in 1.0% cresyl violet acetate (Sigma, St. Louis, MO) for 8 min then dehydrated sequentially in 70%, 95%, and 100% ethanol and xylene baths at room temperature. Percent brain loss was calculated as the area of the contralateral hemisphere minus the ipsilateral hemisphere/whole brain section × 100 [14].

2.4. Histology and Immunofluorescence and Cell Counting. Mice were deeply anesthetized, cardially perfused with normal saline, and decapitated, and brains were snap frozen in liquid nitrogen for cryostat sectioning at 14 days and 28 days after MCAO. Cryosections (20 μm) were fixed with acetone/methanol for 10 min at −20°C and washed 3 times for 5 min with PBS. For BrdU double staining, sections were incubated with 2 N HCl for 30 min at 37°C, followed by an incubation in 0.1 M borate buffer for 10 min at room temperature, washed 6 times for 10 min with TBS, blocked in TBS with 10% normal goat serum and 0.1% Triton for 30 min, incubated with primary antibody against BrdU (1 : 800, Sigma-Aldrich), rabbit anti-Nestin (1 : 100, Sigma-Aldrich), mouse anti-NeuN (1 : 500, Chemicon), and mouse anti-Tuj1 (1 : 50, Abcam) at 4°C overnight in TBS with 1% normal goat serum and 0.1% Triton overnight, washed 2 times for 5 min with TBS and 1 time for 15 min with TBS with 1% normal goat serum and 0.1% Triton, incubated with secondary antibody (1 : 400) for 2 h washed 2 times for 5 min with PBS, incubated 3 min with DAPI, and washed 2 times with water. Sections were mounted and visualized in a confocal microscope (Leica), and photomicrographs were taken for further analysis. For the negative staining of NeuN and Tuj1, the sections were incubated without the primary antibody.

The quantification of antigen-positive cells was performed in six 10 μm coronal sections per animal, spaced 200 μm apart (4 fields per mouse; 4–6 mice per group). Cells were counted under high power (40 objective) on a microscope (Olympus IX51, Japan). Data were represented as a number of positive cells/mm^2 of BrdU or double-positive cells per section. All quantifications were performed with the ImageJ image analysis software.

2.5. Western Blot Analyses. The ipsilateral cortex tissue of the mouse brain was homogenized in prechilled buffer containing 50 mM Tris-HCl (pH 7.4), 2.0 mM EGTA, 2 mM Na3VO4, 50 mM NaF, 0.5 mM AEBSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 4 μg/ml pepstatin A. Protein concentrations of the homogenates were determined by using Pierce 660 nm Protein Assay kit (Thermo Fisher Scientific Inc.). The samples were resolved in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto PVDF membrane (Millipore, Bedford, MA). After blocking with 5% fat-free milk, the blots were then probed with a primary antibody, such as VEGF (1 : 1000; Abcam, USA), anti-AKT (1 : 1000; Cell Signaling Technology, USA), anti-pSer473-AKT (1 : 1000; Cell Signaling Technology, USA), anti-pSer9-GSK3β (1 : 1000; Cell Signaling Technology, USA), anti-GSK3β (1 : 1000; Cell Signaling Technology, USA), or anti-GAPDH (1 : 2000; Sigma, USA),-washed and then incubated with a corresponding HRP-conjugated secondary antibody. The protein-antibody complex was visualized by using the Pierce ECL Western Blotting Substrate (Thermo Scientific) and exposed to a HyBlot CL autoradiography film (Denville Scientific, Inc. Metuchen, NJ). Specific immunostaining was quantified by using the Multi Gauge software V3.0 (Fuji Photo Film Co. Ltd.).

2.6. Statistical Analysis. The data were analyzed by one-way ANOVA followed by Tukey's post hoc tests or unpaired two-tailed t test using software Graphpad Prism 5. Numerical data were presented as means ± SD, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Long-Term Outcome of Delayed Kallikrein Treatment at Different Time Points in Mouse Cerebral Ischemic Models. To compare the efficacy of kallikrein starting at different time points, the neurological function of mice was assessed at 14 d and 28 d after MCAO/reperfusion injury (Figure 1(a)). We found all exogenous tissue kallikrein treatments starting at 8 h, 24 h, and 36 h (i.e., KLK 8 h, KLK 24 h, and KLK 36 h) after ischemic stroke can ameliorate the neurological deficits, but the KLK 8 h group has lower scores (better neurological outcome) than the KLK 24 h and KLK 36 h groups (Figures 1(b)–1(d)). There was no significant difference in neurological severity score between KLK 24 h and KLK 36 h groups.

After removing and photographing the whole brains of some mice in different groups at 28 d after MCAO, brain loss was analyzed by using cresyl violet staining (Figure 1(e)). The results showed that the brain loss areas in the KLK 8 h and KLK 24 h groups but not in the KLK 36 h group were significantly reduced compared with the MCAO group. Consistent with the neurological function, the brain loss area in the KLK 8 h group is much more smaller than that in the KLK 24 h and KLK 36 h groups (Figures 1(e) and 1(f)). These data indicate that though three kallikrein therapeutic regimens all can attenuate the neurological deficits in cerebral ischemic mouse model, kallikrein treatment starting at 8 h has the best efficacy.

3.2. Kallikrein Treatment Starting at 8 h after Ischemia Enhances More Cell Proliferation in the Peri-Infarction Area and the Ipsilateral SVZ. Several studies have reported delayed kallikrein treatment after ischemia enhances neurogenesis and angiogenesis, which are involved in the improvement of neurological function. We next asked whether the long-term outcome difference among the three kallikrein therapeutic regimens are associated with neurogenesis. Using BrdU to label proliferative cells, we found the numbers of BrdU-positive cells in the ipsilateral SVZ and peri-infarction of ischemic animals were higher than those in the sham group (Figures 2 and 3). Compared with the MCAO group, the administration of exogenous tissue kallikrein starting at 8 h, 24 h, and 36 h after ischemic stroke can promote cell proliferation in the ipsilateral SVZ (Figures 2(a, C–E) and 2(b)) and in the peri-infarction area (Figures 3(a, C–E) and 3(b)). More importantly, we also

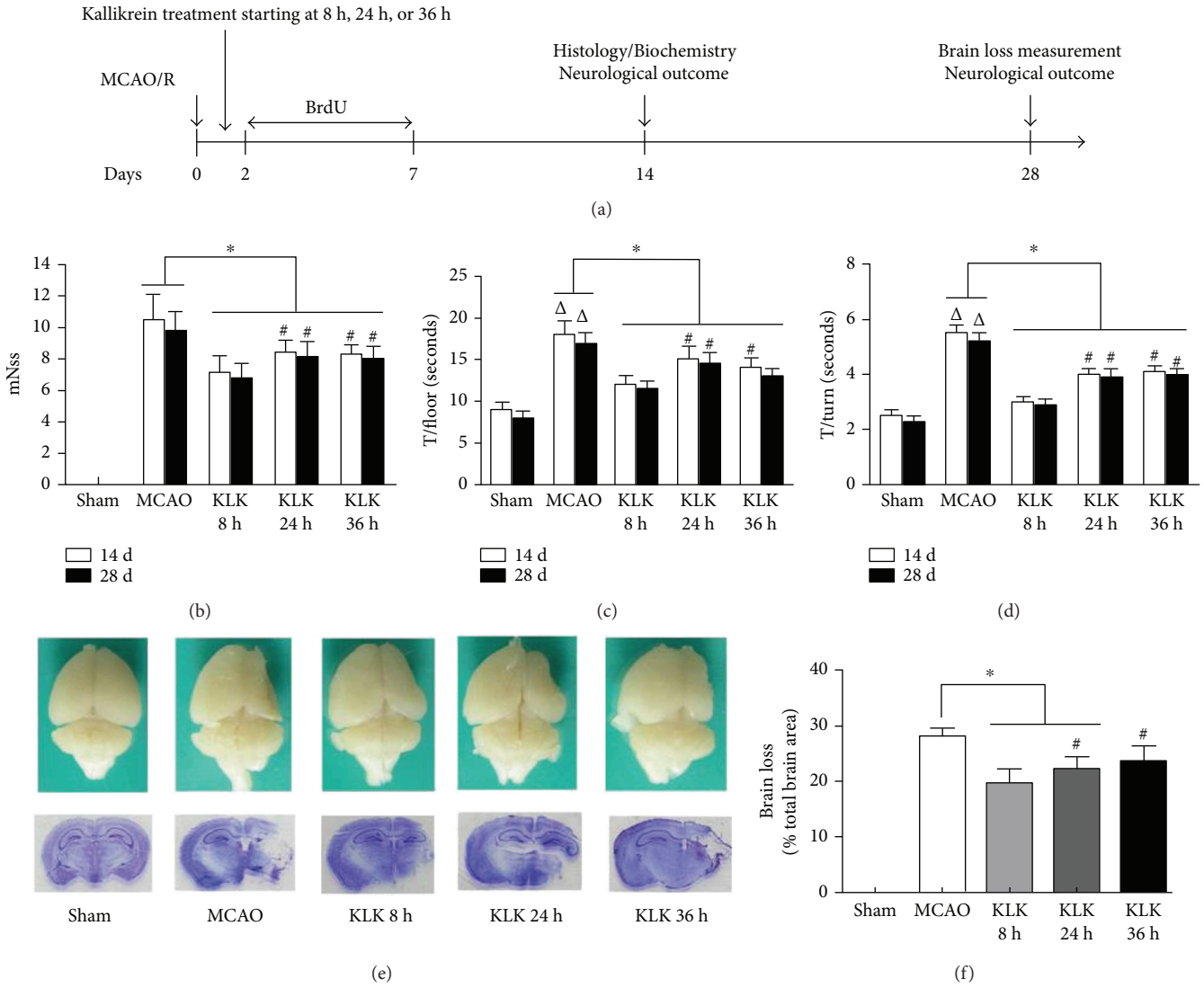

FIGURE 1: The effect of delayed kallikrein treatments on long-term outcome of brain ischemia. (a) Schematic presentation of the experimental design. (b, c, d) Neurological function (b) evaluated with mNSS and movement function (c, d) assessed by pole test at 14 d and 28 d after reperfusion injury. (e, f) Brain loss measurement at 28 d after reperfusion injury. Representative picture of intact brain and brain section stained by cresyl violet (e). Quantitative data of brain loss (f). $^{*}p < .05$ compared with the MCAO group; $^{\triangle}p < 0.05$ compared with the sham group; $^{\#}p < 0.05$ compares with the KLK 8 h group.

found that there were more BrdU-positive cells in the KLK 8 h group than in the KLK 24 h and KLK 36 h groups both in the ipsilateral SVZ and in the peri-infarction area (Figures 2(b) and 3(b)). However, there was no significant difference between the KLK 24 h and KLK 36 h groups (in the ipsilateral SVZ, 127.17 ± 12.54 versus 131.67 ± 9.58, $p > 0.05$; in the peri-infarction area, 99.33 ± 9.93 versus 85.5 ± 4.04, $p > 0.05$). The results suggest that more cell proliferation after focal cerebral infarction in the KLK 8 h group might be associated with its better neurological efficacy.

3.3. Kallikrein Treatment Starting at 8 h after Ischemia Induces More Neural Stem Cell Proliferation. Nestin, an intermediate filament protein, is a marker of a neuroepithelial stem cell. As Nestin is a marker of neuroepithelial stem cell, we used double immunolabeling of Nestin and BrdU to show the proliferation of neural stem cells. All kallikrein treatment regimens significantly elevated the number of $BrdU^{+}/Nestin^{+}$ cells in the ipsilateral SVZ compared with the MCAO group (Figures 2(a, H–J) and 2(c), all $p < 0.05$). The more neural stem cells were observed in the KLK 8 h group than in the KLK 24 h and KLK 36 h groups (Figure 2(c), $p < 0.05$). However, there was no significant difference between the KLK 24 h group and KLK 36 h group (47.83 ± 3.76 versus 44.83 ± 2.71, $p > 0.05$).

3.4. Kallikrein Treatment Starting at 8 h after Ischemia Enhances More Differentiation of Neural Stem Cell into Mature Neuron. Next, we used NeuN and Tuj-1, two markers of mature neurons, to check whether kallikrein treatment can induce mature neuron differentiated from proliferative neural stem cells. Compared with the MCAO group, the therapy

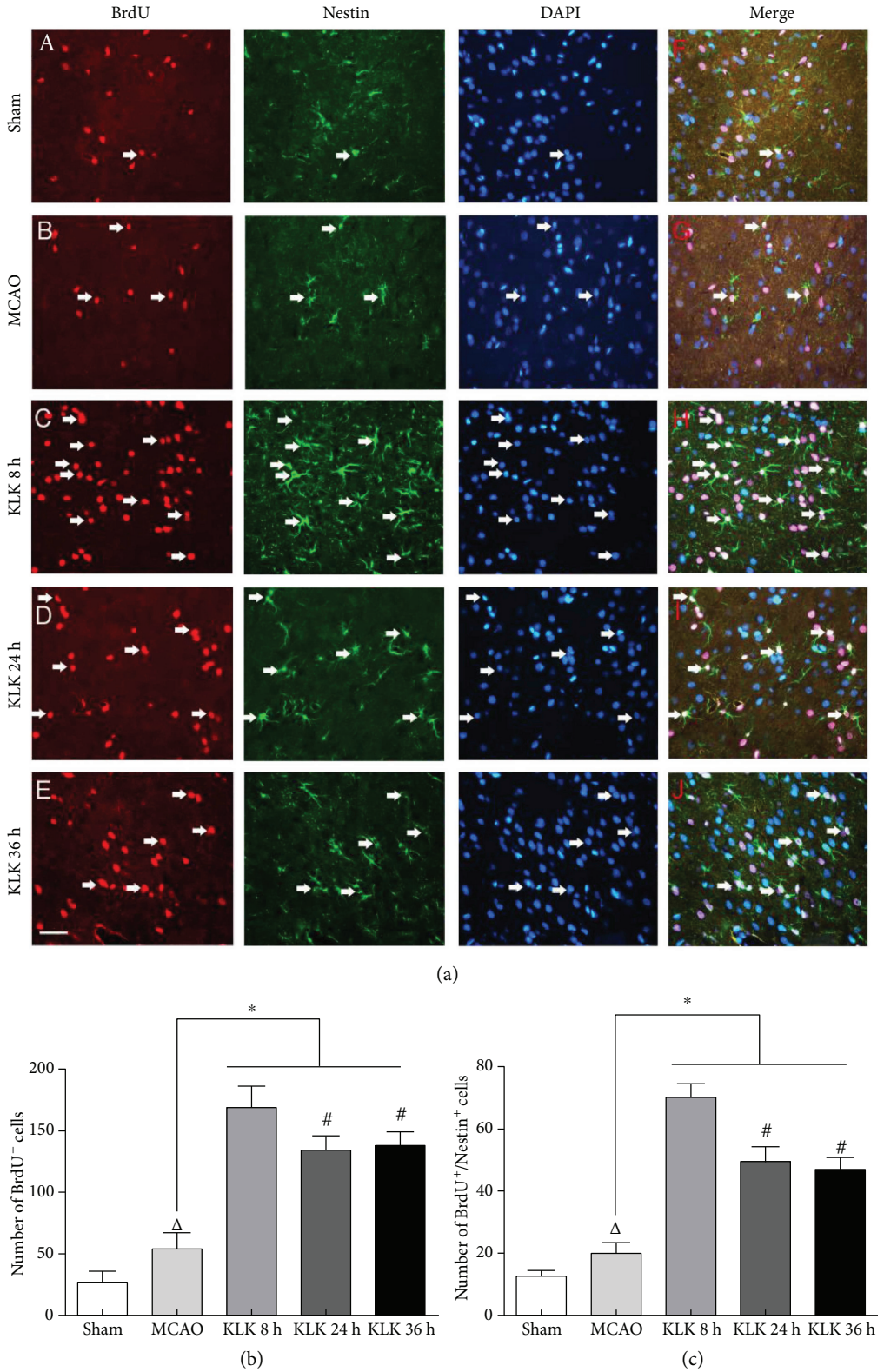

Figure 2: Administration of exogenous tissue kallikrein starting at 8 h, 24 h, and 36 h after ischemic stroke can increase $BrdU^+$ cells (a, C–E) and $BrdU^+/Nestin^+$ cells (a, H–J) in the ipsilateral SVZ. Quantitative data of $BrdU^+$ cells (b) and $BrdU^+/Nestin^+$cells (c) in the ipsilateral SVZ. $^*p < 0.05$ compared with the MCAO group. $^\triangle p < 0.05$ compared with the sham group. $^\#p < 0.05$ compared with the KLK 8 h group. The scale bar represents 40 μm.

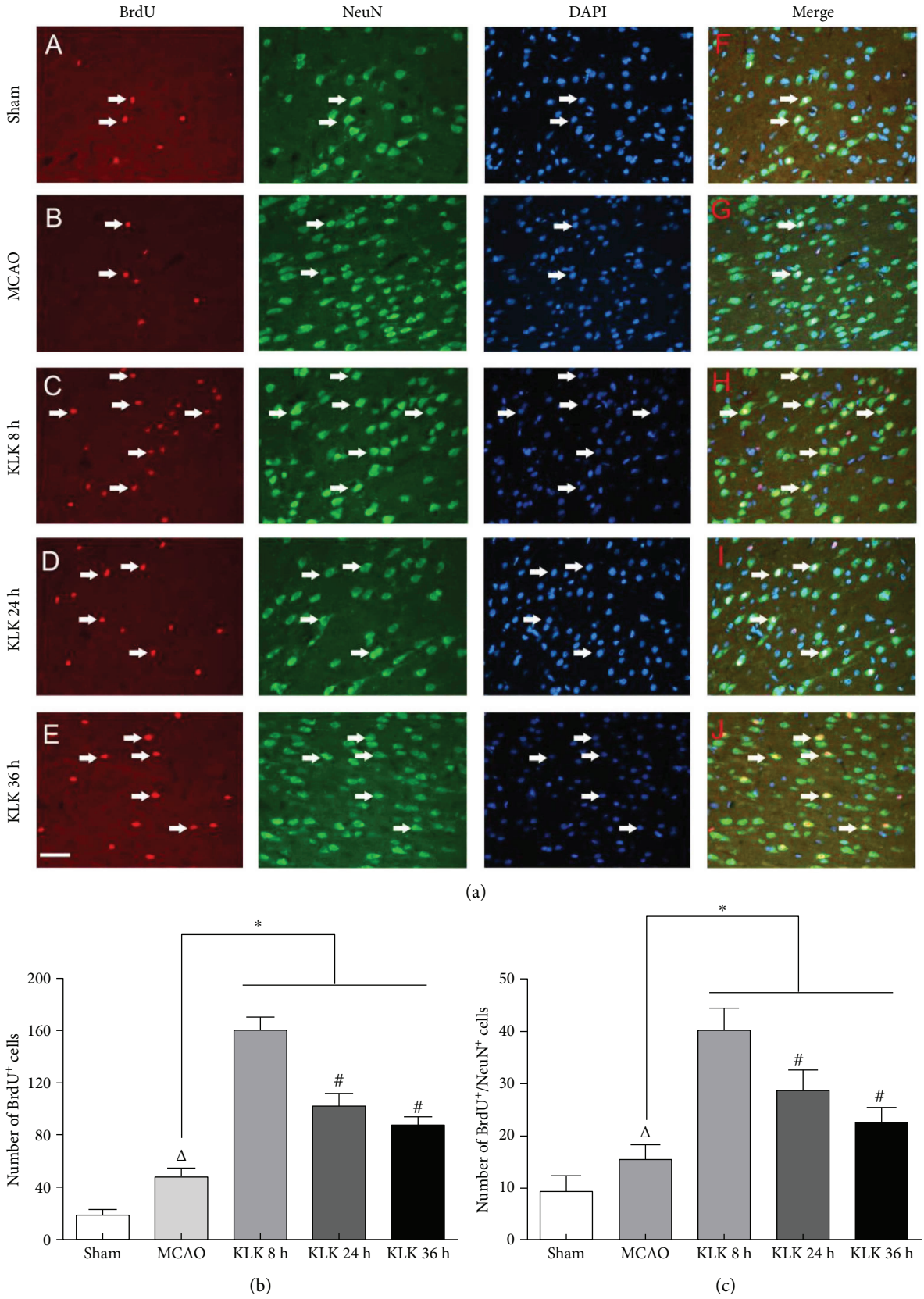

FIGURE 3: Administration of exogenous tissue kallikrein starting at 8 h, 24 h, and 36 h after ischemic stroke can increase $BrdU^+$ cells (a, C–E) and $BrdU^+/NeuN^+$cells (a, H–J) in the peri-infarction region. Quantitative data of $BrdU^+$cells (b) and $BrdU^+/NeuN^+$cells (c) in the peri-infarction region. $^*p < 0.05$ compared with the MCAO group. $^\triangle p < 0.05$ compared with the sham group. $^\#p < 0.05$ compared with the KLK 8 h group. The scale bar represents 40 μm.

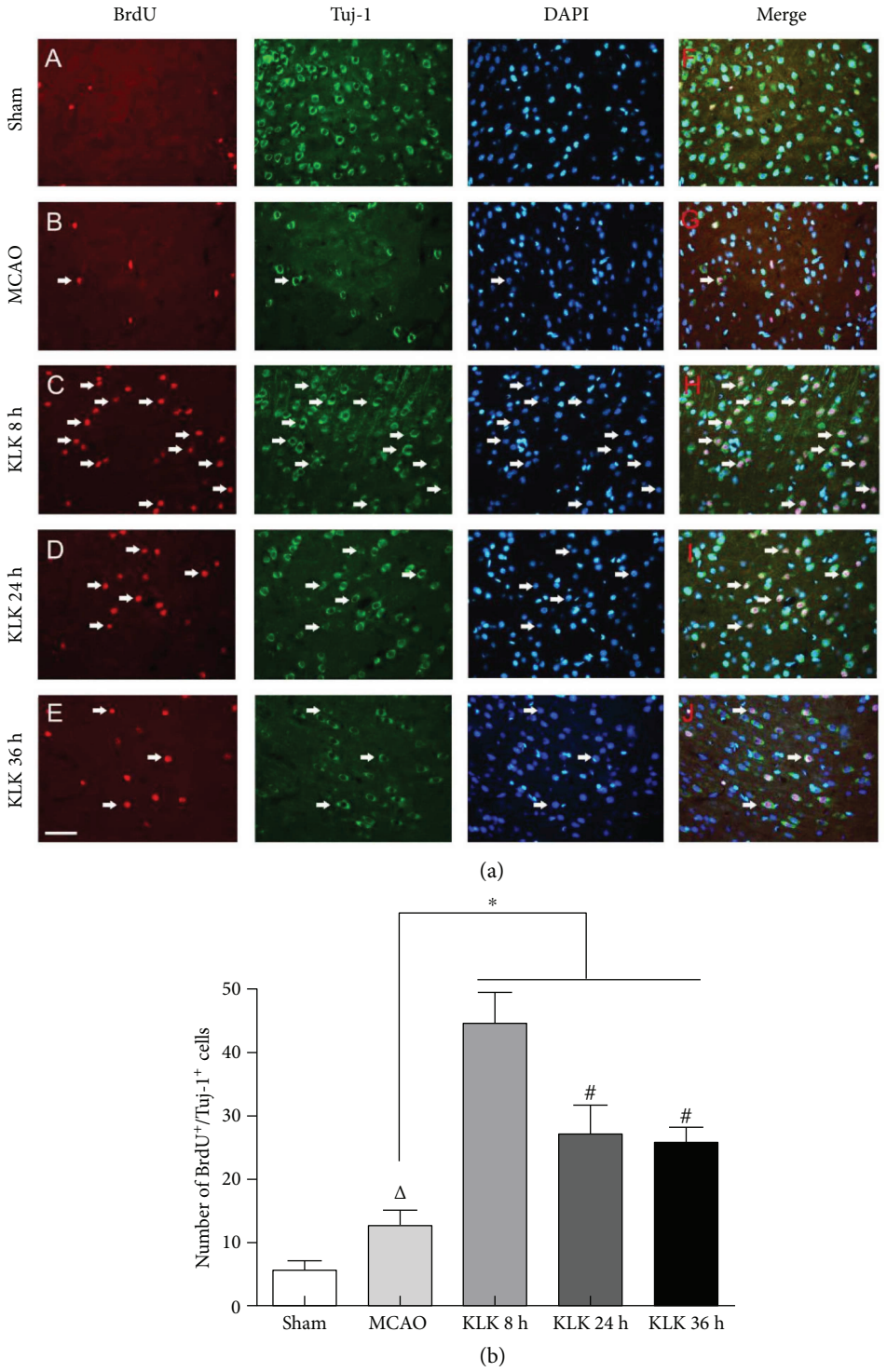

FIGURE 4: Administration of exogenous tissue kallikrein starting at 8 h, 24 h, and 36 h after ischemic stroke can increase $BrdU^+$ cells (a, C–E) and $BrdU^+/Tuj\text{-}1^+$ cells (a, H–J) in the peri-infarction region. Quantitative data of $BrdU^+/Tuj\text{-}1^+$cells (b) in the peri-infarction region. $^*p < 0.05$ compared with MCAO group. $^\triangle p < 0.05$ compared with the sham group. $^\#p < 0.05$ compared with the KLK 8 h group. The scale bar represents 40 μm.

of exogenous tissue kallikrein did augment both $BrdU^+/NeuN^+$ cells (Figures 3(a, H–J) and 3(c), all $p < 0.05$) and $BrdU^+/Tuj\text{-}1^+$ cells (Figures 4(a, H–J) and 4(b), all $p < 0.05$) in the peri-infarction region. Negative control staining of NeuN or Tuj-1 was shown in the supplementary material (available here). The $BrdU^+/NeuN^+$ cells and $BrdU^+/Tuj\text{-}1^+$ cells appeared more in the KLK 8 h group than in the KLK 24 h and KLK 36 h groups (Figure 3(c), all $p < 0.05$; Figure 4(b), all $p < 0.05$). Nevertheless, there was no significant difference between the KLK 24 h group

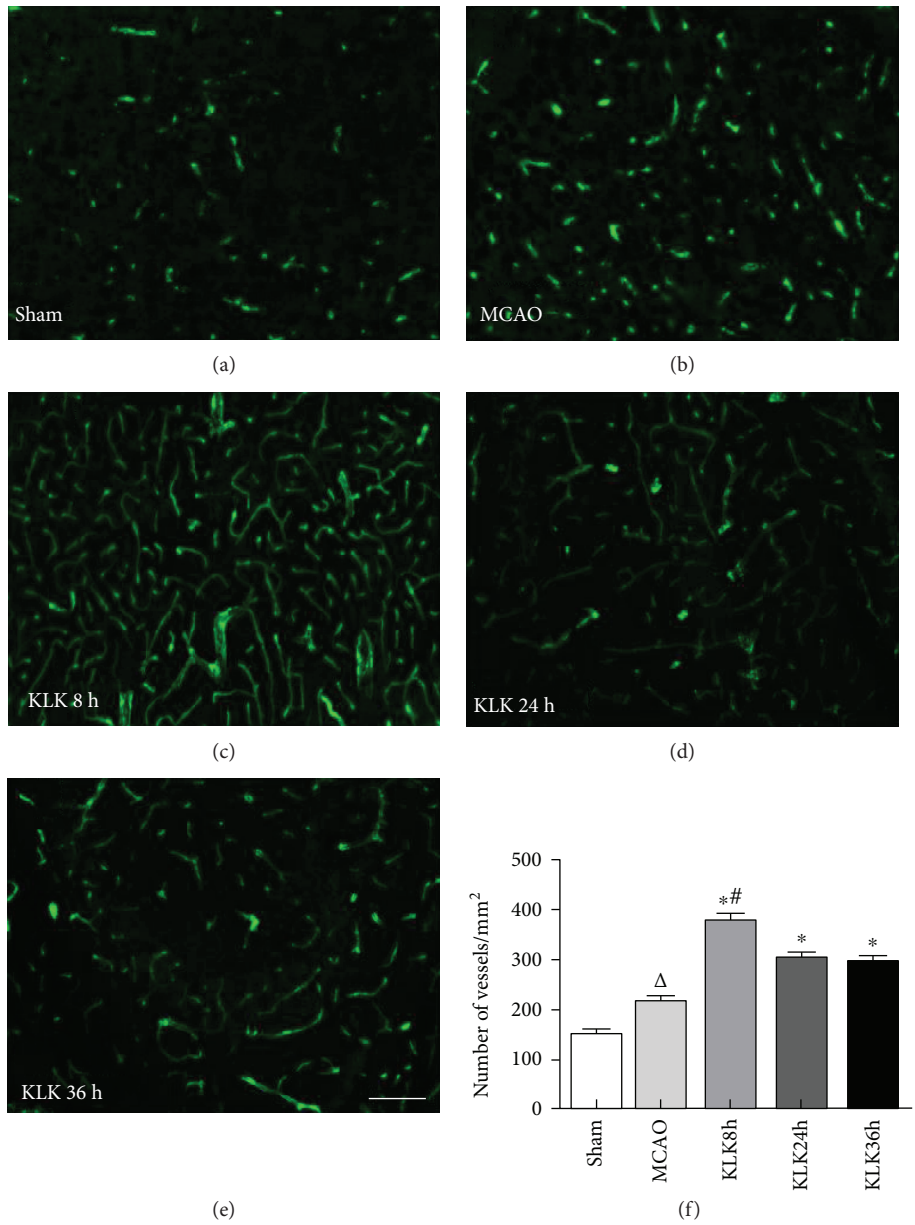

Figure 5: Administration of exogenous tissue kallikrein starting at 8 h, 24 h, and 36 h after ischemic stroke can increase vWF$^+$ cells (c–e) compared with the MCAO group in the peri-infarction region. (f) Quantitative data of vWF$^+$ cells in the peri-infarction region (Mean ± SD). $^*p < 0.05$ compared with the MCAO group. $^\triangle p < 0.05$ compared with the sham group. $^\#p < 0.05$ compared with the KLK 24 h group and KLK 36 h group. The scale bar represents 50 μm.

and KLK 36 h group (BrdU$^+$/NeuN$^+$ cells, 27.17 ± 2.32 versus 25.17 ± 2.41, $p > 0.05$; BrdU$^+$/Tuj-1$^+$ cells, 27.67 ± 2.8 versus 25.33 ± 2.25, $p > 0.05$).

3.5. Kallikrein Treatment Starting at 8 h after Ischemia Promotes More Angiogenesis in Peri-Infarct Area. Angiogenesis also plays an important role in the long-term outcome after ischemia. In this study, the angiogenesis in the peri-infarction region was analyzed by immunostaining with Von Willebrand factor (vWF), an endothelial cell marker. Compared with the MCAO group, three kallikrein therapeutic regimens significantly increased the expression of markers of vessel in the peri-infarction region (Figures 5(c)–5(f), all $p < 0.05$), indicating kallikrein may have stimulated endothelial cell proliferation and promoted the new vessel formation. Among three kellikrein treatment groups, the KLK 8 h group had more new vessel than the KLK 8 h and KLK 36 h groups (Figure 5(f), $p < 0.05$). However, there was no significant difference between the KLK 24 h group and KLK 36 h group (301 ± 21.09 versus 295 ± 23.7, $p > 0.05$).

3.6. Delayed Kallikrein Treatment Promotes Angiogenesis through AKT-GSK3β-VEGF Signaling Pathway. Several studies had reported that inhibiting inflammation, oxidative stress, and increasing NO and VEGF formation are in angiogenesis enhanced by kallikrein. To figure out the

molecular mechanisms, by which the kallikrein treatment starting at 8 h after ischemia has more angiogenesis, we explored the AKT-GSK3β-VEGF signaling pathway after kallikrein treatment. The results show that kallikrein treatment significantly promoted VEGF expression at peri-infarct zone compared with the MCAO group (Figures 6(a) and 6(b)). Consistent with the angiogenesis change tendency in different kallikrein treatment groups, KLK 8 h have more VEGF expression level than the other two treatment groups. Furthermore, we found that the upstream signal molecules in AKT-GSK3β-VEGF pathway were also activated after kallikrein treatment, that is, increased AKT (Ser473) and GSK3β (Ser9) phosphorylation, and thus GSK3β activity was reduced. The data suggest that delayed kallikrein treatments can activate AKT-GSK3β-VEGF signaling pathway, which is involved in agiogenesis enhancement. More importantly, the better effect of kallikrein treatment at 8 h after ischemia was associated with more activation of AKT-GSK3β-VEGF pathway.

4. Discussion

Previous studies have showed delayed kallikrein gene delivery or protein infusion at 8 h, 24 h, or less than 48 h after ischemia was effective in reducing neurological deficit [10, 15]. However, it is still unclear of which time point for the delayed kallikrein treatment will produce better protective effect. In this study, we confirmed that delayed administration of exogenous kallikrein protein starting at 8 h, 24 h, and 36 h after ischemic stroke onset protects against neurological dysfunction. More importantly, kallikrein treatment starting at 8 h after ischemia rendered better long-term outcome than kallikrein treatment starting at 24 h and 36 h. Besides, the molecular mechanisms underlying the protection of delayed kallikrein treatment are associated with neurogenesis and angiogenesis.

In this study, two neurological function tests were applied to evaluate the effect of the delayed kallikrein on the neurological recovery at 14 days and 28 days after ischemia. The first one is modified neurological severity score (mNSS) including motor, sensory, balance, and reflex tests. Another is pole test, which can further assess the motor function. Although the delayed kallikrein treatment starting at 8 h, 24 h, and 36 h all can reduce the mNSS and floor time and turn time, which was consistent with the previous study, the treatment starting at 8 h had better neurological recovery than the other two time points. Furthermore, we found kallikrein at 8 h also significantly reduced brain loss compared with the treatment at 24 h and 36 h. It is interesting that kallikrein treatment starting at 36 h after stroke only reduced neurological deficit and did not decrease brain loss. It may be explained that the cerebral infarct lesions were formed and irreversible within 36 h after stroke onset. Indeed, several therapeutic agents treating ischemic stroke can ameliorate neurological function instead of reducing infarction volume [16–18].

After comparing the effect of a delayed kallikrein treatment starting at different time points, we next explored the mechanisms accounting for the protection difference among

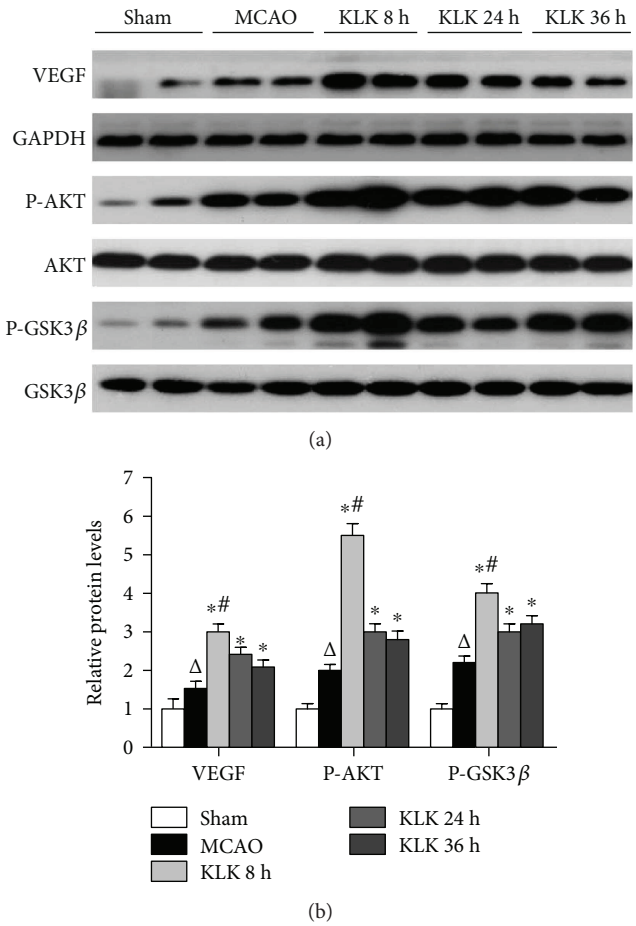

Figure 6: Administration of kallikrein starting at 8 h, 24 h, and 36 h after ischemic stroke activated AKT-GSK3β-VEGF signaling pathway. (a) Western blots of ipsilateral cerebrocortical homogenates from mice at 14 days after ischemia. (b) The blots were quantified, and the relative levels of VEGF, P-AKT, and P-GSK3β (Mean ± SD) after normalization with the GAPDH, AKT, and GSK3β, respectively. $^{*}p < 0.05$ compared with the MCAO group; $^{\triangle}p < 0.05$ compared with the sham group; $^{\#}p < 0.05$ compared with the KLK 24 h group and KLK 36 h group.

three kallikrein therapeutic regimens. Several molecular mechanisms are associated with kallikrein-enhanced protection, such as inhibiting apoptosis and inflammation and reducing oxidative stress. The neurogenesis and angionesis play a critical role in the long-term neurological recovery. For the neurogenesis mechanism, we used the BrdU, a thymidine analog, to label proliferative cells. The BrdU-positive cells in the SVZ and the peri-infarction cortex were induced by cerebral ischemia. Delayed kallikrein treatment starting at 8 h, 24 h, and 36 h significantly raised the number of BrdU-positive cells compared with the MCAO group suggesting that delayed kallikrein protection against stroke was associated with cell proliferation enhancement. Paralleling with the increase of BrdU-labeled cells, BrdU/Nestin^{-}-positive cells were also increased in the SVZ in all delayed kallikrein treatment groups, which suggested that delayed kallikrein treatment also promotes endogenous neural stem

cell (NSCs) proliferation. NSCs will play a role after differentiation into mature neurons.

By the use of colabeling of BrdU with a mature neuronal marker NeuN or Tuj-1, we found that exogenous kallikrein treatment starting at 8 h, 24 h, or 36 h significantly elevated the number of $BrdU^+/NeuN^+$ and $BrdU^+/Tuj\text{-}1^+$ cells in the peri-infarction cortex. These data indicate that delayed treatment can enhance proliferation and differentiation of NSCs into neuron. Although delayed kallikrein treatment at all time points can promote the neurogenesis, more importantly, kallikrein treatment starting at 8 h after ischemia increased more NSC cells in the SVZ and mature neurons in peri-infarction region than the other two time point treatments. These results suggest that more neurogenesis for kallikrein treatment starting at 8 h after ischemia might contribute to its better long-term outcome.

Postischemic angiogenesis also plays an important role in the long-term functional recovery [19]. Previous studies have shown that kallikrein promotes angiogenesis in diverse peripheral tissue, such as hindlimb ischemia, cardiac infarction, and renal ischemia [20, 21]. In this study, we found that, compared with the MCAO group, delayed treatment with kallikrein starting at all time points significantly increased vascular density at 14 days after ischemia in peri-infarction area, suggesting that delayed kallikrein treatment can promote angiogenesis of brain ischemia. These results are consistent with the previous reports. Similar to neurogenesis, kallikrein treatment at 8 h can induce more angiogenesis than that at 24 h and 36 h. Kallikrein gene has been documented that it can promote neovascularization in limb ischemia and myocardial infarction [20, 22–24]. Kallikrein has also been proven to stimulate angiogenesis after local brain infarction [10, 15]. Our data demonstrate that the beneficial effects of delayed kallikrein treatment are exerted via enhanced angiogenesis in the peri-infarction zone, and the distinct therapy effect of kallikein at different times after stroke is in accordance with the degree of vascular proliferation.

PI3K-AKT activation plays an important role in angiogenesis by regulating VEGF expression [25]. VEGF is a well-known endothelial cell-specific angiogenic factor that regulates angiogenesis through the stimulation of proteolytic activity as well as endothelial cell proliferation and migration [26].

Indeed, previous reports showed kallikrein gene transfer protected against acute phase myocardial infarction by promoting neovascularization and improving cardiac function by increasing AKT and GSK3β phosphorylation and thus reducing GSK3β activity. In this study, we found all delayed kallikrein protein treatment significantly upregulated VEGF expression in peri-infarct area, paralleling to AKT and GSK3β phosphorylation. Notably, delayed kallikrein treatment at 8 h induced more activation of this pathway than kallikrein treatment starting at other two time points. These results suggest that delayed kallikrein protein treatment can activate the AKT-GSK3β-VEGF pathway, which might be associated with vascular proliferation enhancement.

In this study, we showed that kallikrein starting at 8 h after ischemic onset is more effective than that at 24 h and 36 h. the results suggested that, for delayed kallikrein administration, earlier injection is better. But, to determine appropriate delivery time, further research should be done by comparing delayed treatment with early treatment (e.g., 4 h or earlier).

In summary, our research show that although delayed systemic delivery of exogenous kallikrein starting at 8 h, 24 h, and 36 h provides protection against cerebral infarction, kallikrein treatment starting at 8 h after ischemia has better long-term outcome than kallikrein starting at 24 h and 36 h. Besides, the long-term outcome difference of exogenous kallikrein treatment starting at three time points is closely associated with the degree of neurogenesis and angiogenesis. These findings will contribute to choose the appropriate time for better clinical application of exogenous kallikrein.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yaohui Ni and Kefu Cai contributed equally to this work.

Acknowledgments

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Supplementary Materials

Negative control for Figure 3 and Figure 4. *(Supplementary Materials)*

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Review Article

A Look into Stem Cell Therapy: Exploring the Options for Treatment of Ischemic Stroke

Cesar Reis,[1] Michael Wilkinson,[2] Haley Reis,[2] Onat Akyol,[1] Vadim Gospodarev,[1] Camila Araujo,[1] Sheng Chen,[3] and John H. Zhang[1,4]

[1]*Department of Physiology and Pharmacology, Loma Linda University School of Medicine, 11041 Campus Street, Risley Hall, Room 219, Loma Linda, CA 92354, USA*
[2]*Loma Linda University School of Medicine, Loma Linda, CA 92354, USA*
[3]*Department of Neurosurgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China*
[4]*Department of Neurosurgery, Loma Linda University School of Medicine, Loma Linda, CA 92354, USA*

Correspondence should be addressed to John H. Zhang; johnzhang3910@yahoo.com

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Neural stem cells (NSCs) offer a potential therapeutic benefit in the recovery from ischemic stroke. Understanding the role of endogenous neural stem and progenitor cells under normal physiological conditions aids in analyzing their effects after ischemic injury, including their impact on functional recovery and neurogenesis at the site of injury. Recent animal studies have utilized unique subsets of exogenous and endogenous stem cells as well as preconditioning with pharmacologic agents to better understand the best situation for stem cell proliferation, migration, and differentiation. These stem cell therapies provide a promising effect on stimulation of endogenous neurogenesis, neuroprotection, anti-inflammatory effects, and improved cell survival rates. Clinical trials performed using various stem cell types show promising results to their safety and effectiveness on reducing the effects of ischemic stroke in humans. Another important aspect of stem cell therapy discussed in this review is tracking endogenous and exogenous NSCs with magnetic resonance imaging. This review explores the pathophysiology of NSCs on ischemic stroke, stem cell therapy studies and their effects on neurogenesis, the most recent clinical trials, and techniques to track and monitor the progress of endogenous and exogenous stem cells.

1. Introduction

Ischemic stroke accounts for 87% of all stroke events and is the 5th leading cause of death in the United States. The National Stroke Association estimates that there are nearly 7 million stroke survivors and though functional mobility impairments exist on a spectrum, it is a leading cause of adult disability [1]. It is well understood that stem cells are the building blocks of life. Achieving guidance of stem cells towards regenerating neurons and damaged tissue caused by ischemic stroke is a new and innovative area of research currently being investigated [2]. Endogenous neural stem and progenitor cells (NSPCs), also described in this review as neural stem cells (NSCs), persist in the subventricular zone (SVZ) lining the ventricles and the subgranular zone (SGZ) of the hippocampus in the adult brain. Finding ways to mobilize and induce neurogenesis in an area of focal ischemia is an area of current research [3]. Though not yet FDA approved for treatment of acute and chronic stroke, clinical trials are well underway to demonstrate their therapeutic benefits.

Various methods of stem cell therapy are being explored using animal models including the use of endogenous and exogenous stem cells. Interestingly, exogenous stem cells have been shown to induce endogenous NSCs towards neuronal differentiation [4, 5]. Cotransplantation therapy is another aspect of stem cell research that offers promising effects on neuronal differentiation and survival. One study looked at transplanting astrocytes with NSCs and found a higher ratio of survival and proliferation compared with transplanting NSCs alone [6]. Embryonic stem cells show

positive therapeutic effects in animal models, as studies have determined that they can focus on regions that support neural differentiation within the adult brain, such as the substantia nigra pars compacta. [7] This aspect of stem cell therapy has unique benefits worth translating into the clinical setting.

Lastly, finding a tracking method to follow the stem cells on their path to neurogenesis provides clinicians with knowledge on the progress of the stem cells, including where they are mobilizing and proliferating [8]. In light of the vast amount of animal model research conducted in recent years, progressing to clinical trials has shown to be challenging, yet promising. The Pilot Investigation of Stem Cells and Stroke (PISCES) clinical trial injected a NSC drug into the ipsilateral putamen following ischemic insult and recorded images and clinical progress over a two-year span. The study found improvement in neurological function and no major adverse events [9]. Uncovering the intricacies and challenges of stem cell therapy using animal models for a variety of stem cell types prepares the medical community for more clinical trials like PISCES and future use of stem cells as a primary treatment option for patients recovering from ischemic stroke.

2. Pathophysiology of Ischemic Stroke

Stroke is caused by a critical disruption of blood supply in a specific area of the brain, resulting from either a sudden or slowly progressing obstruction of a major brain vessel, often leading to death or permanent neurological deficits [10]. Hemorrhagic stroke is caused by rupture of blood vessels in the brain, while ischemic stroke from embolism, thrombolysis, or cryptogenic mechanisms interrupts blood supply to the brain and is responsible for the vast majority of strokes seen in patients (87%) [11]. A lack of blood supply to the ischemic area of the brain known as the penumbra initiates an ischemic cascade whereby brain function stops if oxygen deprivation exceeds 60 to 90 seconds and brain tissue dies within 3 hours of anoxia leading to cerebral infarction. It is within the penumbra that many therapeutic interventions are targeted since its salvage is directly related to recovery [12]. Of the different types of cells found within the brain, neuronal cells are the most vulnerable to changes in oxygen content and can quickly become dysfunctional and die [13]. In an attempt to maintain cellular energy levels, neurons resort to anaerobic metabolism producing substantially less energy in the form of adenosine triphosphate than they would with normal aerobic glycolytic mechanisms. In addition, toxic byproducts including lactic acid are released, further disrupting the acid/base balance leading to additional cellular stress and death [14].

The highly coordinated cellular consequences after ischemic stroke include excitotoxicity, mitochondrial dysfunction, and oxidative stress due to a large intracellular influx of Ca^{2+} ions following disruption of transmembrane protein channels. Ischemia-induced reductions in nutrient availability for neuronal cells lead to the overproduction of excitatory amino acids, namely, glutamate, due to a disruption in the ionic gradients. N-Methyl-D-aspartate (NMDA) glutamate receptors induce increased amounts of intracellular Ca^{2+} influx leading to activation of Ca^{2+}-dependent enzymes including proteases, calpain, and caspases, thereby setting off mitochondrial mechanisms of apoptosis and necrosis [15]. Neural circuitry is subsequently disrupted due to chronic stimulation of glutamate that can persist for months. This overabundant Ca^{2+} influx leads to activation of caspase-dependent cellular death pathways involving caspase-12, caspase-9, and caspase-3 due to the release of cytochrome C from mitochondria. Furthermore, important reactive oxygen species upregulated by Ca^{2+} influx in the mitochondria are implicated in reperfusion injury after ischemia leading to necrosis [16]. Free radicals, including the NO byproduct peroxynitrite, leads to oxidative damage through inhibition of signal transduction cascades favoring cell death mechanisms and inhibiting recovery from ischemic injury [17]. Dying neural cells release signals activating proinflammatory pathways leading to post ischemic inflammation that plays a role in activating the immune response.

3. Understanding Endogenous NSCs

Within the last decade, neurogenesis from endogenous NSCs has shown potential in ameliorating ischemic brain tissue following ischemic stroke through regenerative efforts. The fate of endogenous NSPC is quite complex and depends on many factors but takes on four general processes including proliferation, migration, cell survival, and neuronal differentiation [18]. Here, we discuss the process of how endogenous NSPCs provide neural progenitors for hippocampal and olfactory neurogenesis under normal physiological conditions.

3.1. Proliferation. Neurogenic regions in the adult brain have been localized in two areas, the SVZ of the lateral ventricles and SGZ in the dentate gyrus of the hippocampus [19, 20]. It has been shown that the specific microenvironment in which the progenitor cell is located plays a major role in neurogenesis, as those residing in the SVZ and the SGZ are the only cells capable of becoming neurons without the use of extrinsic factors to assist (Figure 1). In addition, should these cells be relocated to another region of the brain, they differentiate into oligodendrocytes and astrocytes, further supporting the idea that NSPCs outside the SVZ and SGZ will most likely undergo glial rather than neuronal differentiation [21].

3.2. Migration. Understanding the migration pathway of NSCs allows for comparison of the migration process that happens under ischemic conditions. In the adult SVZ, radial glia-like cells lead to the production of transient amplifying cells which produce neuroblasts that will form a chain and migrate through the rostral migratory stream (RMS) towards the olfactory bulb within an astrocyte-derived tube [22]. Stromal cell-derived factor-1 (SDF-1), also known as an angiogenetic cytokine, has been reported to function in conducting neuroblast migration within the RMS. Chemokine-induced NSPC migration necessitates extracellular matrix remodeling via activation of matrix metalloproteinases that are functionally active along the SVZ-olfactory bulb pathway [23]. The main olfactory bulb includes principal neurons as well as local circuit neurons, and the location where olfactory axon terminals contact the principal and local circuit

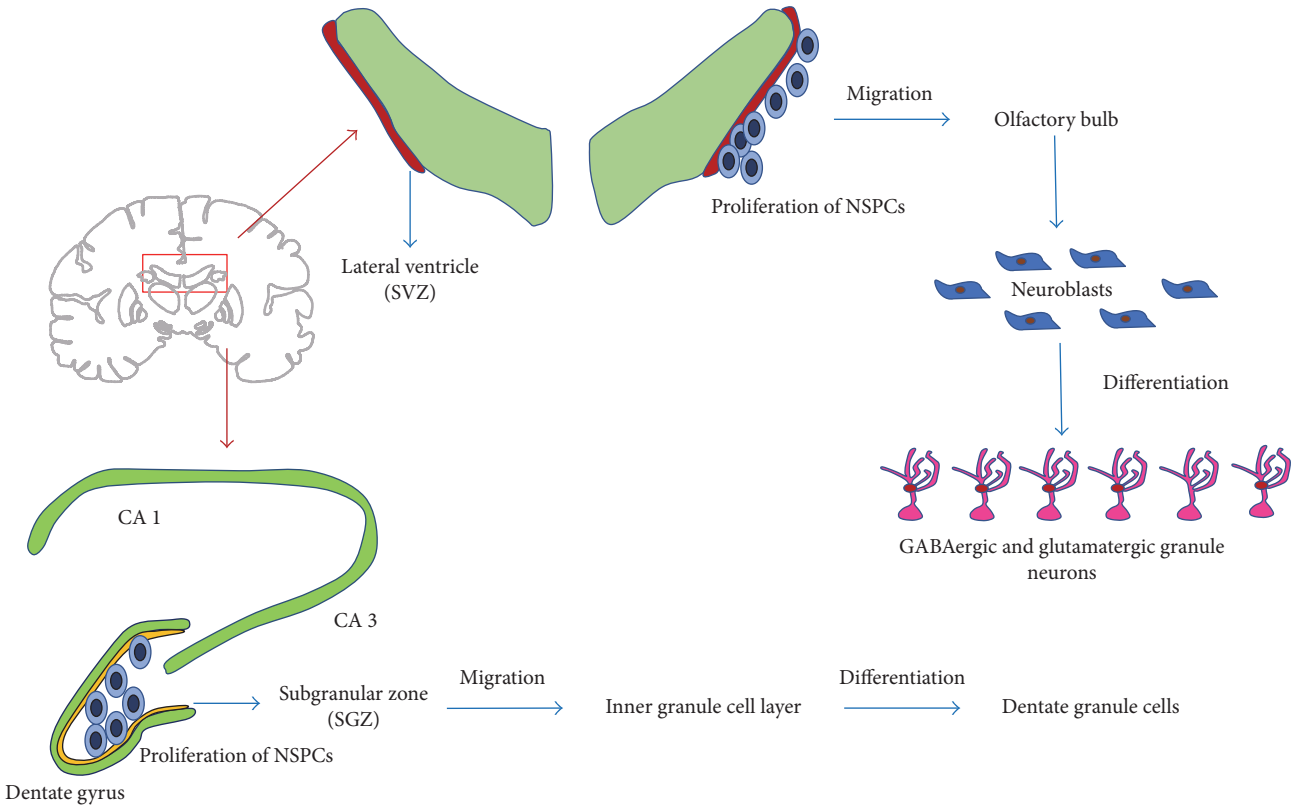

Figure 1: This figure demonstrates neurogenesis of endogenous neural stem cells. (1) Neurogenesis and proliferation occur in the SVZ and SGZ on the lateral ventricle and hippocampus, respectively. (2) NSPC migration occurs through the rostral migratory stream to the olfactory bulb, where neuroblasts migrate as interneurons through specific cell layers. From the SGZ, NSPCs migrate to the inner granule cell layer. (3) Differentiation occurs once neuroblasts reach glomeruli within the olfactory bulb or the inner granule cell layer. The majority of SVZ-derived neuroblasts become GABAergic granule neurons. After complete differentiation and maturation of neuroblasts from the SGZ, new neurons possess GABAergic and glutamatergic characteristics.

neurons is called the glomeruli [24]. In the olfactory bulb, the neuroblasts migrate as interneurons through specific cell layers towards glomeruli where differentiation ultimately occurs [25]. In the adult SGZ, both radial and nonradial precursors generate neuroblasts that then migrate to the inner granule cell layer of the hippocampus where they have been shown to differentiate into dentate granule cells (Figure 1) [26].

3.3. Survival and Differentiation. Recent studies suggest that the SVZ is highly organized, with each area having specific stem cells with unique neuronal fates. In the olfactory bulb, the majority of SVZ-derived neuroblasts become axon-less GABAergic granule neurons while a minority become GABAergic periglomerular neurons and even fewer become short-axon glutamatergic juxtaglomerular neurons [25]. Once arriving in the inner granule cell layer of the hippocampus from the SGZ, new dentate granule cells are generated. Local interneurons tonically release GABA, activating dendritic formation and extensions into the molecular layer. GABAergic synaptic inputs and glutamatergic synaptic inputs further develop. After complete maturation, the new neurons possess similar firing behavior, amplitude, and kinetics of both GABAergic and glutamatergic inputs (Figure 1) [27].

4. Neurogenesis following Ischemia: Endogenous NSCs

Endogenous NSCs, aside from providing new neurons for olfactory and hippocampal neurogenesis under normal physiological conditions, also proliferate and migrate to areas after ischemic brain injury. Cerebral ischemia evokes a proliferation and migration response of NSCs towards the area of injury where they then differentiate into oligodendrocyte progenitors, astrocytes, and neuroblasts (Figure 2) [28].

It has been established that brain ischemia induces neurogenesis by activating neuronal migration through the injured area via secretion of neurotrophic factors such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), cytokines like monocyte chemoattractant protein (MCP-1), and macrophage inflammatory protein (MIP-1). In addition, the natural inflammatory process in response to injury is able to induce NSC enrollment.

Neuroinflammation following ischemic stroke augments chemokine production by astrocytes and microglia [29, 30]. A recent study by Magnusson et al. suggests the reduction in NOTCH signaling pathways by astrocytes after recent ischemic stroke induces latent neurogenic programs. NOTCH1 is a gene which encodes a single transmembrane protein that plays a major role in cell fate. Attenuating

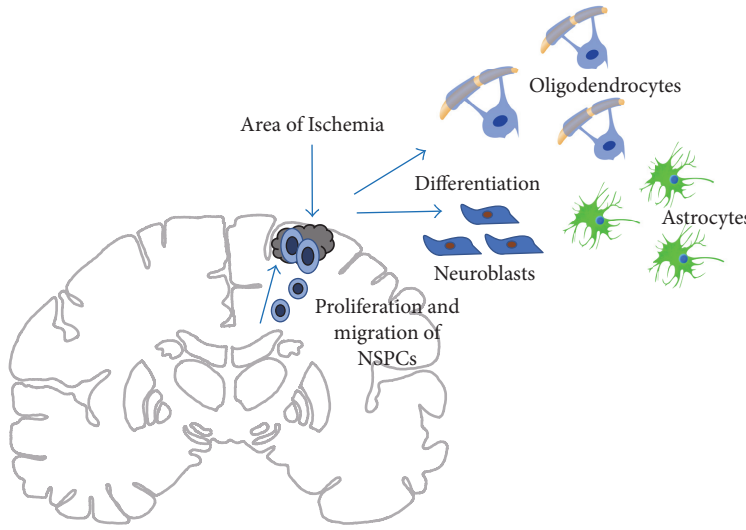

Figure 2: This figure demonstrates the process of neurogenesis following ischemic injury in a coronal section of the brain. Endogenous NSCs proliferate and migrate from the SVZ to areas of ischemic injury. Once they are outside the SVZ, they are able to undergo differentiation into oligodendrocyte progenitors, astrocytes, and neuroblasts.

NOTCH1 signaling to allow neurogenesis by striatal astrocytes may be useful for neuronal replacement following injury or cell death [31]. NSPCs were cultured from a gestational day 14 mouse embryo to study hypoxia-inducible factor-1α (HIF-1α). NSPCs were able to sustain a continuous level of HIF-1α, a crucial element of neural progenitor cells in responding to hypoxic events and affording neuroprotection from hypoxic events. In addition, HIF-1α was expressed in the SVZ and SGZ, suggesting adult neurogenic zones share similar characteristics of a developing embryonic brain [32].

Harms et al. showed that HIF-1α is necessary for NSC-induced neuroprotection in an oxygen-glucose deprivation culture model. HIF-1α gene deletion proceeds diminished VEGF expression along with Notch-β-catenin expression, negatively impacting endogenous NSC resistance to oxygen-glucose deprivation [33]. For the aspect of association between neurogenesis through angiogenesis or gliosis, an experimental study by Thored et al. exhibited how two hours of middle cerebral artery occlusion (MCAO) induced neural progenitor proliferation and neurogenesis in the SVZ, exhibiting permanency for four months after ischemic insult. Additionally, the route of neuroblast migration towards the damaged cortex demonstrated a higher vessel density than other areas (Figure 2) [34].

A study by Li et al. confirmed that endogenous stem cell response six weeks after cerebral ischemia in the SVZ involves production of oligodendrocyte progenitors and astrocytes. They also found that survivability of neuroblasts two weeks post-MCAO was less than oligodendrocytes and astrocytes (10% versus 15–20% and 59%, resp.) [28]. Another study by Kadam et al. using a rodent model in neonatal stroke also found similar results in the amount of SVZ-derived cells able to survive and become neurons [35]. These studies highlight the intricate steps to NSC proliferation, migration, and differentiation after ischemic injury.

5. Neurogenesis following Ischemia: Exogenous Stem Cells

In addition to using endogenous NSCs to induce neurogenesis in areas of ischemic injury, researchers can harvest, expand, and reimplant human stem cells in the area of damaged brain parenchyma as a form of cell replacement, regeneration, and repair. Ischemic insult causes damage to multiple different specialized cell types, and finding an option to repair and regenerate the entire neurovascular unit is the focus of ongoing studies. This section looks at the capabilities of exogenous stem cells in their ability to proliferate, migrate, survive, and differentiate. Large categories of exogenous stem cells include embryonic, neural, mesenchymal, and inducible pluripotent stem cells.

6. Embryonic Stem Cells

Embryonic stem cells have been considered a source for promoting neuronal replacement because of their ability to respond to both extrinsic and intrinsic signaling towards specific neuronal differentiation [36]. Though it is somewhat unclear as to whether differentiation into neurons depends on the transplantation site, brain damage, or a default mechanism, researchers have studied which regions of the adult brain support neuronal differentiation of embryoid body cells derived from embryonic stem cells. Maya-Espinosa et al. compared neurogenesis in adult rat brain and postnatal day 24 rat. Neural uncommitted embryoid body cells differentiated into glia, neural precursors, and neurons in the adult rat brain in both neurogenic and nonneurogenic regions. They found that in neurogenic areas of the adult rat brain, including in the vicinity of the RMS and the cortex, neuronal differentiation, as opposed to astrocytes, was the preferred fate of the embryoid body cells. These results were helpful in determining that neurogenic conditions were

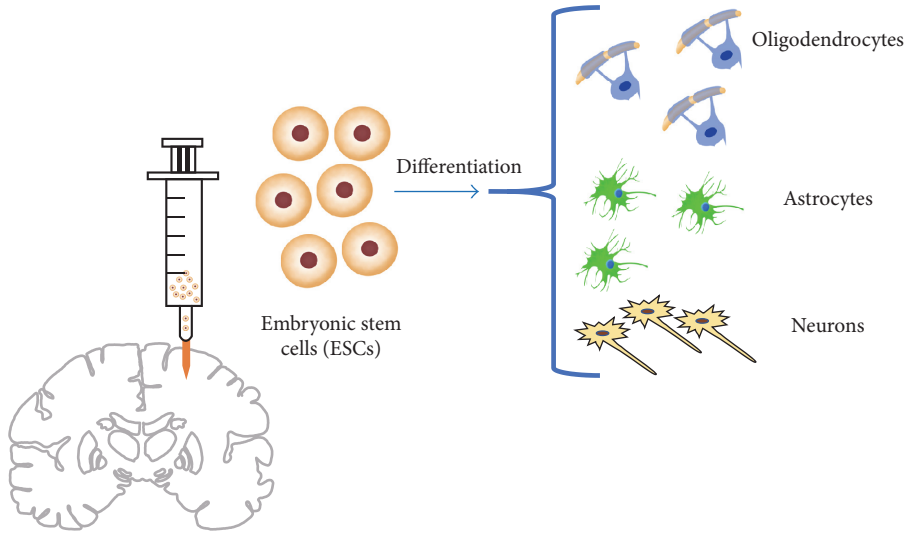

Figure 3: This figure demonstrates the effects of ESC transplantation into the brain. When ischemic stroke occurs, ESCs promote neuronal differentiation in both neurogenic and nonneurogenic regions, such as the striatum. ESCs are able to respond to environmental changes after ischemic insult and improve the capacity to promote neurogenesis.

not exclusively associated with juvenile brains. However, in contrast with the striatum of a young brain, there was less cell expression of neural biomarkers in the adult striatum. They found that after MCAO, regions that were not appropriate for neural differentiation were now able to promote differentiation, as evidenced by embryoid body cells giving rise to both astrocytes and neurons (Figure 3). These findings suggest that ischemic events promote neuronal differentiation of embryoid body cells in addition to attracting endogenous neural precursor cells to the injured area in adult rat brain [7].

Understanding the role of human embryonic-derived NSPCs has been investigated for improving stroke outcomes. A study by Rosenblum et al. sought to pretreat NSCs with BDNF, a growth factor that, as stated earlier, promotes nerve cell survival and function, and compare functional recovery with and without pretreatment of BDNF. They found that the BDNF-treated NSC group showed increased sensorimotor and neural recovery compared to the untreated and control groups over a one-month period following transplantation. They also found that the hippocampal region had a higher percentage of neuronal differentiation signaling, as well as increased overall neuroprotection due to the secretion of both VEGF, which promotes angiogenesis, and BDNF [37]. A study by Liu et al. found that after ischemic injury, transplantation of human embryonic NSCs into the lateral ventricle showed differentiation into neurons in the peri-infarct parenchyma and into oligodendrocytes and astrocytes in the corpus callosum. The human embryonic NSCs decreased ischemia-induced infarction after MCAO in rats and improved neural function [38]. In light of the benefits provided by embryonic stem cells in improving stroke outcomes and inducing neuronal differentiation, the general use of embryos for clinical stem cell transplantation poses more ethical obstacles than does the use of adult NSCs.

7. Exogenous Neural Stem Cells

Huang et al. experimented with NSCs in order to determine whether NSCs migrate into ischemic regions following stroke. Using a murine model, stroke was induced via MCAO. Subsequently, NSCs were injected into the hippocampus one day after stroke onset. The results indicated that only after one day of treatment, the cells migrated into the site of injury and the infarct volume was reduced. Treated mice fared better than their control counterparts in behavioral tests. The researchers hypothesized that an anti-inflammatory pathway led to this result. This study is one of the first demonstrating the short-term benefits of NSCs on behavior [39].

Using NSCs extracted from the hippocampus of fetal rats at 14 days gestation, scientists were able to isolate and culture the NCSs and, after inducing ischemic injury using a cerebral ischemia and reperfusion rat model, stereotaxically inject them into the left striatum. Immunofluorescent labeling showed proliferation of endogenous NSCs beginning day 3 poststroke. When comparing the amount of neurons to glial cells, the NSC transplantation group had less glial cell differentiation and more positive labeled cells for neurons compared to the control group with phosphate-buffered saline (Figure 4). Additionally, this study found functional improvements including less hemiplegia, smaller infarct volume, decreased nerve cell damage, and less apoptotic-positive cells [5].

A study by Cheng et al. found significant improvement in neurological deficits in MCAO rats compared to control using neonatal derived NSCs from mouse cerebellum and transplanting them via an intravenous grafting procedure to avoid surgical trauma. This study did not find a reduction in infarct size or volume, but they found migration abilities to the damaged area as well as increased proliferation of endogenous cells compared to control. They also noted that

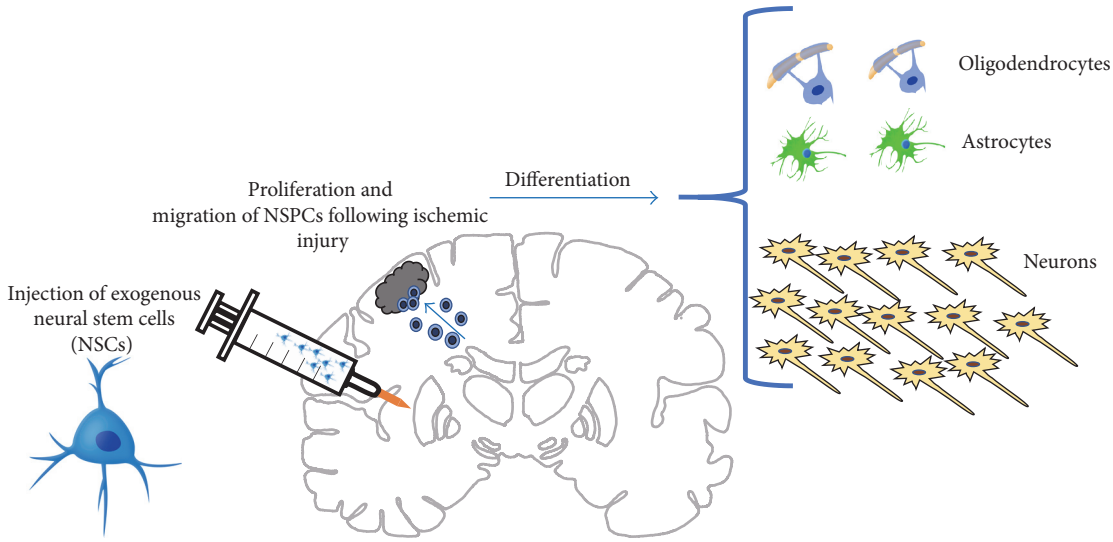

Figure 4: This figure demonstrates the effects of NSC transplantation following ischemic injury in a rat brain. Exogenous NSCs promote increased migration and proliferation of endogenous NSCs. In addition, there is increased differentiation into neurons compared to glial cells.

by day 28, though some NSCs had proteins specific for astrocytes or neurons, most had not yet differentiated [40]. These studies show promising capabilities for exogenously derived NSCs on improving the detrimental effects of ischemic stroke, including their important role in recruiting and activating more endogenous NSCs to assist with regeneration and repair.

8. Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are multipotent cells, with the unique capacity to differentiate into mesodermal, endodermal, and ectodermal cell types, including neurons. These cells are typically derived from mesenchymal tissues, including bone marrow and adipose tissue. This heterogeneous mixture of cells contributes to their ability to differentiate and proliferate (Figure 5) [41]. They are able to cross the blood-brain barrier and preferentially travel to damaged areas and reduce apoptosis, increase basic fibroblast growth factor, and promote endogenous cellular proliferation [42]. One study developed differentiated neuronal MSCs using the NOTCH intracellular domain and transplanted the committed cells into an ischemic area of adult gerbils, comparing their effects to noncommitted MSCs. Significant improvements were seen in recovery using neuronal MSCs; however, no synaptic connection occurred in endogenous cerebral cells [43].

A recent study by Liu et al. sought to improve the migratory capacity of bone marrow-derived MSCs after ischemic injury. It is well known that chemokines orchestrate cellular migration and SDF-1 contributes to recruitment of stem cells in ischemic areas of the brain with its receptor CXC chemokine receptor 4 (CXCR4) to aid in migration of bone marrow stem cells towards the injured area [44]. However, the majority of MSCs have intracellular CXCR4, and few express this receptor on the cell surface [45]. Though CXCR4 is highly expressed in the bone marrow, culture-expanded MSC's lose CXCR4 expression and responsiveness to chemokines, leading to decreased migration [46]. It is with this understanding in mind that Lin et al. performed preconditioning with tetramethylpyrazine (TMP), a pharmacologically active component extracted from a Chinese herb used for treatment of cerebrovascular and cardiovascular disease. It is known not only for its neuroprotective effect but also for its ability to regulate cellular migration, including neural precursor cells [47]. Preconditioning with TMP improved not only bone marrow-derived MSC migration towards ischemic areas but also increased CXCR4 mRNA and protein expression in vitro resulting in increased SDF-1 expression. Improved behavioral performance and angiogenesis in the region of the cortex undergoing ischemic insult was observed. The use of pharmacological agents may provide a more feasible way to improve the use and effectiveness of MSCs in the clinical setting in the treatment and recovery of neurological function after ischemic stroke [48].

Park et al. aimed to investigate the effects of multiple doses of MSCs compared to a single dose of stem cells following stroke. MSCs derived from human umbilical cord blood were transplanted following MCAO in a rodent model. 5 μl of MSCs was administered on the second day after focal cerebral ischemia. A second treatment group received a total of 5 μl in separate dosages on the second and ninth days post cerebral ischemia. Functional outcomes were assessed and although motor dysfunction was found in both MSC groups, there was a decrease in infarct volume and an increase in neurons within the penumbral region. The repeated treatment did not show clear and significant advantages over the single treatment, suggesting that treatment is most effective when administered within the therapeutic window following stroke [49].

MSCs have been shown to play an important therapeutic role in modulating the immune response during transplantation. MSCs seem to inhibit antigen-specific T-cells and promote regulatory T-cells. Aggarwal et al. analyzed the

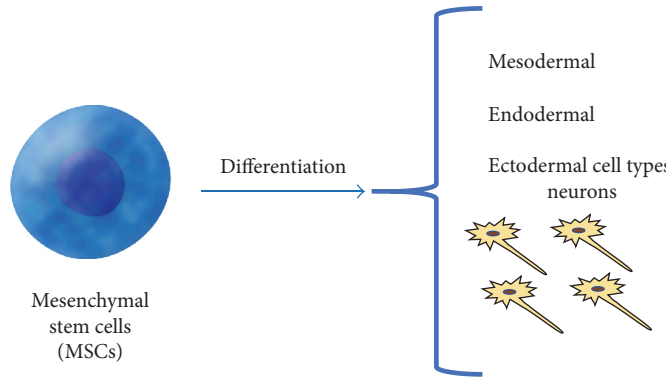

Figure 5: This figure demonstrates the differentiation capacities of mesenchymal stem cells. They are multipotent cells and can differentiate into mesodermal, endodermal, and ectodermal cell types. This includes the ability to become neurons, an important characteristic for the study of ischemic stroke and stem cells.

immunomodulatory functions of human MSCs on different types of immune cells and showed that MSC induction in vitro resulted in a reduction in the proinflammatory cytokines TNF-α and IFN-γ, with increased production of the suppressive cytokine IL-10. Suppressive effects of MSCs were elicited through inhibitors of PGE_2 synthesis which suggests that the increased PGE_2 production from MSCs may play an important role in the mechanism of immunomodulation. An in vivo animal model is currently under investigation to better understand the complete mechanism of immunomodulation by MSCs [50]. The findings of this study show potential benefit in allogenic transplantation where recipients often develop graft-versus-host-disease due to reactive T-cells in the allograft [51].

Survival of MSCs is a significant hurdle to overcome for its intended use in regeneration of cells after ischemic insult. Retention rates of cells posttransplant in a porcine ischemic heart were no more than 6% after 10 days [52]. MSC survival in an immunodeficient rodent heart model was less than 0.4% after only four days [53]. The mechanism of poor survival is multifactorial but can be concisely explained by the harsh microenvironment in which the area of ischemia creates. MSCs struggle with inflammation, hypoxia, and oxidative stress due to inadequate nutrients and oxygen in the region [54]. Strategies aimed at improving survival include developing better ways to deliver MSCs to the ischemic area, preconditioning cells to better tolerate the microenvironment, and modifying the cells by genetic means [52]. Further investigation is needed to analyze and justify the most suitable improvements so that MSC therapy can remain a viable therapeutic option following ischemic stroke.

9. Inducible Pluripotent Stem Cells

Human inducible pluripotent stem cells (iPSCs) encourage potential restorative capabilities after ischemic stroke through their neuroprotective and neuroregenerative properties. However, application technique, adaptation, and optimization of iPSCs including their ability to differentiate may affect outcomes [55]. They are primarily generated from dermal fibroblasts, keratinocytes, lymphocytes, and hematopoietic stem cells by induced expression of transcription factors. Using gene expression and neuronal biomarkers, iPSCs were reported to generate cortical neural precursors in vitro [56]. An in vivo study was performed to assess whether iPSC-derived cortical neuronal progenitors, generated in vitro, survive transplantation and adequately differentiate in an injured adult brain. Human skin-derived iPSCs were found to have a neuronal phenotype differentiation capability in the somatosensory cortex and partially restored injured areas in an adult rat ischemic stroke model. At 2 months after transplantation the iPSCs not only proliferated but also survived, generating neurons with the same cortical phenotype observed in vitro. They expressed a cortex-specific biomarker TBR_1 and exhibited layered patterning and projections, suggesting successful integration into the host brain. Electrophysiological data showed that the grafted cells acquired electrical properties, with the ability to fire action potentials [57]. Another study confirmed how grafted iPSCs can collect functional synaptic connections over a 6-month timeframe [58].

Jensen et al. aimed to test the survivability of NSCs derived from human iPSCs for treatment in an ischemic stroke model. The human iPSCs, derived from postnatal skin fibroblasts, were directed in vitro to the NSC phenotype and then were injected intracerebrally approximately one week following ischemia in adult rats. The amount of surviving graft cells was nearly double the number transplanted and expressed several neuronal biomarkers and neurite-like processes. Grafted cells integrated well and differentiated primarily into neurons in all members of the treatment group and rare astrocytes in half of the treatment group (Figure 6). No tumorgenesis was noted, and continued proliferation occurred one month after transplantation. Though this might suggest that the cell line was immature, the cells demonstrated remarkable survivability [59]. As a follow-up study regarding the optimal differentiation status of the stem cells, Jensen et al. in 2016 hypothesized whether grafting iPSCs depends on neural differentiation maturity status before treatment. The iPSC line was derived from postnatal human skin fibroblasts and differentiated to neural linages. 8-week old rats received intracerebral cell grafts at four different time points. The authors reported no significant difference among iPSCs at days 7, 28, 42, and 56 on the infarct

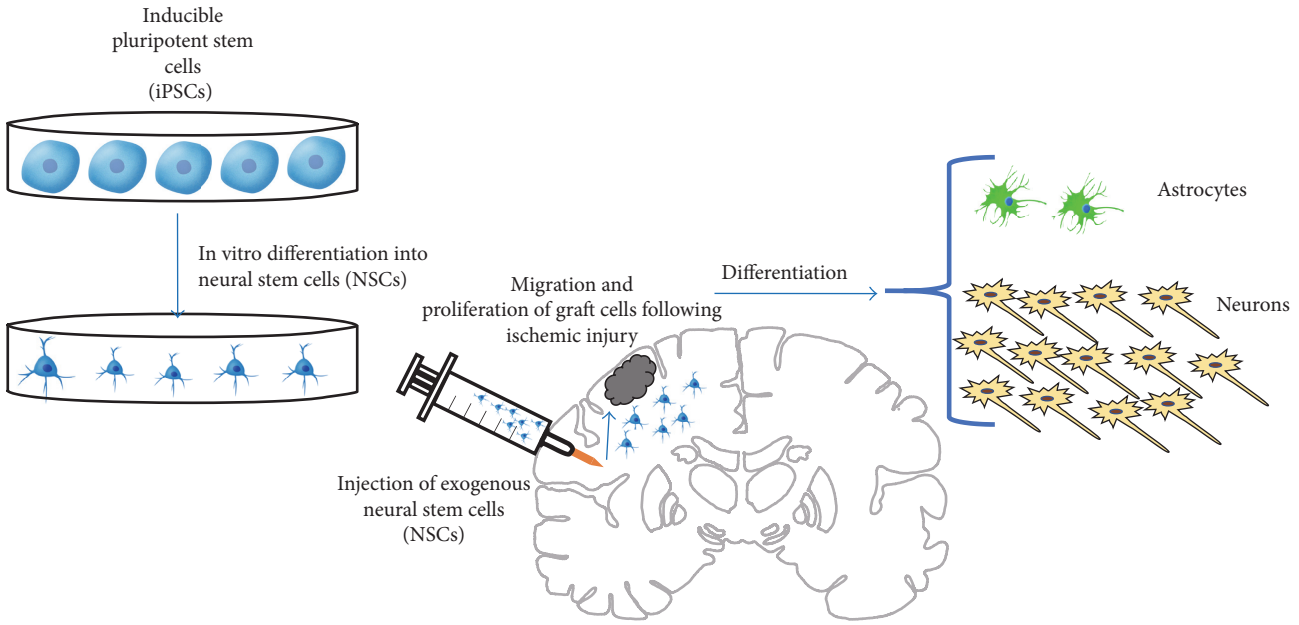

Figure 6: This figure demonstrates the capabilities of inducible pluripotent stem cells (iPSCs). Human iPSCs can be directed in vitro to the NSC phenotype. They are then injected into the cerebrum following MCAO and ischemic injury in rats. iPSCs are able to proliferate and differentiate primarily into neurons.

size, behavioral recovery, microgliosis, astrocytosis, or neurologic outcome [60].

Neurotrophic factor-induced neuroregeneration has been associated with iPSCs. Chung et al. demonstrated how differentiation capabilities of mouse iPSCs also rely upon hypoxia-induced BDNF expression [61]. Cerebral transplantation of iPSCs showed beneficial outcomes in experimental stroke models, as evidenced by neuroinflammatory alterations, neuroplasticity enhancement, and neuronal replacement [62]. A recent study transplanted human iPSC-derived neuroepithelial-like stem cells into the area of ischemic injury in adult male rats and demonstrated the differentiation capacity through functional neurons as well as iPSC-derived cortical neurons. Ultrastructural signs indicated functional activity of synapses, including abundant synaptic vesicles and a wide range of synaptic contacts between grafted and host neurons. The majority of in vivo electrophysiological recordings at 5 months poststroke was made from grafted neurons and exhibited the same properties of mature neurons. The transplanted stem cells were able to gain afferent synaptic inputs from both the injured and uninjured cerebral areas and effectively maintained function from 6 weeks to 6 months following iPSC-derived stem cell transplantation [63].

Chen et al. examined the effects of iPSCs, generated from 13.5-day-old mouse embryonic fibroblasts, when transplanted in the subdural region of rodents with the help of fibrin glue. 8-week old adult rats underwent MCAO to induce ischemia. Direct injection of iPSCs into the brains showed 100% incidence of teratoma formation 4 weeks after transplantation. However, in the iPSC-fibrin glue group, no tumor formation or survival was observed 6 weeks after subdural transplantation in the ipsilateral cerebral hemisphere with MCAO. The results in the iPSC-fibrin glue group also included a smaller infarct size and improved motor function. In-depth analysis of cytokine expression demonstrated a decrease in proinflammatory cytokines coupled with an increase in anti-inflammatory cytokines, providing evidence for the efficacy of the iPSCs in the treatment of stroke using fibrin glue [64].

10. Combination/Cotransplantation Therapy

There has been a keen interest in using combination and cotransplantation therapies in treatment of ischemic stroke. A growing number of studies have shown encouraging results when combining single therapies to treat the aftermath of this often-debilitating phenomenon. As previously mentioned, it has been established that NSCs hold great potential in replacing cells that were lost due to ischemic stroke; however, the central nervous system (CNS) does not naturally provide an optimal microenvironment for transplanted NSCs to properly establish their intended purpose. To solve this issue of exogenous NSC survival, cotransplantation studies have been performed to evaluate whether there is an improvement in grafting efficacy of exogenous NSCs when other cells, such as astrocytes and/or microvascular endothelial cells, are cotransplanted.

Luo et al. used an animal model to explore whether astrocytes act to make the CNS microenvironment more suitable for differentiation of exogenous NSCs in an ischemic brain. Their results were encouraging, showing that animals who received cotransplantation of astrocytes exhibited a higher likelihood of exogenous NSC survival [6]. Another important study was conducted by Wang et al. who revealed an understanding of a potential mechanism behind the synergistic effect of endothelial progenitor cells (EPCs) and NSCs protecting cerebral endothelial cells (CECs) from hypoxia/

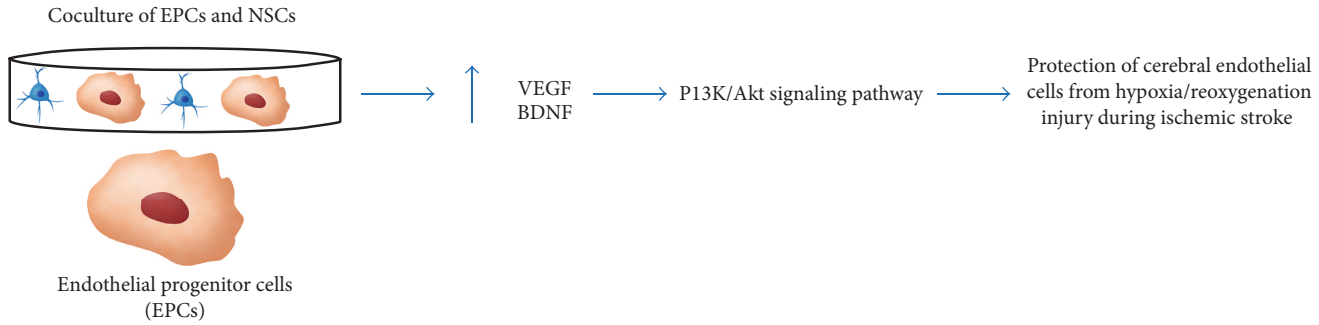

Figure 7: This figure demonstrates the effects of using cotransplantation as a method for stem cell therapy. An example of this approach is using endothelial progenitor cells (EPCs) and NSCs. Together, they create a synergistic effect that results in an increase in VEGF and BDNF. This activates the PI3K/Akt pathway that is thought to protect cerebral endothelial cells from hypoxia/reoxygenation injury during ischemic stroke.

reoxygenation-induced injury associated with ischemic stroke. This in vitro study demonstrated that coculturing EPCs with NPCs resulted in an increase in VEGF and BDNF, which further activated the phosphatidylinositol-3-kinase (PI3K)/Akt pathway that is thought to protect CECs during ischemic stroke (Figure 7) [65].

To explore the potential benefits of cotransplantation further, an animal model study was conducted by Cai et al. who revealed that cotransplanting astrocytes and brain microvascular endothelial cells (BMECs) together with hippocampal NSCs improved memory deficits in ischemic stroke animal models. Furthermore, this improvement in memory was greater in animals that received both astrocytes and BMCEs compared to those that received one or the other along with the exogenous NSCs [66].

Using a tissue engineering approach, Zhang et al. demonstrated the beneficial effects of a combined treatment of plasma scaffold with MSCs. A rodent model of ischemic stroke was performed via MCAO. Three weeks later, the treatment was administered. The combined treatment group, consisting of the scaffold and MSCs, and the single treatment group of only MSCs showed better signs of recovery than the vehicle group. However, it is notable that the combined scaffold-MSCs showed more positive results than the single treatment group. This unique approach led to an improvement of motor function and a reduction in the amount of atrophy, further supporting the therapeutic benefits of MSCs [67]. A 2017 study by Augestad et al. studied the effects of cografting NSCs with olfactory ensheathing cells. These cells are a special type of glial cell with neuroprotective and angiogenic capabilities that may assist in graft survival. Using an MCAO rat model, they found extensive vascular remodeling and even more NSC movement towards the infarct border [68].

Stem cell clinical trials are well under way and showing beneficial outcomes. Qiao et al. assessed safety and feasibility of cotransplanting NSCs and MSCs into the brains of patients that experienced ischemic stroke. Although only eight patients were enrolled in this study, the results were encouraging as the patients exhibited an improvement in neurological function and disability levels. Furthermore, none of the patients experienced tumorigenesis when reevaluated during their two-year follow-up appointments [69]. The results of this study are encouraging; however, larger samples, extensive follow-up, and standardized study design methods, such as utilization of control groups, are required to further explore these observations (Table 1).

11. Stem Cell Tracking Using Magnetic Resonance Imaging

It is of specific importance to be able to track and monitor the dynamics of endogenous stem cells in ischemic stroke. Typically, these NSCs are located in the SVZ of the lateral ventricle and the SGZ in the dentate gyrus of the hippocampus [70]. After ischemic stroke, NSCs are triggered to proliferate and migrate towards the injured region of the cortex, and it is this process that in vivo tracking aims to visualize, as well as provide insights into the SVZ under ischemic conditions [71]. In order to effectively use stem cells as a reliable tool in the clinical setting, there needs to be a method of tracking and long-term monitoring of cell acceptance, growth, distribution, differentiation, and cell survival of the transplanted stem cells [8].

Intracellular magnetic labels such as superparamagnetic iron oxide nanoparticles (SPIONs) have their surface modified to facilitate cellular uptake, and they work well for tracking experiments because of their molar relaxivity and can induce internalization of the contrast medium without interrupting cellular functions [72]. Zhong et al. found that in vivo targeted magnetic resonance imaging (MRI) of endogenous NSCs in a normal adult rodent brain could be achieved using anti-CD15 antibody-conjugated superparamagnetic iron oxide nanoparticles (SPIONS) as the molecular probe [73]. This method is able to overcome the shortcomings of using a nonspecific SPION or a ferritin-based reporter gene, including low imaging sensitivity, nontargeting, and toxicity [74].

Zhang et al. explored the use of anti-CD15 mAB SPIONS, which previously showed benefits in being able to monitor endogenous NSC migration, as the imaging probe in targeted tracking of activated endogenous NSCs expressing the CD15 antigen on the surface of NSCs after cerebral ischemia. Their findings included proliferation of endogenous NSCs without

Table 1: Current completed phase I and II clinical trials on stem cell and ischemic stroke.

Stem cell completed clinical trials for ischemic stroke					
Authors	NCT	Stage of trial	Type of stem cells used/mode of delivery	Primary outcomes	Results
Kalladka et al. 2016 [9]	01151124	Phase I	NSCs: CTX-DP drug product/stereotactic ipsilateral putamen injection	Incidence of adverse events	No adverse events were seen NIHSS improvement ranged from 0–5 (secondary outcome)
Qiao et al. 2014 [69]	NA	Phase I	Cotransplantation with neural stem/progenitor cells and mesenchymal stromal cells/IV	Safety and feasibility	No evidence of neurological deterioration or neurological infection
Prasad et al. 2014	02425670	Phase II	BM mononuclear cells/IV	Functional ability-modified Barthel Index score	No significant difference between BMSC versus control in Barthel Index score
Banerjee et al. 2014	00535197	Phase I	$CD34^+$ stem cells/intra-arterial	Safety	Safe and feasible
Savitz and Sean 2014	00859024	Phase I	Autologous mononuclear bone marrow cells/IV	Adverse events	No study-related adverse events

IV: intravenous; BMSC: bone marrow stem cells; NIHSS: National Institute of Health Stroke Scale; NCT: National Clinical Trial Number.

migration towards the infarcted lesion, possibly due to an 8-day follow-up post ischemic stroke, or a lower imaging sensitivity of SPIONS compared to micron-sized particles of iron oxide (MPIOs). In addition, by using the CD15-positive subpopulation of NSCs, not as many NSCs were visualized, as there are far less of this subtype than the nonspecific MPIO-labeled cells, which includes NSCs as well as neuroblasts, astrocytes, progenitor cells, and mature neurons [71]. MPIOs are found in microglia, ependymal cells, and oligodendrocyte progenitor cells in addition to being in NSCs, thereby providing a nonspecific tracking method [75]. Despite several limitations of anti-CD15 mAB SPIONS for tracking stem cells, this is a more recent approach to effectively track and monitor endogenous stem cells in vivo.

iPSC-derived neural precursors offer an exogenous source for stem cell transplantation therapy in CNS disorders. A study investigated the effect of two different contrast agents on neural precursor cell proliferation and differentiation capability using silica-coated cobalt zinc ferrite nanoparticles and SPIONs coated with poly-l-lysine (PLL). They found that PLL-coated SPIONs did not have any significant negative effects on cell proliferation or differentiation in any dose, as opposed to the silica-coated cobalt zinc ferrite nanoparticles that negatively impacted cell proliferation. PLL-coated SPIONs were found effective for noninvasive cell tracking and show promising use in future neural cell therapy-based in vivo applications for different disease models [76].

A study in 2013 designed a mesoporous silica-coated SPION to utilize for neural progenitor cell MRI. Compared to fluorescent dense silica-coated SPIONs, a commercially available contrast agent, the mesoporous silica-coated SPIONs had improved uptake efficiency potentially due to their less negative surface charge. It also had improved cell internalization over SHU555A, another commercially available contrast agent used for cell imaging [77]. After an incubation period of 3 hours, no direct cytotoxic effects could be found in the short term, but viability did decrease after 24 hours of incubation, with similar cytotoxicity levels as noted in SHU555A, likely due to high intracellular iron concentrations [78]. Using the noncytotoxic conditions of 10 micrograms Fe/ml for 2 hours, cell proliferation was not impacted. Researchers then used these criteria to perform intracerebral and intravenous injections of labeled progenitor cells in MCAO mouse model. Both methods were able to show that the transplanted progenitor cells migrated to the ischemic site, with cell clusters detected near the lesion boundary [79] (Table 2). Advances in stem cell tracking using MRI following ischemic injury are well underway, and new molecular probes are being testing in vitro with hopes of providing additional tracking options. Currently, after searching on clinicaltrials.gov using search terms "stroke and stem cell tracking," no clinical trials on tracking methods are being performed at this time. Studies that elucidate consistent results regarding effectiveness and safety in animal models will continue to lay the groundwork for future clinical trials.

12. Conclusion

Basic science research on stem cell treatment of stroke is a necessity and may change the lives of millions around the world burdened by the effects of ischemic stroke. The progress done so far in animal research has led to multiple clinical trials showing the safety and benefits of stem cells with recovery from ischemic stroke. Clinical trials are underway, most within phase I or phase II and focusing on MSCs or cotransplantation methods. Basic science studies continue to publish results on the benefits of transplanting stem cells after stroke to improve recovery, infarct size, and reduce apoptotic events and neurodegeneration. Various new methods are being tried, including cotransplantation and preconditioning with pharmacologic agents known to induce angiogenesis and improve receptor binding for neuronal migration and inducing more endogenous stem cells to migrate. Finding consistent methods to promote not only neuronal differentiation but also adequate migration to the area of infarct is a critical issue in the field of stem cell transplantation. The challenges

Table 2: The 3 most common studies that tested various molecular probes to track stem cells in both normal and ischemic brain.

In vivo tracking using magnetic resonance imaging						
Author	Target	Probe delivery method	Molecular probe	Location	Signal time	Findings
Zhang et al. 2016 [71]	Endogenous NSCs in vivo ischemic stroke adult rats	Stereotactic injection	Anti-CD15 antibody SPIONS	Intraventricular SVZ and RMS regions in adult mouse brain following MCAO	Detected day 1–day 8	(i) Proliferation of endogenous NSCs but no migration towards infarction lesion (ii) Findings may be due to short-term follow-up of only 8 days (iii) SPIONs have less imaging sensitivity than MPIOs (iv) Migration to OB along RMS observed after ischemic stroke (v) Introduction of heterologous antibody risk host immunological response (vi) Many other phenotypes undetected
Zhong et al. 2015 [73]	Endogenous NSCs in vivo normal adult rats	Stereotactic injection	Anti-CD15 antibody SPIONs	Intraventricular SVZ and RMS regions in adult mouse brain	Detected 1 day after injection, lasted 7 days total	(i) Small size, low artifact (ii) Increased specificity for NSCs (iii) Can track highly active areas such as OB surface binding less likely to affect biological behavior of cells (iv) Introduction of heterologous antibody risks host immunological response (v) Many other phenotypes undetected
Zhang et al. 2013 [79]	Exogenous neural progenitor cells in vivo ischemic stroke adult mice	Intravenous and implantation into hemisphere contralateral to stroke	Fluorescent mesoporous silica-coated superparamagnetic iron oxide nanoparticles	Right hemisphere following MCAO	Detected and analyzed 1–3 days after injection	(i) Cells injected both intracerebrally and intravenously could be seen migrating to ischemic sites of MCAO mice (ii) Migrated cells/cell clusters were detected nearby the lesion boundary (iii) Migrated cells reduced the MR signal intensity in ischemic region 3 days after injection

in the area of improving tracking methods include length of tracking using current methods and the risk of provoking an immune response or affecting intrinsic properties of stem cells. Monitoring the process of cell acceptance, growth, migration, differentiation, and cell survival of injected cells is important to be able to analyze and understand the process after stem cell transplantation. Noninvasive strategies to study neurogenic mechanisms of stem cells will benefit the development of future studies and therapies in the field in search of improving the overall utilization of NPSCs in the treatment and recovery of ischemic stroke.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Cesar Reis and Michael Wilkinson contributed equally to this work.

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Research Article

FGF2 Attenuates Neural Cell Death via Suppressing Autophagy after Rat Mild Traumatic Brain Injury

Chonghui Tang, Yudong Shan, Yilan Hu, Zhanjian Fang, Yun Tong, Mengdan Chen, Xiaojie Wei, Xiaojun Fu, and Xinlong Xu

Department of Neurosurgery, Affiliated Cixi Hospital, Wenzhou Medical University, No. 999 Nanerhuandong Road, Ningbo 315300, China

Correspondence should be addressed to Xinlong Xu; xuxinlong2001@126.com

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Traumatic brain injury (TBI) can lead to physical and cognitive deficits, which are caused by the secondary injury process. Effective pharmacotherapies for TBI patients are still lacking. Fibroblast growth factor-2 (FGF2) is an important neurotrophic factor that can stimulate neurogenesis and angiogenesis and has been shown to have neuroprotective effects after brain insults. Previous studies indicated that FGF2's neuroprotective effects might be related to its function of regulating autophagy. The present study investigated FGF2's beneficial effects in the early stage of rat mild TBI and the underlying mechanisms. One hundred and forty-four rats were used for creating controlled cortical impact (CCI) models to simulate the pathological damage after TBI. Our results indicated that pretreatment of FGF2 played a neuroprotective role in the early stage of rat mild TBI through alleviating brain edema, reducing neurological deficits, preventing tissue loss, and increasing the number of surviving neurons in injured cortex and the ipsilateral hippocampus. FGF2 could also protect cells from various forms of death such as apoptosis or necrosis through inhibition of autophagy. Finally, autophagy activator rapamycin could abolish the protective effects of FGF2. This study extended our understanding of FGF2's neuroprotective effects and shed lights on the pharmacological therapy after TBI.

1. Introduction

Traumatic brain injury (TBI), the leading cause of death and disability nowadays, is a major health problem all over the world [1, 2]. Even mild TBI can cause delayed physical and cognitive deficits [3]. Although lots of randomized controlled trials (RCTs) were done in recent years, no intervention had shown to be beneficial [4–6]. Thus, it is imperative to further elucidate the complicating pathophysiological mechanisms of TBI and develop effective pharmacological intervention targets. It is generally acknowledged that TBI has two injury phases—primary injury and secondary injury. The primary injury is directly caused by trauma itself, while the secondary injury is more complex with a series of pathological responses, including blood-brain barrier disruption, oxidative stress, neuroinflammation, autophagy, apoptosis, and necrotic cell death. These processes in the secondary injury are directly related with long-term neurological deficits and also provide us multiple therapeutic targets in TBI management.

Autophagy is a lysosomal degradation pathway that protects organisms against diverse pathologies [7]. In most circumstances, autophagy could promote cell survival by maintaining cellular homeostasis, but there are a number of studies that demonstrated that autophagy could also trigger cell death in certain pathological situations [8–10]. Researchers had already observed autophagy's existence in TBI model several years ago, and modulation of this process could result in neurological improvements [11–13]. However, up until recent years, whether inducing or inhibiting autophagy can result in neuroprotection remains controversial [14–16]. The relationship between autophagy and other forms of cell death such as apoptosis after TBI is worth studying.

Fibroblastic growth factors (FGFs) are small polypeptide growth factors which play a pivotal role in morphogenesis [17]. Fibroblast growth factor-2 (FGF2), also known as basic fibroblast growth factor (bFGF), is an important member of this family. FGF2 is highly expressed in the central nervous system and exhibits early decline during the course of aging [18]. Previous studies showed that it had a variety of neuroprotective effects, including supporting neural stem and progenitor cell proliferation in vitro and in vivo [19, 20], maintaining vascular integrity and angiogenesis [21, 22], and helping cognitive recovery [20]. Studies have demonstrated the neuroprotective roles of FGF2 in various pathological conditions in the central nervous system, such as TBI, spinal cord injury (SCI), ischemic brain injury, subarachnoid hemorrhage (SAH), and neurodegenerative diseases [21, 23–26]. Most of the previous studies focused on neurogenesis effects of FGF2, but several recent studies have discovered FGF2's novel effect as an autophagy inhibitor, in which the activation of PI3K/Akt/mTOR signaling pathway may take an important part [27, 28]. As the molecular mechanisms of FGF2 in treatment of TBI has not been fully understood, we hypothesized that FGF2 could also inhibit autophagy and attenuate cell death in TBI treatment.

In the present study, we found that FGF2 could act as a neuroprotective agent after rat mild TBI, alleviating brain edema, reducing cerebral lesion volume, and promoting functional recovery. Meanwhile, FGF2 also inhibited autophagy and decreased neural apoptosis and necrotic cell death. Furthermore, autophagy activator rapamycin could abolish the protective effects of FGF2. These results provide us a new perspective about FGF2's neuroprotective role after TBI.

2. Materials and Methods

2.1. Animals and Study Design. Adult male Sprague-Dawley rats (250–300 g) obtained from SLAC Laboratory Animal Co. Ltd. (Shanghai, China) were used in this study. The animals were maintained under controlled temperature and humidity conditions at a 12 hr light/dark cycle place. All procedures involving animals were strictly conformed to the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University.

2.2. CCI Model. The procedure details used for creating CCI model were described previously [29]. Briefly, after the rats were anesthetized with pentobarbital (40 mg/kg) through intraperitoneal injection, the head of them was fixed on a stereotaxic frame. A 5×5 mm craniotomy was performed approximately midway between the bregma and lambda on the right frontoparietal cortex after a midline incision. The bone flap was then removed carefully without disturbing the underlying dura. Then the CCI was performed using a PinPoint™ Precision Cortical Impactor (Cary, NC, USA) perpendicular to the brain surface (12° from the vertical). The impact tip was 4 mm in diameter, with an impact velocity of 3 m/s, a duration time of 120 ms, and a deformation depth of 2 mm below the dura, to mimic a mild focal TBI in rats. The bone flap was put back and sealed immediately, and the wound was sutured. Rats in the sham group received the same surgical procedure only without CCI. During the surgery, body temperature was monitored and maintained at 37.0 ± 0.5°C with a rectal probe coupled to a heating pad. For arterial pH, pO_2, pCO_2, and blood glucose level monitoring catheterize the right femoral artery before the procedure. The rats were returned to their home cages after completely recovered from anesthesia in a heated chamber.

2.3. Experimental Design

2.3.1. Experiment 1. To explore the effects of FGF2 in rat TBI models, ninety rats were randomly assigned into three groups: the sham group ($n = 30$), TBI + vehicle group ($n = 30$), and TBI + FGF2 group ($n = 30$). A dose of 250 μg/kg recombinant human FGF2 (PeproTech Inc., Rocky Hill, NJ, USA) was administrated intranasally 1 h before TBI induction in the TBI + FGF2 group. Intranasal administration could deliver FGF2 directly to the brain through nasal epithelium [30, 31]. The dosage and time point of FGF2 pretreatment were based on a previous study [22]. The sham group and TBI + vehicle group received the same volume of sterile saline intranasally at the same time point before TBI induction. The endpoints of the experiments were 6 h (PI staining) and 48 h (other experiments) after TBI induction, based on a previous study [32]. Six rats in each group were used for brain water content assessment, lesion volume assessment, Western blot, immunofluorescence staining, and PI staining.

2.3.2. Experiment 2. To explore whether autophagy mediates FGF2 effects, fifty-four rats were randomly assigned into three groups: the TBI + vehicle group ($n = 18$), TBI + FGF2 group ($n = 18$), and TBI + FGF2 + Rap group ($n = 18$). The dosage and time point of FGF2 pretreatment were the same as in experiment 1. A dosage of 2 mg/kg rapamycin (Selleck Chemicals, Houston, TX, dissolved in 2% DMSO) was administrated intraperitoneally 30 min after TBI induction in the TBI + FGF2 + Rap group based on a previous study [33]. The TBI + vehicle group received same volume of sterile saline intranasally 1 h before TBI induction. The TBI + vehicle group and TBI + FGF2 group received the same volume of 2% DMSO intraperitoneally 30 min after TBI induction. Six rats in each group were used for Western blot, immunofluorescence staining, and PI staining. The endpoints of the experiments were the same as in experiment 1.

2.4. Evaluation of Neurological Deficits. To examine the effects of FGF2 on the neurological deficits of animals after TBI, the modified neurological severity scores (mNSS) were used. The neurological scores of the animals in each group were evaluated by an independent observer at 48 h after TBI induction using motor, sensory, reflex, and balance tests. A total score ranging from 0 to 18 was calculated by adding the scores together, and a higher score indicated a worse neurological function [34]. Most mNSS of the rats were lower than 6, which indicated a mild injury after TBI. Foot fault test was examined in experiment 2 to evaluate the cognitive functional deficits by an independent observer at 48 h after TBI induction. The rats were put on an elevated grid and allowed

to move freely for 5 minutes or until 50 steps were taken with the left forelimb. The total number of steps and fell time of the left forelimb were counted, and the percentage of left forelimb foot faults was calculated.

2.5. Brain Water Content Measurement. Rats were sacrificed under deep anesthesia after 48 h of TBI. The brains were removed, and the right hemispheres were collected immediately. After weighing for the wet weight, the samples were then dried at 105°C for 24 h to obtain the dry weight. The brain water content was calculated using the following formula: (wet weight − dry weight)/wet weight × 100%.

2.6. Hematoxylin and Eosin (HE) Staining and Lesion Volume Assessment. At 48 h after TBI, the rats were sacrificed and perfused intracardially with 0.1 mmol PBS (pH, 7.4) and 4% paraformaldehyde (pH, 7.4). The brains were then removed and immersed in the same perfusate at 4°C for 72 h. Coronal sections started from one millimeter which was anterior to the lesion margin, to the posterior lesion margin, were obtained and then paraffin embedded. The lesion volumes were calculated by integrating 10 sections (3 μm thick) with 300 μm intervals. After HE-staining with standard methods (0.5% hematoxylin, 25°C, 5 min and 0.5% eosin, 25°C, 1 min), the brain sections were imaged using a stereomicroscope (Olympus BX51, Olympus, Tokyo, Japan) in a bright-field illumination and measured using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA). Lesion volume was calculated as follows: $\Sigma(A_n + A_{n+1}) \times d/2$, where A is the ventricular area and d is the distance between sections, according to a previously published work [35].

2.7. Western Blot. Western blotting was performed as previously described [36]. Briefly, the cortical regions of the brains were collected and homogenized. The samples were centrifuged at 1000g for 10 min at 4°C. The supernatant was further centrifuged, and the protein content was measured using a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA, USA). Equal quantities of protein (40 μg) from each samples were resuspended in loading buffer, denatured at 95°C for 5 min, and loaded into the walls of the sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the protein was transferred onto polyvinylidene fluoride membranes. The membranes were blocked with nonfat dry milk buffer for 2 h and subsequently incubated overnight at 4°C with the following primary antibodies: LC3 (1 : 1000, Cell Signaling Technology CST#4108), Beclin-1 (1 : 1000, Abcam ab62557), p62 (1 : 1000, Abcam ab56416), and β-actin (1 : 2000, Santa Cruz SC-47778). The membranes were processed using the appropriate secondary antibodies (1 : 5000) for 1 h at room temperature. The protein band densities were detected using X-ray film, and the densitometric signals were quantified using ImageJ software (NIH, Bethesda, MD, USA).

2.8. TUNEL and Immunofluorescence Staining. The rats were sacrificed at 48 h after TBI induction in deep anesthesia and perfused intracardially with 0.1 mmol PBS (pH, 7.4) and 4% paraformaldehyde (pH, 7.4). The brains were then removed and immersed in the same perfusate at 4°C for 72 h and then dehydrated with 30% sucrose solution until they sank to the bottom (about 2 days). Coronal sections (7 μm) were collected from the right hemisphere. The brain sections were incubated with 10% normal goat serum and 0.3% Triton X-100 to prevent nonspecific binding for 1 h at room temperature. Then the sections were incubated at 4°C overnight with the primary antibodies: NeuN (1 : 250, Millipore MAB377) or LC3 (1 : 200, Cell Signaling Technology CST#4108). After washing with 0.01 mmol PBS several times, the sections were incubated with the secondary antibodies for 2 h at 4°C in the dark. A TUNEL staining kit (In Situ Cell Death Detection Kit, Fluorescein, Roche, Switzerland) was used to analyze apoptotic cell death. The brain sections were incubated with TUNEL reaction mixture (90 min) at 37°C. After washing with PBS again, the sections were mounted onto slides with Fluoroshield™ with DAPI (Sigma-Aldrich, St. Louis, MO, USA). Immunostaining was observed using a fluorescent microscope (Olympus, Tokyo, Japan). The total number of NeuN-positive, LC3-positive, or TUNEL-positive cells were counted in three different slices per animal around the lesion area or in the CA1 and CA3 regions of the ipsilateral hippocampus by an independent observer.

2.9. Propidium Iodide (PI) Labeling. At 5 h after TBI induction, PI (10 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was diluted in normal saline and injected to rats intraperitoneally at a dose of 30 mg/kg. After 1 h, the rats were sacrificed and perfused intracardially with 0.1 mmol PBS (pH, 7.4) and 4% paraformaldehyde (pH, 7.4). Then the brains were removed and immersed in 4% paraformaldehyde for 72 h. After dehydrated with 30% sucrose solution, coronal sections (7 μm) were collected from the right hemisphere and PI-positive cells were quantitated around the lesion area or in the CA1 region from 200x cortical fields in three brain sections per rats. The counting task was also done by an independent observer.

2.10. Statistical Analysis. To facilitate comparisons between three groups, the Western blotting results were expressed as relative density of the band as compared β-actin and then normalized to the mean value of the sham group. The data were expressed as mean ± SD. SPSS Statistics (version 22.0) was used for statistical analysis. All the data were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was inferred at $P < 0.05$.

3. Results

3.1. Physiological Evaluation. All the physiological parameters were monitored during the TBI procedure. The body temperature, arterial pH (7.35–7.45), pO_2 (85–95 mmHg), pCO_2 (35–45 mmHg), and blood glucose level (95–125 mg/dL) were monitored and analyzed between different groups, and no significant difference exists among groups (data not shown). No mortality was recorded during the experiment.

3.2. FGF2 Alleviates Neurological Deficits and Brain Edema. The representative pictures of the brain samples in each group

are presented in Figure 1(a). Significant neurological function deficits were observed 48 h after TBI induction ($P < 0.05$, Figure 1(b)). The mNSS were significantly lower in the TBI + FGF2 group than in the TBI + vehicle group ($P < 0.05$, Figure 1(b)). The brain water content of the injured hemisphere was also significantly increased at 48 h post-TBI, pretreatment with FGF2 could attenuate this increase ($P < 0.05$, Figure 1(c)).

3.3. FGF2 Prevents the Loss of the Brain Tissue and Promotes Neuronal Survival after TBI. The cerebral lesion volume and the neuronal survival were assessed at 48 h post-TBI. The representative coronal sections in each group are shown in Figure 2(a). The lesion volumes of the TBI + FGF2 group were significantly smaller than those of the TBI + vehicle group at 48 h ($P < 0.05$, Figure 2(b)). The numbers of NeuN-positive cells in the ipsilateral cortex and the CA1 and CA3 regions of the ipsilateral hippocampus were significantly decreased 48 h after TBI, and this process could be significantly reversed when pretreated with FGF2 ($P < 0.05$, Figures 2(c) and 2(d)).

3.4. FGF2 Prevents the Increase of LC3-Positive Cell Numbers after TBI. Autophagy takes an important part in brain injury after TBI, but the role of FGF2 in regulating autophagy after TBI has not been studied. As LC3 is an ideal biomarker for autophagy activation, we chose LC3 to mark the autophagic cells. The immunofluorescence staining showed that LC3-positive cells could be hardly observed in the cortex of the control group but significantly increased 48 h after TBI induction ($P < 0.05$, Figures 3(a) and 3(b)). In the TBI + FGF2 group, the number of LC3-positive cells was less than that in the TBI + vehicle group, which was statistically significance ($P < 0.05$, Figures 3(a) and 3(b)).

3.5. FGF2 Regulates the Autophagy-Related Protein Levels. To confirm the inhibition effects of FGF2 on autophagy post-TBI, we further measured the protein levels of Beclin-1 and p62 and the ratio of LC3II/I in each group. As shown in Figure 4, the protein levels of Beclin-1 was significantly upregulated in the injured cerebral cortex 48 h after TBI ($P < 0.05$, Figures 4(a) and 4(b)), and FGF2 pretreatmet significantly decreased the Beclin-1 level compared with the TBI + vehicle group ($P < 0.05$, Figures 4(a) and 4(b)). TBI significantly decreased the protein level of p62 at 48 h ($P < 0.05$, Figures 4(a) and 4(c)), while FGF2 could significantly prevent this process ($P < 0.05$, Figures 4(a) and 4(c)). FGF2 pretreatment also significantly upregulated the ratio of LC3II/I compared with the TBI + vehicle group ($P < 0.05$, Figures 4(a) and 4(d)). These results indicated that FGF2 pretreatment could inhibit the level of autophagy at 48 h after TBI.

3.6. FGF2 Prevents Cell Apoptosis. TUNEL staining was used to detect the apoptotic cell death in the injured cortex and CA1 region of the ipsilateral hippocampus. In the sham group, TUNEL-positive cells barely exist (Figure 5(a)). TBI significantly increased the apoptotic index compared with the sham group, while pretreatment of FGF2 could significantly prevent the increase of the apoptotic index compared with the TBI + vehicle group ($P < 0.05$, Figures 5(a) and 5(b)).

3.7. FGF2 Decreased the Number of Necrotic Cells after TBI. To evaluate the effect of FGF2 in protecting cells from injury, PI was used to identify the plasma membrane disrupted cells. As plasma membrane disruption is one of the key hallmarks of necrotic cell death, PI-positive cells could represent necrotic cell death. The number of PI-positive cells was significantly increased in the injured cortex and CA1 region of the ipsilateral hippocampus in the TBI + vehicle group 6 h after TBI ($P < 0.05$, Figures 6(a) and 6(b)). Pretreatment with FGF2 could significantly prevent the increase of the percentage of PI-positive cells ($P < 0.05$, Figures 6(a) and 6(b)).

3.8. Rapamycin Abolished the Neuroprotective Effects of FGF2. To explore whether autophagy mediates FGF2 effects, rapamycin was used to counteract the autophagy inhibition caused by FGF2. The mNSS and foot faults were significantly lower in the TBI + FGF2 group than those in the TBI + vehicle group ($P < 0.05$, Figures 7(a) and 7(b)). The increase of the neurological function with FGF2 management was significantly suppressed by rapamycin ($P < 0.05$, Figures 7(a) and 7(b)). The protein level of Beclin-1 was significantly downregulated in the injured cortex in the TBI + FGF2 group ($P < 0.05$, Figures 7(c) and 7(d)), and rapamycin management significantly increased Beclin-1 level compared with the TBI + FGF2 group ($P < 0.05$, Figures 7(c) and 7(d)). FGF2 significantly increased the protein level of p62 at 48 h ($P < 0.05$, Figures 7(c) and 7(e)), while rapamycin could significantly prevent this process ($P < 0.05$, Figures 7(c) and 7(e)). Rapamycin also significantly upregulated the ratio of LC3II/I compared with the TBI + FGF2 group ($P < 0.05$, Figures 7(c) and 7(f)). Rapamycin could also significantly decrease the NeuN-positive cells in the injured cortex and CA1 region, compared with the TBI + FGF2 group ($P < 0.05$, Figures 8(a), 8(d), and 8(g)). The apoptosis index and necrosis index in the injured cortex were also increased after rapamycin management ($P < 0.05$, Figures 8(b), 8(c), 8(e), and 8(f)). The necrosis index of ipsilateral CA1 region in the TBI + FGF2 + Rap group was also significantly higher than that in the TBI + FGF2 group ($P < 0.05$, Figures 8(c) and 8(i)). These results indicated that rapamycin could reverse the downregulation of the autophagy level caused by FGF2 and abolish the neuroprotective effects of FGF2, demonstrated that reduced autophagy mediates FGF2-induced benefit effects after rat mild TBI.

4. Discussion

In the present study, we explored the neuroprotective role of FGF2 in rat mild TBI model and studied the potential mechanisms. We found that pretreatment with FGF2 had neuroprotective effect in rat mild TBI by alleviating neurological deficits, decreasing brain water content and reducing lesion volume. FGF2 also had the ability to increase the number of surviving neurons at the site of injury. Furthermore, we

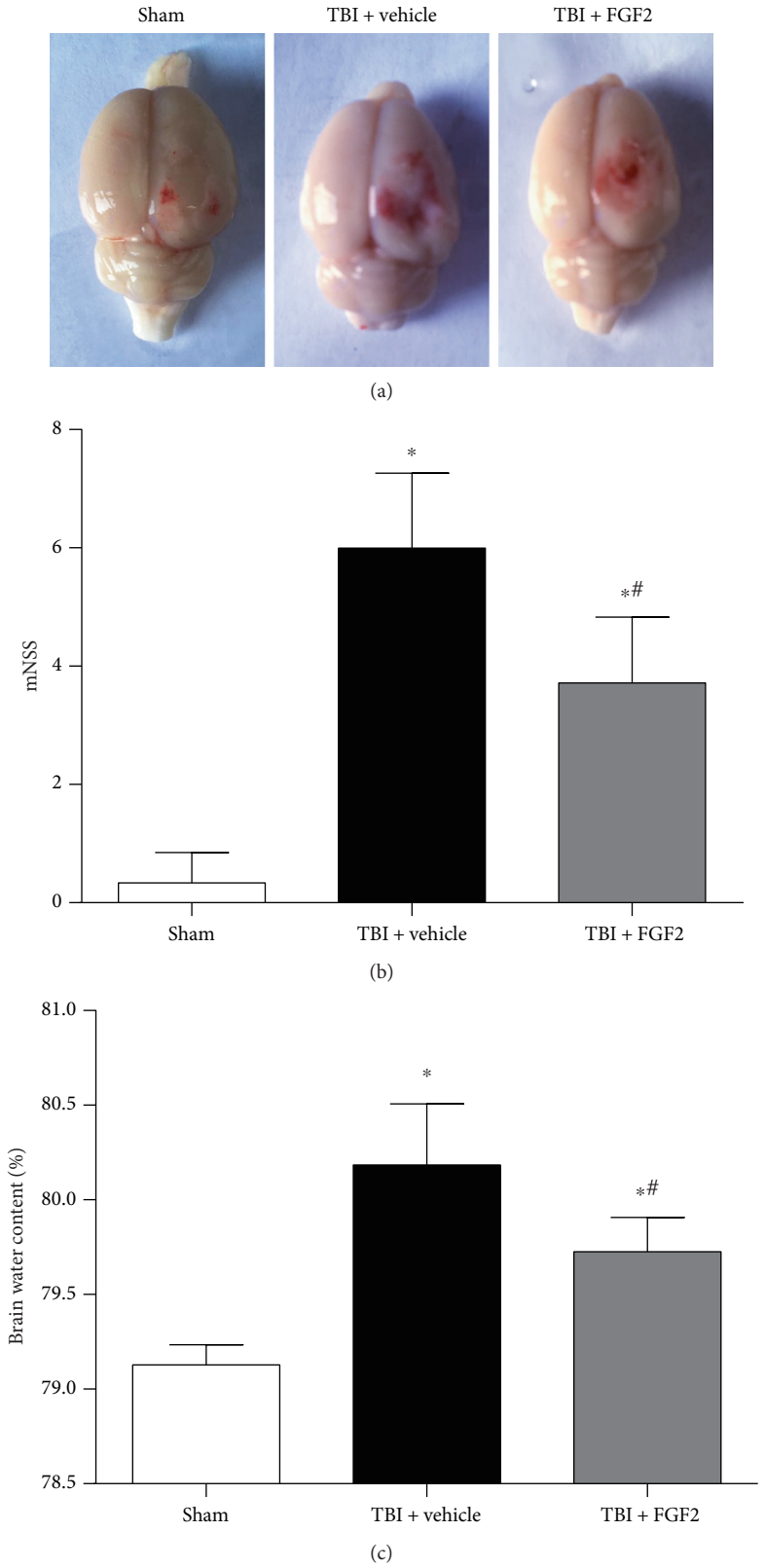

Figure 1: Representative pictures of the brains from each group, mNSS, and brain water content at 48 h after rat mild TBI. (a) Typical brains from the sham, TBI + vehicle, and TBI + FGF2 groups. (b) The quantification of mNSS at 48 h after TBI induction. The *bars* represent the mean ± SD. $n = 24$. $^{*}P < 0.05$ versus sham and $^{\#}P < 0.05$ versus TBI + vehicle. (c) The quantification of brain water content of the right hemisphere. The *bars* represent the mean ± SD. $n = 6$. $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus TBI + vehicle.

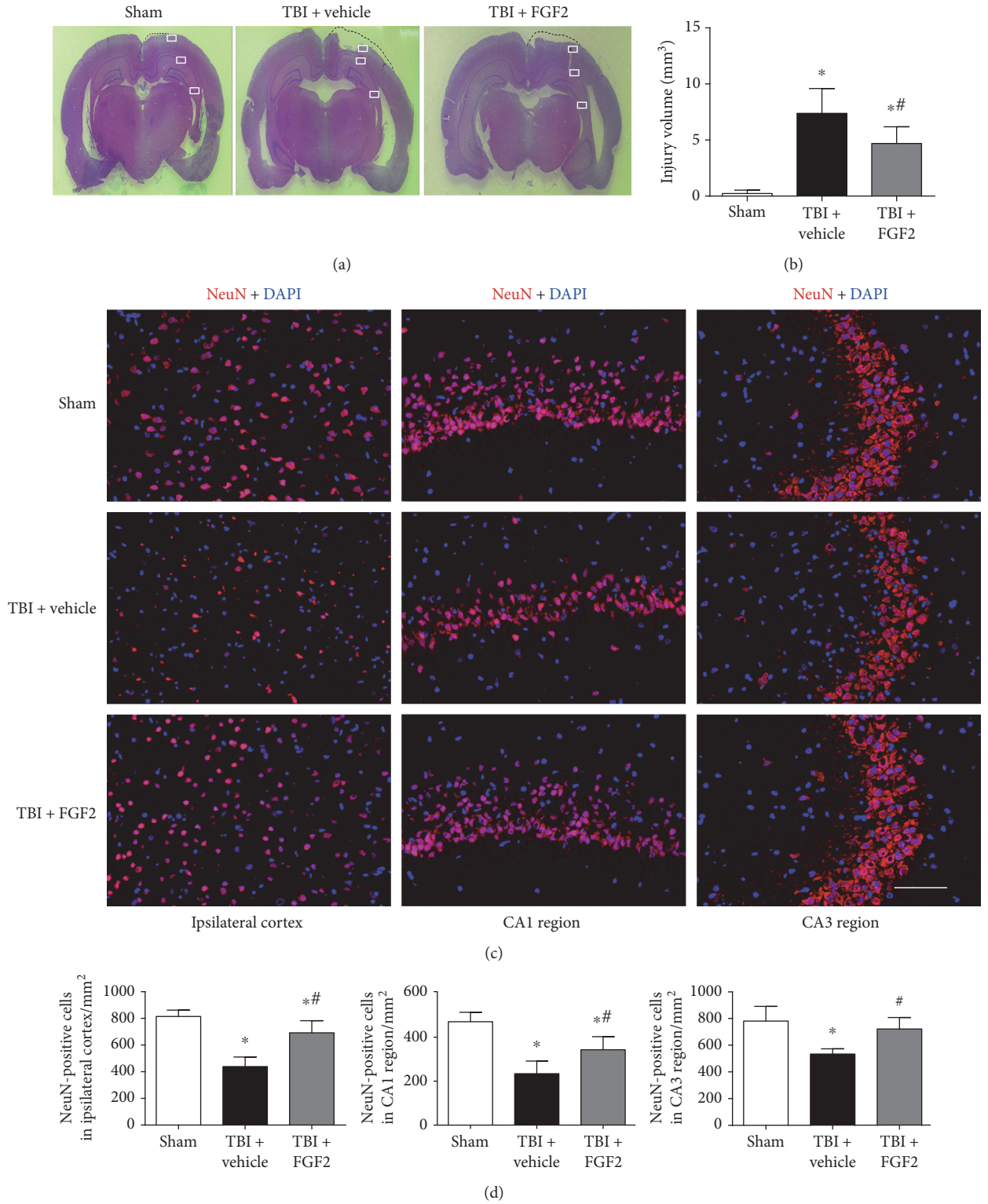

Figure 2: FGF2 prevents the loss of the brain tissue and promotes neuronal survival after TBI. (a) Representative images of HE-stained coronal sections 48 h post-TBI. The white squares indicated the region of the images presented in Figure 2(c). (b) Quantitative analysis of cerebral lesion volume revealed a significant decrease in loss of tissue following treatment with FGF2. The *bars* represent the mean ± SD. $n = 6$. $^{*}P < 0.05$ versus sham and $^{\#}P < 0.05$ versus TBI + vehicle. (c) Representative images of the ipsilateral cortex (left) and CA1 and CA3 regions of the ipsilateral hippocampus (middle and right) on NeuN-stained coronal sections from each group 48 h post-TBI. *Scale bar* = 100 μm. (d) Quantitative analysis revealed significant increases in the numbers of NeuN-positive cells in the ipsilateral cortex (left) and CA1 and CA3 regions of the ipsilaterial hippocampus (middle and right) 48 h post-TBI following pretreatment with FGF2. The *bars* represent the mean ± SD. $n = 6$. $^{*}P < 0.05$ versus sham and $^{\#}P < 0.05$ versus TBI + FGF2.

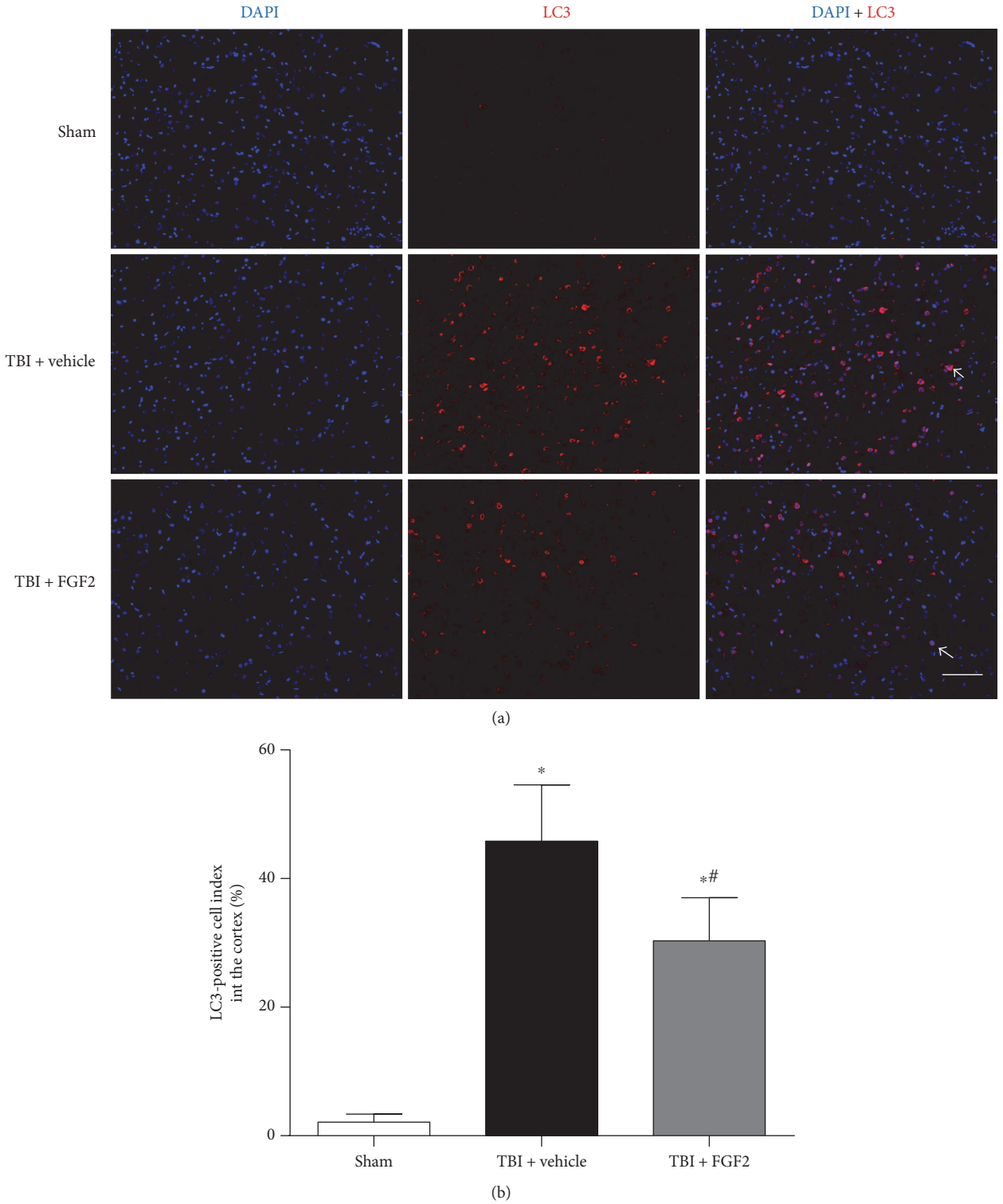

Figure 3: Effects of FGF2 on the expression of LC3 in the ipsilateral cortex 48 h post-TBI. (a) Representative microphotographs showed the colocalization of DAPI (blue) with LC3 (red) positive cells in the ipsilateral cortex 48 h post-TBI. *Scale bar* = 100 μm. (b) The quantification of the LC3-positive cells as a percent of the total DAPI+ cells. The *bars* represent the mean ± SD. $n = 6$. $^{*}P < 0.05$ versus sham and $^{\#}P < 0.05$ versus TBI + FGF2.

discovered the role of autophagy in FGF2 management after rat mild TBI for the first time. FGF2 could significantly decrease the apoptotic cell death and necrotic cell death through inhibiting autophagy after TBI. As an autophagy activator, rapamycin could reverse the neuroprotective effects of FGF2.

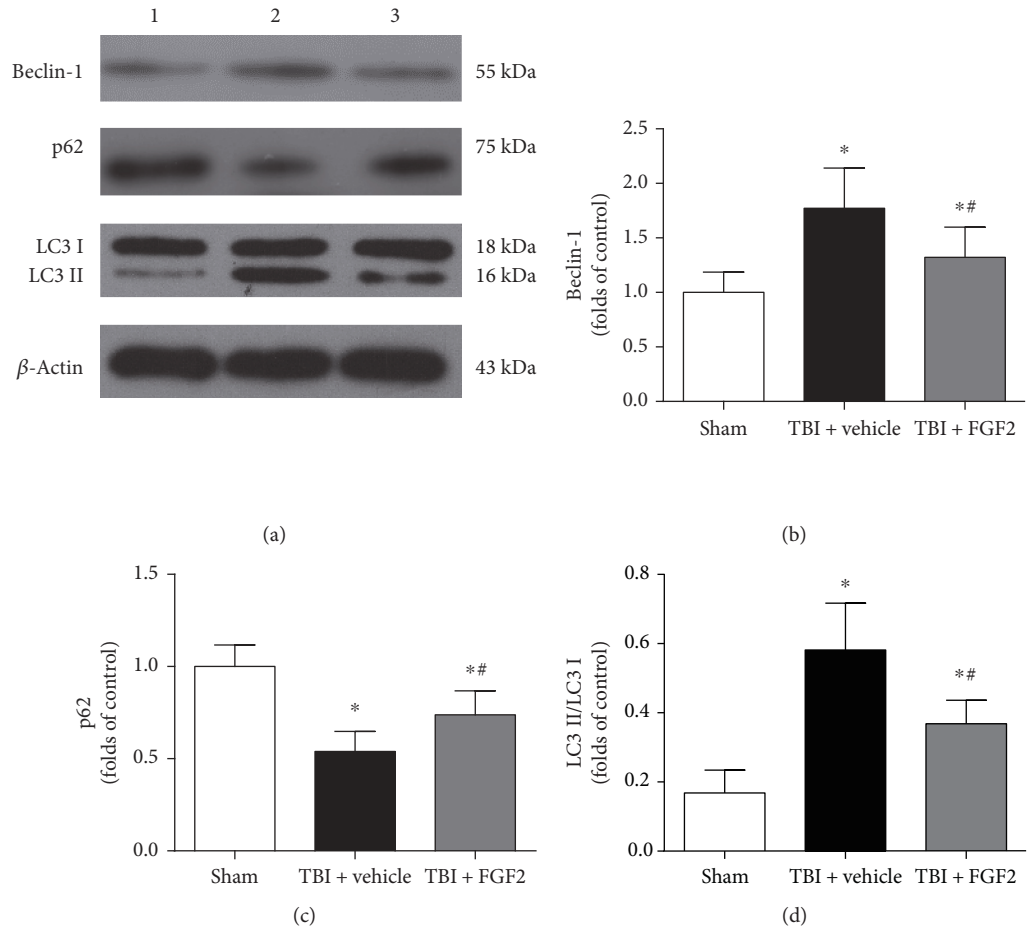

Figure 4: Effects of FGF2 on the expression of autophagy-related proteins in the ipsilateral cortex 48 h post-TBI in different groups. (a) Representative Western blots showing levels of Beclin-1, p62, and LC3 in the ipsilateral cortex 48 h post-TBI. Lane 1, sham group; lane 2, TBI + vehicle group; lane 3 TBI + FGF2 group. (b, c) The relative band densities of Beclin-1 and p62. The densities of the protein bands were analyzed and normalized to β-actin. The data are expressed as a percentage of the sham group. The *bars* represent the mean ± SD. $n = 6$. $^{*}P < 0.05$ versus sham and $^{\#}P < 0.05$ versus TBI + vehicle. (d) The band density ratio of LC3 II to LC3 I. The *bars* represent the mean ± SD. $n = 6$. $^{*}P < 0.05$ versus sham and $^{\#}P < 0.05$ versus TBI + vehicle.

As previously mentioned, the long-term consequence of TBI was dominated by the secondary injury, so the key to successful treatment had to focus on how to alleviate secondary damage after TBI [37]. Brain edema and the increased intracranial pressure in the early stage of TBI are the main causes of mortality, even a minor increase of brain water content can lead to a significant increase of intracranial pressure and the poor outcome [38]. Thus, alleviate brain edema is a promising pharmacological therapeutic direction in TBI management.

The concentration of FGF2 significantly increases in the interstitial space in the first week after TBI [39]. FGF2 binds to the fibroblast growth factor receptor 1 (FGFR1) which is located on the cell membrane of neurons [40] and stimulates the neural stem/progenitor cell proliferation and differentiation [20]. However, the fact is that for TBI patients, spontaneous improvement of neurologic function is limited and almost all of them will keep a stable condition after 12 months. Exogenous FGF2 had been used as a pharmacological intervention method in experimental TBI since many years ago [41]. However, most of the previous studies focused on its delayed effect of stimulating neurogenesis [20, 21], and to our knowledge, there is only one research about FGF2's effects in the early stage of TBI that is focused on its effect of alleviating blood-brain barrier disruption through activation of PI3K/Akt/Rac1 pathway [22]. Although disruption of blood-brain barrier permeability is the main cause of vasogenic edema [37], no direct evidence about FGF2's effect in reducing brain water content after TBI could be found in the literature. So, in this study, we tested FGF2's neuroprotective effects in the first 48 h after TBI and found that it could significantly alleviate brain water content of the injured hemisphere and promote mNSS of the injured rats. These results might be due to the alleviation of blood brain barrier disruption and were consistent with previous studies about FGF2's neuroprotective effects after intracerebral hemorrhage and ischemic brain injury [24, 42]. We also found that FGF2 could prevent tissue loss and increase the number of surviving neurons in injured cortex, CA1, and CA3 regions of the ipsilateral hippocampus in the early stage of TBI. These data indicated that FGF2 could assist in preventing neuronal cell death after TBI.

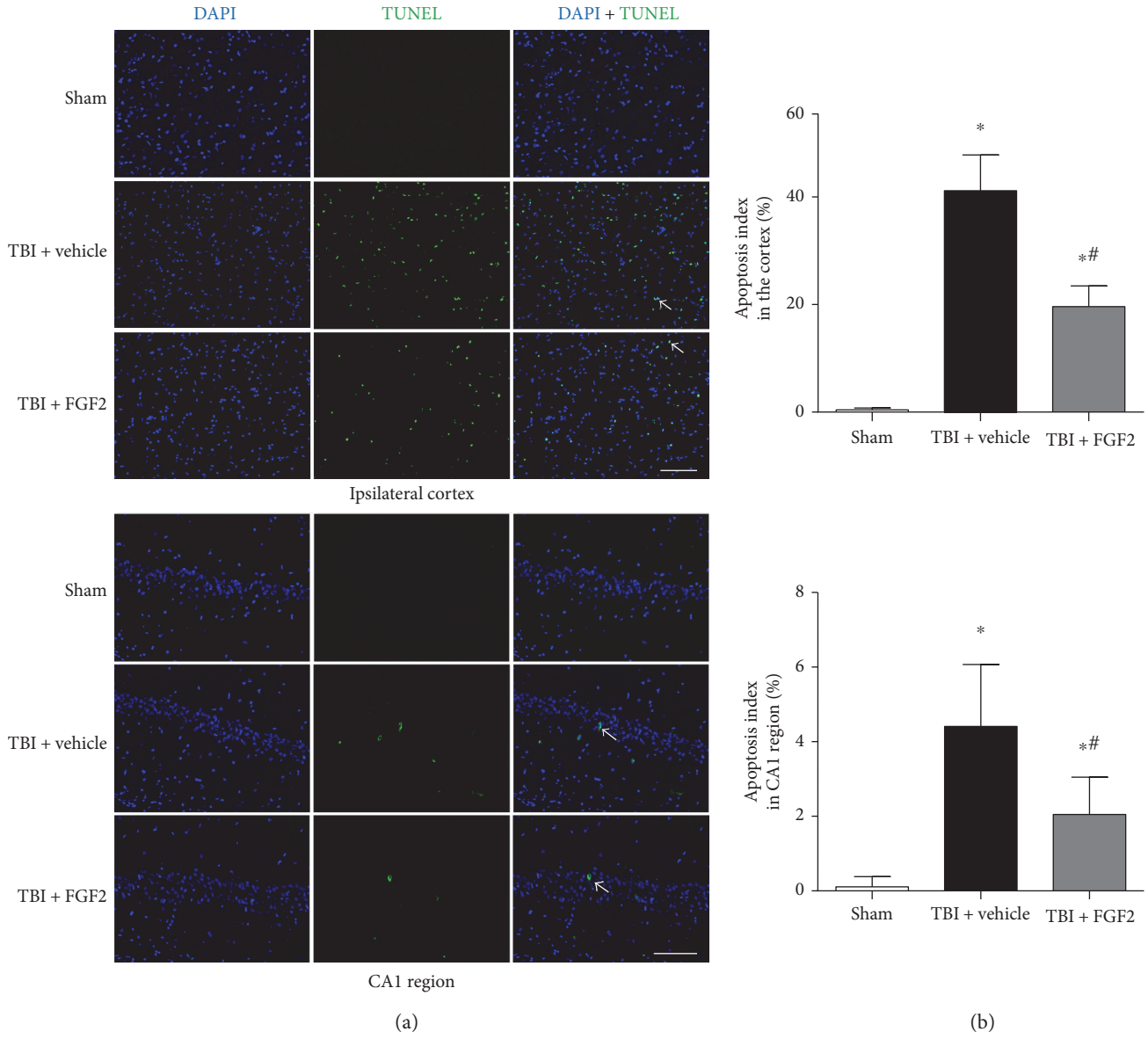

Figure 5: Cortical cellular apoptosis in the ipsilateral cortex 48 h post-TBI in different groups. (a) Representative microphotographs showed the colocalization of DAPI (blue) with TUNEL-positive (green) cells in the ipsilateral cortex and CA1 region 48 h post-TBI. Scale bar = 100 μm. (b) The quantification of the TUNEL-positive cells as the apoptosis index as a percent of the total DAPI+ cells. The bars represent the mean ± SD. $n = 6$. $^{*}P < 0.05$ versus sham and $^{\#}P < 0.05$ versus TBI + vehicle.

Several years ago, researchers had discovered that FGFs could regulate autophagy in cardiac stem cell differentiation and mouse embryonic fibroblasts [43, 44]. Recently, Wang et al. discovered that FGF2 could regulate autophagy and ubiquitinated protein accumulation in myocardial ischemia mice and spinal cord injury rats [27, 28]. In this study, we further explored the relationship between autophagy and the neuroprotective function of FGF2 in the early stage of TBI. The immunofluorescence study showed that the immunoreactivity of LC3 was downregulated by FGF2, suggesting that FGF2 pretreatment could suppress autophagic cell death. LC3 I and LC3 II are the two forms of LC3, and the ratio of LC3 II to LC3 I is generally used as a biomarker of autophagy [45]. Western blot showed that FGF2 pretreatment can prevent this ratio from increasing. Beclin-1, which played an important role in autophagic cell death, was also downregulated by FGF2. p62 links ubiquitinated protein bodies to LC3 and mediates the autophagic protein degradation, and its accumulation inversely related with autophagic activity [46]. In this study, p62 level was found to be significantly decreased after TBI, while FGF2 could reverse this change. These results suggested that FGF2 could act as an autophagy inhibitor in TBI management.

Autophagy takes an important part in many neurological conditions, including the secondary damage after TBI. Appropriate autophagy can eliminate the aberrant cell components and protein aggregates, so as to maintain cellular homeostasis [14]. In some situations such as subarachnoid

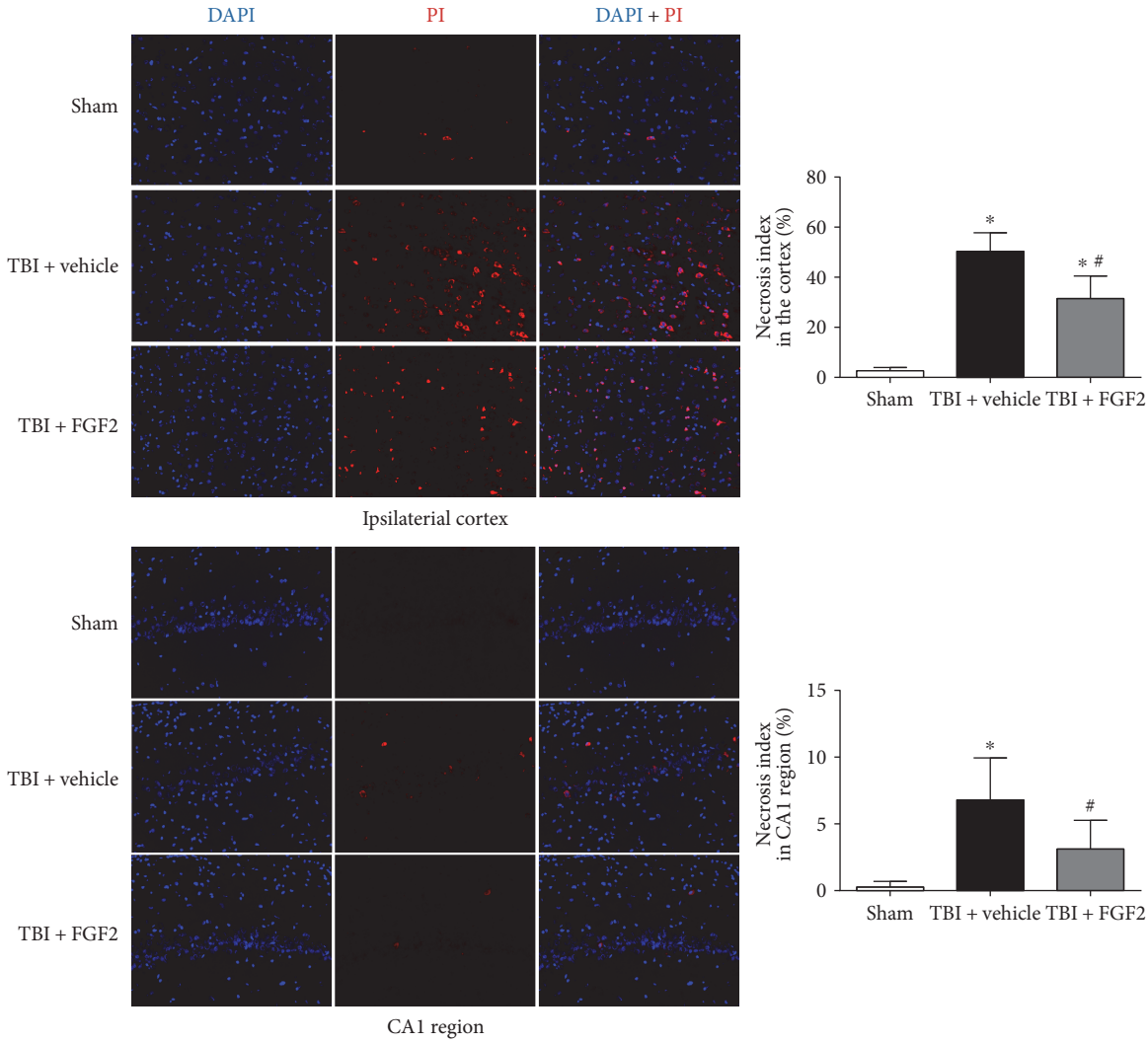

Figure 6: PI-positive cells in the ipsilateral cortex 6 h post-TBI in different groups. (a) Representative microphotographs showed the colocalization of DAPI (blue) with PI-positive (red) cells in the ipsilateral cortex and CA1 region 6 h post-TBI. *Scale bar* = 100 μm. (b) The quantification of the PI-positive cells as the necrosis index as a percent of the total DAPI+ cells. The *bars* represent the mean ± SD. $n = 6$. $^{*}P < 0.05$ versus sham and $^{\#}P < 0.05$ versus TBI + vehicle.

hemorrhage, autophagy can play a neuroprotective role and activation of autophagy may protect cortical cells against apoptosis [47]. In TBI, however, whether inducing or inhibiting autophagy can result in neuroprotection is still controversial. On the one hand, the autophagy activator rapamycin or melatonin can protect the brain from TBI-induced damage [13, 48], but on the other hand, the autophagy inhibitor 3-MA or resatorvid could also have similar neuroprotective effects [15, 16]. In the present study, FGF2 could inhibit autophagy and at the same time promoting neurological function, alleviating brain edema, reducing lesion volume, and promoting neuronal survival. This result tallied with previous studies about the autophagy inhibitor 3-MA's neuroprotective effects after TBI [15]. Although the autophagy activator rapamycin may exert a neuroprotective effect after TBI, the results in our experiment showed that coadministration of FGF2 and rapamycin cannot exert a synergistic effect. On the contrary, rapamycin could reverse the benefit effects of FGF2. This result may be related with the dose of rapamycin, and worth further study.

Apoptosis and necrosis are the two major ways of cell death after TBI, and both of them have been linked to autophagy, playing either a prosurvival or prodeath role [49]. Autophagy and apoptosis are the two main types of programmed cell death, and there are closed relations between autophagy and apoptosis. Beclin-1, the essential mediator of autophagy tested in this study, could also be inhibited by antiapoptotic proteins Bcl-2 or Bcl-xl [50, 51]. In addition, autophagy-related gene 5 (Atg5) could increase the susceptibility to apoptotic stimuli [52], and caspase-mediated cleavage of Atg5 and Beclin-1 could switch autophagy into apoptosis [49]. In our study, pretreatment with FGF2 could decrease the number of apoptotic cells and plasma membrane-disrupted cells in injured cortex. These results

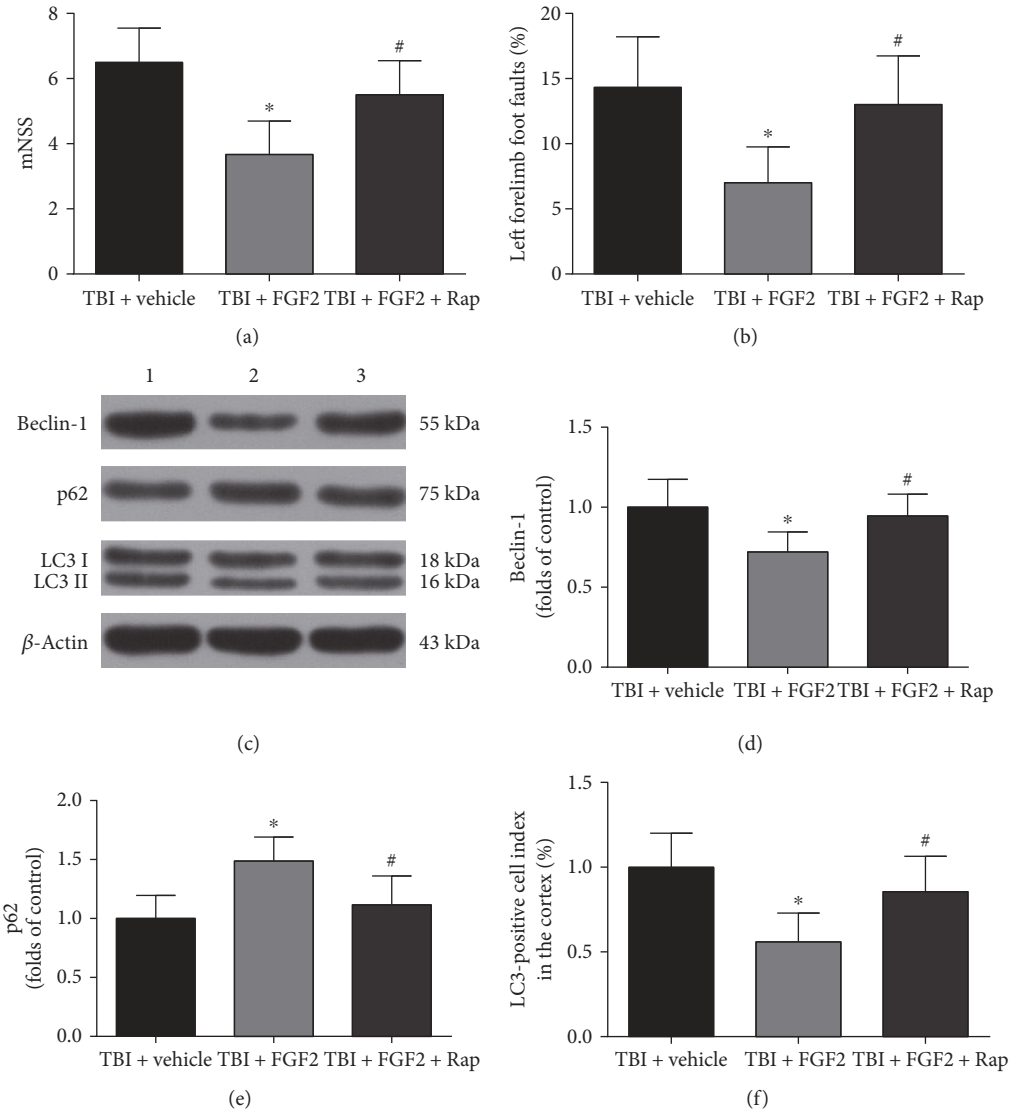

Figure 7: Rapamycin reversed the beneficial effects of FGF2 on neurological function and changed the expression of autophagy-related proteins in the ipsilateral cortex 48 h post-TBI. (a, b) The quantification of mNSS and the percentage of left forelimb foot faults at 48 h after TBI induction. The *bars* represent the mean ± SD. $n = 24$. $^{*}P < 0.05$ versus TBI + vehicle and $^{\#}P < 0.05$ versus TBI + FGF2. (c) Representative Western blots showing levels of Beclin-1, p62, and LC3 in the ipsilateral cortex 48 h post-TBI. Lane 1, TBI + vehicle group; lane 2, TBI + FGF2 group; lane 3 TBI + FGF2 + Rap group. (d, e) The relative band densities of Beclin-1 and p62. The densities of the protein bands were analyzed and normalized to β-actin. The data are expressed as a percentage of the sham group. The *bars* represent the mean ± SD. $n = 6$. $^{*}P < 0.05$ versus TBI + vehicle, $^{\#}P < 0.05$ versus TBI + FGF2. (f) The band density ratio of LC3 II to LC3 I. The *bars* represent the mean ± SD. $n = 6$. $^{*}P < 0.05$ versus TBI + vehicle and $^{\#}P < 0.05$ versus TBI + FGF2.

indicate that FGF2 could alleviate autophagy, apoptosis, and necrosis thus protect cells from various forms of death, at least in rat mild TBI models. Further studies are needed to explore the crosstalk among autophagy, apoptosis, and necrosis after TBI.

In this study, we explored the neuroprotective effects of FGF2 in the early stage of rat mild TBI and discussed the potential mechanisms. Nevertheless, there are several limitations. First, FGF2 is not a specific inhibitor of autophagy, actually it might play neuroprotective roles through various ways such as alleviating blood-brain barrier disruption, as mentioned previously. However, at least, the inhibition of autophagy might take part in FGF2's antiapoptotic and antinecrotic effects. Second, FGF2 was administrated 1 h before TBI, which limited the translational relevance. Whether FGF2 could exert the same effects when delivered after TBI needs further study. Finally, we only investigated the role of FGF2 on autophagy in the first 48 h after TBI, whether FGF2's effect of inhibiting autophagy is associated with its main function of stimulating neural stem/progenitor cell proliferation and differentiation still requires further research.

In summary, the results of this study indicated that FGF2 plays a neuroprotective role in the first 48 h of rat mild TBI

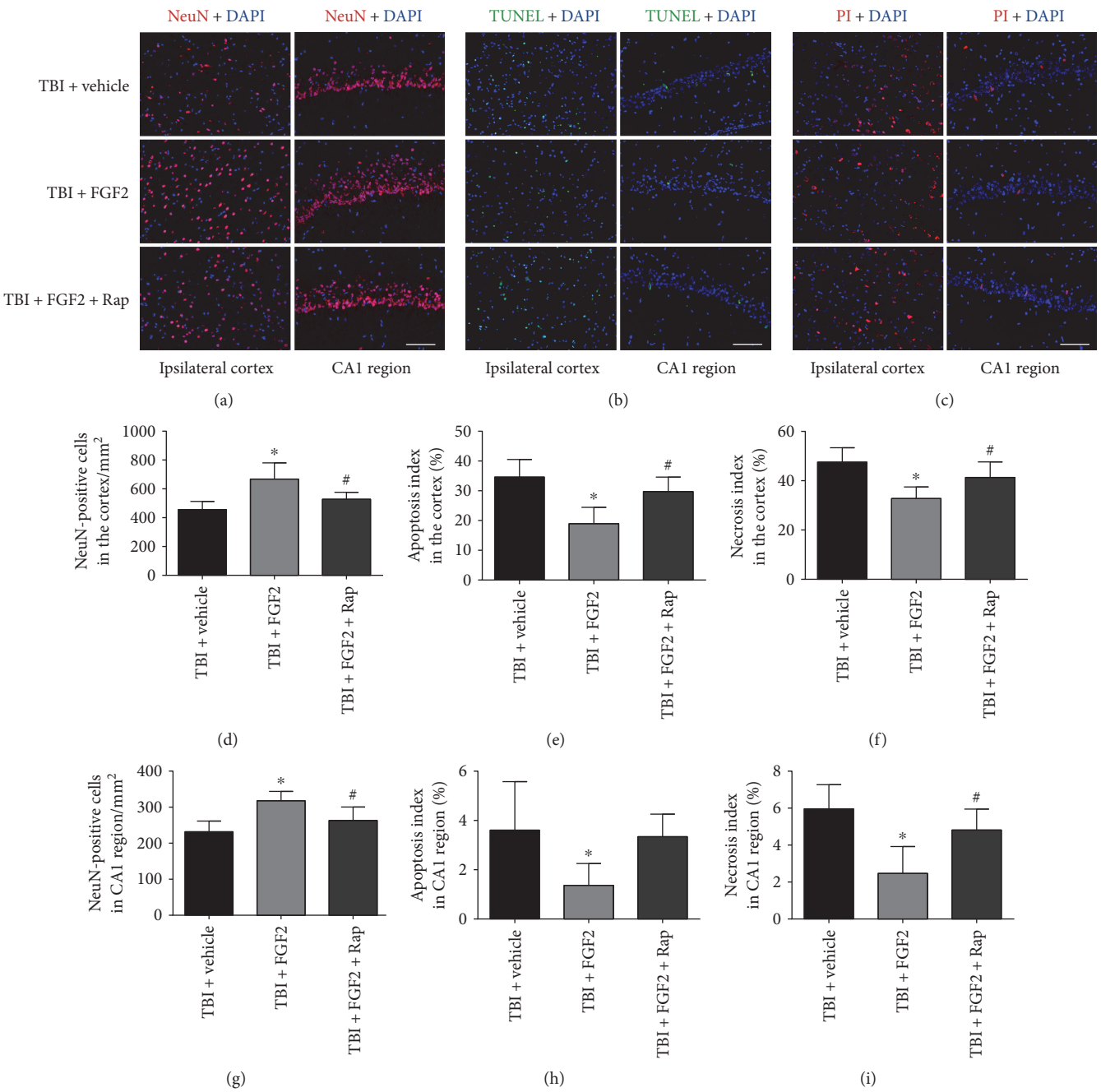

Figure 8: Rapamycin reversed the beneficial effects of FGF2 on neuronal survival and cell death after TBI. (a–c) Representative images of the ipsilateral cortex (left) and CA1 region of the ipsilateral hippocampus (right) on NeuN-stained, TUNEL-stained, and PI-stained coronal sections from each group 48 h post-TBI. *Scale bar* = 100 μm. (d–i) Quantitative analysis of the numbers of NeuN-positive, TUNEL-positive, and PI-positive cells in the ipsilateral cortex and CA1 region of the ipsilateral hippocampus 48 h post-TBI in each group. The *bars* represent the mean ± SD. $n = 6$. $^{*}P < 0.05$ versus TBI + vehicle and $^{\#}P < 0.05$ versus TBI + FGF2.

through alleviating brain edema and neurological deficits. Pretreatment with FGF2 could also protect cells from various forms of death such as apoptosis or necrosis through inhibition of autophagy. This study extended our understanding of FGF2's neuroprotective effects after TBI. As FGF2 can both attenuate cell death in the early stage after TBI and stimulate neural regeneration and functional recovery later, it may be a promising pharmacological intervention in TBI management.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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Research Article

Maternal Sevoflurane Exposure Causes Abnormal Development of Fetal Prefrontal Cortex and Induces Cognitive Dysfunction in Offspring

Ruixue Song, Xiaomin Ling, Mengyuan Peng, Zhanggang Xue, Jing Cang, and Fang Fang

Department of Anesthesiology, Zhongshan Hospital, Fudan University, 180 Fenglin Rd, Shanghai 200032, China

Correspondence should be addressed to Jing Cang; cangjing_zs@sina.com and Fang Fang; jerryfang81@hotmail.com

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Maternal sevoflurane exposure during pregnancy is associated with increased risk for behavioral deficits in offspring. Several studies indicated that neurogenesis abnormality may be responsible for the sevoflurane-induced neurotoxicity, but the concrete impact of sevoflurane on fetal brain development remains poorly understood. We aimed to investigate whether maternal sevoflurane exposure caused learning and memory impairment in offspring through inducing abnormal development of the fetal prefrontal cortex (PFC). Pregnant mice at gestational day 15.5 received 2.5% sevoflurane for 6 h. Learning function of the offspring was evaluated with the Morris water maze test at postnatal day 30. Brain tissues of fetal mice were subjected to immunofluorescence staining to assess differentiation, proliferation, and cell cycle dynamics of the fetal PFC. We found that maternal sevoflurane anesthesia impaired learning ability in offspring through inhibiting deep-layer immature neuron output and neuronal progenitor replication. With the assessment of cell cycle dynamics, we established that these effects were mediated through cell cycle arrest in neural progenitors. Our research has provided insights into the cell cycle-related mechanisms by which maternal sevoflurane exposure can induce neurodevelopmental abnormalities and learning dysfunction and appeals people to consider the neurotoxicity of anesthetics when considering the benefits and risks of nonobstetric surgical procedures.

1. Introduction

Advances in prenatal imaging and innovations in surgical techniques have resulted in a wide range of fetal interventions [1]. Because of the relatively long duration of such procedures and the necessity of general anesthesia, long-time inhalation of anesthetic such as sevoflurane is administered to help uterine quiescence and lower the premature birth risk. However, inhalation anesthetics could be powerful regulators of brain development and have been reported to contribute to detrimental behavioral deficits [2]. Several large cohort studies have investigated the neurotoxicity of anesthesia to the developing brain [3–5], but the data remain elusive. Recently, the "Drug Safety Communication" has issued a warning that general anesthesia used in pregnant women in their third trimester may affect the development of the children's brain [6]. Sevoflurane is one of the most prevalent inhalation anesthetics in nonobstetric surgeries. Although sevoflurane has smaller potency to cause neurotoxicity to the developing brain compared with other general anesthetic such as isoflurane [7], there were still some preclinical studies reported that sevoflurane could cause neurological deficits [8, 9]. While neurogenesis abnormality is thought to play a vital role [10–12], the concrete impact of sevoflurane on fetal brain development remains poorly understood.

Most studies on the sevoflurane-induced neurotoxicity have focused on the change in the development of the hippocampus [10, 13]. It is worth noting that the third trimester is a stage at which there are high levels of neurogenesis throughout the cortex and that the development of the prefrontal cortex (PFC), a seat of the highest-order cognitive functions, plays critical roles in the onset and development of many neurodevelopmental deficits [14]. Three main types of neural progenitors, neural stem cell, radial glial cell, and intermediate progenitor cell, have been identified to be involved in the proliferation and differentiation of the

PFC [15]. The neurogenesis of the PFC is accomplished by a regular production and migration of neurons in a deep to superficial order [16]. Former *in vitro* studies have shown that self-renewal capacity and the subsequent differentiation of neural progenitors could be disturbed by sevoflurane [12, 17]. Our earlier study has also shown a significant proliferation inhibition in neural progenitors after sevoflurane exposure [10]. Cell cycle dynamics, including the progression and exit of cell cycle, is important in cell fate decisions during neurogenesis [18]. Our former study has found that sevoflurane could lead to postoperative cognitive dysfunction in aged mice through interfering cell cycle dynamics in neurons [19].

All of the knowledge mentioned above prompted us to determine whether the sevoflurane-induced neurotoxicity could be attributed to the cell cycle-related abnormality in the development of the fetal PFC. Thus, we hypothesized that maternal sevoflurane exposure may disturb the differentiation and proliferation of neural progenitors by interfering the cell cycle dynamics, which finally lead to learning deficits in offspring. Our results demonstrated that maternal sevoflurane exposure induced cell cycle arrest in neural progenitors of the fetal PFC, lead to decrease in neuronal output and inhibition in neural progenitor replication, and finally resulted in learning deficits in offspring.

2. Materials and Methods

2.1. Mice Anesthesia. All procedures were approved by the Animal Care and Use Committee of Fudan University and followed institutional guidelines. Four-month old C57BL/6J female mice were mated with four-month old C57BL/6J male mice, and the pregnant mice were housed individually after identified. All of the animals were raised in a temperature-controlled (22°–23°C) room under a 12 h light/dark period; water and standard mouse chow were available ad libitum. The pregnant mice were randomly assigned to a control group or a sevoflurane group at gestational day 15.5 (G15.5). Pregnant mice in the sevoflurane group received 2.5% sevoflurane in 100% oxygen for 6 h in an anesthetizing box, while the pregnant mice in the control group received 100% oxygen for 6 h. The size of the anesthetizing box was $20 \times 20 \times 20\,\text{cm}^3$. The gas flow rate was 2 L/min in the first 5 min for induction and then 1 L/min for maintenance. The concentrations of sevoflurane and oxygen were continuously monitored with a gas analyzer (Drager Inc.). Sevoflurane anesthesia was discontinued by terminating sevoflurane supply. The mortality rate was <1% in the present study.

2.2. Morris Water Maze (MWM) Test. The MWM test was performed as described in our former study [10]. For grouping, the male offspring at postnatal day 30 were delivered to the same group as their mothers. Specifically, the offspring were tested in the MWM four trials per day for five consecutive days (from P30 to P34). Each mouse was given 60 s to search the platform. The platform was then removed at P35, and the mice were placed in the opposite quadrant to swim for 60 s. The swimming speed, escape latency, platform crossing times, and the percentage of time target quadrant were recorded with a video tracking system (Shanghai Jiliang Software Technology Co. Ltd., China). All the mice were dried under a heat lamp for 5–8 min after each trail.

2.3. Measurement of Proliferation, Cell Cycle Exit, and S-Phase Duration of Neural Progenitors with Bromodeoxyuridine (BrdU) and Iododeoxyuridine (IdU). For the determination of proliferation, pregnant mice were injected i.p. with a single BrdU (Sigma, B5002) dose (50 mg/kg of body weight) at the start of experiment and the pregnant mice were sacrificed 6 h later (at the end of the sevoflurane/oxygen exposure). The percentage of proliferating cells was calculated as $\text{BrdU}^+/\text{DAPI}$ [10]. For cell cycle exit assay, the pregnant mice were also injected i.p. with the same dose of BrdU at the start of experiment but sacrificed 18 h later. Cortices from embryos that had been labelled with BrdU for 18 h were visualized for both Ki67 reactivity and BrdU incorporation. Those neural progenitors that had divided in the previous 18 h and subsequently exited the cell cycle would have taken up BrdU but would not stain for Ki67; therefore, the evaluation of $\text{BrdU}^+\text{Ki67}^-/\text{total BrdU}^+$ can serve as an effective detection method for cell cycle exit [20]. To determine the S-phase duration, pregnant mice at G15.5 were injected i.p. with an IdU (Sigma, I7125) dose (50 mg/kg of body weight) 4 h after the start of the sevoflurane/oxygen exposure, followed by BrdU injection (50 mg/kg of body weight) 1.5 h later. The pregnant mice were then killed at the end of the 6 h sevoflurane/oxygen exposure (0.5 h after BrdU injection), and the embryos were processed to immunofluorescence to reveal IdU/BrdU. The length of S-phase (T_s) was calculated with the following paradigm described by Quinn et al. [21]: the number of cells labeled with IdU but not BrdU was regarded as L_{cells}, referring to the cells that have taken up IdU but left S-phase and failed to take up BrdU during the interval between IdU and BrdU injection ($T_i = 1.5$ h). The number of cells labeled with BrdU is designated S_{cells}. Then T_s can be calculated with the following formula: $T_s/T_i = S_{\text{cells}}/L_{\text{cells}}$.

2.4. Immunofluorescence. A cesarean section was performed to extract the embryos, and the fetal brains were then fixed overnight in 4% paraformaldehyde. For cryosectioning, fixed brains were equilibrated in 20% (wt/vol) sucrose in PBS followed by 30% sucrose in PBS overnight at 4°C. Brains were then embedded with Tissue-TEK (O.C.T., Sakura Finetek) and cryosectioned at 12 μm. For immunofluorescence, the cryosections were first washed with PBS and then incubated with blocking solution (10% goat serum in PBS, 0.03% Triton X-100) for 2 h at 37°C. Sections were next incubated with primary antibodies diluted in blocking solution overnight at 4°C. The tissues were then washed in PBS and incubated with appropriate secondary antibodies for 1 h at room temperature. Cell nucleus was counterstained with DAPI (Sigma, 1 : 1000). For BrdU and IdU detection, an additional antigen retrieval step was performed before blocking by using HCl (2 N HCl, 15 min incubation at 37°C) [10]. The following primary antibodies were used: Tbr1 (Abcam, ab31940, 1 : 200), Satb2 (Abcam, ab51502, 1 : 200), NeuN (Abcam, ab104224, 1 : 200), GFAP (Abcam, ab10062, 1 : 200), BrdU only (Abcam, ab6326, 1 : 1000), Ki67 (Abcam, ab16667, 1 : 500), caspase-3 (Abcam, ab13847, 1 : 200), nestin (Abcam,

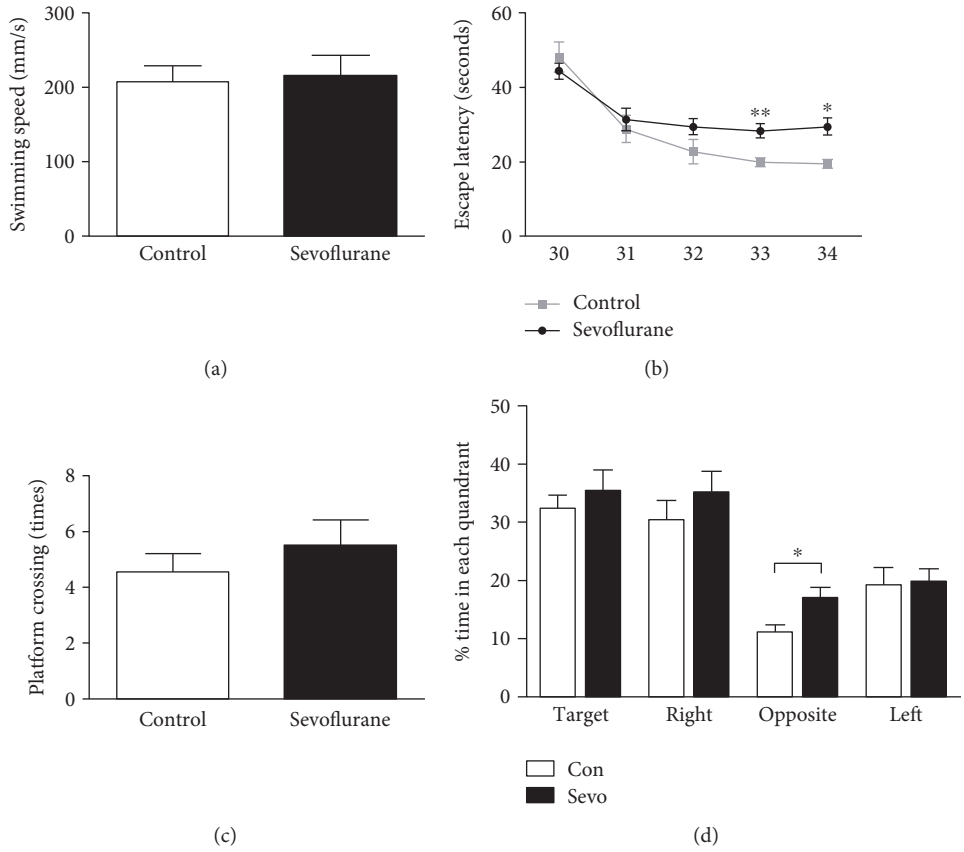

Figure 1: Maternal sevoflurane exposure impaired learning and memory ability in offspring. (a) No significant differences in swimming speed were found between the control group and the sevoflurane group. (b) The escape latency of MWM in the sevoflurane group was longer than that in the control group. (c) No significant differences in platform crossing times were found between the control group and the sevoflurane group. (d) The %time in the opposite quadrant in the sevoflurane group was longer than that in the control group. Data are expressed as the mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$.

ab6142, 1 : 200), Pax6 (Abcam, ab5790, 1 : 200), Tbr2 (Abcam, ab23345, 1 : 200), BrdU and IdU (BD Biosciences, 347580, 1 : 200), Ccnd1 (Abcam, ab6134175, 1 : 200), and PH3 (Abcam, ab5176, 1 : 200). Secondary antibodies used were goat anti-mouse Alexa Fluor 488, goat anti-mouse Alexa Fluor 594, goat anti-rat Alexa Fluor 594, and goat anti-rabbit Alexa Fluor 488 (all from Abcam, diluted at 1 : 200).

2.5. Image and Cell Count. Immunofluorescence analysis was performed on data collected from cortexes of at least 3 ($n \geq 3$) embryos of each group. Fluorescence images were acquired using a Leica TCS SP2 confocal microscope, and all images showing the target parameters for the control group versus the sevoflurane group were acquired with the same settings during each microscope session. Cells were counted in four 100 μm-wide strips through the prefrontal cortex, in a minimum of three nonadjacent sections from each embryo, with the image J pro plus software.

2.6. Statistical Analysis. Values are presented as means ± SEM. Two-way ANOVA with repeated measurements was used to analyze the difference of escape latency in the MWM test, and the Bonferroni method was used to adjust the multiple comparisons. Two-tailed Student's t-test was performed for statistical evaluation of immunofluorescence. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Maternal Sevoflurane Exposure Impaired Spatial Learning and Memory Ability in Offspring. All of the pregnant mice delivered offspring at G20.5–G22.5, and the offspring were reared for 30 days before being assigned to the MWM test. There was no statistical significance in the swimming speed between the control group and the sevoflurane group (Figure 1(a)) ($n = 9$, $F = 1.590$, and $P = 0.525$), which excluded the possibility that the learning changes observed in the current study were influenced by sensorimotor disturbances. A two-way ANOVA with repeated measurement on the escape latency (the time that each mouse took to reach the platform) revealed a statistical interaction between the time and the group (Figure 1(b)) ($n = 9$, $F = 7.740$, and $P = 0.013$) in the cued trials. Specially, the offspring in the sevoflurane group had significantly longer escape latency compared with those in the control group at P33 and P34 (Figure 1(b)) ($P = 0.001$ and 0.013, resp.). Moreover, we found that %time in the opposite quadrant was longer in

the sevoflurane group compared to the control group in the probe test at P35 ($n = 9$, $F = 2.417$, $P = 0.025$). However, we found no significant difference in platform crossing times (Figure 1(c)) ($n = 9$, $F = 2.250$, and $P = 0.423$). These data indicated that maternal sevoflurane exposure impaired spatial learning and memory ability in offspring.

3.2. Sevoflurane Decreased the Production of Deep-Layer Immature Neurons in the Fetal PFC. To investigate whether sevoflurane disturbs embryonic brain development, we first examined the numbers of neural cells in the fetal PFC. To distinguish between the upper-layer and deep-layer newborn neurons, we used the established Tbr1 to label layers V–VI immature neurons and Satb2 to label layers II–IV immature neurons [22]. We also selected NeuN and GFAP to identify mature neurons [23] and mature astrocytes [20], respectively. $Tbr1^+$ immature neurons in the fetal PFC were significantly decreased after sevoflurane exposure (Figures 2(b), 2(c), and 2(d)) ($n = 15$, $F = 1.369$, and $P = 0.001$) while there were no significant differences in the numbers of $Satb2^+$ immature neurons (Figures 2(b), 2(c), and 2(d)) ($n = 15$, $F = 1.106$, and $P = 0.789$), $NeuN^+$ mature neurons (Figures 2(e), 2(f), and 2(g)) ($n = 8$, $F = 6.278$, and $P = 0.406$), and $GFAP^+$ mature astrocytes (Figures 2(h), 2(i), and 2(j)) ($n = 6$, $F = 1.656$, and $P = 0.796$). Together, these data showed that maternal sevoflurane exposure decreased the generation of deep-layer immature neurons.

3.3. Sevoflurane Suppressed the Proliferation of the Fetal PFC. Reduced numbers of deep-layer $Tbr1^+$ immature neurons could result from reduced proliferation or increased apoptosis of the fetal PFC, so we next investigated the influence of sevoflurane on these processes. The fetal PFC of the sevoflurane group showed reduced numbers of $Ki67^+$ (Figures 3(b), 3(c), and 3(d)) ($n = 6$, $F = 7.115$, and $P = 0.0001$) and $BrdU^+$ neural cells (Figures 3(e), 3(f), and 3(g)) ($n = 6$, $F = 1.111$, and $P = 0.0001$), emphasizing the inhibition of proliferation. However, we have not detected any differences in the number of cells undergoing apoptosis between the two groups as judged by staining with caspase-3 (Figures 3(h), 3(i), and 3(j)) ($n = 5$, $F = 1.817$, and $P = 0.809$).

3.4. Sevoflurane Inhibited the Expansion of Neural Progenitors in the Fetal PFC. Reduced labeling index of Ki67 and BrdU in the PFC suggested a decrease of neural progenitor pool after sevoflurane exposure, so we further performed nestin, Pax6, and Tbr2 immunostaining to label neural stem cell, radial glial cell, and intermediate progenitor cell, respectively [15]. We found a weak and sparse staining of nestin (Figures 4(b), 4(c), and 4(d)) ($n = 8$, $F = 2.728$, and $P = 0.003$), Pax6 (Figures 4(e), 4(f), and 4(g)) ($n = 6$, $F = 1.649$, and $P = 0.001$), and Tbr2 in the sevoflurane group (Figures 4(h), 4(i), and 4(j)) ($n = 4$, $F = 3.209$, and $P = 0.025$), which indicated that the neural progenitor abundance in the fetal PFC was inhibited after maternal sevoflurane exposure.

3.5. Sevoflurane Decreased Cell Cycle Exit and Increased S-Phase Duration of Neural Progenitors in the Fetal PFC. Since cell cycle dynamics could affect the proliferation and differentiation of the developing brain [24], we postulated sevoflurane-induced reduction in neural progenitor proliferation and newborn neuron production could reflect cell cycle dysregulation. With the assessment of cell cycle exit, we found that the fetal PFCs in the sevoflurane group contained significantly less Ki67-negative neural progenitors that had incorporated BrdU in the previous 18 h (Figures 5(b), 5(c), and 5(d)) ($n = 13$, $F = 1.029$, and $P = 0.0001$). Moreover, our double-labeling experiments of IdU/BrdU revealed a significant increase in the S-phase duration after sevoflurane exposure (Figures 5(f), 5(g), and 5(h)) ($n = 15$, $F = 19.63$, and $P = 0.004$). Taken together, our data indicated that sevoflurane decreased cell cycle exit and increased the S-phase duration of neural progenitors in the fetal PFC.

3.6. Sevoflurane Did Not Influence the Duration of G1-, M-, and G2-Phases of Neural Progenitors in the Fetal PFC. In addition to cell cycle exit and S-phase duration, the dysregulation of G1, M, and G2 may also alter cell fate in the developing brain [25]. In the current study, no significant differences were found between the control group and the sevoflurane group when analyzing the proportion of neural progenitors that expressed the Ccnd1, a cyclin expressing from mid-G1 to late G1 (Figures 6(b), 6(c), and 6(d)) ($n = 8$, $F = 2.073$, and $P = 0.670$). PH3, a specific indicator of late G2- and M-phases, has been used to investigate the duration of G2- and M-phases in neural progenitors [26, 27]. We did not found any significant difference in the expression of PH3 (Figures 6(e) and 6(f)) ($n = 10$, $F = 1.744$, and $P = 0.420$). These data have indicated that sevoflurane did not influence the duration of G1-, M-, and G2-phases of neural progenitors in the fetal PFC.

4. Discussion

In this study, we evaluated the *in vivo* toxic effects of maternal sevoflurane exposure via investigating the learning ability of offspring and the development of the fetal PFC. Our data indicated that maternal gestational exposure to sevoflurane was associated with increased risk for learning deficits in offspring. The sevoflurane neurotoxicity may be due to the decrease in cell cycle exit and increase in the S-phase duration of neural progenitors, which consequently lead to proliferation inhibition and differentiation abnormality in the fetal PFC.

Anesthetics can be toxic to brain development, and the vulnerability mainly depends on three factors: the stage of brain development and the concentration and duration of the exposure [28]. In human, maternal and fetal procedures are usually performed in the second or early third trimester, a critical time for the proliferation and differentiation of the fetal brain [29]. From a developmental perspective, these processes in rodents, unlike human beings, begin from the middle of the second trimester and continues to the time of birth [29]. In the present study, we chosen the pregnant mice in early third trimester (G15.5) to study the neurotoxicity of sevoflurane. While low concentration of sevoflurane such as 1.5% has been reported to do no harm to the brain development [30], our previous study [10] has found 2.5% sevoflurane could lead to learning deficits in offspring. Additionally, a

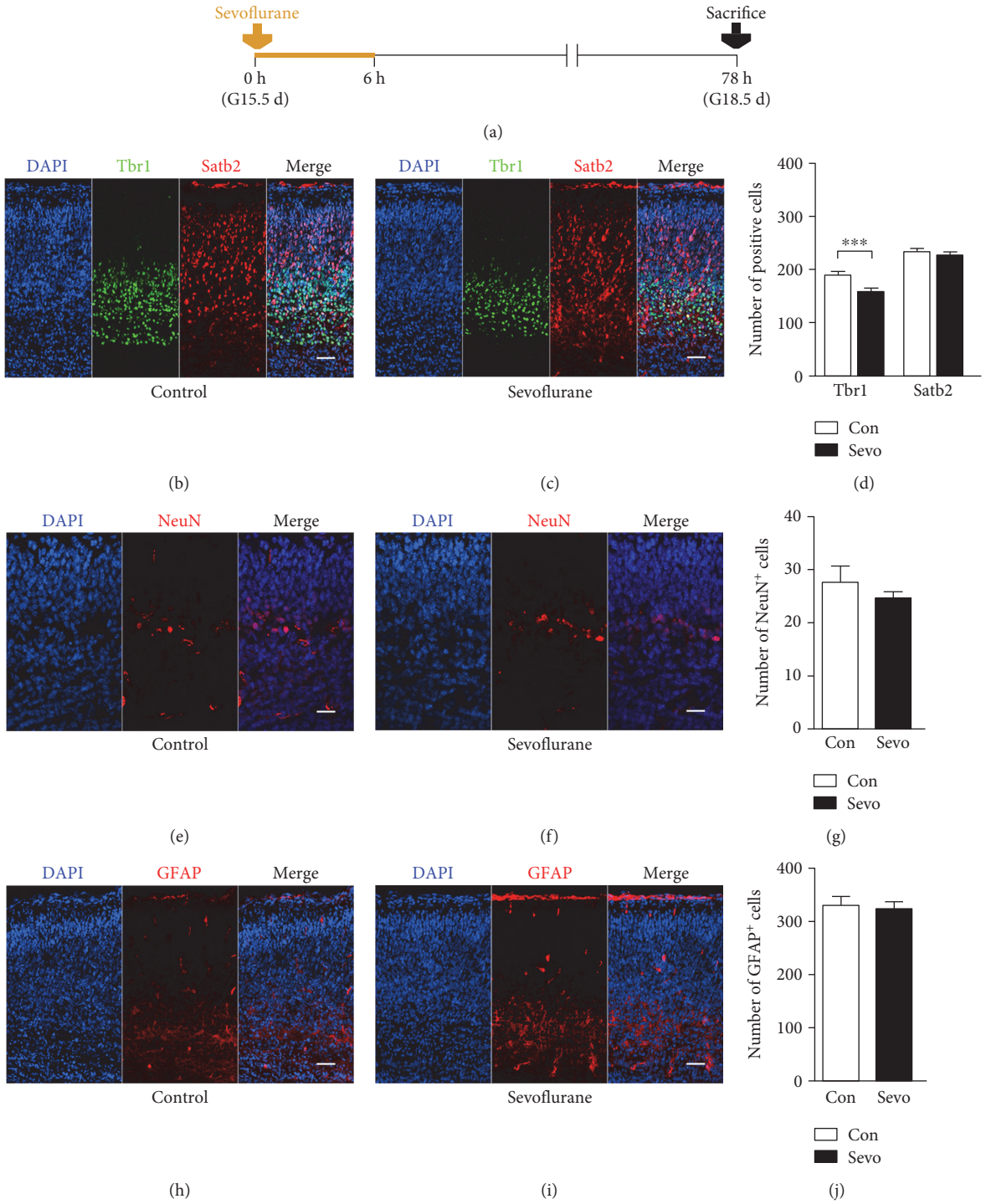

Figure 2: Maternal sevoflurane exposure decreased the production of deep-layer immature neurons. (a) Schematic diagram of the timing of sevoflurane exposure and sacrifice to assess the differentiation in the fetal PFC. (b, c) Tbr1 (green) and Satb2 (red) immunofluorescence, combined with DAPI staining (blue) in the cortical plate at G18.5. Scale bars, 20 μm. (d) Quantification of the Tbr1$^+$ and Satb2$^+$ cells of the control and sevoflurane groups. (e, f) NeuN (red) immunofluorescence and DAPI staining (blue) in the cortical plate at G18.5. Scale bars, 20 μm. (g) Quantification of the NeuN$^+$ cells of the control and sevoflurane groups. (h, i) GFAP (red) immunofluorescence and DAPI staining (blue) in the cortical plate at G18.5. Scale bars, 20 μm. (j) Quantification of the GFAP$^+$ cells of the control and sevoflurane groups. Data are expressed as the mean ± SEM. $^{***}P < 0.001$.

prolonged exposure of sevoflurane such as 6 h has been reported to suppress the proliferation of neural progenitors [17] and caused learning impairments in offspring [8]. Therefore, the pregnant mice of the sevoflurane group in the current study were exposed to 2.5% sevoflurane for 6 h to study the neurodevelopmental change of the fetal brain.

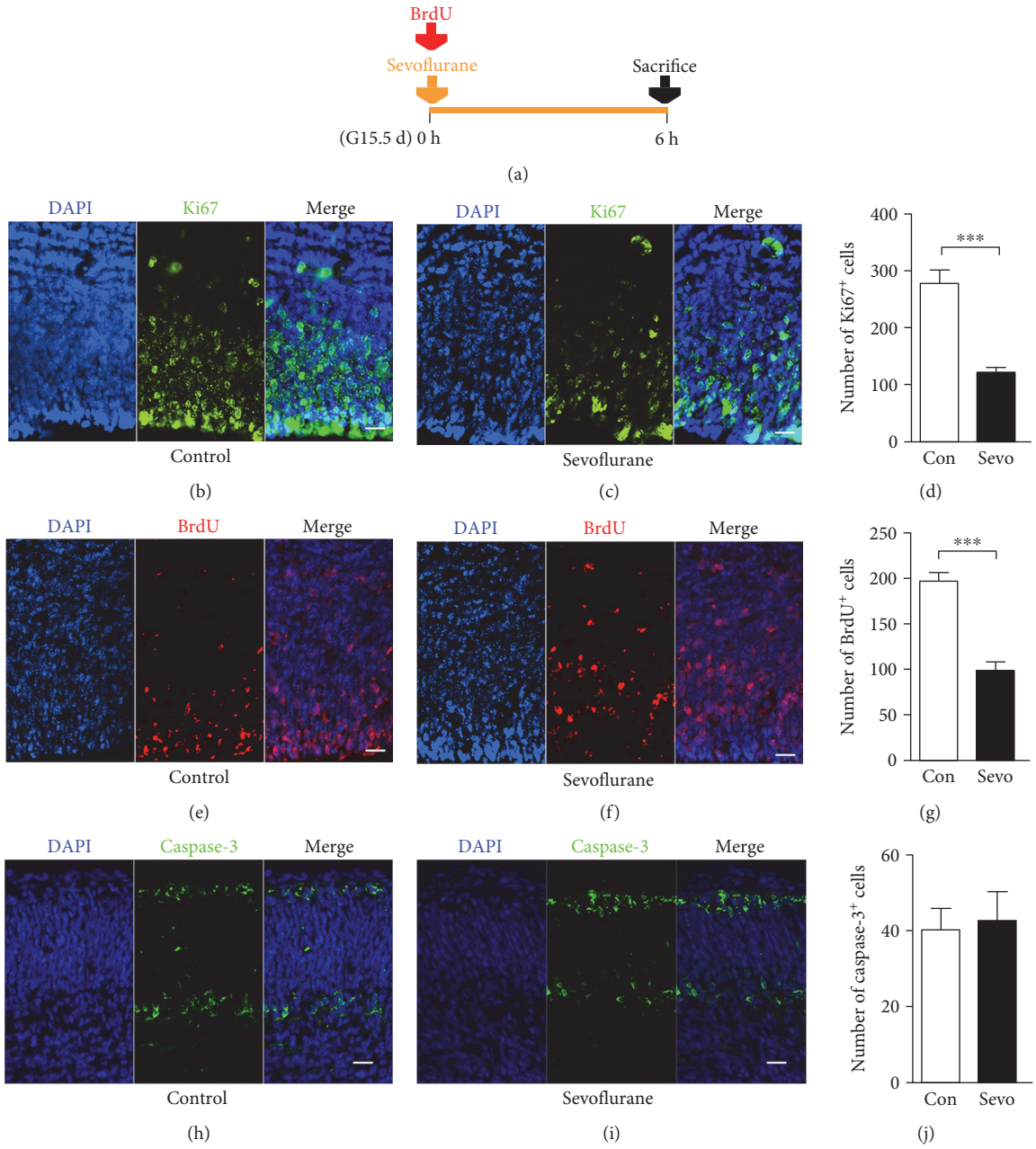

Figure 3: Maternal sevoflurane exposure suppressed the proliferation of the fetal PFC. (a) Schematic diagram of the timing of sevoflurane exposure, BrdU injection, and sacrifice to assess the proliferation and apotosis of the fetal PFC. (b, c) Ki67 (green) immunofluorescence and DAPI staining (blue) in the cortical plate at G15.5. Scale bars, 20 μm. (d) Quantification of the Ki67$^+$ cells of the control and sevoflurane groups. (e, f) BrdU (red) immunofluorescence and DAPI staining (blue) in the cortical plate at G15.5. Scale bars, 20 μm. (g) Quantification of the BrdU$^+$ cells of the control and sevoflurane groups. (h, i) Caspase-3 (green) immunofluorescence and DAPI staining (blue) in the cortical plate at G15.5. Scale bars, 20 μm. (j) Quantification of the GFAP$^+$ cells of the control and sevoflurane groups. Data are expressed as the mean ± SEM. $^{***}P < 0.001$.

The Morris water maze is a reliable method for assessing the ability of learning and memory [31]. In this test, spatial learning ability is determined by the escape latency in cued trials and reference memory is assessed with preference for the platform area in the probe test [31]. We have found a significant increase of the averaged escape latency in the offspring of the sevoflurane group (Figure 1(b)), indicating the impairment in spatial learning ability after prenatal sevoflurane exposure. In analyzing the probe test, we did not find any significant differences in the platform crossing times and the %time in the target, right and left adjacent quadrants (Figures 1(c) and 1(d)). However, we have found that the %time in the opposite quadrant was longer in the sevoflurane group than in the control group (Figure 1(d)), indicating that maternal sevoflurane exposure could lead to memory impairment in the offspring. Our finding was

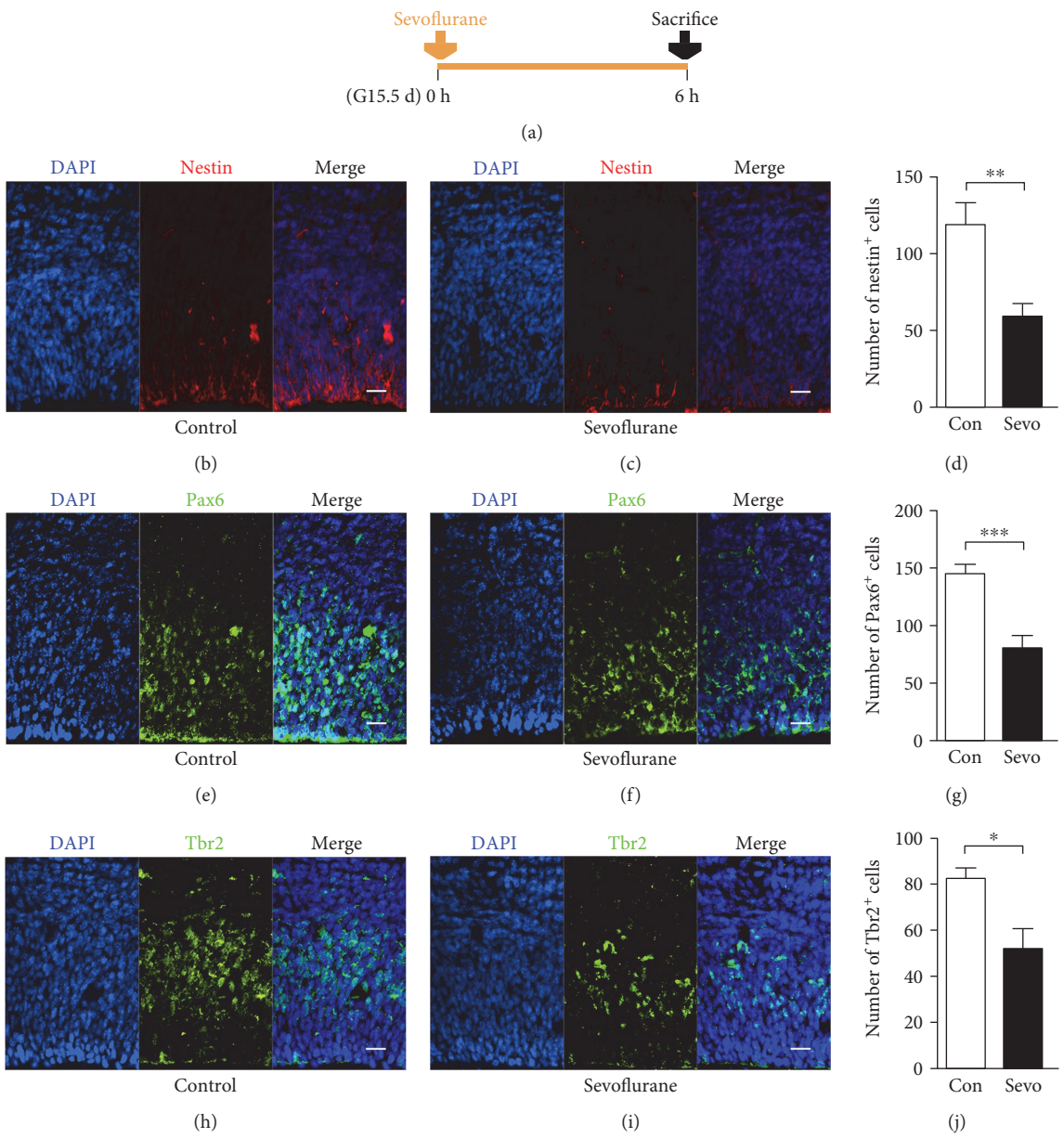

Figure 4: Maternal sevoflurane exposure inhibited the expansion of neural progenitors in the fetal PFC. (a) Schematic diagram of the timing of sevoflurane exposure and sacrifice to assess the abundance of neural progenitors. (b, c) Nestin (red) immunofluorescence and DAPI staining (blue) in the cortical plate at G15.5. Scale bars, 20 μm. (d) Quantification of the nestin$^+$ cells of the control and sevoflurane groups. (e, f) Pax6 (green) immunofluorescence and DAPI staining (blue) in the cortical plate at G15.5. Scale bars, 20 μm. (g) Quantification of the Pax6$^+$ cells of the control and sevoflurane groups. (h, i) Tbr2 (green) immunofluorescence and DAPI staining (blue) in the cortical plate at G15.5. Scale bars, 20 μm. (j) Quantification of the Tbr2$^+$ cells of the control and sevoflurane groups. Data are expressed as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

consistent with prior studies reporting that sevoflurane anesthesia used in pregnant mice could affect the cognitive function in offspring [8, 10].

Learning is a highly dynamic process, and the PFC has been reported to play vital roles in this process [32]. Some mental diseases with symptoms of cognitive dysregulation are usually contributed to structural and pathophysiological abnormalities in the PFC [33]. A fundamental feature of fetal brain neurogenesis is that the positioning of neurons into vertical arrays specifies their functions [34]. As neurogenesis proceeds, newborn neurons migrate radially from the proliferative zone, past neurons generated earlier, settle in more outer layers, and finally form a six-layered cortex [34]. Upper layers (layers II–IV) are composed of late-born neurons while deep layers (layers V-VI) are of early-born neurons [35]. Dysregulation in the neurogenesis of the prefrontal cortex, such as incomplete clustering and abundance of newly generated neurons, has been reported in neurological disorders [36]. In the present study, the production of immature deep-layer neurons in the PFC, identified by

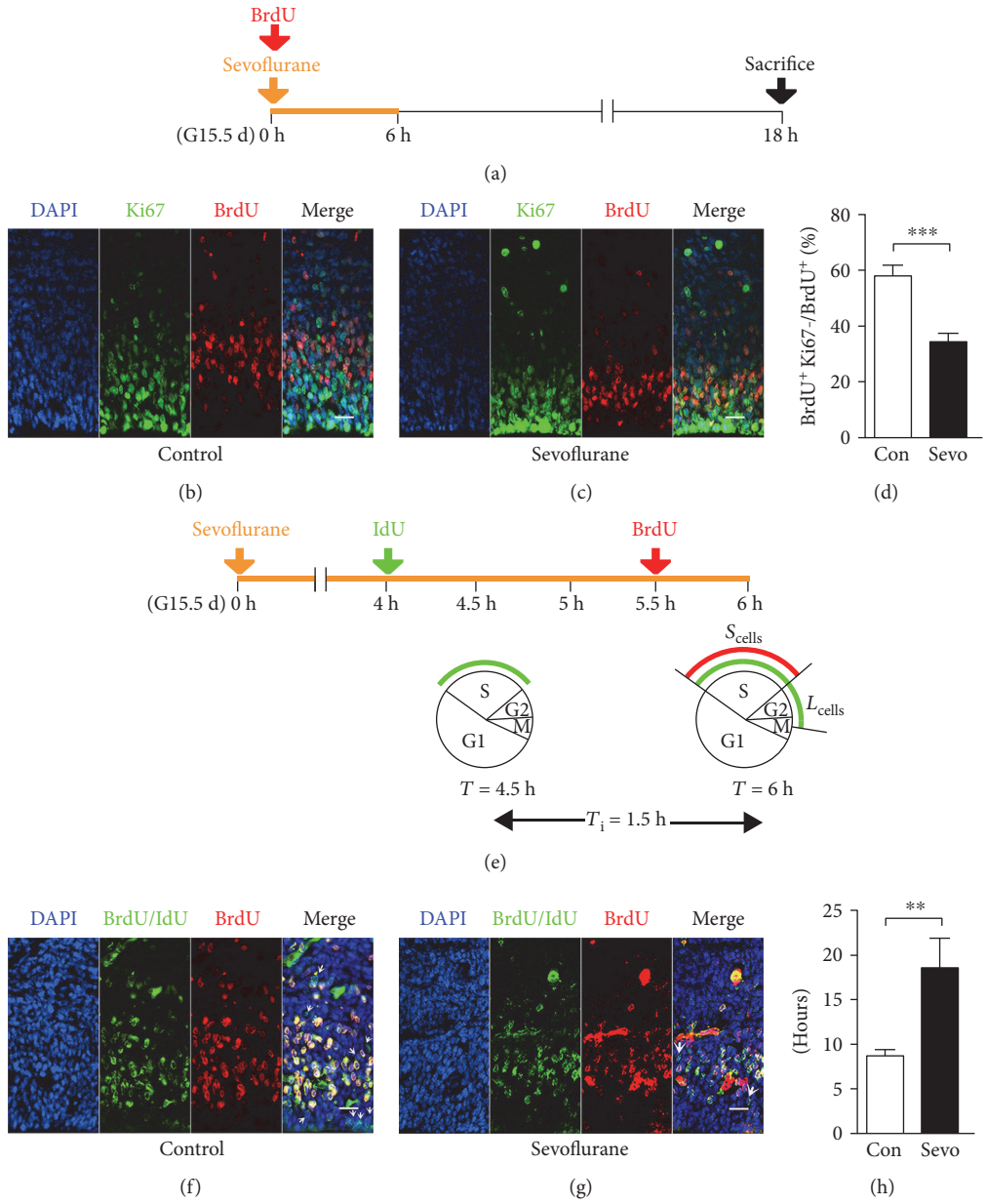

FIGURE 5: Maternal sevoflurane exposure decreased cell cycle exit and increased S-phase duration of neural progenitors in the fetal PFC. (a) Schematic diagram of the timing of sevoflurane exposure, BrdU injection, and sacrifice to assess the proportion of the cell cycle exit. (b, c) Coronal sections of the PFC from G16.5 mice were immunostained for BrdU (red) and Ki67 (green) at G16.5. Scale bars, 20 μm. (d) Numbers of $BrdU^+Ki67^-$ cells are expressed as the numbers of $BrdU^+$ cells. (e) Schematic diagram of the timing of sevoflurane exposure, IdU injection, and BrdU administration to assess the S-phase duration. S_{cells} = cells labeled with BrdU; L_{cells} = cells labeled with IdU but not BrdU. (f, g) Coronal section through the cortex of the G15.5 fetal brain immunostained with antibodies specific for both BrdU and IdU (green) and BrdU alone (red) to identify L_{cells} (green-only cells) and S_{cells} (red and green double-labeled cells). Arrowheads indicate L_{cells}. Scale bars, 20 μm. (h) Quantification of the length of S-phase in G15.5 embryos. Data are expressed as the mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$.

Tbr1, was significantly inhibited after sevoflurane exposure (Figures 2(b), 2(c), and 2(d)). Tbr1 is a transcription factor necessary for directing immature neurons to a glutamatergic phenotype, and the downregulation of $Tbr1^+$ neurons in the developing brain could lead to neurological disorders [37]. Therefore, the reduction in $Tbr1^+$ neuron production may partly attribute to the learning impairment in offspring after sevoflurane exposure. Satb2 is involved in specifying callosal projection neurons [38], and the results of Satb2 staining (Figures 2(b), 2(c), and 2(d)) may suggest that the callosal connectivity of the fetal brain was not significantly affected by prenatal sevoflurane exposure. Moreover, we have not found any significant differences in the number of mature neurons (identified by NeuN staining) and mature astrocytes

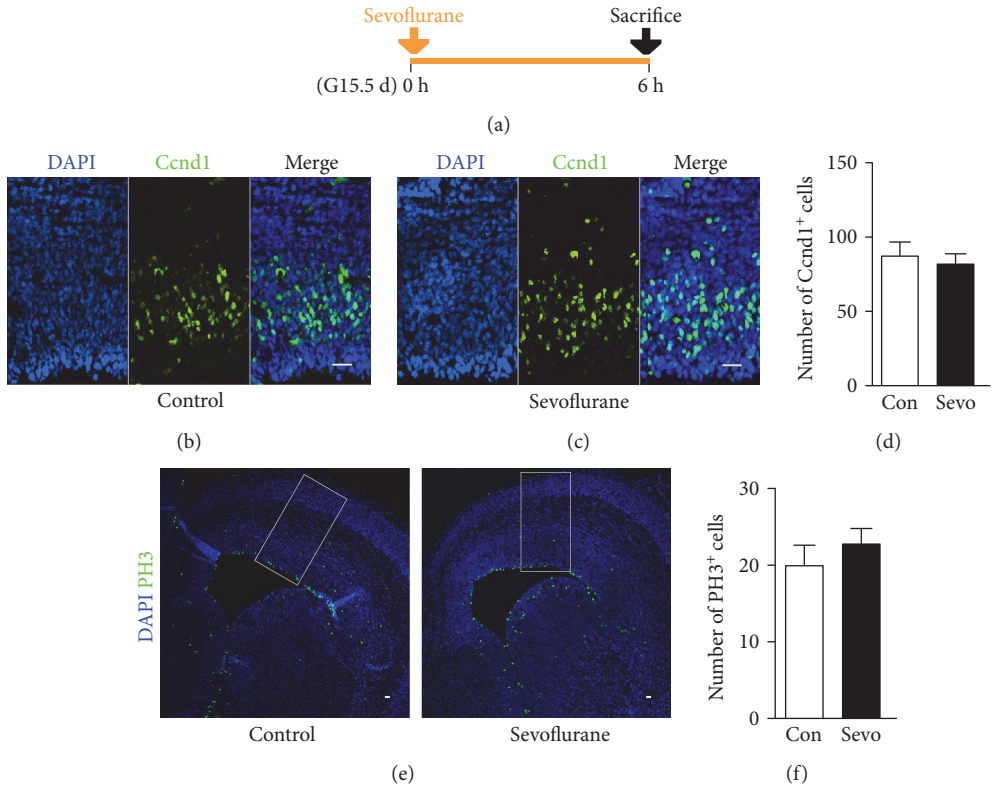

FIGURE 6: Maternal sevoflurane exposure did not influence the duration of G1-, M-, and G2-phases of neural progenitors in the fetal PFC. (a) Schematic diagram of the timing of sevoflurane exposure and sacrifice to assess the expression of Ccnd1 and PH3. (b, c) Ccnd1 (green) immunofluorescence and DAPI staining (blue) in the cortical plate at G15.5. Scale bars, 20 μm. (d) Quantification of the Ccnd1$^+$ cells of the control and sevoflurane groups. (e) PH3 (green) immunofluorescence and DAPI staining (blue) in the cortical plate at G15.5. PH3-positive cells were counted in radially arranged 200 mm-wide boxes, as illustrated. Scale bars, 20 μm. (f) Quantification of the PH3$^+$ cells of the control and sevoflurane groups. Data are expressed as the mean ± SEM.

(identified by GFAP staining) in the PFC (Figures 2(e), 2(f), 2(g), 2(h), 2(i), and 2(j)). Given that neural progenitors give rise to different types of neurons and glial cells according to the intrinsic time course [39], the sevoflurane exposure time of G15.5 selected in our study may be just at the particular temporal window when the deep-layer immature neurons emerge.

The decrease in the production of deep-layer immature neurons in the PFC may be attributed to decreased neurogenesis and/or increased neurodegeneration. In the present study, we clearly showed that sevoflurane decreased the number of BrdU-labeled and Ki67-positive cells in the PFC of fetal mice (Figures 3(b), 3(c), 3(d), 3(e), 3(f), and 3(g)). This is consistent with a previously published report showing that the proliferation of cultured neural progenitors was decreased significantly after sevoflurane exposure [12]. Zheng et al. [8] have reported that sevoflurane activates caspase-3 in the total fetal brain, but we did not find any significant changes in the number of caspase-3$^+$ cells in the PFC (Figures 3(h), 3(i), and 3(j)). The different findings of our study and Zheng's study may suggest that maternal sevoflurane exposure has different impacts on different regions of the fetal brain.

At the onset of neurogenesis, neural stem cells, the primary cortical stem cells, express nestin and undergo mitosis at the apical surface of the cortex. Radial glial cells are transformed from neural stem cells and express the transcription factor Pax6 [40, 41]. Radial glial cells also undergo divisions to generate intermediate progenitor cells, expressing Tbr2 [15, 41]. These neural progenitors repeatedly undergo self-renewal mitosis and form a progenitor pool at the apical surface of the cortex [15]. As we noticed that the proliferation inhibition mainly occurred in the apical surface of the cortex (Figures 3(b), 3(c), 3(d), 3(e), 3(f), and 3(g)), the region of neural progenitors, we supposed that maternal sevoflurane exposure might disturb the expansion of the neural progenitor pool. Therefore, we estimated the number of neural progenitor with immunofluorescence and found a significant decrease in the number of nestin$^+$, Pax6$^+$, and Tbr2$^+$ cells (Figures 4(b), 4(c), 4(d), 4(e), 4(f), 4(g), 4(h), 4(i), and 4(j)). As the main source of PFC neurons, it is easy to deduce that this reduction in neural progenitor is one of the reasons leading to the decrease in the production of deep-layer immature neurons.

The proliferation and differentiation of neural progenitors are influenced by not only cell cycle exit but also cell cycle progression [40]. With the staining of BrdU and Ki67, we found a significant decrease in the proportion of cell cycle exit after sevoflurane exposure (Figures 5(b), 5(c), and 5(d)), indicating that the neural progenitors in the sevoflurane

group are incapable of exiting cell cycle and differentiating to immature neurons. Both the decrease in the cell cycle exit and the downregulation of deep-layer neuron production (Figures 2(b), 2(c), and 2(d)) have indicated an abnormality in fetal brain differentiation. The length of S-phase has been reported to be the main cell cycle parameter associated with the proliferative behavior, and the self-renewal neural progenitors exhibited a relatively longer S-phase than that committed to neuron production [27]. We have found a significant increase in the S-phase duration of the neural progenitors exposed to sevoflurane (Figures 5(f), 5(g), and 5(h)), further indicating the downregulation of differentiation in the fetal PFC. The S-phase duration is the main time for neural progenitors to control the quality of replicated DNA [27], and sevoflurane has been reported to significantly increase DNA damage in rodents [42]. Therefore, our observation of prolonged S-phase may also indicate that DNA replication of neural progenitors in the sevoflurane group is abnormal, pending further study. Interestingly, the decreased cell cycle exit and increased S-phase duration were not accompanied by an expansion of the neural progenitor pool (Figure 4). This prompted us to think whether maternal sevoflurane exposure could lead to cell cycle arrest in neural progenitors. To verify our hypothesis, we further tested the change in other cell cycle parameters, including G1-, M-, and G2-phases. In a recent study, sevoflurane was reported to delay G1-phase and lead to cell cycle arrest in embryonic stem cells [43]. However, in our study of Ccnd1 labeling, the G1-phase of neural progenitors was not affected by prenatal sevoflurane exposure (Figures 6(b), 6(c), and 6(d)). It is reasonable that sevoflurane may affect G1-phase in different cell types through different mechanisms. Altered G2- and M-phase duration has been reported to directly alter cell fate in neural progenitors [44, 45], but the effect of sevoflurane on these two cell cycle phases of neural progenitors has not yet been studied in detail. In the present study, we did not find any significant differences in the expression of PH3 (Figures 6(e) and 6(f)), an indicator of M-phase and late G2-phase [26]. Together, the assessment of cell cycle exit and progression have indicated that maternal sevoflurane exposure could cause cell cycle arrest at S-phase in neural progenitors, which was associated with disrupted differentiation and proliferation of the fetal PFC.

Our findings indicated that the cell cycle disturbance of the neural progenitors in the fetal PFC contributed to aberrant proliferation and differentiation after maternal sevoflurane exposure, which may finally lead to the functional neurological impairments in adult offspring. Our study helped to understand the mechanism of postoperative neurological impairments after prenatal sevoflurane exposure and appealed people to consider the neurotoxicity of anesthetics when considering the benefits and risks of nonobstetric surgical procedures.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

BMP4/LIF or RA/Forskolin Suppresses the Proliferation of Neural Stem Cells Derived from Adult Monkey Brain

Xinxin Han,[1] Liming Yu,[1] Qingqing Chen,[1] Min Wang,[2] Jie Ren,[3] Guangming Wang,[3] Yihong Chen,[4] Lixia Lu,[3,5] Haibin Tian,[3,5] Li Chen,[1] Ying Zhang,[1] Yuehua Liu,[1] Hua He,[4] and Zhengliang Gao[3,5]

[1]*Shanghai Stomatological Hospital, Fudan University, Shanghai 200001, China*
[2]*School of Medicine, Jiaxing University, Zhejiang 314001, China*
[3]*Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200092, China*
[4]*Changzheng Hospital, Second Hospital Affiliated with Second Military Medical University, Shanghai 200003, China*
[5]*Tongji University Advanced Institute of Translational Medicine, Shanghai 200092, China*

Correspondence should be addressed to Xinxin Han; xxhan@fudan.edu.cn, Hua He; panda1979hh@sina.com, and Zhengliang Gao; zhengliang_gao@tongji.edu.cn

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Monkeys are much closer to human and are the most common nonhuman primates which are used in biomedical studies. Neural progenitor cells can originate from the hippocampus of adult monkeys. Despite a few reports, the detailed properties of monkey neural stem cells (NSCs) and their responses to cytokine are still unclear. Here, we derive NSCs from an adult monkey brain and demonstrate that BMP4 inhibits cell proliferation and affects cell morphology of monkey NSCs. Combined treatment of BMP4 and LIF or RA and Forskolin represses the proliferation of monkey NSCs. We also show that BMP4 may promote monkey NSC quiescence. Our study therefore provides implications for NSC-based cell therapy of brain injury in the future.

1. Introduction

An adult mammalian brain shows amazing plasticity by regenerating new neural cells after injury or damage [1, 2]. In the brain, neural regeneration mainly arises from the differentiation of endogenous neural stem cells (NSCs), which exists in subventricular zone (SVZ) and subgranular zone (SGZ). SVZ is in lateral ventricle, and SGZ is in the dentate gyrus (DG) of the mammalian brain [3]. The DG area in hippocampus constantly produces new cells throughout the life. Newborn neuron cells are activated to support the memory and cognition particularly in their plasticity phase [4].

Stem cells possess the ability to self-renew and differentiate into diverse progeny cells [2, 5]. NSCs belong to multipotent cells and can differentiate into neurons, astrocytes, and oligodendrocytes [6, 7].

Neural regeneration always requires neuron protection and axon regeneration [8]. NSCs are responsible for brain plasticity and repair by producing, restoring, and modifying central nervous system (CNS) [9]. Due to the limited number of NSCs in CNS, one of the key strategies of brain repair is transplanting NSCs into CNS. Three decades ago, fetal tissue was grafted into Parkinson's patients for brain repair [10]. However, the wide application of fetal tissue transplantation is hampered by various ethical issues [11]. Induced pluripotent stem cells (iPSCs) have been considered as a new approach for cell therapy [12, 13]. Tissue damage provides critical signals for cellular reprogramming [14]. Fibroblast and astroglial cells also have been transdifferentiated into neurons for CNS repair [15, 16]. However, iPSC-based cell therapy also encounters problems such as low efficiency and safety issues.

One way of neural regeneration is utilizing endogenous NSCs to generate newborn neurons. Endogenous NSCs survive in stem cell niches which receive the support from microenvironments [17, 18]. When damage or disease (such as stroke) occurs, NSCs' proliferation in adult brain niches increases and migrates to brain ischemic areas [3, 19, 20]. Newborn endogenous neurons can be recruited and integrated into local circuits [21]. However, the in vivo neurogenesis ability is restricted and only a few new neurons could be produced, which are inadequate for brain repair [22, 23].

NSCs can survive in the DG regions of hippocampus throughout an individual's life-span, but human VZ and SVZ regions stop to produce neurons at 2 years old [24]. Despite NSCs have long time activity in adult hippocampus, their amount decreases with age and significantly declines in Alzheimer's disease (AD) transgenic mouse [25]. This reduction of NSCs causes learning and memory loss [25]. It is vital that NSCs maintained the proliferating activity by the stem cell niche which are consisted by various cytokines [9]. For example, IGF (insulin-like growth factor), FGF (fibroblast growth factor), and Noggin (a BMP inhibitor, encoded by the NOG gene) increase NSC proliferation [26]. Dkk1 (Wnt antagonist Dickkopf-1) is increased along with aging, and loss function of Dkk1 can enhance neurogenesis in the hippocampus [27].

Mouse and rat often are used as a model organism for mammalian development research. Nonetheless, the growth mechanisms of mammals are different among species [28]. Monkeys, especially rhesus macaque, are the most universal nonhuman primates used in biomedical research, particularly for disease modeling which are special for advanced animals (such as HIV, poliomyelitis, and and aging) due to a close evolutionary and genomic relationship with humans [29, 30].

At present, most researches of monkey neural stem cells focus on embryonic stem cells differentiating into neural stem cells. There are very few studies on adult monkey neural stem cells. For example, monkey neural stem and progenitor cells can differentiate into immature oligodendrocytes [31]. Brain-derived neurotrophic factor (BDNF) promotes NPC proliferation and induces cynomolgus monkey neural progenitor differentiation into neurons [32]. Study on transplantation of adult monkey neural stem cells also showed that monkey NSCs can be injected into a contusion spinal cord injury model in rhesus macaque monkeys [33]. However, detailed cell properties of adult monkey NSCs and factors except BDNF that can regulate monkey NSC proliferation are still unknown.

In this study, we generated NSCs from monkey brain and investigated the proliferation ability. We found bone morphogenetic protein 4 (BMP4) inhibited monkey NSC proliferation and changed the morphology of monkey NSCs. Combined application of BMP4 and LIF (leukocyte inhibitor factor) or RA (retinoic acid) and Forskolin suppressed cell proliferation. We also examined the differentiation tendency under these cytokine treatments. These results may provide useful information for brain injury repair using stem cell-based therapy.

2. Materials and Methods

2.1. Experimental Monkey. Animals were fed according to the requirements of the Animal Welfare Act, and protocols were followed based on the permission implemented by the animal ethics committee of JOINN Laboratories (Suzhou). The animals were fed under conditions approved by the Association for the Assessment and Accreditation of Laboratory Animal Care International. A male rhesus monkey (*Macaca fascicularis*, 4 kg, 3 years old) was used for studies.

2.2. Derived NSCs from Monkey Brain. A monkey's brain was removed and collected in accordance with Regulations for the Administration of Affairs Concerning Experimental Animals. Brain tissue was washed in HBSS—Hank's buffer (Gibco) for 8 times. Then, the hippocampus and temporal cortex were dissected into the dishes. These selected tissues were crushed by a sterile scalpel and surgical scissors and then added 30 mL phosphate-buffered saline (0.01 M PBS, pH 7.2) to the tissues. Tissue suspension was passed through 70 μm sieve, and filtered tissue liquid was collected to tubes. The tissue mixture was digested with 1 U/mL dispase II (Roche) at 37°C for 45 minutes and then was centrifuged at 1000g for 3 minutes. The centrifuged suspension was discarded, and a precipitate was washed by NSC growth medium (DMEM/F12/N2/B27/GlutaMAX/penicillin/streptomycin/ (20 ng/mL FGF)/(20 ng/mL EGF)/(20 ng/mL heparin)). Then, cell suspension mixture was centrifuged at 1000g for 3 minutes. The precipitate containing primary cells was resuspended and seeded into 100 mm dishes at different concentration in medium. Then, the medium was half refreshed every 2 days. After 2 months of culture, NSCs were obtained which originated from an adult monkey brain.

2.3. Neurosphere Formation and NSC Culture. After 2 months of careful culture, we first observed neurospheres originating from an adult monkey brain. Then, NSCs climbed out from the neurospheres. When the NSC clone was large, NSC clones were washed twice by using DMEM/F12 then added 2 mL dispase II (1 U/mL dissolved in DMEM/F12) to the NSC clones. Cell dishes were incubated at 37°C for 5 minutes then added 3 mL growth medium. Cells were collected into tubes and centrifuged at 1000g for 3 minutes. Then, cells were resuspended and planted to new 100 mm dishes at 1 : 4 ratios. NSCs were passaged and expanded depending on this protocol.

2.4. Factor Treatment. NSCs were passaged and cultured in growth medium containing lower FGF (5 ng/mL) for 12 hours. Then, the NSCs were treated with different factors in 6 groups as follows: (1) control; (2) BMP4 (100 μg/mL); (3) BMP4 (100 μg/mL) and LIF (leukocyte inhibitor factor, 50 ng/mL); (4) RA (retinoic acid, 1 μM) and Forskolin (5 μM); (5) 10% fetal bovine serum (FBS) in DMEM/F12/GlutaMAX/FGF/EGF/heparin/penicillin streptomycin; and (6) no factor growth medium (DMEM/F12/N2/B27/GlutaMAX/FGF/EGF/heparin/penicillin streptomycin). These NSCs were cultured at 37°C in 5% CO_2 incubator for 6 days.

2.5. Antibodies and Immunostaining. For antibody staining, cells were fixed by using 4% PFA and washed with PBS for 3 times. Then, they were permeabilized with 2.5% Triton X-100 for 10 minutes. Cells were blocked with 5% BSA for 1 hour at room temperature. According to the general immunofluorescence procedure, cells were washed with 0.1% Tween-20 in PBS and incubated with primary antibody at 4°C for 48 hours. Primary antibodies used for immunostaining were Sox2 (R&D), Nestin (R&D), and Ki67 (Thermo Fisher). The dilution buffer of primary antibody was 2.5% BSA in PBS. Cells were washed with 0.1% Tween-20 in PBS for 3 times and incubated with a second antibody at room temperature for 1.5 hours. At last, we used 4′,6-diamidino-2-phenylindole (DAPI, Sigma) to mark the nucleus of monkey NSCs. Additional attention, when using DAPI, we must treat the cells for 10 minutes at room temperature after second antibody incubation.

2.6. Microscopic Image. Cells were observed by using an inverted fluorescence microscope (Nikon TE2000). Images were acquired under a color CCD camera and digitized by a PC-based frame grabber. Then, photos were analyzed and checked in ImageJ software. Data collected from ImageJ were calculated by Excel. Calculation results were analyzed by the GraphPad Prism 6 software and then were organized into charts.

2.7. Statistical Analysis. We manually drew the frames of neurosphere, NSC clones, or differentiation cells of NSCs based on morphology. Then, we recorded areas or lengths by ImageJ software. Meanwhile, we drew bar's area or lengths in the same image as a ruler. Compared with the bar, actual size was counted and recorded. All data were showed as mean ± standard deviation of the mean (SD). Data was calculated by Excel and p value was measured for statistical significance by two-tailed Student's t-test.

3. Results and Discussion

3.1. Results

3.1.1. Improved Process and Cell Proliferation Rate of NSCs from an Adult Monkey. To obtain the development secret of NSCs in nonhuman primates, we designed a process of isolating monkey NSCs from hippocampus and temporal cortex of an adult monkey brain (Figure 1). The male adult monkey (*Macaca fascicularis*) (Figure 1(a)) was used as a donor. A flow chart of the method was designed for deriving neural stem cell-like cells from an adult monkey (Figure 1(b)). Cells originated from primary culture of the monkey brain. The detail of isolating process was shown. The hippocampus and cortex of the monkey brain were digested by enzyme and planted in different concentration for 2 months. During these 2 months, fresh medium was changed to cells every 2 days.

In order to test the proliferation status and rate of monkey NSC-like cells, we recorded and analyzed the proliferation of monkey neurospheres (Figure 1(c)). Monkey neurosphere appeared after 2 months of monkey brain culture (Figure 1(c), A). Neural stem cell-like cells were proliferated, and cells climbed out the neurosphere like waterfalls (Figure 1(c), B). Massive neural stem cell-like cells formed an independent clone around neurosphere (Figure 1(c), C). Monkey NSC-like cells proliferated rapidly based on the growth curve (Figure 1(c), D).

3.1.2. Growth Process of Neural Stem Cell-Like Cells. To examine the proliferation and development potential of cells derived from an adult, we fostered and observed these cells. Firstly, some visible cell balls appeared in dishes and these suspended floating balls (sphere) became bigger with time and culture process (Figure 2(a)). Subsequently, the suspended floating balls and spheres started to stick to the bottom of the dishes and sporadic cells climbed out from the adherent spheres (Figure 2(b)). Then, more and more cells emerged from adherent spheres and a stem cell clone was formed on the surface of the dish bottom (Figure 2(c)). As time went by, the clone extended quickly and cells which climbed out from the clone increased rapidly (Figure 2(d)). Finally, monkey neural stem cell-like cells emerged (Figure 2(e)).

3.1.3. Proliferation and Division Potential Analysis of Neurospheres and NSCs. To thoroughly investigate neural stem cells, we made use of neural stem cell marker protein antibodies for immune staining. SRY- (sex-determining region Y-) box 2, also known as Sox2, is a transcription factor that is essential for maintaining embryonic and neural stem cells. Nestin is a neuroectodermal stem cell marker and a type VI intermediate filament (IF) protein. We fixed NSC-like cells from the monkey brain and discovered these cells were Sox2 (Figure 3(a)) and Nestin (Figure 3(b)) positive. Cells expressing both Sox2 and Nestin were considered as the characteristics of NSCs.

To study the dynamic proliferation change and differentiation potential of neurospheres and NSCs, we calculated the sphere growth pattern as shown in yellow circles (Figure 3(c)). The core of the sphere grew slowly during the 4 sphere development progresses: sphere formation (SF), sphere adherence (SA), clone formation (CF), and clone extension (CE) (Figure 3(c)). We drew up cells area surging from spheres in a big yellow border line and measured cell's extending areas (Figure 3(d)). Yellow circles present spheres (Figure 3(d)). The data showed that cell's extending area was significantly increased through the 4 sphere development processes (Figure 3(d)). There was a slightly declining trend of sphere size but there were no significant differences (Figure 3(e)). Cells grew very quickly from the sphere, and the multiplication rate was more than a thousand times when compared with the initial stage (Figure 3(f)).

3.1.4. BMP4 Inhibited Monkey NSC Proliferation and Affected Their Morphology. Bone morphogenetic proteins (BMPs) are members of transforming growth factor-β (TGF-β) super family [34]. BMP4 and BMP type I receptor (BMPRIA) are overexpressed in coronal phase of a rat molar. BMP4 can rescue the absence of molar germ which is caused by homeobox-containing transcription factor 1 (Msx1) knockout [35]. BMP also plays a major role in

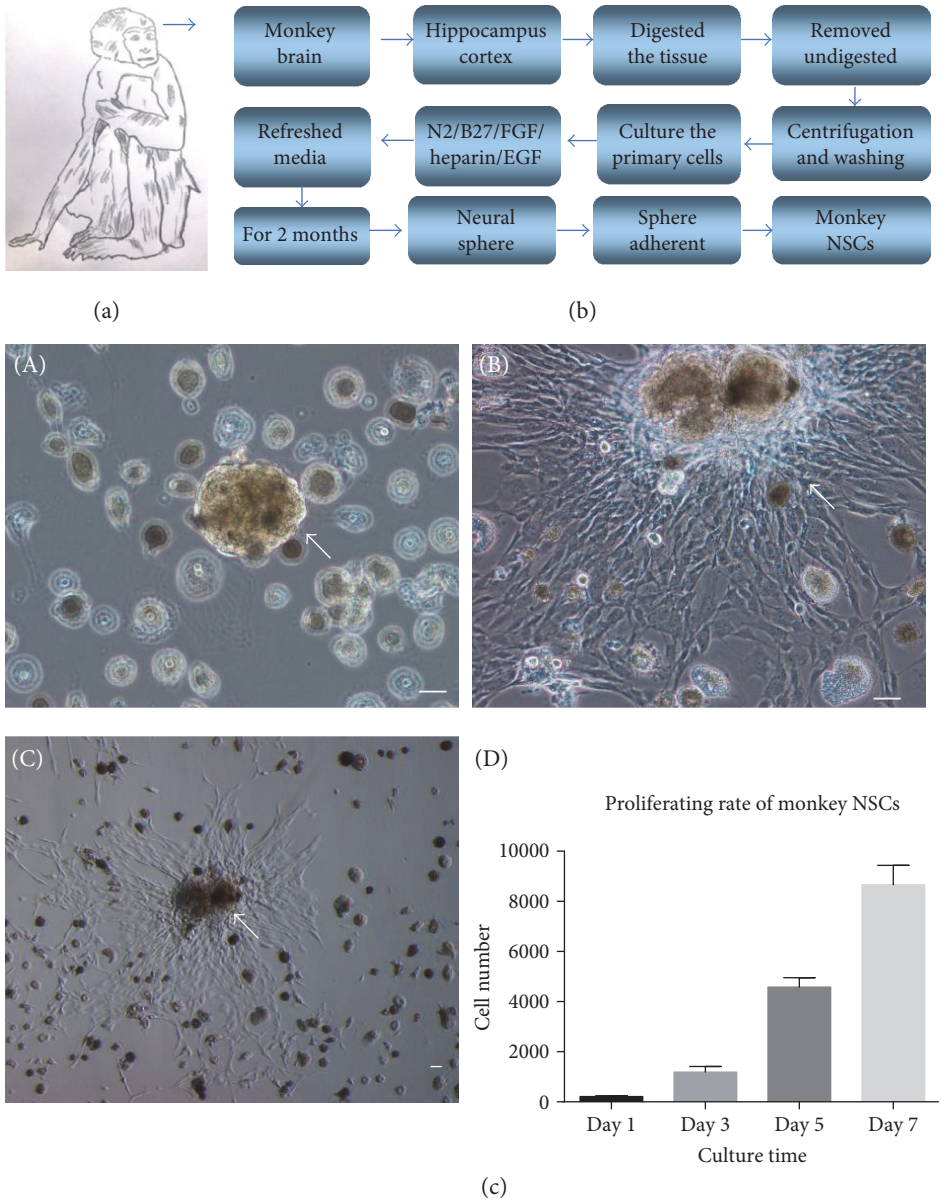


Figure 1: A flow chart of a method used in neural stem cell-like cell isolation from the monkey brain (*Macaca fascicularis*). (a) Primary cells were obtained from the cultured monkey brain. (b) The detail of cell-isolating process was described. The monkey brain cortex was treated with enzyme and cultured for two months. Within these months, fresh-specific medium was changed to neural stem cells (cell sedimentation after centrifugation) every two days. (c) Monkey neurospheres occurred, and neural stem cell-like cells proliferated from neurospheres. (A) Monkey neurospheres appeared in adult monkey brain culture medium. (B) Neural stem cell-like cells proliferated from neurospheres. (C) Large number of neural stem cell-like cells formed an independent clone, and this clone was seen at low-magnification microscope. (D) The proliferating rate of monkey NSC-like cells was shown. Scale bar = 50 μm.

the formation and maintenance of a variety of tissues, such as induction of osteogenesis, cartilage, kidney, muscle, and fat [34].

In consideration of the important functions of BMP in development, we detected the influence of BMP4 factor in NSCs. We found that BMP4 remarkably affected cell morphology (Figure 4(a)) and the cell's body became flat at high magnification (Figure 4(b)). Ki67, a marker protein of ribosomal RNA transcription, is a nuclear protein which is necessary for cellular proliferation. In previous reports, scientists adopted Ki67 to investigate cell proliferation and quiescence. We also used Ki67 to detect proliferation and quiescence of monkey NSCs. The photos showed that Ki67-positive cells obviously reduced after immunofluorescence staining in monkey NSCs (Figure 4(c)).

It has been showed that BMP4 suppressed monkey NSC proliferation (Figure 4(d)). Single cell exhibited a larger size after BMP4 treatment (Figure 4(e)). The length-width ratio suggested that cells changed into oblateness from leptosomic type after BMP treatment (Figure 4(f)). Cells appeared shorter after BMP treatment when compared with the untreated group (Figure 4(g)).

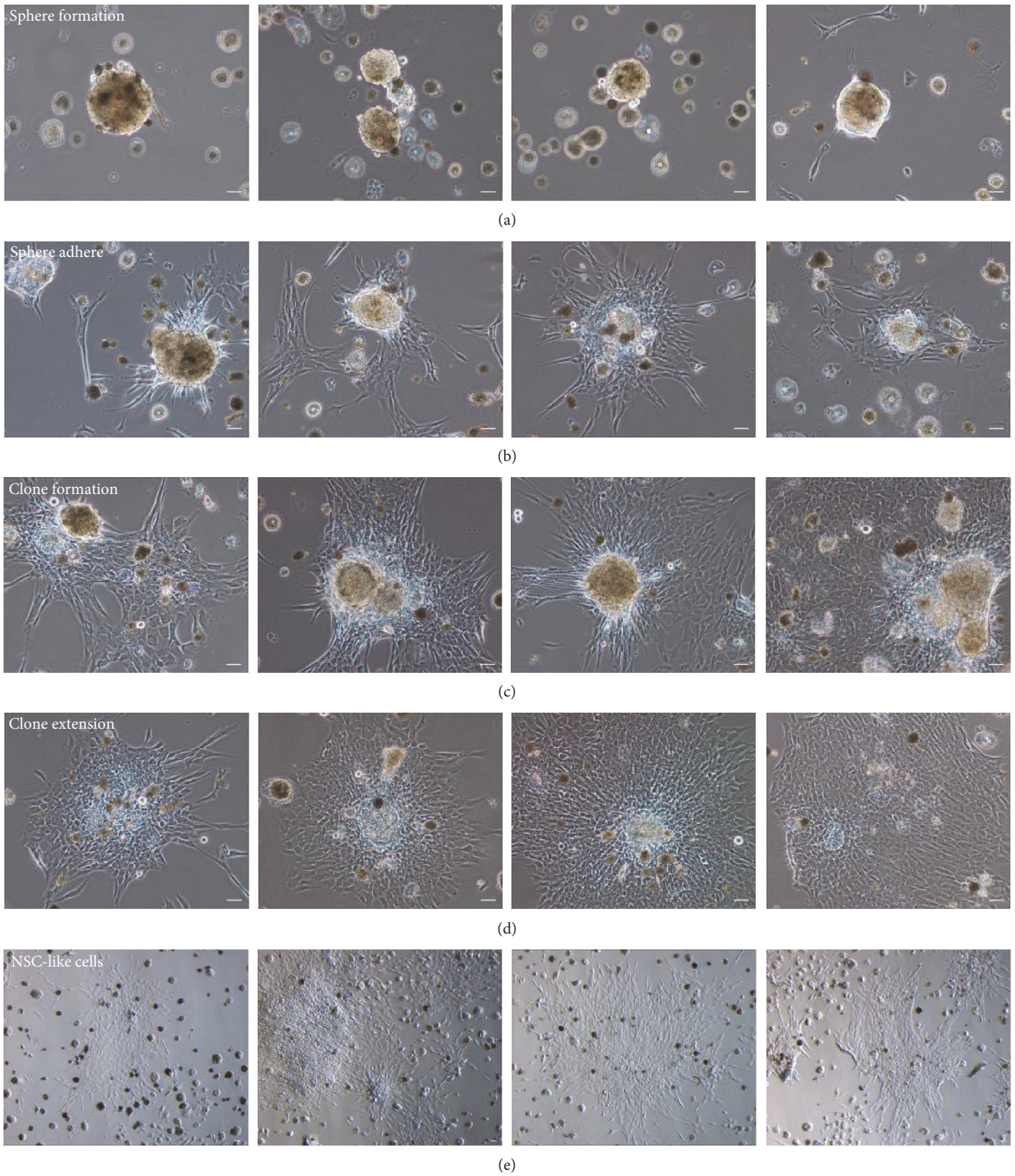


Figure 2: Growth process of neural stem cell-like cells derived from monkey. (a) Neurospheres formed in neural stem cell selective media with factors. (b) Spheres attached the plastic surface of the cell culture dish. (c) Cells climbed from the spheres and cell clone appeared. (d) Clone expanded the territory, and new cells are increased. (e) Monkey neural stem cell-like cells emerged. Scale bar = 50 μm.

3.1.5. BMP4/LIF and RA/Forskolin Suppressed Monkey NSC Proliferation. Both BMP and LIF promote the differentiation of mouse neural stem cells into mature astrocytes and other glial fibrillary acidic protein (GFAP) immunoreactive cells [36]. RA can induce human neuroblastoma cell differentiation into neuronal-like cells [37]. Forskolin, a cyclic adenosine 3′,5′-monophosphate (cAMP) activator, increases neuregulin receptors in human Schwann cells [38, 39].

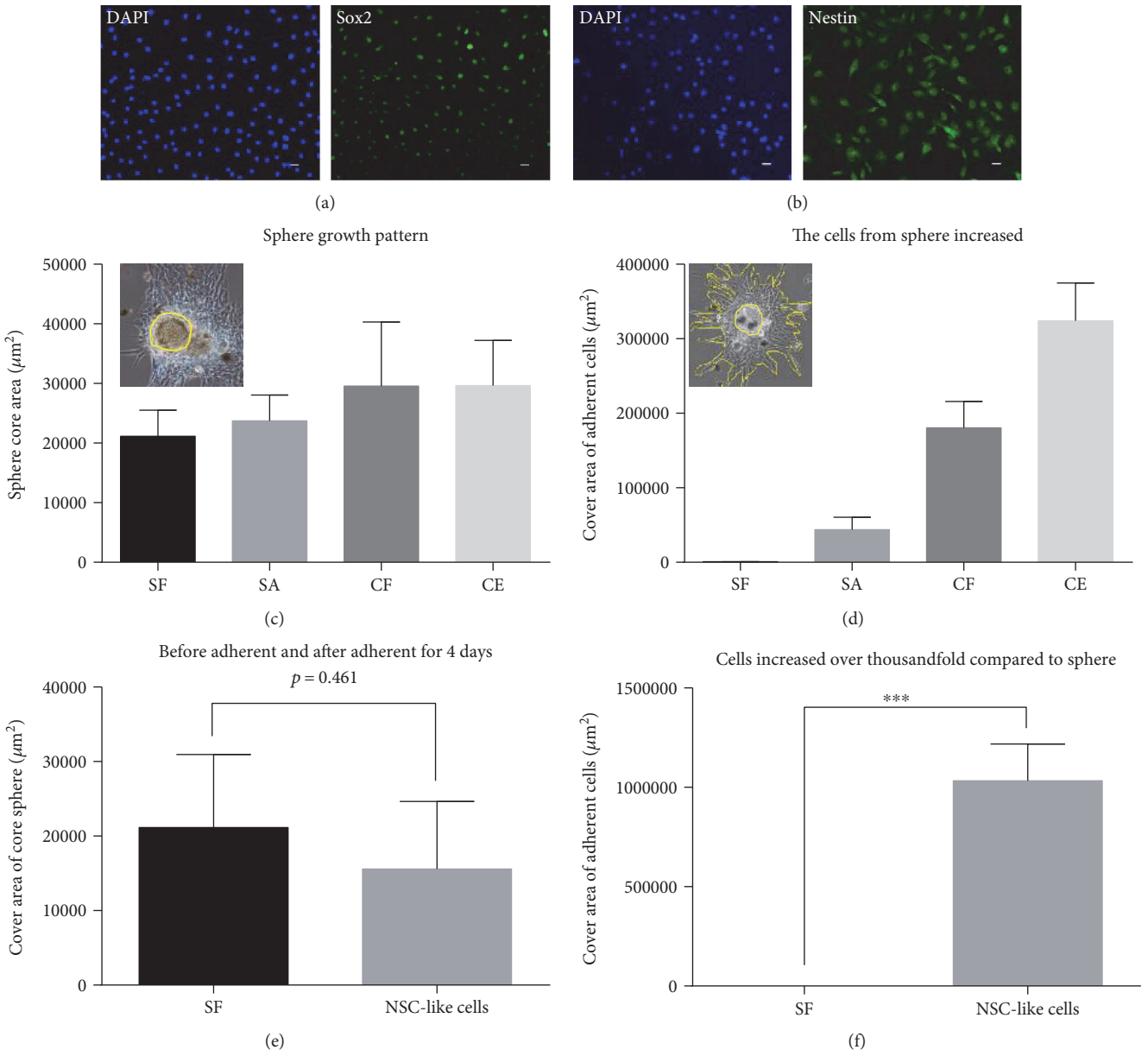


Figure 3: Characteristics of the neurospheres and neural stem cells. (a) Cells expressed neural stem cell marker protein Sox2. (b) Cells expressed neural stem cell maker protein Nestin. (c) Sphere growth pattern showed in yellow circles. Sphere core was slowly growing during the progress of sphere formation (SF), sphere adherence (SA), clone formation (CF), and clone extension (CE) in neural stem cell selective media with factors. (d) Cells proliferated from the spheres and adherent cell increased. Cells climbed from the spheres, and cover area of adherent cell pattern was showed in a big yellow border line. Yellow circles present the spheres. (e) Sphere size slightly seemed to be a downward trend but there was no significant difference. p value = 0.461. (f) Cell growth area from sphere was more than a thousand times versus sphere. Scale bar = 25 μm. Data were shown as mean ± SD; $^{***}p$ value < 0.001.

According to the function of above factors, we used different combination of these factors testing their influence on monkey NSCs. The phenotype under different factor conditions was presented, and BMP4/LIF or RA/Forskolin dramatically changed cell morphology (Figure 5(a), A–F). BMP4/LIF (Figure 5(a), C) and RA/Forskolin (Figure 5(a), D) groups showed significant difference compared with control (Figure 5(a), A). BMP4 dramatically changed cell morphology (Figure 5(a), B) as detected before (Figure 4(b)). The changes in fetal bovine serum are not obvious within a short time (Figure 5(a), E). We also exposed monkey NSCs to spontaneous differentiation condition in order to test their differentiation abilities (Figure 5(a), F). We had observed that the neuron-like cell and astrocyte-like cell appeared in spontaneous differentiation condition (Figure 5(a), F). BMP4 and LIF seemed to promote the differentiation of neural stem cells into astrocytes (Figure 5(a), C). RA and Forskolin may advance the process of monkey NSCs differentiating into neuron (Figure 5(a), D). Cell amount suggested that BMP4/LIF and RA/Forskolin suppressed the proliferation of monkey NSCs (Figure 5(b)).

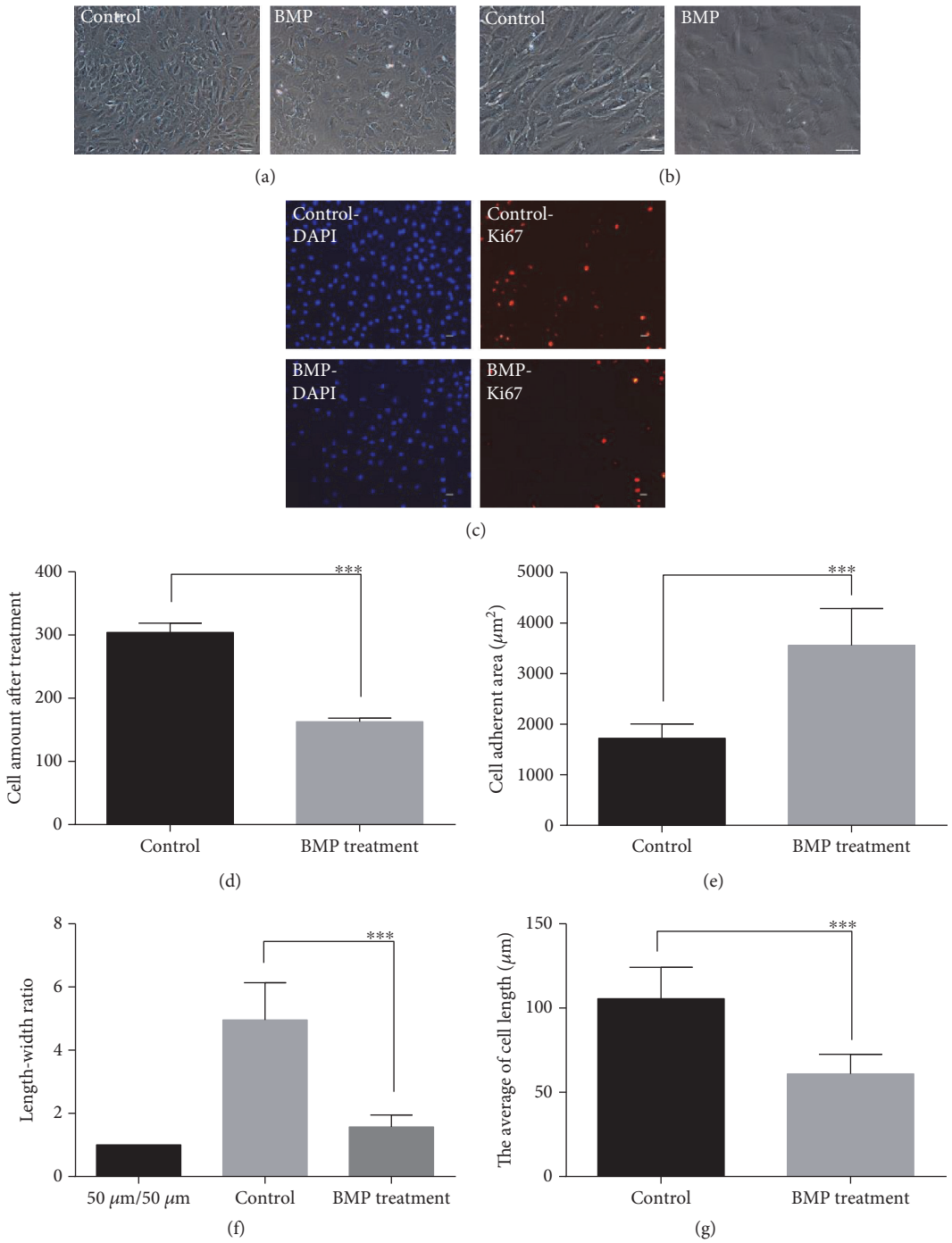
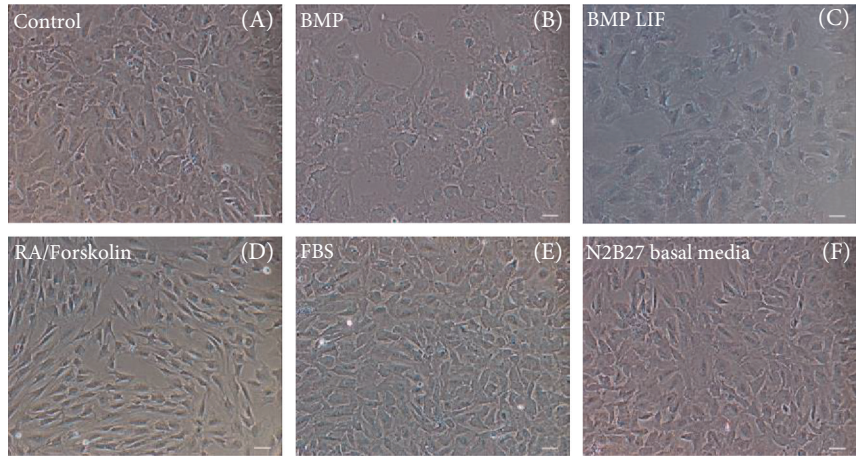
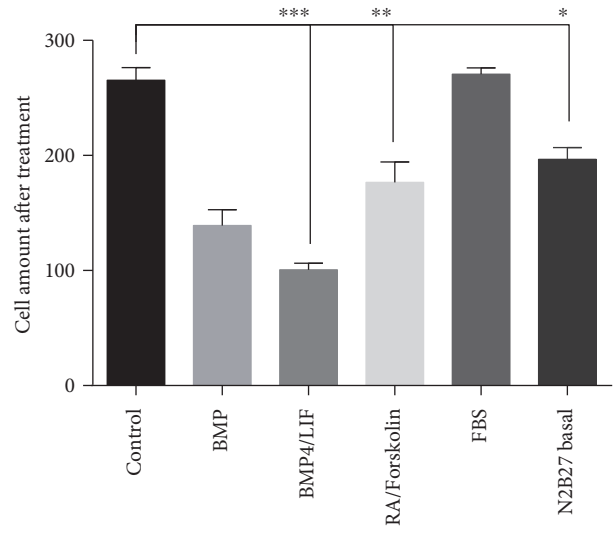


Figure 4: BMP4 inhibited cell proliferation and effected cell morphology. (a) Monkey NSCs treated in control media or BMP4 media for 6 days. (b) Cell morphology displayed the different phenotypes after BMP4 treatment under enlarged view. (c) Ki67-positive cells greatly reduced after BMP treatment compared with the control group. Ki67 was observed by immunofluorescence staining. (d) Cell amount presented that BMP4 suppressed the proliferation of monkey NSC-like cells. (e) The increase of cell adherent area showed that single cell became large after BMP4 treatment. (f) The length-width ratio suggested the cells became oblate from leptosomic type after BMP treatment. (g) Cells appeared shorter than control after BMP treatment. (a) and (b) Scale bar = 50 μm; (c) scale bar = 25 μm. Data represent the mean values ± SD; $^{***}p$ value < 0.001.

3.1.6. BMP4/LIF and RA/Forskolin Promoted the Change of Cell Morphology. The phenotype of single cells showed significant changes after BMP4/LIF or RA/Forskolin treatment (Figure 6(a)). BMP4 and LIF seemed to promote the differentiation of neural stem cells into astrocytes (Figure 6(a), B) compared with control (Figure 6(a), A). RA and Forskolin may advance monkey NSCs differentiating into the neuron (Figure 6(a), C). The length-width ratio suggested that BMP4/LIF had an effect on the cell morphology and may promote monkey NSC differentiation to mature astrocytes



(a)



(b)

Figure 5: BMP4/LIF and RA/Forskolin suppress cell proliferation. (a) Monkey NSCs were treated in control medium or other rich with factors medium for 6 days. (A) Control, (B) BMP group, (C) BMP4/LIF group, (D) RA/Forskolin groups, (E) FBS group, and (F) N2B27 group. BMP4/LIF and RA/Forskolin groups showed significant difference. (b) Cell amount presented that BMP4/LIF and RA/Forskolin suppressed the proliferation of monkey NSC-like cells. Bar = 50 μm. Data represent the mean values ± SD; $^{*}p$ value < 0.001; $^{**}p$ value < 0.01; $^{***}p$ value < 0.001.

(Figure 6(b)). RA/Forskolin may promote the differentiation of neural stem cells into neurons according to the phenotype of cells and cell bodies (Figure 6(b)).

4. Discussion

Our study firstly demonstrated that BMP4 inhibits cell proliferation and affects cell morphology of monkey NSCs. We also discovered that combining the application of BMP4 and LIF or RA and Forskolin represses the proliferation of monkey NSCs. We also observed that BMP4 may promote quiescence of monkey NSCs. It may be essential to identify the influence of these factors to monkey NSCs, and our results may supply some helpful information to cell transplantation in practical clinical trials. Our study suggested monkey NSCs could be utilized as a useful platform for translational research. This study also may bring some theoretical support for cell therapy of human brain injury.

Brain injuries such as a traumatic injury, stroke, or other neurodegenerative disorders are life-threatening damage and leading causes of death and disability in the population worldwide, with an extensive range of symptoms and disabilities [40–42]. Brain injury causes neural cell death when the damage occurs and tissue lacks blood oxygen supply. The effective treatment for stroke is quite limited [43]. There are numerous challenges and hurdles in both academic and preclinical trials of translational stroke research [44–46].

At present, there is no particularly effective treatment for brain injuries. The capability of an adult mammalian brain to remedy the neuronal defeat which causes injury or disease is very limited [47]. Cell transplantation such as NSCs, neural progenitor cells, or mesenchymal stem cells has been considered as possible therapies for brain injury [47]. Transplantation cells aim to replace lost neurons. There is a report that embryonic neurons are transplanted into the visual cortex

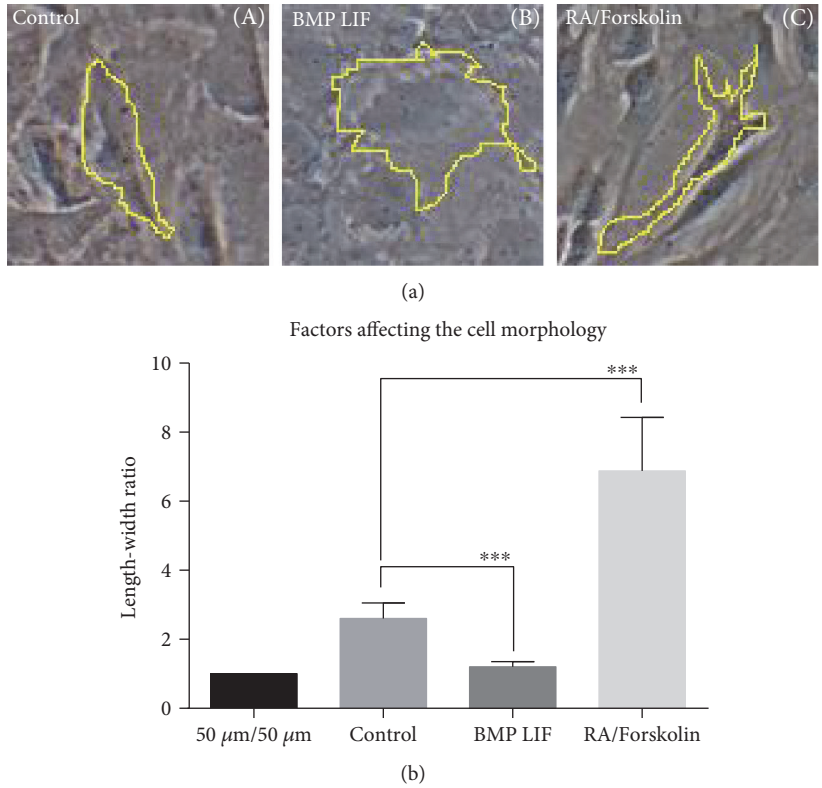


Figure 6: BMP4/LIF and RA/Forskolin affected the cell morphology. (a) Monkey NSCs were treated in control media or BMP4/LIF or RA/Forskolin factors media for 6 days. (A) Control; (B) BMP4/LIF group; (C) RA/Forskolin groups. The phenotype of single cells in BMP4/LIF and RA/Forskolin groups showed significant changes. (b) The length-width ratio suggested that BMP4/LIF and RA/Forskolin affected the cell morphology and may induce monkey NSC-like cells differentiation. Scale bar = 50 μm. Data represent the mean values ± SD; $^{***}p$ value < 0.001.

of adult mice; then, these grafted neurons can mature into bona fide pyramidal cells and integrate with neocortical circuits in the adult brain [48].

iPS cells can differentiate into a broad variety of neural type cells, and we may take them as an attractive donor source for autogenously neural transplantation therapies for brain injury repair [49]. However, iPS cell transplantation faces ethical barriers and risk of cancer formation. At the same time, transplanted iPS cells in vivo are difficult to produce a clear result in an extensive diversity of preclinical models for brain injury.

NSC cells sustaining neuron regeneration discovered in SGZ and SVZ are considered to be an endogenous neuroprotective device for these brain injuries. However, present strategies cannot suitably improve functional recovery after brain injury like stroke because NSCs and their microenvironment are very complex and multiple [41]. Searching for the fate-determining mechanism and studying the NSCs' performance are extremely important, including cell proliferation, migration, and differentiation.

Monkeys are the most common nonhuman primates which are employed in biomedical study. They are closer to human and suitable as a model of human disease. Moreover, monkeys such as rhesus macaque (*Macaca fascicularis*) have lower cost compared to other nonhuman primates. Neural stem and progenitor cells isolated from adult rhesus were found that these cells can differentiate into immature oligodendrocytes [31]. BDNF can support NPC proliferation and induce monkey neural progenitor differentiation into neurons, and these cells are isolated from adult cynomolgus monkeys [32]. Here, we showed detailed property analysis of monkey NSCs and we firstly investigated the inhibition effects to monkey NSCs such as BMP4, BMP4 and LIF, RA, and Forskolin.

We found BMP4 inhibited the cell proliferation and influenced cell morphology of monkey NSCs. The proliferation of cells was suppressed by application of BMP4 and LIF or RA and Forskolin. We also observed the changes of cell fate after factor treatment. Through analysis, we discovered that BMP4 alone may promote the monkey NSC quiescence. But this was just a speculation, and we needed to do more tests.

After BMP4, BMP4 and LIF, or RA and Forskolin treatment, what is the final fate of the monkey NSCs? Are the factor actions reversible? What is the impact of these factors to the NSC transplantation rate? In the following study, we will pay more attention to these questions and explore the intrinsic signaling mechanism that regulates cell proliferation, differentiation potential, and cell cycle of monkey NSCs after cytokine treatment. Identification of these mechanisms may be helpful to understand the application foundation of cell transplantation and may provide some useful information to further targeted cell therapies.

5. Conclusions

We firstly demonstrated the effects of BMP4, BMP4 and LIF, RA, and Forskolin on the monkey NSCs and showed detailed property analysis of monkey NSCs. We found that BMP4 inhibited the proliferation and affected monkey NSC morphology. BMP4 and LIF or RA and Forskolin suppressed proliferation of monkey NSCs. Identification of these factors' functions to monkey NSCs may be helpful to understand cell transplantation application and may provide some useful information to guide the cell therapy progress.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

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Research Article

Efficient and Fast Differentiation of Human Neural Stem Cells from Human Embryonic Stem Cells for Cell Therapy

Xinxin Han,[1] Liming Yu,[1] Jie Ren,[2] Min Wang,[3] Zhongliang Liu,[2] Xinyu Hu,[2] Daiyu Hu,[2] Yihong Chen,[4] Li Chen,[1] Ying Zhang,[1] Yuehua Liu,[1] Xiaoqing Zhang,[5] Hua He,[4] and Zhengliang Gao[5]

[1]*Shanghai Stomatological Hospital, Fudan University, Shanghai 200001, China*
[2]*Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200092, China*
[3]*School of Medicine, Jiaxing University, Zhejiang 314001, China*
[4]*Changzheng Hospital, Second Affiliated Hospital of Second Military Medical University, Shanghai 200003, China*
[5]*Shanghai Tenth People's Hospital, Tongji University School of Medicine, Tongji University Advanced Institute of Translational Medicine, Shanghai 200092, China*

Correspondence should be addressed to Xinxin Han; xxhan@fudan.edu.cn, Hua He; panda1979hh@sina.com, and Zhengliang Gao; zhengliang_gao@tongji.edu.cn

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Stem cell-based therapies have been used for repairing damaged brain tissue and helping functional recovery after brain injury. Aberrance neurogenesis is related with brain injury, and multipotential neural stem cells from human embryonic stem (hES) cells provide a great promise for cell replacement therapies. Optimized protocols for neural differentiation are necessary to produce functional human neural stem cells (hNSCs) for cell therapy. However, the qualified procedure is scarce and detailed features of hNSCs originated from hES cells are still unclear. In this study, we developed a method to obtain hNSCs from hES cells, by which we could harvest abundant hNSCs in a relatively short time. Then, we examined the expression of pluripotent and multipotent marker genes through immunostaining and confirmed differentiation potential of the differentiated hNSCs. Furthermore, we analyzed the mitotic activity of these hNSCs. In this report, we provided comprehensive features of hNSCs and delivered the knowledge about how to obtain more high-quality hNSCs from hES cells which may help to accelerate the NSC-based therapies in brain injury treatment.

1. Introduction

Neurogenesis is defined as progress of new neuron generation from neural stem cells (NSCs) or usually named neural progenitor cells (NPCs) [1, 2]. Neurogenesis exists in both embryonic stages and adult stages. In adult, there are two distinct regions occurring neurogenesis in the central neural system (CNS): subventricular zone (SVZ) of lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in mammalian hippocampus [3, 4]. Embryonic neurogenesis taking place in the ventricular zone (VZ) and SVZ originates from the differentiation of neuroepithelial cells into radial glial cells (RGCs) in the mouse brain [4, 5].

Adult neurogenesis was firstly reported 50 years ago in the hippocampus of dentate brain (dentate gyrus, DG area) [6]. Before that, scientific community generally had believed for a long time that the adult brain cannot produce new neurons. Now, the idea is widely acknowledged that adult neurogenesis exists in the DG of human brain [7, 8]. Adult neurogenesis occurs in most mammals and several other vertebrates [9].

NSCs are multipotential stem cells with the capability to self-renew and can generate neurons, astrocytes, and oligodendrocytes [9]. NSCs play an important role both in basic research of neural development and wide potential for stem cell-based therapy in neurological diseases such as stroke,

Parkinson's disease, and spinal cord injury [10, 11]. It has been reported that an immortalized human NSC line, HB1.F3 (F3), was constructed from a 15-week gestational human fetal brain but this cell line is overexpresses v-myc oncogene with a retrovirus vector [10]. Previous studies show that human NSCs which transplant by intravenous injection can differentiate into diverse neural cell types and reduce the neurological damage after stroke [12, 13].

At present, research on hES cells to neural differentiation is mainly focused on direct differentiation of mature functional neurons from hES cells or neural crest stem cells for clinical application [14, 15]. Noticeably, it is reported that a good manufacturing practice (GMP) differentiation procedure is devised for efficient production of dopamine progenitors from hES cells [16]. There is also research about obtaining GABA neurons from human embryonic stem cell [17] and cerebral cortex neurons by directing differentiation of human pluripotent stem cells [18]. Meanwhile, several groups successfully investigated that they can induce mature cortical neuron production from hES cells by applying some small molecular compounds [19–22].

Due to the potential of neural stem cells for cell therapy, the importance of developing and optimizing approaches was realized for production of hNSCs. Although the above studies can model cortical development well, most of the cells differentiated from hES cells are a mature mixed population including the upper layer and deep layer cortical neurons. It is unclear whether highly enriched hNSCs have been generated from hES cells. We like to develop differentiation protocols which eliminate the use of undefined factors.

Noggin, known as bone morphogenesis protein (BMP) inhibitor, is a critical neural-inducing factor both in frog and mammalian [23, 24]. Recombinant Noggin has been applied in different neural induction protocols for hES cell differentiation [25, 26]. Recently, SB431542 presents to increase neural induction ability in an embryoid body-based neural induction protocol from hES cells by suppressing the Lefty/Activin/TGFb pathways [14, 27]. Although Noggin or SB431542 treatment can prompt the efficiency of neural induction, treatment alone is not valid for neural induction by converting hES cells under defined or adherent conditions [14].

Multipotential stem cells from hES offer great promise for cell replacement therapies. Better differentiation protocols are necessary for reducing undefined factors in order to investigate the potential of these approaches in neural cell production. However, the qualified procedure is scarce and detailed features of hNSCs originated from hES cells are still unclear.

Here, we developed a method to obtain hNSCs from hES cells, by which we could harvest abundant hNSCs in a relatively short time. Most hES cells differentiated into NSCs according to this protocol. Then, we characterized the separating NSCs by detecting the expression of marker protein and identified their differentiation potential into astrocytes and neurons. Finally, we analyzed the mitotic activity and cell division cycle ratio of hNSCs and found that these hNSCs were healthy populations. Our study will provide detailed characteristics of hNSCs and improve the knowledge of how to obtain more hNSCs from hES cells. Our study may shed light on the therapeutic potential of these cell populations for the treatment of brain injury and disease.

2. Materials and Methods

2.1. hES Cells and Cell Culture Condition. hES cells (H9) were cultured for 30–32 passages on mouse embryonic fibroblasts (MEF) cells. MEF cells were obtained from 16-day pregnant mice and cultured in 10% foetal bovine serum (FBS) in high glucose DMEM (Gibco). Before using, MEF cells were treated with mitomycin C to block the MEF division. hES cells were planted on MEF cells at $1.8 \times 10^3/\text{cm}^2$ densities. And then, hES cells were cultured on MEF cells under the following conditions: DMEM/F12 (Gibco) containing 20% knockout serum replacement (Gibco), 1 × nonessential amino acids (Gibco), 1 × GlutaMAX (Gibco), 1/2 × penicillin/streptomycin (Gibco), 0.1 mM beta-mercaptoethanol (Sigma), and 6 ng/mL FGF-2 (HumanZyme) on 6-well plates. Medium was changed freshly every day. When cells needed to passage every 8 days, we used 1 U/mL dispase II (Roche) in DMEM/F12 (Gibco) for digesting the cells. Finally, we seeded the cells at 1 : 9 proportions into new 6-well plates.

2.2. Matrigel, Poly-L-ornithine, and Laminin-Coated Plates. The plates were coated with gelatin (Sigma) or Matrigel (BD) for neural stem cell induction. Other plates were precovered with poly-L-ornithine (Sigma) and laminin (Thermo Fisher) for human NSC culture and passage. The dishes are freshly coated with gelatin or Matrigel and must be kept overnight at 4°C for better package effect. The dishes were treated by 0.5 μg/mL poly-L-ornithine (dissolved in water) at room temperature for 16 hours. Then, we applied 1x PBS to wash the dishes. Finally, 5 μg/mL laminin was added to the dishes for at least 16 hours. The coated dishes can be centrally stored in the refrigerator for −20 degrees, and before using them, we needed to thaw and discard the supernatant liquid.

2.3. Neural Stem Cell Induction. hES cells were digested by applying StemPro Accutase (Thermo Fisher) for 20 min at 37°C. Then, cells were collected and washed with hES cell growth medium. Cell sedimentation was centrifuged and collected. The cells were put into the gelatin-coated plates for 1 hour at 37°C. Because hES cells were suspended and the MEF cells were adherent in existence of ROCK inhibitor (Tocris), we could collect the suspended cells and separated hES cells from MEF cells. The nonadherent hES cells were washed and plated at 5×10^4 cells/cm^2 density on Matrigel-precoated dishes in MEF-conditioned medium (DMEM/F12 containing 20% suspended medium collected from MEF cells which were not treated by mitomycin C, 20% KSR, 1 × nonessential amino acids, 1 × GlutaMAX, 1/2 × penicillin/streptomycin, 0.1 mM beta-mercaptoethanol, 10 ng/mL FGF-2, and ROCK inhibitor). After 1 day, the medium containing ROCK inhibitor was changed. Single adherent hES cells expanded in cell medium until they were almost confluent. Fresh medium was changed every 2 days. Noggin (500 ng/mL, R&D) and TGF-beta inhibitor (10 mM, Tocris) were added to confluent cells.

Fresh medium was changed every 2 days using KSR medium. On the 6th day, TGF-beta inhibitor was removed from differentiation medium and changed the cells into the medium with 25% N2 and 75% KSR with 500 ng/mL Noggin. Two days later, 50% N2 medium and 50% KSR medium with 500 ng/mL Noggin were given to cells. After 2 more days, 75% N2 medium and 25% KSR medium with 500 ng/mL Noggin were brought to cells. Nearly after 10 days of differentiation, NSCs were cultured with 100% N2 medium (DMEM/F12/N2/B27/GlutaMAX/FGF/EGF/heparin/penicillin/streptomycin) for 1 day and then transferred into a 100% N2/B27 medium (DMEM/F12/N2/B27/GlutaMAX/FGF/EGF/heparin/penicillin/streptomycin). hNSCs were passaged every 5 days at a 1 : 4 ratio onto the poly-L-ornithine and laminin-coated plates.

2.4. Neural Stem Cell Character and Differentiation. hNSCs were cultured in 100 mm dishes and passaged to poly-L-ornithine and laminin-coated 24-well plates at the 4th passage in N2/B27 medium supplemented with FGF/EGF/heparin factors. Three days later, hNSCs in 24-well plates were fixed with 4% paraformaldehyde (PFA) for 12 minutes.

To characterize the potential ability of NSCs, spontaneously differentiation was initiated. Spontaneously differentiation assay could induce NSCs into neurons, astrocytes, and oligodendrocytes. hNSCs were planted in the poly-L-ornithine and laminin-coated 24-well plates at a lower density in 100% N2/B27 medium with 3 factors (FGF/EGF/heparin) for 16 hours. On the second day, we changed fresh N2/B27 medium without FGF, EGF, and heparin factors. Then, we refreshed the medium without factors every 2 days. This process of spontaneous differentiation lasted for 21 days at least. Then, differentiated cells were fixed by using a method which was described above.

2.5. Antibodies, Immunostaining, and Microscopy. For BrdU immunostaining, the living cells needed to incubate with growth medium including 10 μM BrdU for 24 hours in the dark CO_2 incubator and then fixed cells by 4% PFA in PBS as follows. Fixed cells were permeabilized by 1% saponin in PBS for 8 minutes and then washed by 0.1% saponin in PBS. 2 mol/L HCl (2N) treated the cells for 18 minutes. Cells were washed by 0.1% saponin in PBS for three times and blocked with 5% bovine serum albumin (BSA) for 1 hour at room temperature. Cells were incubated with BrdU primary antibody at 4°C for 48 hours and second antibody at room temperature for 1.5 hours according to the general process.

For other antibody staining, cells fixed by 4% PFA were washed with PBS for 3 times and then were permeabilized with 2.5% Triton X-100. Cells were blocked with 5% BSA for 1 hour at room temperature. According to the general immunofluorescence technique, cells were washed with 0.1% Tween-20 in PBS and incubated with primary antibody at 4°C for 48 hours. Primary antibodies used for immunostaining were Oct4 (Biovision), Sox2 (R&D), Pax6 (Covance), Nanog (R&D), Nestin (R&D), Tuj1 (Covance), S100-beta (R&D), Ki67 (Thermo Fisher), and BrdU (Santa Cruz). The dilution buffer of primary antibody was 2.5% BSA in PBS. Cells were washed with 0.1% Tween-20 in PBS for 3 times and incubated with a second antibody at room temperature for 1.5 hours. At last, we used 4′,6-diamidino-2-phenylindole (DAPI, Sigma) to mark the nucleus of hNSCs. Additional attention, when we used DAPI, we must treat the cells for 10 minutes at room temperature after the second antibody incubation.

Cells were observed by using an inverted fluorescence microscope (Nikon). The images were acquired under a color CCD camera and digitized by PC-based frame grabber. Then, photos were analyzed by ImageJ, which was a powerful image analysis software. Then, the data collected from ImageJ were calculated by Excel. Calculation results were input into GraphPad Prism 6 and then organized as charts.

2.6. Flow Cytometry and Statistical Analysis. Cells were collected after digestion and centrifuged at 1000g/min for 3 minutes. Cell pellet was washed with 3 mL PBS and gently suspended in 50 μL PBS. Cells were dropped to 1 mL precooled 70% ethanol in PBS. Cells were kept at −20 degrees overnight. On the second day, tubes containing cells were centrifuged at 500g/min. The supernatant was discarded, and the cells were resuspended gently with 400 μL PBS containing 0.5 μg/mL Hoechst 33258 (Sigma) and 10 μg/mL RNAse A. The cell tubes were kept at room temperature in the dark. Lastly, we added Pyronin Y to the tubes at the final concentration of 0.5 μg/mL. After 20 minutes, tubes were placed on ice in the dark and analyzed by flow cytometry (BD). The results from flow cytometry were calculated and analyzed by FlowJo.

All results were showed as mean standard deviation of the mean (SD). Data were calculated by Excel and p value was measured for statistical significance of two-tailed Student's t-test.

3. Results and Discussion

3.1. Results

3.1.1. Induction of Neural Stem Cell from hES Cells. In order to develop an optimal scheme and detect the detailed features of hNSCs which differentiate from hES cells, we expanded the differentiation method to obtain hNSCs (Figure 1). Using this new protocol, hNSCs were isolated through a simple process and a relatively short time (Figure 1(a)). Concrete state of cells was showed during different differentiation stages (Figure 1(b)). During this differentiation process, cells needed to stay in a different medium for a specific culture time (Figure 1(c)). The overall process was hNSCs originally were cultured on MEF cells and were harvested to digest into single cells. After single ES cells grew to confluent layer, inducing factors were added to the confluent ES cells. Then, cells were cultured until the end of differentiation. The whole procedure lasted for 4 weeks, and during this period, it is necessary to continuously change medium details described as follows.

With the purpose to demonstrate the process of inducing differentiation in detail, we documented various stages of cell differentiation. hES cells were cultured and digested into single cell as showed in the first two pictures (Figure 2(a)). In

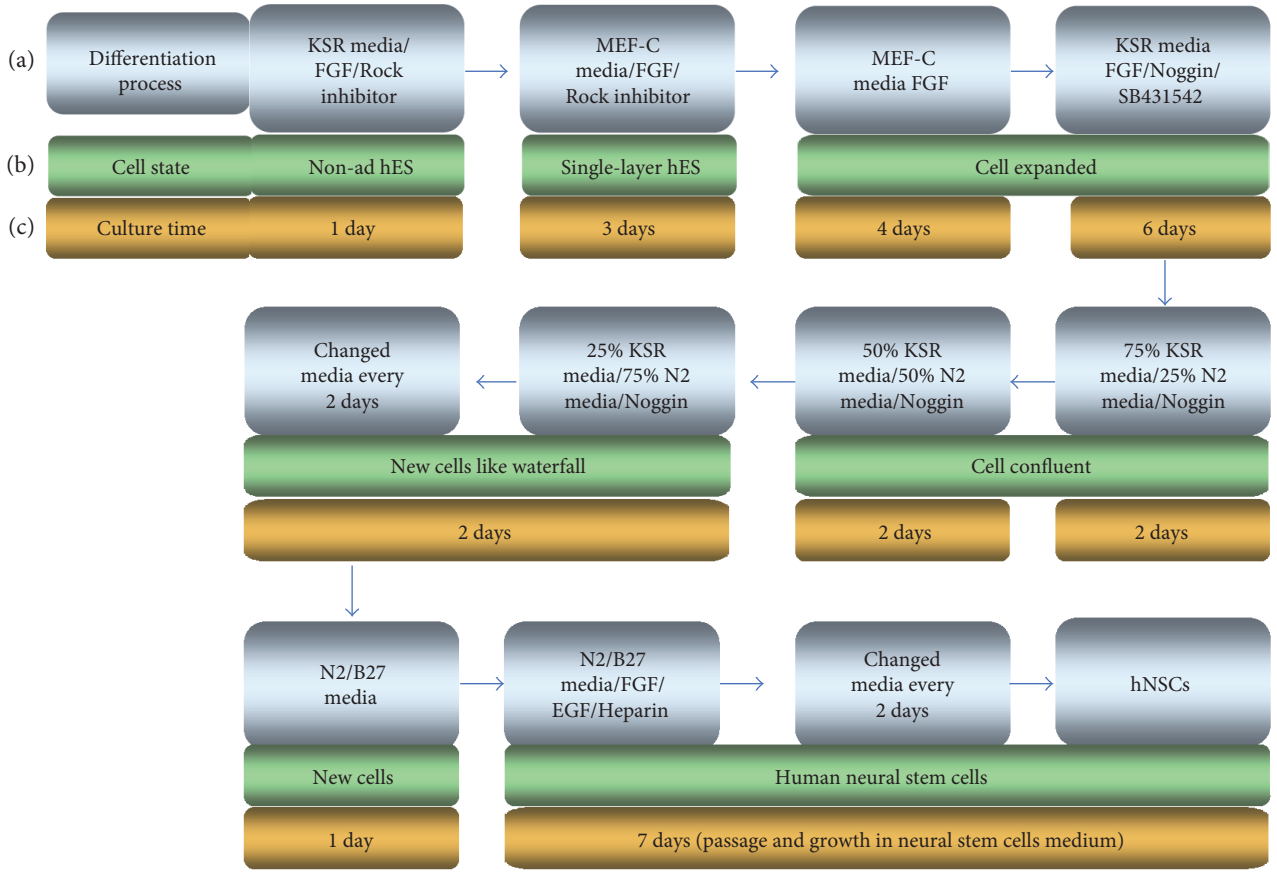

Figure 1: Flow chart of method used to obtain neural stem cell-like cells derived from human embryonic stem cells. (a) Differentiation process of human embryonic stem cells to neural stem cells. (b) State of cells in different differentiation stages. Human embryonic stem cells originally grew on feeder cells, and then cells were digested to single cell and removed the feeder cells. Single hES cells stuck to the dish bottom surface and grew to single-layer hES cells. Reduced medium and the factors were added to the single-layer hES cells. The cells were cultured until the end of differentiation. The process lasted about 4 weeks, and the cells were kept carefully in a fresh medim. KSR media: knockout serum replacement media; MEF-C media: mouse embryonic fibroblast cell conditional medium; non-ad hES: nonadherent human embryonic stem cells.

order to get enough cells, hES cells were cultured and amplified on MEF feeder cells (Figure 2(a), A). hES cells had grown on MEF feeder cells for 7 days and will be digested immediately (Figure 2(a), B). For removing MEF feeder cells, hES cells were digested with gelatin-coated plates (Figure 2(a), C). Nonadherent hES cells are planted into Matrigel-coated dishes and expanded in MEF-C medium about 4 days until confluent (Figure 2(a), D).

Cell morphology was presented during induction progress especially at days 1, 5, 10, and 12 with Noggin and SB431542 added to human ES cells (Figure 2(b)). Confluent single-layer ES cells were cultured in differentiation medium including KSR medium with TGF-beta inhibitor and Noggin for 1 day (Figure 2(b), A). State of Cells was showed after 5 days differentiation (Figure 2(b), B). Cells were differentiated for 10 days and changed to KSR medium only with Noggin from the 6th day (Figure 2(b), C). After 12 days, cells grew in 25% N2 media with 75% KSR medium for 2 days (Figure 2(b), D).

To track the cell differentiation state, we used imaging to record the birth of new cells during differentiation. Cells could be observed that there were many cells crawling out from the assembled hES cells (Figure 2(c)). Then, we passaged cells into growth medium which was prepared for hNSCs (Figure 2(c)). A large number of newborn cells climbed from hES cells in 15 days and 21 days (Figure 2(c), A-B). After cell passage, the hNSCs clone formed as showed (Figure 2(c), C-D). Differentiated cells from hES cells grew homogeneously and looked very healthy (Figure 2(d)). The morphology of cells may suggest that the new born cells had a healthy state and good proliferative ability (Figure 2(d), A-B). Different generations were shown from passage 0 to passage 1 (Figure 2(d), C-D).

3.2. Cells Differentiated from hES Cells Were Sox2 and Pax6 Positive. To identify the differentiation tendency and potential of cells from embryonic stem cells, we used specific markers to test cell properties. We found that cells were Octamer-binding transcription factor 4 (Oct4) negative and Sox2 positive (Figure 3(a)). Oct4 was critically involved in the self-renewal of undifferentiated embryonic stem cells [28]. SRY- (sex-determining region Y-) box 2, also known as Sox2, is a transcription factor that is essential for maintaining embryonic and neural stem cells [29]. Nanog is a transcription factor critically

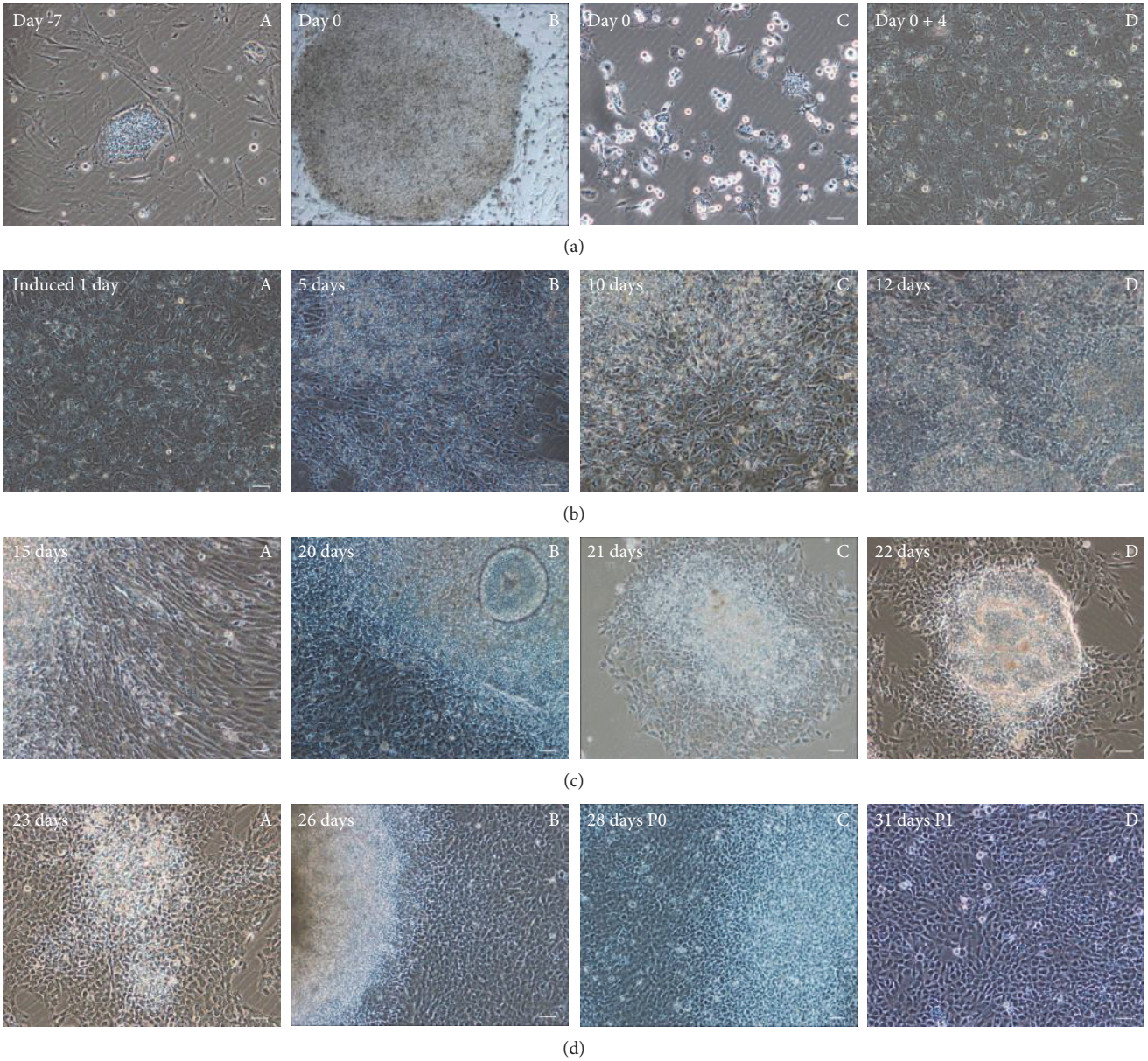

Figure 2: Neural stem cell-like cells were differentiated from human embryonic stem cells. (a) Human embryonic stem cells were cultured and digested into single cell, then the feeder cells (MEF cells) were removed, and single-layer cells were cultured in MEF conditional media until they were confluent. (A) hES cells grew on MEF feeder cells and will be digested after 7 days culture to start differentiation. (B) hES cells had grown on MEF feeder cells for 7 days and will be digested immediately. (C) hES cells were digested to gelatin-coated plates to remove MEF cells. (D) The nonadherent hES cells expanded in MEF-C medium about 4 days until confluent. (b) Noggin and SB431542 induced human ES cells into hNSCs. (A) The confluent single-layer hES cells were cultured in differentiation medium including KSR medium with TGF-beta inhibitor and Noggin for 1 day. (B) Differentiation for 5 days in differentiation medium. (C) Differentiation for 10 days and changing to KSR medium only with Noggin from the 6th day. (D) After 12 days, cells grew in 25% N2 media with 75% KSR medium for 2 days. (c) Cells crawled out from the assembled ES cells. After cell passage, the clone was visible. (A) Cells climbed from hESCs at 15 days. (B–D) State of cells around passage. (d) Differentiated cells from human ES grew homogeneously and fast. Scale bar = 50 μm. (A, B) Different generations from passage 0 to passage 1.

involved with self-renewal [30]. Pax6 achieves its key roles in neurogenesis and proliferation [31]. Here, we found hNSCs expressed Pax6, the neurogenesis factors (Figure 3(b)). However, the expression of Nanog was decreased but still not fully withdrawn (Figure 3(b)). Persistence of transcription factor Nanog may be due to its critical role in the self-renewal of neural stem cells as well as in embryonic stem cells. In our study, although most hES cells could differentiate into hNSCs, there were a small population hES cells that refused to differentiate into hNSCs (Figure 3(c)). These little undifferentiating hES cells also showed embryonic cells character—Oct4 positive (Figure 3(d)) and died soon in the specific culture in hNSCs medium (data not shown).

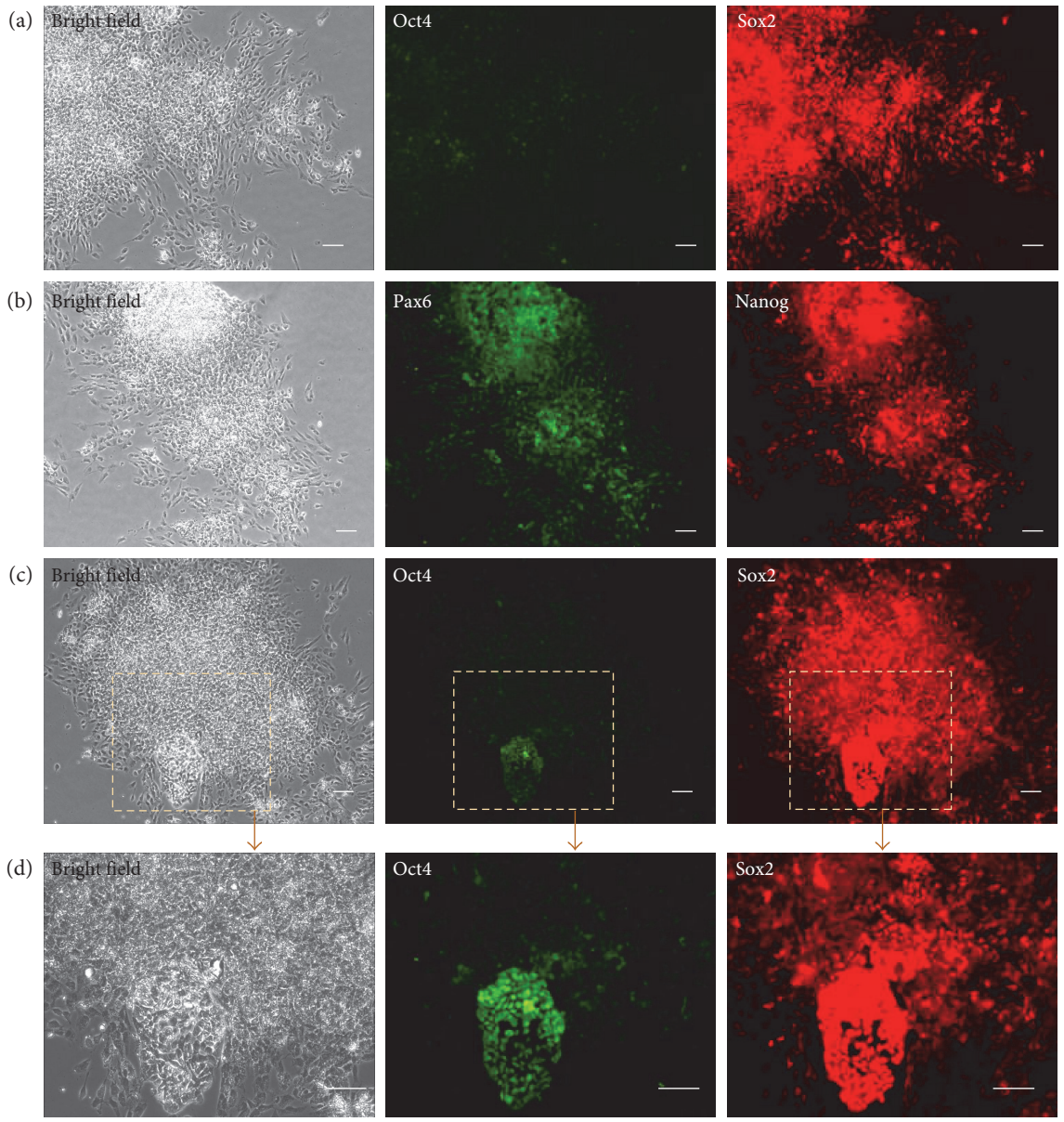

Figure 3: Human neural stem cell-like cells differentiated from hES cells were Sox2 and Pax6 positive. (a) Cells differentiated from hES cells were Oct4 negative and Sox2 positive. (b) Pax6 and Nanog were also expressed in human neural stem cell-like cells. (c, d) Most hES cells differentiated into hNSCs, but a small population hES cells did not differentiate into human neural stem cell-like cells and also showed embryonic cell character—Oct4. (d) It was an enlarged field of (c). Scale bar = 50 μm.

3.3. Cells Expressed Marker Proteins of NSCs and Could Differentiate into Astrocyte and Neuron. To thoroughly investigate neural stem cells, we used the neural stem cell marker protein antibodies for immunostaining. We fixed the 4th passage cells differentiated from hES cells and discovered that these cells were Sox2 (Figure 4(a)) and Nestin (Figure 4(b)) positive. Cells expressed that both Sox2 and Nestin were considered as the characteristics of hNSCs. In order to detect the potential of cell differentiation, we used spontaneous differentiation to identify whether hNSCs could generate into glial cells and neurons. We found that cells could differentiate into S100-beta-positive cells (astrocyte) (Figure 4(c)) and neuron-specific class III beta-tubulin- (Tuj1-) positive cells (neuron) (Figure 4(d)).

3.4. Mitotic Activity Analysis of hNSCs. To analyze the activity of cells, we utilized 5-bromo-2′-deoxyuridine (BrdU) antibodies to detect the division status of neural stem cells. BrdU is always used as a thymidine analogue in the identification of DNA synthesis. BrdU-positive cells were observed by immunofluorescence staining (Figure 5(a)), and almost half of the cells (40.2%) were BrdU positive in hNSCs (Figure 5(b)). Ki67, a marker protein of ribosomal RNA transcription, is a nuclear protein that is necessary for cellular proliferation. We also explored Ki67 to further investigate the cell proliferation. Data showed that Ki67-positive cells were observed by immunofluorescence staining (Figure 5(c)) and nearly half of the cells (52.2%) were Ki67 positive in hNSCs (Figure 5(d)).

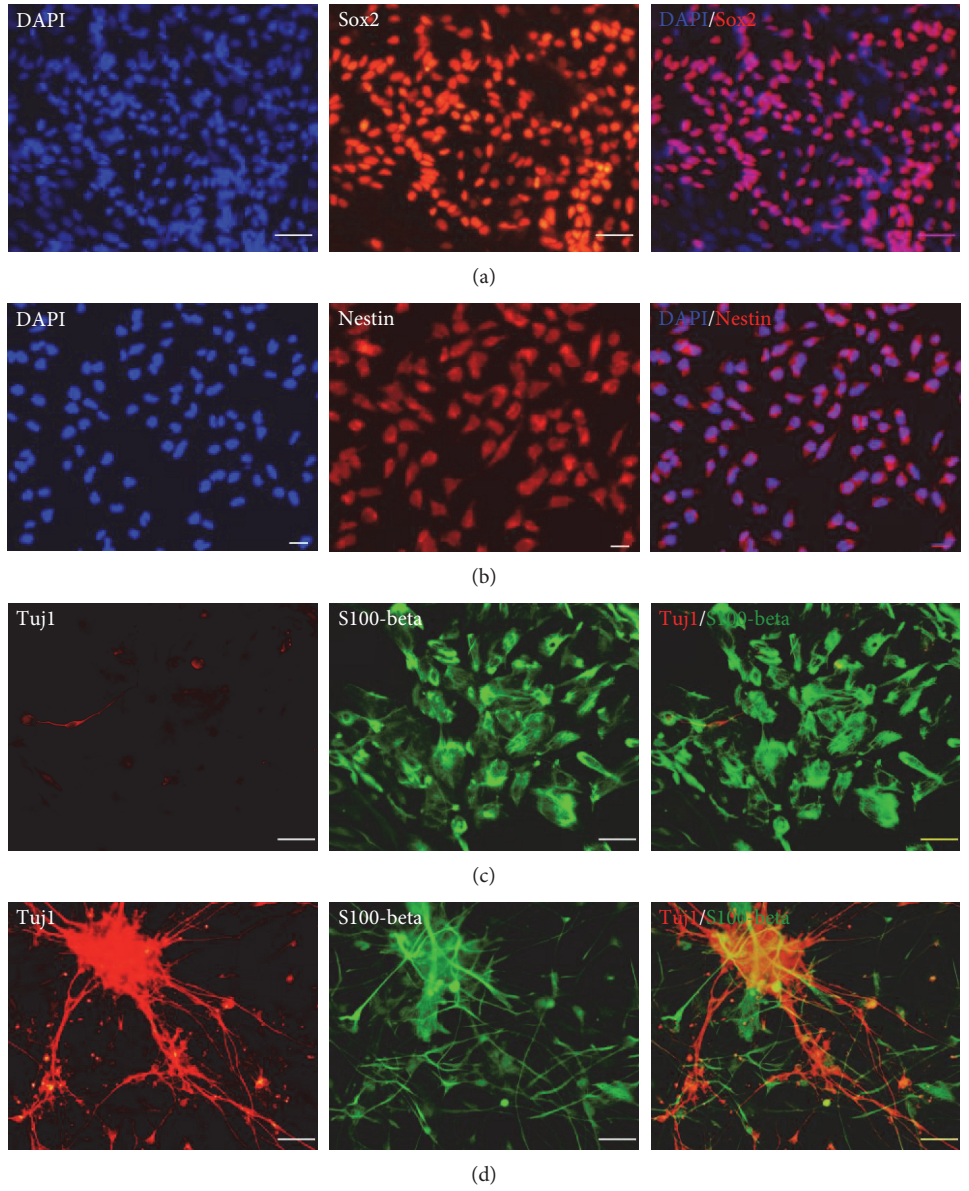

Figure 4: Cells expressed neural stem cell marker proteins and can differentiate into astrocyte and neuron. (a, b) Cells differentiated from hES cells expressed Sox2 and Nestin protein. (c, d) Cells express neural stem cell marker proteins and can differentiated into S100-beta-positive cells (astrocyte) and Tuj1-positive cells (neuron). (a) Scale bar = 50 μm; (b–d) scale bar = 25 μm.

3.5. Cell Division Phase Assay of hNSCs. To obtain the character of cell division and cell phase, we extinguished hNSC flow cytometry analysis using Pyronin Y and Hoechst 33258 staining. Hoechst 33258 is a blue fluorescent dye using to stain DNA. Pyronin Y is used for RNA staining. G0/G1 phase DNA always kept at 2N but RNA started to replicate in G1 phase. DNA of S phase cells changed between 2N and 4N, and RNA continued to replicate. DNA content reached to 4N in G2/M phase. Almost half of hNSCs were in division (Figure 6(a)). Morphology of hNSCs was shown before flow cytometry analysis (Figure 6(b)). Data showed that 54% of cells were distributed in G0-G1 phase, 31% in S phase, and 14% in G2-M phase (Figure 6(c)).

4. Discussion

Our study provides detailed characteristics of hNSCs and improves the knowledge of how to obtain more high-quality hNSCs from hES cells. These results may help to prompt the therapeutic potential of these cell populations for cell therapy.

In this paper, we adopted a double-inhibiting method to obtain hNSCs from hES cells by combined applying of Noggin and SB431542. We harvested hNSCs through a simple procedure in a relatively short time. We identified the potential of cells from embryonic stem cells. Most hES cells could differentiate into NSCs, except a small population of Oct4-positive hES cells. These little nondifferentiation hES cells died quickly in the following culture (data not shown).

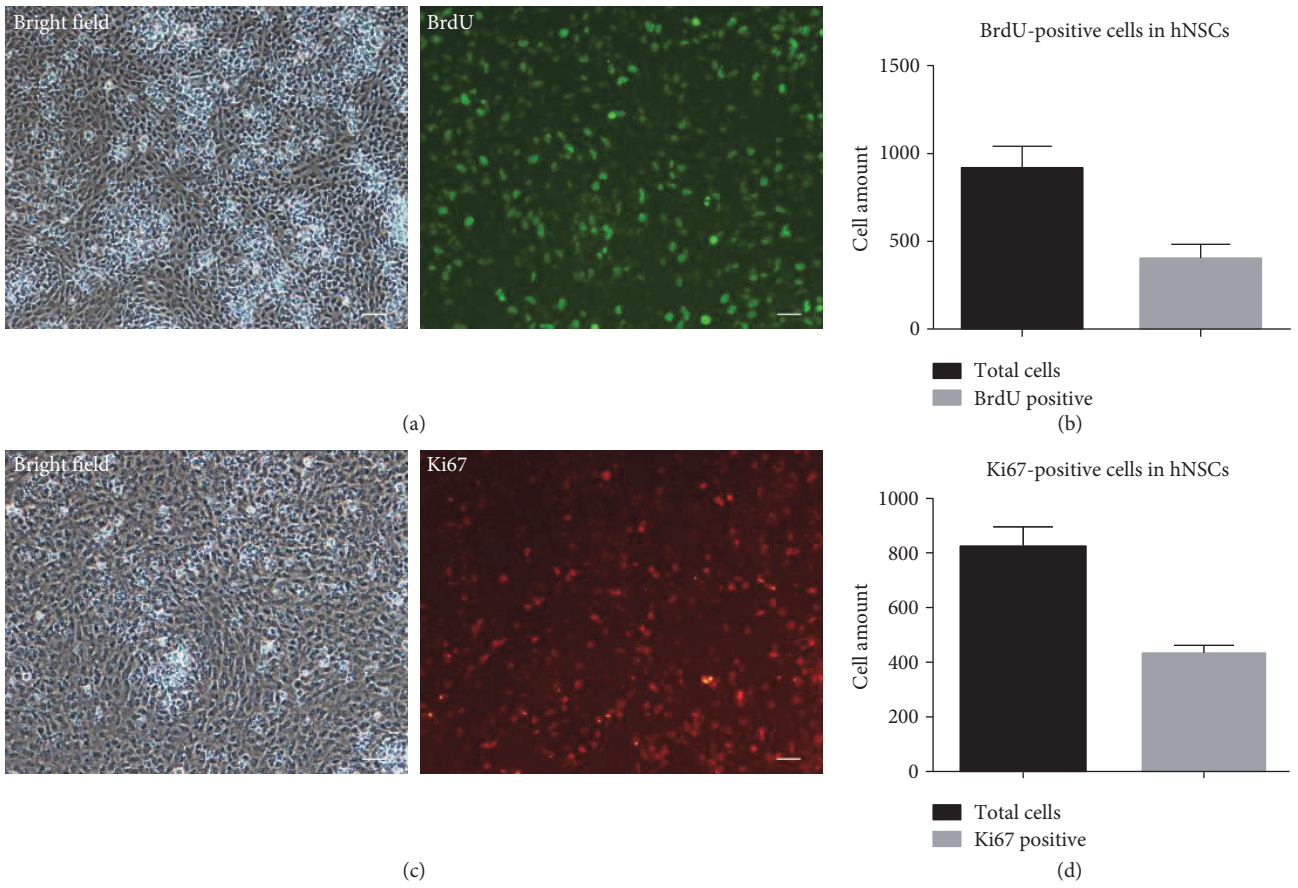

Figure 5: Mitotic activity analysis of human neural stem cells by using BrdU and Ki67 antibodies. (a) BrdU-positive cells were observed by immunofluorescence staining. (b) Nearly 40.2% (396/985) cells were BrdU-positive in hNSCs. (c) Ki67-positive cells were observed by immunofluorescence staining. (d) Nearly 52.2% (427/817) cells are Ki67-positive in hNSCs. Scale bar = 50 μm.

Then, we identified hNSCs by detecting the expression of marker protein and confirmed their differentiation potential into astrocytes and neurons. Furthermore, we analyzed the mitotic activity and cell division cycle ratio of hNSCs and found these hNSCs were active residents. To examine the activity of cells, we exploited Ki67 and BrdU antibodies to detect the division of neural stem cells.

Currently, studies of promoting hES cells to neural differentiation are mostly focused on differentiation of mature functional neurons or neural crest stem cells for cell therapy [15, 16]. Such differentiation procedure is developed for efficient production of dopamine progenitors from hES cells [16]. GABA neurons and cerebral cortex-specific neurons are derived from hES cells [17, 18]. At the same time, mature cortical neurons generated from hES cells by some small molecules [19–22]. Although the above studies can model cortical development fine, most of the cells which are produced from hES cells are a mixed population including mature neurons.

It is indistinct whether highly enriched hNSCs which had high self-renewal and proliferation capabilities have been generated from hES cells. Here, we developed differentiation protocols which eliminate the use of undefined factors. The defined differentiation factors will reduce the application obstacles of hNSCs in cell therapy. We investigated an efficient and fast differentiation approach to obtain hNSCs from hES cells successfully through a simple process in a shorter time compared to the usual method [25, 32].

Then, we identified the separating hNSCs by detecting the expression of marker protein and identified their differentiation potential into astrocytes and neurons. Finally, we analyzed the mitotic activity and cell division cycle ratio of hNSCs and found that these hNSCs were healthy populations. From hES cells to hNSCs, our differentiation method only needs about 3 weeks (from Figure 2(a),B) to Figure 2(c),C). Three weeks is a very short time because of previous reports. It frequently takes 5–7 weeks when inducing differentiation in the presence of MEF cells [25, 32].

Brain injuries such as traumatic injury, ischemic stroke, Parkinson's disease, or other neurodegenerative disorders are major causes of death and disability in the worldwide and brought serious social and economic burden [33–36]. Hence, brain injury therapies aimed to reduce neurological deficit are needed and cell therapies are crucial [37].

Stem cell transplantation plays a great potential to reduce the disability and promotes brain function after central nervous system (CNS) trauma and disease [38, 39]. Preclinical data points out that those cell-based therapies can enhance

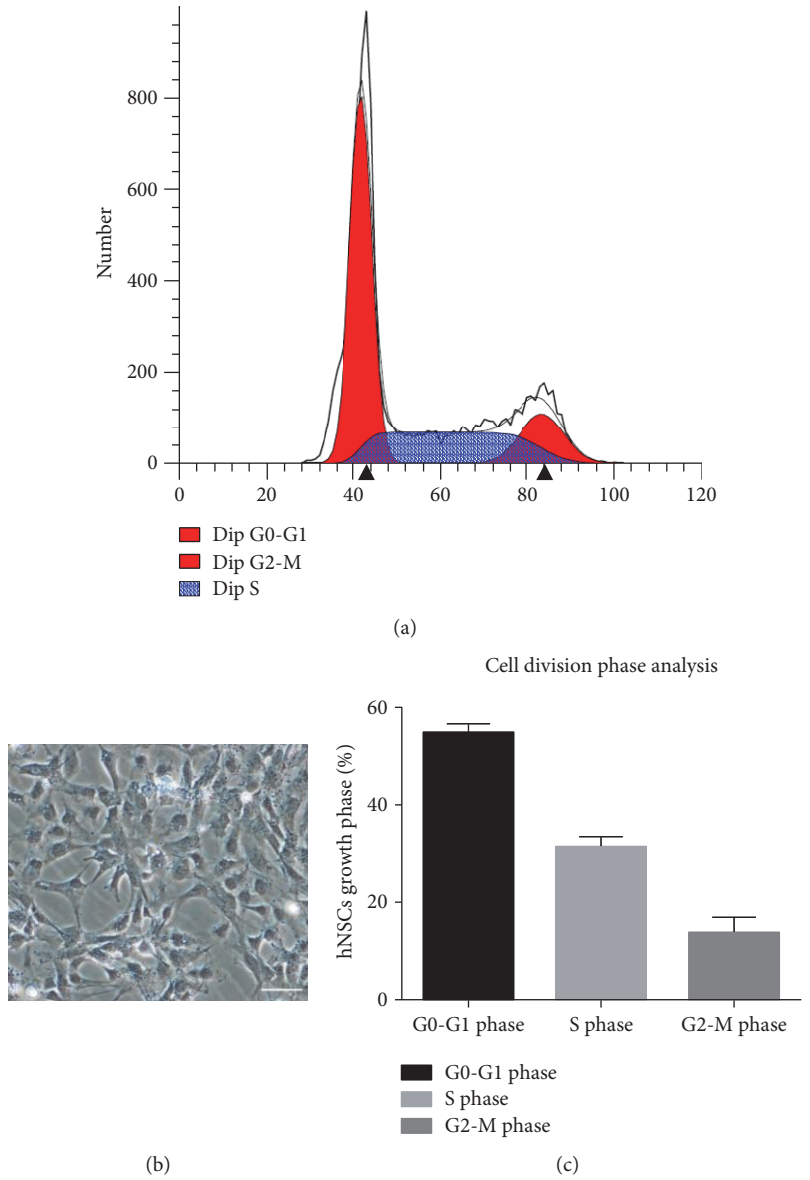

Figure 6: Flow cytometry analysis of human neural stem cells by using Pyronin Y and Hoechst 33258. (a) Flow cytometry analysis showed hNSCs and almost half of the cells were in division. (b) The morphology of hNSCs before flow cytometry. (c) Cell division phase analysis of hNSCs. 54% cells were distributed in G0-G1 phase, 31% in S phase, and 14% in G2-M phase. Scale bar = 50 μm.

brain repair and significantly improve functional recovery after stroke or other brain injury [40]. They were proved safe and efficient both in stroke experimental animal and stroke patients [41, 42].

Cell-based therapies especially stem cell therapy also have been tested in some neurological disorders and get hopeful results proposing that it is maybe an efficient stroke therapy [42]. The reagents which target to protect neural stem cells, cerebral endothelial cells, astrocytes, oligodendrocytes, and neurons also can improve neurological function after stroke [37].

NSCs are immature precursors of the CNS and on self-renewal and multipotential differentiation abilities. Hormonal and local factors can directly regulate their proliferation and differentiation capability [43]. Alteration in neurogenesis is associated with many neurological disorders. Research results show that NSCs can be a potential therapy for brain injury [43].

Noggin is a BMP antagonist and plays an important role in neural tube development [44, 45]. Recombinant Noggin in mammalian also performs neural-inducing role [24]. Recombinant Noggin has been applied to several different neural induction protocols for hES cell differentiation [25, 26]. Lately, the drug SB431542 presents to support neural induction from hES cells [27]. SB431542 destroys the Lefty/Activin/TGFb pathways by inhibiting activities of ALK4, ALK5, and ALK7 receptors [14]. Although Noggin or SB431542 treatment prompts the efficiency of neural induction, treatment alone is not valid for neural induction by converting hES cells under defined or adherent conditions [14].

Multipotential stem cells from hES cells offer great promise for cell replacement therapies. Better differentiation protocols are necessary for reducing undefined factors in order to apply these approaches for the production of neural cells. However, detailed features of hNSCs which differentiate from hES cells are still unclear.

We also did the oligodendrocyte differentiation assay for 3 weeks, and cells presented the phenotype of oligodendrocytes. Oligodendrocyte differentiation medium consists of neurobasal medium supplementation with B-27, GlutaMAX-I, and T3. But finally, our immunostaining test did not obtain the ideal results and maybe, we need more tests. There was a difference between Ki67 and BrdU detecting division of neural stem cells. BrdU was generally used as a thymidine analogue in the identification of DNA synthesis. Proliferation marker protein Ki67 stops chromosomes from collapsing into a single chromatin mass and acts as a biological surfactant to separate mitotic chromosomes [46]. We should design more assays to discover the proliferation information. Additional work will be required to identify the mechanisms after double inhibition of hNSC differentiation and needed to further decrease the quantity close to zero of undifferentiated hES cells.

5. Conclusions

Cell-based therapy can increase functional recovery and help neurological brain injury. NSCs can be a potential therapy for brain injury. Multipotential stem cells from hES cells provide great promise for cell replacement therapies. Better differentiation protocols are necessary in order to produce more neural stem cells for therapies. However, the qualified procedure is scarce and detailed features of hNSCs originated from hES cells are still unclear. In this study, we developed a procedure to get hNSCs from hES cells, by which we could harvest abundant hNSCs in a pretty short time. We provided comprehensive features of hNSCs and delivered the knowledge about how to obtain more high-quality hNSCs from hES cells. These results may help to accelerate the therapy by using these stem cells to treat brain injury.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

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Research Article

Optogenetic Inhibition of Striatal Neuronal Activity Improves the Survival of Transplanted Neural Stem Cells and Neurological Outcomes after Ischemic Stroke in Mice

Yifan Lu,[1] Lu Jiang,[1] Wanlu Li,[1] Meijie Qu,[2] Yaying Song,[2] Xiaosong He,[3] Zhijun Zhang,[1] Guo-Yuan Yang,[1,2] and Yongting Wang[1,4]

[1]*Neuroscience and Neuroengineering Research Center, Med-X Research Institute and School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai, China*
[2]*Department of Neurology, Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200030, China*
[3]*Department of Human Anatomy, Guangzhou Medical University, Guangzhou 510182, China*
[4]*Brain Science and Technology Research Center, Shanghai Jiao Tong University, Shanghai, China*

Correspondence should be addressed to Guo-Yuan Yang; gyyang0626@163.com and Yongting Wang; yongting.wang@gmail.com

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Neural stem cell (NSC) transplantation is a promising treatment to improve the recovery after brain ischemia. However, how the survival, proliferation, migration, and differentiation of implanted NSC are influenced by endogenous neuronal activity remains unclear. In this work, we used optogenetic techniques to control the activity of striatal neurons and investigated how their activity affected the survival and migration of transplanted NSCs and overall neurological outcome after ischemic stroke. NSCs cultured from transgenic mice expressing fluorescent protein were transplanted into the peri-infarct region of the striatum after transient middle cerebral artery occlusion (tMCAO) surgery. The striatal neurons were excited or inhibited for 15 minutes daily via implanted optical fiber after tMCAO. The results revealed that mice which received NSC transplantation and optogenetic inhibition had smaller brain infarct volume and increased NSC migration compared to the NSC alone or PBS group ($p < 0.05$). In contrast, mice which received NSC transplantation and optogenetic excitation showed no difference in infarct volume and neurological behavior improvement compared to the PBS control group. In vitro experiments further revealed that the conditioned media from excited GABAergic neurons reduced NSC viability through paracrine mechanisms. *Conclusion*. Optogenetic inhibition of striatal neuronal activity further improved neurological recovery after NSC transplantation at the subacute phase after brain ischemia.

1. Introduction

Ischemic stroke is a cerebrovascular disease which can result in motor, sensory, cognitive deficits and even death. Developing effective treatment and rehabilitation strategies for ischemic stroke remains a challenging task at present [1]. In recent years, studies on animal models showed that neural stem cell (NSC) transplantation holds promise for the treatment of stroke [2–5]. In the early phase after brain ischemia, transplanted NSCs can activate the endogenous repair pathways that are involved in immunomodulation [6, 7], angiogenesis [7–9], neurogenesis [9, 10], and neural plasticity [11, 12], mostly through paracrine mechanisms. In the chronic phase of stroke, transplanted NSCs can replace the lost neurons and integrate into the host circuitry by physically connecting with surrounding host cells, which may contribute to long-term recovery [13–15]. However, the therapeutic efficacy of stem cells is limited by the poor survival of transplanted cells [16]. The previous studies had unveiled the influence of various extrinsic survival factors including growth factors, morphogens, proteoglycans, cytokines, and hormones in the poststroke niche [17] on the survival and proliferation of embryonic and adult NSCs. Adult NSCs also were shown to respond to a variety of

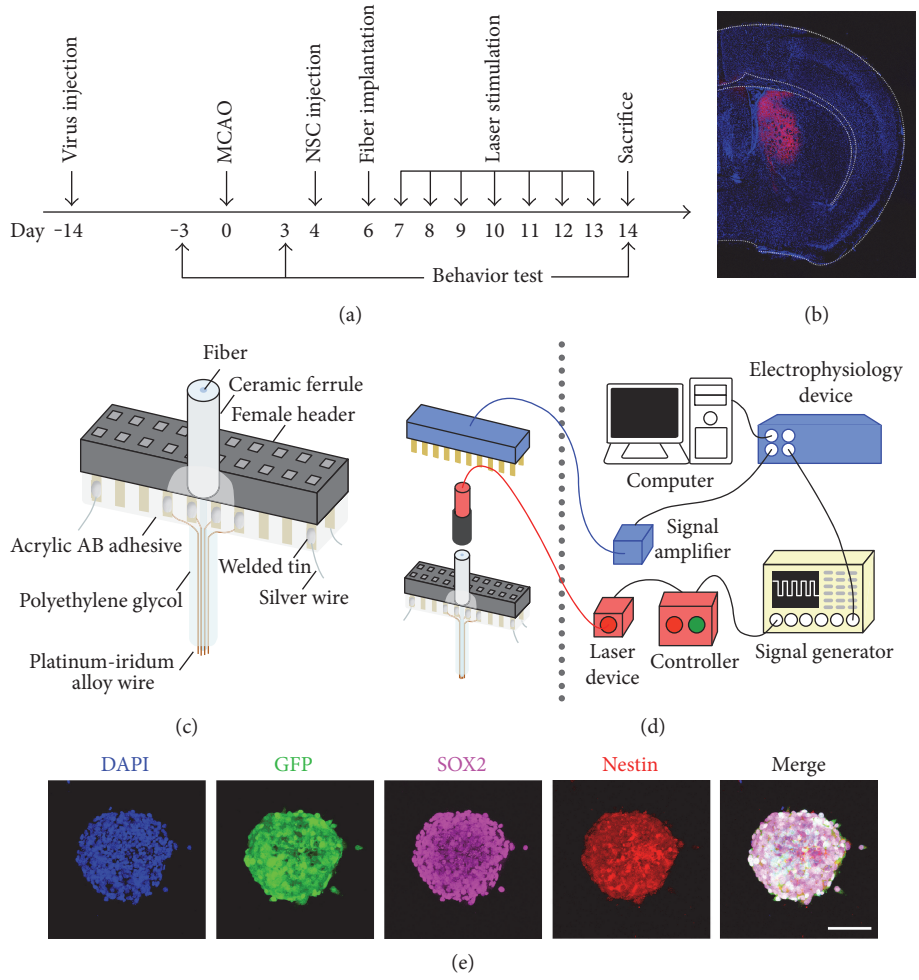

Figure 1: Experimental design and technical set-up. (a) The schedule of animal experiments in this study. (b) The representative expression of ChR2-mCherry opsins in striatal neurons under CaMKII promoter. (c) The schematic representation of the structure of lab-made optrode. (d) The hardware system for synchronous laser stimulation and electrophysiological recording. (e) The characterization of cultured NSC-GFP sphere by immunohistochemical staining with NSC markers SOX2 and Nestin. Bar = 100 μm.

neurotransmitters such as glutamate, dopamine, histamine, GABA, D-Serine, NO, and 5-HT [17]. Action potential can lead to the secretion of neurotransmitters, suggesting that the activity of surrounding intrinsic neurons would influence the survival, proliferation, and differentiation of the transplanted NSCs. However, very limited data is available regarding how intrinsic neuronal activities influence the behavior and fate of transplanted NSCs under pathological conditions after cerebral ischemia.

In this study, we used optogenetic technique to regulate striatal neuronal activity after transient middle cerebral artery occlusion (tMCAO) followed by NSC transplantation in mice to investigate whether exciting or inhibiting striatal neuronal activity in the peri-infarct region could improve the survival and migration of transplanted NSCs.

2. Methods and Materials

2.1. Animal Experimental Design. Animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University, Shanghai, China. Adult male ICR mice were housed in specific-pathogen-free rooms which were maintained on a 12-hour light/dark cycle at room temperature around 25°C. Intraperitoneal ketamine/xylazine (100 mg/10 mg per kg, Sigma, San Louis, MO) was used for anesthesia. The animal experimental design for this study is shown in Figure 1(a). Sixty-four mice received virus injection, thirty-two mice were used for excitatory stimulation experiments, and the others were used for inhibitory stimulation experiments. In the excitatory or inhibitory stimulation experiments, animals were randomly divided into four groups that designed to receive (1) PBS (PBS group); (2) PBS followed by laser stimulation (PBS-E for excitation or PBS-I for inhibition groups, resp.); (3) NSC transplantation (NSC group); and (4) NSC transplantation followed by laser stimulation (NSC-E or NSC-I group, resp.) after 60-minute tMCAO surgery. PBS or NSC was administrated at 4 days after tMCAO surgery. Optical fiber was implanted at day 6. Laser stimulation was carried out from day 7 to day 13, for 15 minutes daily. Behavioral test was performed before tMCAO and at 3 and 14 days after tMCAO. Animals were sacrificed at day 14.

2.2. Virus Transfection. After mice were anesthetized, 0.5 microliter adeno-associated virus (AAV) (Obio technology, Shanghai) carrying ChR2-mCherry or ArchT-eGFP gene under the control of CaMKII promoter was injected into the left striatum (AP = 0 mm, ML = −2.5 mm, DV = 3 mm relative to the bregma) using a minipump (WPI, Sarasota, FL) at a rate of 50 nl/min. The titer of AAV used in this study was 4×10^{12} TU/ml. After finishing injection, the needle was maintained in the brain for additional 5 minutes before it was withdrawn.

2.3. Transient Middle Cerebral Artery Occlusion Surgery. Two weeks after virus injection, mice underwent a 60-minute tMCAO surgery. The protocol of tMCAO surgery was described in the previous study [18]. Briefly, the middle cerebral artery (MCA) was occluded by a 6–0 suture (Covidien, Mansfield, MA) with a silicone-coated round tip. During the surgery, mice body temperature was maintained at 37 ± 0.5°C using a heating pad (RWD Life Science) with a feedback control. The blood flow in MCA was measured by Laser Doppler monitor (Moor instruments, Devon, U.K.) before MCAO, at the beginning of occlusion and 1 minute after reperfusion. Animals whose MCA blood flow did not decrease to 10%~20% of baseline during occlusion or did not recover to 40%~60% of baseline after reperfusion were excluded from this study.

2.4. NSC Culture, Characterization, and Transplantation. NSCs were isolated from the cortex of E14 transgenic mice (Animal Research Center of Nanjing University, Nanjing, China) that express green fluorescent protein (GFP) under the control of beta-actin promoter or red fluorescent protein (RFP) under the control of Tie2 promoter, as previously described [19] and maintained by DMEM/F-12 medium (Gibco, Carlsbad, CA, USA) with B27 supplement (Gibco) and growth factors (20 ng/ml epidermal growth factor, 20 ng/ml fibroblast growth factor-2) (Gibco) at 37°C under 5% CO_2 atmosphere. Culture medium was replaced with fresh medium every 2 days. NSC spheres were passaged at one-week intervals. The NSCs used for experiments were obtained from cultures that underwent 3–5 passages. To characterize the cultured NSCs, the cells were grown on poly-L-ornithine hydrobromide (Sigma, St. Louis, MO) and laminin (Sigma)-coated glass coverslips and immunostained with Nestin (Millipore, Billerica, MA, USA) and SOX2 (Abcam, Cambridge, MA).

NSC transplantation was performed at 4 days after tMCAO. Before transplantation, NSC spheres were incubated with accutase (Gibco) for 20 minutes to disperse into single NSCs. A total of 3×10^5 NSCs dissolved in 10 μl PBS was injected into the left striatum (AP = 0 mm, ML = −2.5 mm, DV = 3 mm relative to the bregma) using a minipump at 1 μl/min. After finishing the injection, the needle was maintained in the brain for additional 5 minutes before it was withdrawn.

2.5. Optrode Preparation and In Vivo Electrophysiology. Optrode preparation and implantation were performed as previously described [20]. The structure of the homemade optrode was shown in Figure 1(c). The optrode was comprised of an optical fiber, with 200 μm diameter and 0.22 numerical aperture (Thorlabs, NEWTON, NJ), surrounded by 8 platinum-iridium alloy microelectrodes with 35 μm diameter (Plexon, Dallas, Texas). The optrode was implanted into the left striatum of the mouse (AP = −0.02 mm, ML = −2.5 mm, DV = 2 mm relative to bregma) at 3 days after tMCAO for *in vivo* electrophysiology recording. Optrode implantation and *in vivo* electrophysiology were performed on 3 mice in each group that express ChR2 or ArchT in the striatum, respectively. Electrophysiological data was synchronously recorded by electrophysiological instrument (Plexon, Dallas, Texas) when the striatal neurons were stimulated by a laser (Figure 1(d)). Single and multiunit activity recordings were sampled at 30 kHz and bandpass filtered at 250 Hz to 3000 Hz. Different laser power conditions ranged from 0.001 mW to 0.1 mW for 473 nm laser pulse in the ChR2 group and from 0.1 mW to 2 mW for 530 nm constant laser in the ArchT group were tested. At the beginning of each recording, five-minute baseline was recorded to ensure stable electrophysiological signal. All recordings were carried out with the animals under anesthesia.

2.6. Optical Fiber Implantation and Laser Stimulation. All animals except the 6 mice for in vivo electrophysiology underwent optical fiber implantation at 6 days after tMCAO, following the protocol as described in the previous study [20]. Laser stimulation was performed once daily from 7 to 13 days after tMCAO. Each stimulation session lasted for 15 minutes. The parameters of lasers were controlled by a waveform generator (Tektronix, Shanghai, China). For the ChR2 group, each 5-second stimulation cycle was composed of 1-second stimulation phase and 4-second rest phase. In the stimulating phase, 473 nm laser pulses with 5 ms pulse width were administrated at 20 Hz. For the ArchT group, 530 nm laser was administrated constantly for 15 minutes. The laser powers were 0.05 mW for 473 nm laser pulses and 1 mW for 530 nm constant laser, measured by an optical power meter (Thorlabs).

2.7. Neurological Behavioral Test and Brain Infarct Assessment. Modified neurological severity score (NSS) scaled from 0 to 14 [21] was used for the estimation of injury caused by ischemic insult and the recovery after NSC transplantation and laser stimulation. In addition, beam walk test was used to assess the neurological functional recovery. Before tMCAO surgery, animals were trained twice daily for 3 days to pass a beam with a length of 1 meter to establish the baseline. The assessment of NSS and beam walk test were performed at 3 and 14 days after tMCAO. Data were collected for three independent trials, and the average time was used in statistical analysis.

Animals were sacrificed at 14 days after tMCAO. Mice were transcardially perfused first with normal saline and then with freshly prepared 4% paraformaldehyde in PBS after deep anesthesia with 10% chloral hydrate (350 mg/kg). Brains were quickly removed into isopentane at −80°C and then frozen sectioned into 20 μm slices. For each brain, five coronal sections that are 2 mm apart were used for the

quantification of infarct volume. The sections were stained with 0.1% cresyl violet (Sigma) for 10 minutes at room temperature followed by gentle rinsing with water for 1 hour. The sections after staining were imaged using a digital camera, and the infarct volume was calculated using NIH ImageJ software as previously described [22]. The volume was assessed as the following formula:

$$V = \sum \frac{h}{3}\left[\Delta Sn + (\Delta Sn * \Delta Sn + 1)^{1/2} + \Delta Sn + 1\right]. \quad (1)$$

In the formula, h represented the distance between two adjacent sections and Sn and Sn + 1 were the infarct areas of two adjacent sections. The infarct ratio equaled to the infarct volume divided by the ipsilateral brain volume in the same coronal plane.

2.8. Fluorescent Immunohistochemical Staining and Quantification. Brain sections or cells grown on glass coverslips were treated with 4% paraformaldehyde for 10 minutes, 0.3% Triton-PBS for 30 minutes, and 5% normal donkey serum for 60 min, followed by incubation with antibody at 4°C overnight. For NSC characterization after cell culture, Nestin and SOX2 antibodies were used for double-staining, both in a dilution of 1 : 200. For vascular density measurement, CD31 antibody (R&D Systems, Minneapolis, MN) was used in a dilution of 1 : 200. After thorough rinsing with PBS, samples were incubated with secondary antibodies (Invitrogen, Carlsbad, CA) in a dilution of 1 : 500 for 60 min and DAPI (Invitrogen) for 5 minutes at room temperature. Photomicrographs were taken with a confocal microscope (Leica, Solms, Germany) under a 40x objective lens. The fluorescent signal was computed by the software ImageJ. After binary conversion of the photomicrographs, the numbers of pixel which had a brightness exceeding threshold value were counted to calculate the proportion of fluorescent signal on each photomicrograph. Data were normalized to the control group. To measure the survival and migration of NSCs, images of the area around the needle passage of three brain slices per animal were collected and quantified to yield the average fluorescent area. In the assessment of local vascular density, three fields around the peri-infarct area were chosen from each brain slice, and four brain slices from each mouse were quantified to yield the average vascular density for each animal.

2.9. Apoptosis Assessment. Cell apoptosis in the peri-infarct area, where NSC was transplanted, was detected by TUNEL-DAB method using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, Bedford, MA). After TUNEL-DAB staining, the sections were counterstained with hematoxylin (Biyuntian Company, Shanghai, China). The areas around the injection track in the striatum were imaged under a 20x objective lens. The DAB-positive cells in each photomicrograph were counted and averaged over 6 brain sections for each animal.

2.10. Primary Neuronal Culture. Primary neuronal culture was prepared from the cortices of GAD2-ChR2-tdTomato transgenic mice at embryonic day 15. The cortices were quickly isolated in ice-cold calcium and magnesium-free Hanks balanced salts solution (HBSS) followed by digestion with 0.05% trypsin (Gibco) for 10 minutes. After three PBS rinses, cells were counted and plated onto poly-d-lysine-coated 6-well plates at 3×10^6 per well in neural basal medium (Gibco) supplemented with 2% B27 (Gibco), 0.3 mM l-glutamine (Sigma), and 1% penicillin streptomycin. At 4 days after cell plating, 15 μg/ml 5-fluoro-2′-deoxyuridine (Sigma) and 35 μg/ml uridine (Sigma) were added to the cultures to inhibit nonneuronal cell proliferation. Half of the medium was replaced by fresh culture medium every 4 days. Neurons used for the experiment of laser stimulation were at 8 to 10 days of culturing.

2.11. Conditioned Medium Generation and NSC Viability Assessment. Before optogenetically stimulating the neurons, the medium was temporarily replaced by that used for NSC culture. The stimulation was carried out using 473 nm laser at 20 Hz pulses with a 5 ms pulse width and 0.05 mW power, for 5 minutes. The medium was immediately collected after stimulation. Standard cultured NSC spheres were dispersed into single cells using accutase and plated on a new well and incubated with the conditioned medium from excited GABAergic neurons for 2 days. The culturing medium was replaced by freshly conditioned medium daily.

The viability of the NSC in vitro was determined by CCK-8 kit (Donjindo, Kumamoto, Japan). The NSCs, cultured in conditioned media from light stimulated or nonstimulated GABAergic neurons, were plated in 96-well plates at 1×10^6 cells/ml. Ten microliter of CCK-8 solution was added to each well and incubated for 2 or 12 hours at 37°C. The absorption data were acquired using a microplate reader (Synergy2 BioTek, Winooski, VT) at 450 nm.

2.12. Real-Time Polymerase Chain Reaction (PCR) Analysis. The total RNA from NSC or neuron was prepared by RNA-TRIZOL extraction (Gibco, Grand Island, NY). RNA concentration was determined by spectrometric methods (ND-3300, NanoDrop Technologies, USA). Reverse transcriptions of RNA to cDNA were performed via a universal cDNA synthesis kit (EXIQON, Vedbaek, Denmark). The amplification was performed by a fast real-time PCR system (7900 HT, ABI, Foster City, CA) using a SYBR Green master mix (EXQION). Primer sequences of the β-actin, NGF, and GDNF were obtained from the PrimerBank. The cycling conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 10 seconds and 60°C for 1 minute. The relative expression level of NGF and GDNF was normalized to the β-actin control in triplicate and was calculated using the 2-Δct method.

2.13. Statistical Analysis. All results were presented as mean ± SD. Data was analyzed by two-way (for behavioral tests only) or one-way (for other analysis) ANOVA followed by Bonferroni post hoc comparison using GraphPad Prism version 3.05 (GraphPad Software, Inc., La Jolla, CA). $p < 0.05$ was considered statistically significant.

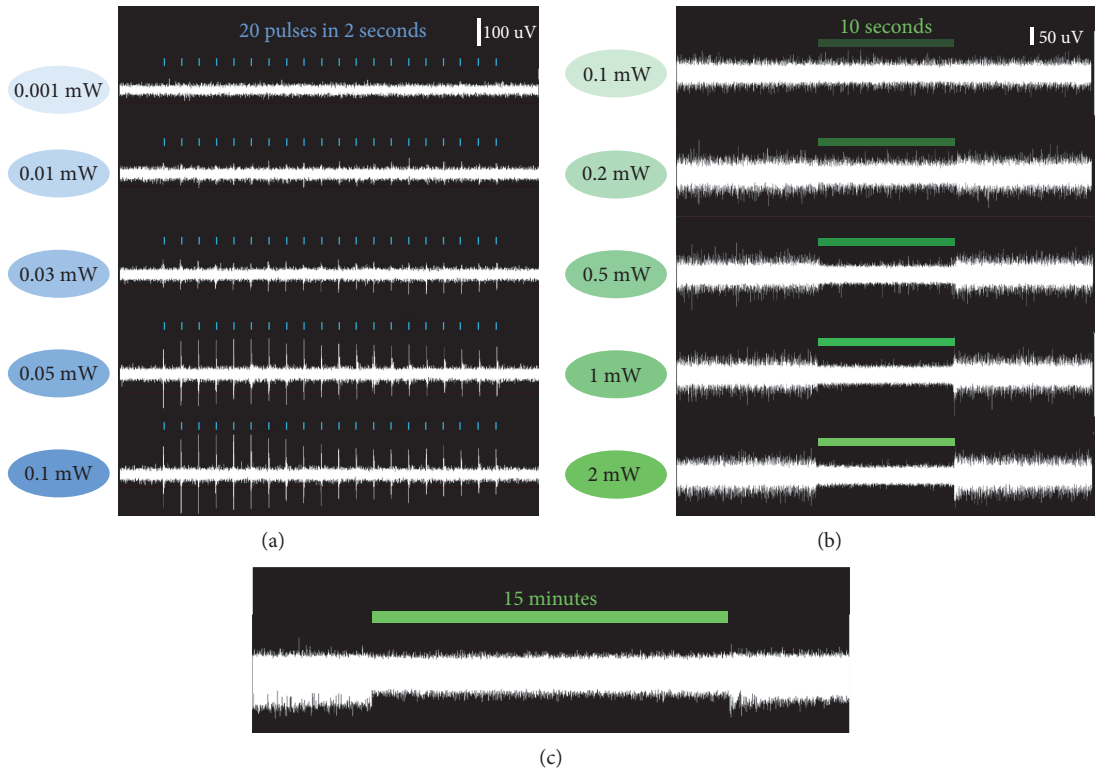

Figure 2: The validation of laser stimulation efficiency and the optimization of laser power based on electrophysiological recording in mouse striatum at 21 days after virus injection. (a) Excitatory stimulation with 473 nm laser pulse in the striatal area expressing ChR2. (b) Inhibitory stimulation with 530 nm constant laser in striatal area expression ArchT. (c) Uninterrupted stimulation with 1 mW 530 nm laser for 15 minutes did not affect the local spontaneous action potentials after the stimulation is finished.

3. Results

3.1. Validation of Optogenetic Control of Neuronal Activity in the Striatum. CaMKII promoter had been proved to be able to drive gene expression in most neurons in the striatum [20]. Consistent to the result of the previous study, in this study, AAV-carrying opsin gene under the control of CaMKII promoter successfully transfected most striatal neurons at 14 days after virus injection (Figure 1(b)).

In vivo electrophysiology data showed that the efficiency of optogenetic excitation or inhibition depended on the power of laser. A 0.05 mW 473 nm laser pulse was sufficient to stably trigger the action potential in striatal neurons expressing ChR2 (Figure 2(a)). For the striatal neurons expressing ArchT, administration of 530 nm constant laser with a power greater than 1 mW eliminated the majority of spontaneous action potentials (Figure 2(b)). The spontaneous action potentials immediately reappeared upon the conclusion of 530 nm laser stimulation (Figure 2(c)). These results demonstrated the successful optical control of striatal neuronal activity in this study.

3.2. The Inhibition of Striatal Neuronal Activity after Transplanting NSC Further Reduced Brain Infarct Volume after tMCAO. The ratio of brain infarct volume in each group at 14 days after tMCAO was measured by cresyl violet staining (Figures 3(a), 3(c), 4(a), and 4(c)) followed by image quantification. In the set of inhibition experiments, the NSC group and NSC-I group showed significantly smaller ratio of brain infarct volume when compared to the PBS control (NSC = 6.3 ± 0.8% versus PBS = 8.6 ± 1.4%, $p < 0.01$; NSC-I = 4.5 ± 0.7% versus PBS = 8.6 ± 1.4%, $p < 0.001$, $n = 7$). The NSC-I group showed significantly smaller brain infarct volume when compared to the NSC group (NSC-I = 4.5 ± 0.7% versus NSC = 6.3 ± 0.8%, $p < 0.05$, $n = 7$), but the PBS-I group showed no statistical difference with the PBS group (PBS-I = 8.1 ± 1.6% versus PBS = 8.6 ± 1.4%, $p > 0.05$, $n = 7$). In the set of excitation experiments, the NSC group showed significantly smaller ratio of brain infarct volume when compared to the PBS control, PBS-E group, and NSC-E group (NSC = 5.4 ± 1.1% versus PBS = 7.9 ± 1.8%, $p < 0.05$; NSC = 5.4 ± 1.1% versus PBS-E = 8.3 ± 2.1%, $p < 0.01$; and NSC = 5.4 ± 1.1% versus NSC-E = 7.8 ± 1.4%, $p < 0.05$, $n = 7$). The NSC-E group showed no statistical difference with the PBS group. These results suggested that inhibition of striatal neuronal activity can further enhance the beneficial effect of transplanted NSC, while excitation of striatal neuronal activity abolished the beneficial effect of NSC in reducing brain infarct.

3.3. Excitation of Striatal Neuronal Activity Reversed the Beneficial Effect of Transplanted NSC in Promoting Neurological Functional Recovery after tMCAO. Neurological function was assessed using neurological severity score as

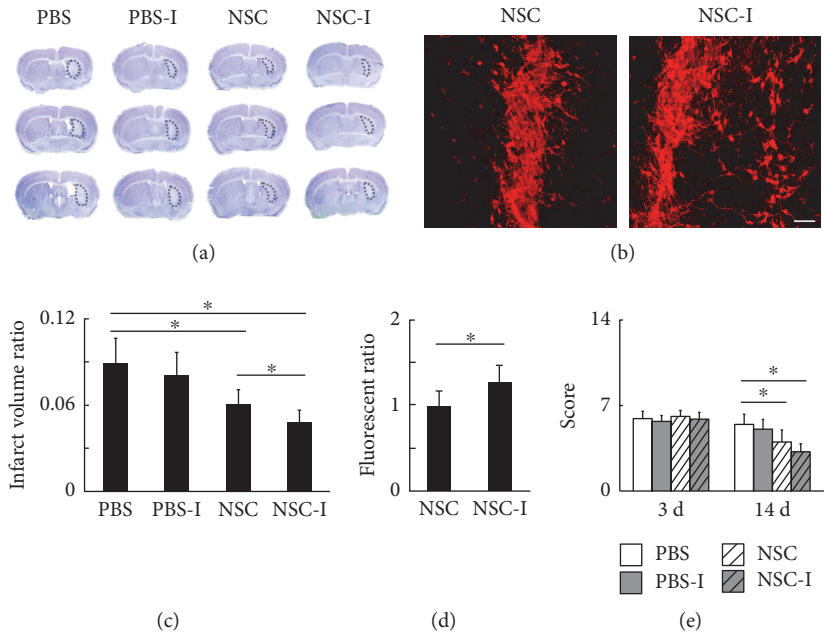

Figure 3: The inhibition of striatal neuronal activity enhanced the survival and migration of transplanted NSC and further reduced brain infarct volume. (a) Cresyl violet staining of the brain infarction at 14 days after tMCAO. (b) The fluorescence of NSC-RFP in the striatum at 14 days after tMCAO, bar = 100 μm. (c) The ratio of infarct volume over the contralateral hemisphere volume (PBS = 8.6 ± 1.4%, PBS-I = 8.1 ± 1.6%, NSC = 6.3 ± 0.8%, and NSC-I = 4.5 ± 0.7%, $n = 7$). (d) The quantification of fluorescence signal from the transplanted NSCs (NSC = 1.00 ± 0.17, NSC-I = 1.29 ± 0.18, $n = 6$). (e) The behavioral outcome evaluated by NSS method at 3 days (PBS = 5.9 ± 0.6, PBS-I = 5.7 ± 0.4, NSC = 6.1 ± 0.4, and NSC-I = 5.9 ± 0.5, $n = 7$) and 14 days (PBS = 5.5 ± 0.9, PBS-I = 5.1 ± 0.8, NSC = 4.0 ± 0.9, and NSC-I = 3.3 ± 1.0, $n = 7$) after tMCAO. $*$ represents $p < 0.05$.

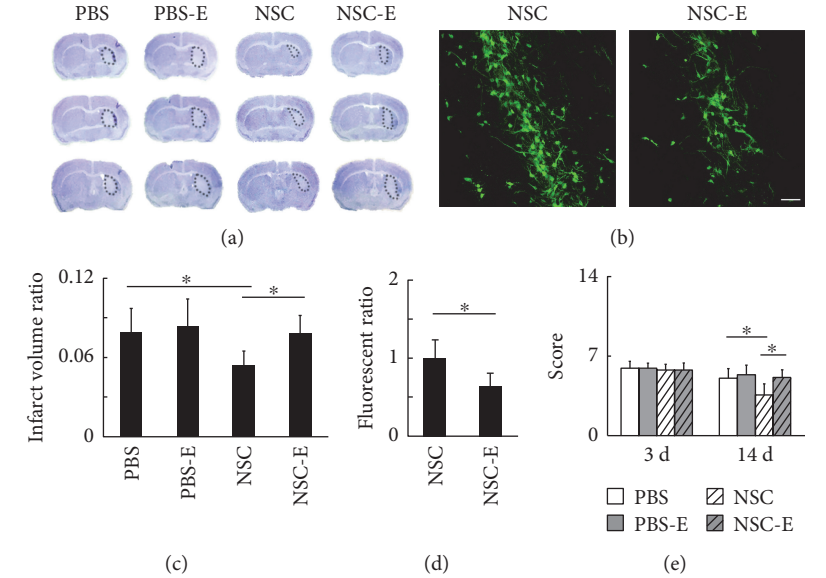

Figure 4: The excitation of striatal neurons after NSC transplantation reversed the beneficial effects of NSC in reducing brain infarction. (a) The brain infarction at 14 days after tMCAO. (b) The fluorescence from NSC-GFP in the striatum at 14 days after tMCAO, bar = 100 μm. (c) The ratio of infarct volume over the contralateral hemisphere volume (PBS = 7.9 ± 1.8%, PBS-E = 8.3 ± 2.1%, NSC = 5.4 ± 1.1%, and NSC-E = 7.8 ± 1.4%, $n = 7$). (d) The quantification of fluorescence signal from the transplanted NSCs (NSC = 1.00 ± 0.23, NSC-E = 0.64 ± 0.16, $n = 6$) (e) The behavioral outcome evaluated by NSS method at 3 days (PBS = 5.7 ± 0.6, PBS-E = 5.7 ± 0.4, NSC = 5.6 ± 0.5, and NSC-E = 5.6 ± 0.6, $n = 7$) and 14 days (PBS = 4.8 ± 0.8, PBS-E = 5.1 ± 0.8, NSC = 3.4 ± 1.0, and NSC-E = 4.9 ± 0.7, $n = 11$) after tMCAO. $*$ represents $p < 0.05$.

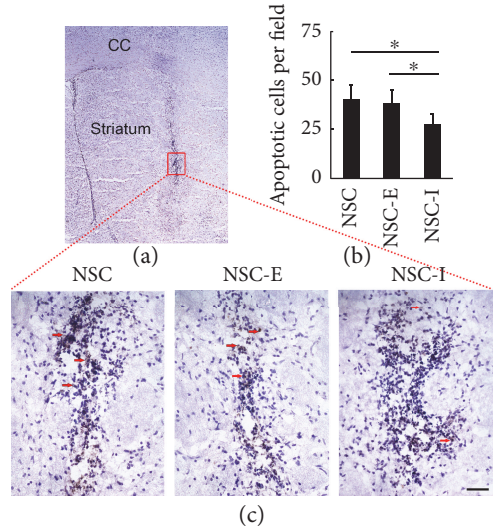

FIGURE 5: Striatal neuronal activity influenced cell apoptosis around the transplantation site. (a) DAB-TUNEL and hematoxylin staining. The area with high cell density in striatum is NSC transplantation site. The red rectangle shows the fields selected for quantifying the apoptotic cell number. (b) The quantitative result of apoptotic cell number in each group (NSC = 40.5 ± 6.6, NSC-E = 38.5 ± 6.2, and NSC-I = 27.7 ± 5.1, $n = 6$). (c) The apoptotic cells in NSC transplantation site. The brown signals indicated by red arrow are apoptotic cells. Bar = 20 μm. * represents $p < 0.01$.

previously reported at 3 and 14 days after tMCAO (Figures 3(e) and 4(e)). At 3 days after tMCAO, there were no statistical differences between any two groups. At 14 days after tMCAO, in the set of inhibition experiments, the NSC group and NSC-I group showed significantly lower NSS score when compared to the PBS control (NSC = 4.0 ± 0.9 versus PBS = 5.5 ± 0.9, $p < 0.01$; NSC-I = 3.3 ± 1.0 versus PBS = 5.5 ± 0.9, $p < 0.001$, $n = 7$). The NSC-I group showed no statistical difference to the NSC group. In the set of excitation experiments, the NSC group showed significantly lower severity score when compared to the PBS control, PBS-E group, and NSC-E group (NSC = 3.4 ± 1.0 versus PBS = 4.8 ± 0.8, $p < 0.01$; NSC = 3.4 ± 1.0 versus PBS-E = 5.1 ± 0.8, $p < 0.01$; and NSC=3.4 ± 1.0 versus NSC-E = 4.9 ± 0.7, $p < 0.01$, $n = 11$). The NSC-E group showed no statistical difference with the PBS group. The data of the beam walk test at 14 days after tMCAO showed that the NSC-I group is 15.1% faster in average to pass the beam when compared to the NSC group (NSC-I = 4.8 ± 0.9, NSC = 5.9 ± 1.4), but the NSC-E group took 20.1% more time in average to accomplish the mission when compared to the NSC group (NSC-E = 7.2 ± 1.7, NSC = 5.9 ± 1.4) (Supplementary Figure 1 available online at https://doi.org/10.1155/2017/4364302). These results suggested that excitation of striatal neuronal activity reversed the beneficial effect of transplanted NSC in promoting neurological functional recovery after tMCAO.

3.4. The Inhibition of Striatal Neuronal Activity Reduced the Apoptosis and Enhanced the Migration of Transplanted NSC. Transplanted NSCs were derived from GFP or RFP transgenic mice and were characterized by staining for markers Nestin and SOX2 before transplantation (Figure 1(e)). The migration of the transplanted NSC was compared between the laser-stimulated group and nonstimulated group by determining the spreading of fluorescence-positive cells in the brain sections (Figures 3(b), 3(d), 4(b), and 4(d)). In the set of inhibition experiments, the NSC-I group showed significantly larger fluorescent area when compared to the NSC group (NSC-I = 1.29 ± 0.18 versus NSC = 1.00 ± 0.17, $p < 0.05$, $n = 6$). In contrast, in the set of excitation experiments, the NSC-E group showed significantly smaller fluorescent area when compared to the NSC group (NSC-E = 0.64 ± 0.16 versus NSC = 1.00 ± 0.23, $p < 0.05$, $n = 6$). The number of apoptotic cells around the needle path was compared between the laser-stimulated group and nonstimulated group after TUNEL-DAB staining (Figure 5). The NSC-I group showed significantly less TUNEL-positive cells per field than the NSC-E group and NSC group (NSC-I = 27.7 ± 5.1 versus NSC-E = 38.5 ± 6.2, $p < 0.01$; NSC-I = 27.7 ± 5.1 versus NSC = 40.5 ± 6.6, $p < 0.01$, $n = 6$). These results suggested that inhibition but not excitation of striatal neuronal activity can reduce the apoptosis and enhance the migration of transplanted NSC.

3.5. The Inhibition of Striatal Neuronal Activity Further Increased the Vascular Density in the Penumbra after NSC Transplantation. To examine whether the improved survival and migration of transplanted NSC contributed to the better outcome after tMCAO through preventing vascular injury or promoting vascular repair, we examined the vascular density in brain sections by staining CD31 (Figure 6). Three photomicrographs were taken in the penumbra area in each brain slice for quantification of the vascular density. The NSC group and NSC-I group showed significantly higher vascular density when compared to the PBS group (NSC = 1.67 ± 0.28 versus PBS = 1.00 ± 0.21, $p < 0.001$; NSC-I = 2.10 ± 0.26 versus PBS = 1.00 ± 0.21, $p < 0.001$, $n = 6$). In addition, the

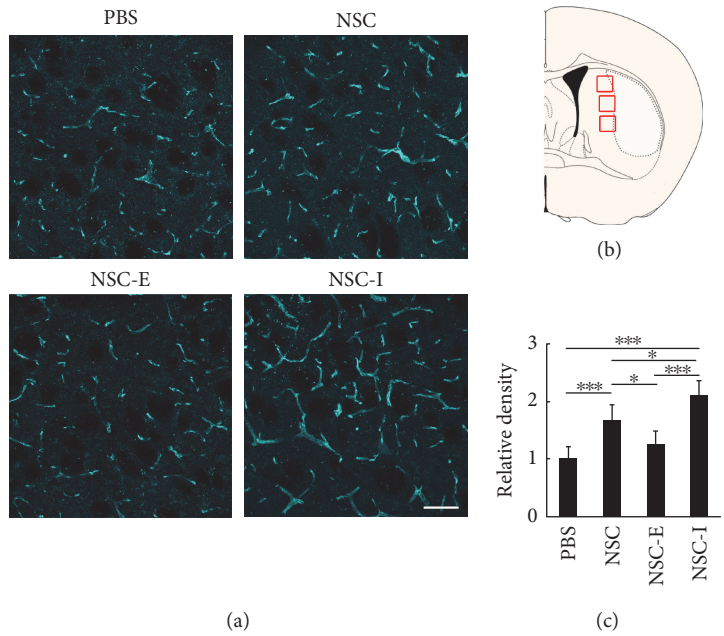

Figure 6: Striatal neuronal activity affected the vascular density in the penumbra after NSC transplantation. (a) The vascular density in the penumbra examined by staining with CD31, bar = 50 μm. (b) Selected fields for imaging evaluation (indicated by red rectangles). The light-colored area represents brain infarct. (c) The quantitative result of vascular density (PBS = 1.00 ± 0.21, NSC = 1.67 ± 0.28, NSC-E = 1.27 ± 0.22, and NSC-I = 2.10 ± 0.26, $n = 6$). $*$ represents $p < 0.05$. $***$ represents $p < 0.001$.

vascular density in the penumbra area of the NSC-I group was higher than that of the NSC group (NSC-I = 2.10 ± 0.26 versus NSC = 1.67 ± 0.28, $p < 0.05$, $n = 6$). In contrast, the NSC-E group exhibited reduced vascular density when compared to the NSC group (NSC-I = 2.10 ± 0.26 versus NSC = 1.67 ± 0.28, $p < 0.05$, $n = 6$). Compared to the PBS group, the NSC-E group showed a little higher vascular density in average, but the difference between the two groups is not significant (NSC-E = 1.27 ± 0.22 versus PBS = 1.00 ± 0.21, $p > 0.05$, $n = 6$). This result revealed a correlation between the enhanced transplanted NSC survival/migration and the increased vascular density in the NSC-I group and suggested that the better outcome after tMCAO brought by inhibiting the striatal neuronal activity after NSC transplantation is associated with higher vascular density.

3.6. Excitation of GABAergic Neuronal Activity Reduced NSC Viability through Paracrine Mechanisms in Cell Culture. To investigate whether striatal neuronal activity could influence the survival of transplanted NSC via paracrine mechanisms, we cultured neurons from GAD2-ChR2-tdTomato transgenic mice, stimulated the neurons by 473 nm laser pulse, collected the conditioned medium, and used the medium to incubate cultured NSC *in vitro* (Figure 7). The control group used medium from unstimulated neuron for NSC culture. The viability of NSC was examined by CCK-8 method. The NSC treated by laser-activated-neuron-conditioned medium showed less viability at 2 hours (Stim = 0.94 ± 0.02 versus Con = 1.00 ± 0.03, $p < 0.05$, $n = 3$) and 12 hours (Stim = 0.88 ± 0.02 versus Con = 1.00 ± 0.01, $p < 0.001$, $n = 3$) after incubation when compared to the control (Stim = 0.94 ± 0.02 versus Con = 1.00 ± 0.03, $p < 0.05$, $n = 3$). To further determine the status of NSC, NSCs incubated by laser-activated-neuron-conditioned medium or unstimulated-neuron-conditioned medium were used for NGF and GDNF mRNA quantification. These two factors could contribute to the survival of NSC. The data showed that NSC treated by laser-activated-neuron-conditioned medium expressed significantly lower NGF (Stim = 0.37 ± 0.06 versus Con = 1.00 ± 0.15, $p < 0.001$, $n = 3$) and GDNF (Stim = 0.54 ± 0.10 versus Con = 1.00 ± 0.07, $p < 0.01$, $n = 3$) in mRNA level. These results indicated that excitation of GABAergic neuorns can induce the secretion of substances, which inhibit NSC viability in vitro, and suggested that striatal neuronal activity could influence the survival of transplanted NSC through paracrine mechanisms.

4. Discussion

It has been reported that transplantation of NSCs, alone or in combination with other progenitor/stem cells, could improve the functional recovery after ischemic stroke [9, 23, 24]. However, how the survival, proliferation, migration, and differentiation of implanted NSC are influenced by endogenous neuronal activity remains unclear. In this study, we used optogenetic technique to investigate the relationship between striatal neuronal activity and the survival of transplanted NSCs after ischemic stroke. Although CaMKII was traditionally considered as a specific promoter for excitatory neuron in the cortex, it had been proven that CaMKII could be used as a forebrain-neuron-specific promoter in striatum to drive the gene expression of inhibitory neurons [25]. The results from this work demonstrated that inhibiting striatal neuronal activities improved the survival and migration of

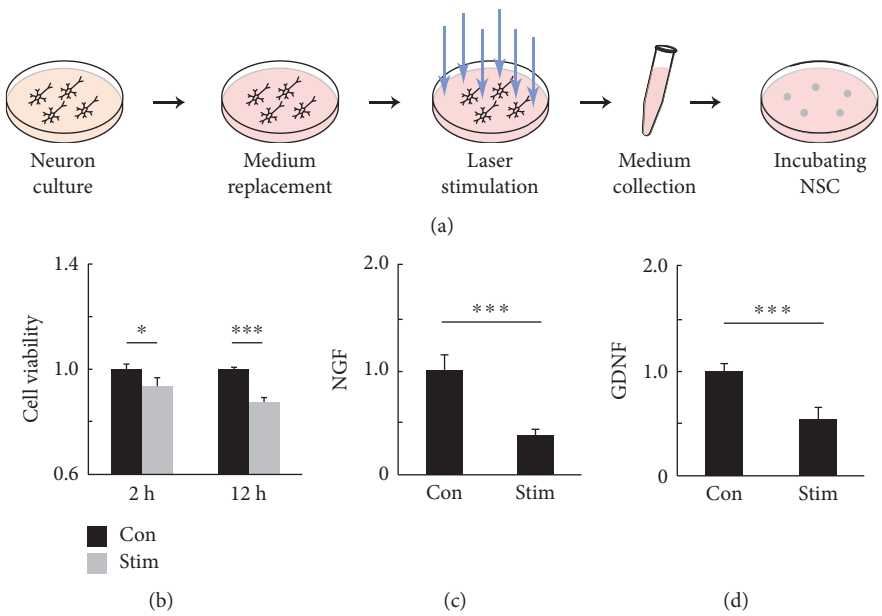

Figure 7: Excitation of GABAergic neurons can release factors that reduce NSC viability *in vitro*. (a) The schematic representation of the *in vitro* experiments. The blue arrows represent the stimulation with 473 nm laser pulse. (b) The viability of NSC, determined by CCK-8 assay at 2 hours (Con = 1.00 ± 0.03, Stim = 0.94 ± 0.02, $n = 3$) and 12 hours (Con = 1.00 ± 0.01, Stim = 0.88 ± 0.02, $n = 3$) after incubating with conditioned medium. (c) The relative mRNA expressions of NGF (Con = 1.00 ± 0.15, Stim = 0.37 ± 0.06, $n = 3$). (d) The relative mRNA expressions of GDNF (Con = 1.00 ± 0.07, Stim = 0.54 ± 0.10, $n = 3$). $*$ represents $p < 0.05$. $***$ represents $p < 0.001$.

transplanted NSCs and further reduced the brain infarct volume at 14 days after transient ischemic stroke. In contrast, activating striatal neuronal activities reversed the beneficial effect of transplanted NSCs in reducing brain infarct volume and promoting functional recovery. To investigate how neuronal activities affect the viability of transplanted NSCs, we cultured primary neurons from GAD2-ChR2-tdTomato transgenic mice and treated NSCs with the laser-activated-neuron-conditioned or unstimulated-neuron-conditioned medium (CM). The results showed that the CM from activated neurons decreased the expression of NGF and GDNF in NSCs. Reports showed that both NGF and GDNF play important roles in supporting of subpopulations of neurons [26–29]. This could be a potential reason why activating striatal neurons could decrease the survival of transplanted NSCs. The primary neurons used in the *in vitro* experiments of this study were cultured from the cortex consisted of approximately 20% GABAergic neurons [30]. The opsin-specific stimulation by laser would therefore activate about 20% of neurons in the culture. Nonetheless, we observed differences between the laser-excited group and the control group. A much significant difference may be observed with pure GABAergic neuron culture.

Studies have shown that exogenous stem cells could promote the functional recovery after ischemic stroke and other neurological diseases [24, 31–34]. However, one of the indisputable facts is that most of the transplanted exogenous stem cell would die in a short time after cell transplantation [16]. Our study demonstrated that inhibiting striatal neurons could promote the survival of transplanted NSCs after ischemia, providing a new sight to further augment the beneficial effect of NSCs.

5. Conclusions

NSC transplantation improved the neurological recovery after ischemic brain injury. Intrinsic neuronal activity impacts the migration and survival of transplanted NSCs. Optogenetic inhibition of striatal neuronal activity at the subacute phase after brain ischemia further augmented the beneficial effects of NSC transplantation, while activation of striatal neurons abolished the benefit of NSC treatment.

Conflicts of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' Contributions

Yifan Lu and Lu Jiang did the conception and design, provision of study material, collection and/or assembly of data, data analysis and interpretation, and manuscript writing. Wanlu Li, Meijie Qu, Yaying Song, Xiaosong He, and Zhijun Zhang did the provision of study material and collection and assembly of data. Yongting Wang and Guo-Yuan Yang did the conception and design, financial support, administrative support, provision of study material, data analysis and interpretation, and manuscript writing. Yifan Lu and Lu Jiang contributed equally to this work. All authors corrected and approved the manuscript.

Acknowledgments

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Review Article

Stem Cell Tracking Technologies for Neurological Regenerative Medicine Purposes

Yongtao Zheng,[1] Jiongwei Huang,[2] Tongming Zhu,[1] Ronggang Li,[1] Zhifu Wang,[1] Fukai Ma,[1] and Jianhong Zhu[1]

[1]*Department of Neurosurgery, Fudan University Huashan Hospital, National Key Laboratory of Medical Neurobiology, The Institutes of Brain Science and the Collaborative Innovation Center for Brain Science, Shanghai Medical College, Fudan University, Shanghai 200040, China*
[2]*Department of Urinary Surgery, Zhongshan Hospital of Fudan University, Shanghai, China*

Correspondence should be addressed to Jianhong Zhu; jzhu@fudan.edu.cn

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The growing field of stem cell therapy is moving toward clinical trials in a variety of applications, particularly for neurological diseases. However, this translation of cell therapies into humans has prompted a need to create innovative and breakthrough methods for stem cell tracing, to explore the migration routes and its reciprocity with microenvironment targets in the body, to monitor and track the outcome after stem cell transplantation therapy, and to track the distribution and cell viability of transplanted cells noninvasively and longitudinally. Recently, a larger number of cell tracking methods in vivo were developed and applied in animals and humans, including magnetic resonance imaging, nuclear medicine imaging, and optical imaging. This review has been intended to summarize the current use of those imaging tools in tracking stem cells, detailing their main features and drawbacks, including image resolution, tissue penetrating depth, and biosafety aspects. Finally, we address that multimodality imaging method will be a more potential tracking tool in the future clinical application.

1. Introduction

Neural stem cells (NSCs) are capable of self-renewing, proliferating, and differentiating into cells of the neural lineage, including neurons, astroglia, and oligodendroglia. NSCs had been used as a novel treatment strategy of brain trauma, stroke, and some neurological disorders, such as Parkinson's disease, both in the preclinical experimental and clinical settings [1–3]. As limited control and tracking of endogenous NSCs, exogenous NSCs or neural progenitor cells (NPCs) were used in cell therapy widely. After implanted in damaged brain regions, PSC-derived neurons could reestablish the damaged long range axonal projections and synaptic connections in the host brain [1]. Specifically, fetal brain-derived human neural progenitor cells (hNPCs), which transplanted in the induced injury striatum of an animal model, demonstrate their ability to protect the striatum and improve functional recovery [4]. Overall, neural progenitor/stem cells present a promising therapy strategy in the treatment of various neuronal diseases.

To ensure cell treatments are effective and successful, it is crucial to track the survival, migration, and differentiation of transplanted cells and to track their capabilities of reconstructing brain function and their biological role. Traditional methods to track implanted NSCs such as fluorescence imaging need to kill animals to test whether the transplanted stem cells survive or differentiate into tissue cells [5]. This translation of cell therapies into clinical settings has prompted a need to track the spatial destination, migration pathway, and final distribution of transplanted cells in vivo longitudinally, noninvasively, and repeatedly. Additionally, the advantages of an ideal imaging modality were as follows: high sensitivity of imaging agent, able to image deep tissues, high resolution, tracking transplanted cells for a long time, and very fast image acquisition [6, 7]. Among the various cell imaging modalities, MRI plays

an important role in the procedure of transferring cell therapies from the animal experiments to the clinical settings, because of its characteristic of noninvasive and good tissue contrast. These methods have had varying success, and they each have their own strengths and weaknesses of applicability in the central nervous system. For example, PET is a high-sensitive tracking method; however, it also has some limitations: low spatial resolution, radiation exposure, and short-term signal production. Optical imaging, which can track stem cells for a long time without radiation, is not feasible for clinic application as the limited penetration depth and low spatial resolution (Table 1). Therefore, a noninvasive method of tracking stem cells for a long time in the human body is a crucial step before translating stem cell research into clinical application.

In this review, we describe recent advances in the development of novel imaging sensors and tools in the field of tracking stem cells, as well as the benefits and drawbacks of each approach. We will address image spatial/temporal resolution, signal sensitivity, and tracking stem cells for a long time, as well as tissue penetrating depth associated with those imaging technique. Finally, we also describe multimodality molecular imaging of NSC transplantation in consideration that each technique has advantages and disadvantages.

1.1. Magnetic Resonance Imaging (MRI). Recently, MRI has become a very important method for real-time, noninvasive tracking stem cell fasting in clinical cell therapy trials, providing high resolution in the field of neurology [8]. The first study of MR tracking of transplanted progenitor cells in the CNS was reported in 1992, in which superparamagnetic contrast agents were used for cell imaging in rat brain [9]. MRI is a well-defined noninvasive cell imaging technique, which has many valuable advantages, for example, it is able to provide an excellent image quality and high sensitivity and spatial 3D resolution, identify labeled cells in their anatomical context, get additional information about the surrounding milieu, and promise clinical applicability with nontoxicity and noninversion (Figure 1).

Gadolinium (III) (Gd^{3+}) is a heavy metal contrast agent widely used in clinical and animal experimental MRI. The contrast-enhanced lesions or labeled transplanted stem cells will appear as hyper intense on T1-weighted and hypointense on T2-weighted images, as Gd^{3+}-based contrast could shorten T1 and T2 relaxation times. Therefore, those Gd^{3+}-based agents were called T1 agents. However, because of their low uptake by cells, many tracking methods, such as coupling of the contrast agent to a membrane translocation peptide or using transfection agents during the process of transfection, are available for increasing the uptake rate of the contrast agent [10]. Next to gadolinium, manganese is another potentially useful "positive" T1 contrast agent which was used widely to study the function of the brain. As its similar ionic property to Ca^{2+}, Mn^{2+} can be taken up by excitable cells of the brain and spinal cord via voltage-gated Ca^{2+} channels and the sodium (Na^{+})/Ca^{2+} exchanger. Also, Mn^{2+} can enter the stem cells through binding with Ca^{2+}- and Mg^{2+}-binding sites on specific proteins and nucleic acids [11]. In general, manganese is a particularly attractive contrast agent for MRI of the brain to study neuronal activity, to monitor neuronal tracts, and to detect transplanted cell functions, as its property of entering the cell conveniently.

Over the past decades, iron oxide particles have been developed for more efficient intracellular labeling, due to their high sensitivity, biocompatibility, and increased paramagnetic per mole of metal compared to manganese or gadolinium. These iron oxide particles act locally to reduce the T2 relaxation via inducing strong field inhomogeneity. When T2-weighted pulse sequences were released, these particles will produce a hypointense or signals on the MRI, allowing to catch the vision of the labeled, transplanted cells. As for the experimental model, after transplanted into adult murine brains, MRI could visualize the migration routine of SPIO-labeled stem cells. The study found that SPIO nanoparticle labeling has no adverse effect on the cell survival, proliferation, self-renewal, and multipotency [12]. The two formally approved iron-oxide-based agents used for stem cell labelling, SPIO nanoparticles coated with dextran or low molecular weight carboxydextran, were subsequently removed from the market in 2009 because of economic considerations. As the example of their clinical use, Zhu et al. reported a case of labeling NSCs with SPIO and tracking their survival, migration, and distribution in a patient with brain trauma in the left temporal lobe. The patient was then imaged with MRI weekly for 10 weeks after transplantation. Marked MRI image of transplanted stem cells was observed in vitro noninvasive [13]. More importantly, many studies have reported that SPIO labeling does not affect the function of stem cells and that tracking effect keeps as long as several weeks (Figure 2(a)) [7, 14].

However, there are several limitations in labeling stem cells with magnetic contrast agents. The label will be diluted due to stem cells continuing to proliferate with a fast speed after transplantations. Therefore, the MR signal will decrease even lost over time because of cellular proliferation. Additionally, SPIO nanoparticles will deposit in extracellular tissues when the dead transplanted cells were engulfed by immune cells, such as microglia in the central nervous system, which can lead to a false signal on MRI [15–17]. Although MRI has a unique advantage in tracing the location of stem cells, it cannot reflect the survival state of stem cells and the changes of microenvironment.

In 2005, a new medical imaging technology, named magnetic particle imaging (MPI), was introduced to track transplanted cells with the advantage of imaging the SPION distribution directly and producing linearly quantitative images of SPIO-labeled cells [18]. Since biological tissue itself does not produce an MPI signal, MPI images are extremely sensitive with a high signal-to-noise ratio [19, 20]. Theoretically, MPI is sensitive enough to image 1 pg Fe, meaning this tool has potential to detect even a single stem cell. Importantly, the MPI signal was linear with iron concentration and cell number, which allows for proper cell quantification. As the SPIO tracers are detected directly with MPI, their quantification is simple and straightforward [21]. This is somewhat analogous to fluorine-19 (^{19}F) MRI, which can

Table 1: Imaging modalities currently available for tracking neural stem cells.

Modality	Source of imaging	Type of probe	Spatial resolution	Temporal resolution	Tissue penetrating depth	Sensitivity	Clinical use	Advantages	Disadvantages
MRI									
In vivo labeling	Radiowave	Para- (Gd3+/Mn2+), SPIO or ^{19}F	>25 μm	Min–hrs	No limit	mM–μM	Yes	No radiation, very good tissue contrast, high resolution	Low sensitivity, agent dilution
Ex vivo labeling	Radiowave	MR reporter genes						Long-term imaging, long-term imaging	Exogenous gene risk
PET									
Direct labeling	High-energy γ-ray	Radionuclides (e.g., ^{18}F, ^{11}C)	>1 mm	Sec–min	No limit	pM	Yes	High sensitivity, high sensitivity, deep tissues	Radiation, radiotracer dilution
Indirect labeling	High-energy γ-ray	Reporter genes (e.g., HSV1-tk)						Long-term imaging, avoid false signal, nontoxicity	Exogenous gene risk
SPECT									
Direct labeling	Low-energy γ-ray	Radionuclides (e.g., ^{111}In, ^{99}mTc)	>1 mm	Min	No limit	pM	Yes	High sensitivity, able to image deep tissues	Radiation, low resolution, radiotracer dilution
Indirect labeling	Low-energy γ-ray	Reporter genes						Long-term imaging, nontoxicity	Exogenous gene risk
Optical imaging									
Fluorescence imaging	Visible light	Fluorescence near-infrared dye, QD light	>2 mm	Sec–min	<1 cm	nM-pM	No	Cheap, simple, high sensitivity, activatable	Deep tissue limited, low resolution, tissue damaging
BIL	Visible light	Reporter genes	>2 mm	Sec–min	<1 cm	nM	No	Simple, high sensitivity	Deep tissue limited, low resolution

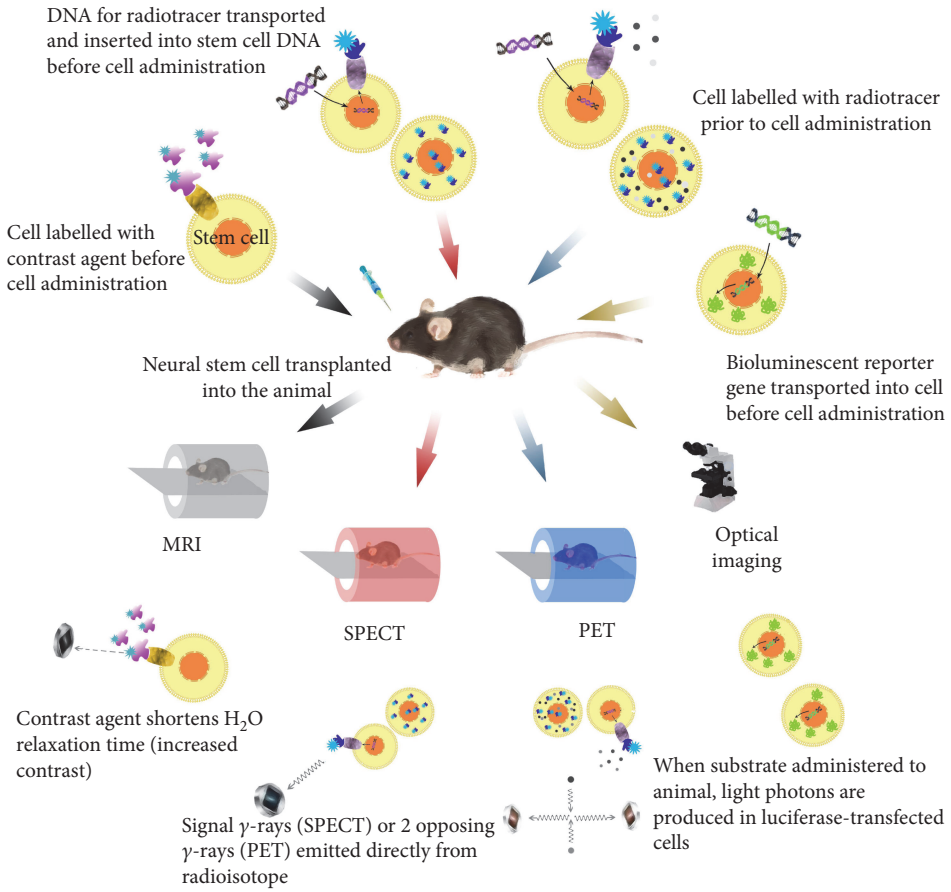

Figure 1: Principles of stem cell labelling for different imaging modalities.

overcome the disadvantages of cell quantification and ambiguity of contrast assignment when used to track stem cells [22]. The directly proportional and linear relationship between the signal strength and concentration of the ^{19}F allows quantification of ^{19}F-labeled stem cells in vivo [23]. Importantly, ^{19}F signal can be overlaid on 1H MR image with a very high quantitative tracking of labeled transplanted cells in vivo because the host tissue is absent in the level of background ^{19}F signal. Particularly, compared with hydrogen, ^{19}F has a nuclear magnetic resonance sensitivity of 83%, which is suitable for labeling cells [24]. Therefore, it is of high sensitive to use ^{19}F MRI for tracking stem cells. In contrast with a diluting process of SPIOs as stem cell proliferation, ^{19}F MRI could monitor the spatial-temporal migration dynamic routine of NSCs transplanted into the central nervous system, with the ability of detecting as low as several cells with a considerable high spatial resolution; even the interest labeling cells migrate within an even small scale.

Recently, more clinical grade studies are needed to overcome some limitations of existing MR cell imaging methods. For instance, MR reporter genes were introduced for stable, robust, and long-lasting tracking of the migration of implanted (stem) cells which does not diminish or decrease along with cell division that was the major limitation of the present MR imaging techniques by using routine contrast agents [25]. Also, transgenic cell lines with inbuilt contrast agents were proposed for stem cell transplantation. In addition to develop more sensitive novel contrast agents, increased resolution is also achieved through various means. The most common method includes increasing the number of coil receiver channels, the magnetic field strength, and image acquisition times. In general, equipping various (stem) cell therapy modalities with noninvasive MR imaging techniques has a great potential for clinic application.

1.2. Nuclear Medicine Imaging. Nuclear medicine imaging techniques, both PET and SPECT, represent another promising imaging modality to track stem cells which have been used in experimental and clinic trials widely (Figure 1). Before the stem cells were transplanted into the host, a radiotracer, such as ^{11}C, ^{13}N, ^{15}O, and ^{18}F, is necessary to label the stem cells in order to detect the transplanted cells though PET/SPECT scanner [26]. The emitted positron from radioisotopes will lose its kinetic energy rapidly while traveling through the surrounding tissue and then interact with electrons resulting in the emission of two high-energy photons of 511 keVat (high-frequency photons) travelling at nearly opposite directions. The PET camera can detect and image these photons in the scanner. SPECT is very similar to PET in its use of radioactive tracer and detection of gamma rays. In general, the operational principle of SPECT is similar to PET; however, what the SPECT scanner detected is signal gamma rays emitted by isotopes. Compared with SPECT, the key characteristic of PET imaging is high sensitivity

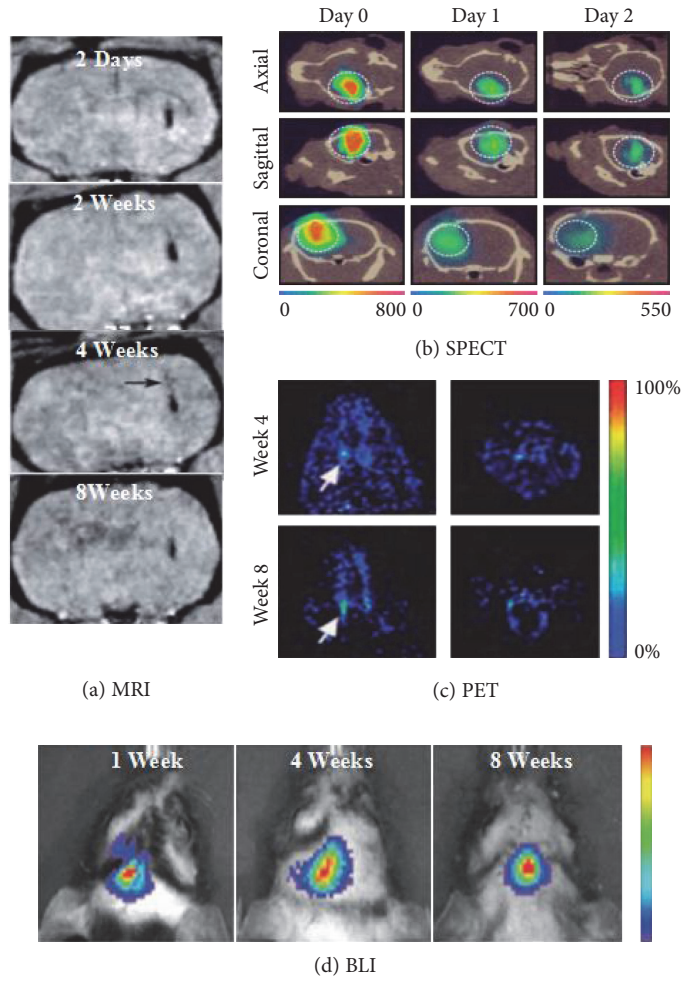

Figure 2: Comparison of imaging techniques for transplanted therapeutic neural stem cells (NSCs). (a) Monitoring of magnetic nanoparticle-labelled NSCs in rat brain using magnetic resonance imaging (MRI). MRI was performed 2 days, 2 weeks, 4 weeks, and 8 weeks after cell transplantation [7]. (b) Single photo emission computed tomography (SPECT) imaging of mouse brain after intracerebral delivery of NSCs loaded with ^{111}In. SPECT was performed immediately, 1 day and 2 days after cell transplantation [28]. (c) 9-(4-[^{18}F]fluoro-3hydroxymethylbutyl) guanine ([^{18}F]FHBG)-labeled embryonic stem cell-derived neural stem cells (NSCs) viewed through positron emission tomography (PET) can be seen localizing in the striatal region of the forebrain [6]. (d) Luciferase photon emission detected through bioluminescence imaging (BLI) 1 week to 8 weeks after transplanting of neural progenitor cells (NPCs) [41].

and temporal resolution. However, a marked advantage of imaging two different radioisotopes at the same time made SPECT an important tracking method. In both techniques, because of their intrinsic tomographic nature, they can present the distribution of labeled stem cells by generating three dimensional images. These images can be used to assess biological features of labelled stem cells, such as blood perfusion, metabolism, and enzymatic activity.

^{111}In oxyquinoline, an FDA-approved radiotracer, has been used to image the accumulation and biodistribution of stem cells/progenitor cells in animal models successfully in the previous studies [27]. Due to the lipophilic nature of the ^{111}In-oxinemolecule, it can "enter" the cell easily by passively diffusing into the cell membrane. It is possible to image the cells as long as 2 weeks after injection because of the long half-life of ^{111}In (2.8 days). Cheng et al. reported that ^{111}In-mesoporous silica nanoparticle (MSN) complex shows minimal toxicity to stability and biological activity of NSCs both in vitro and in vivo (Figure 2(b)) [28]. In a rat model of middle cerebral artery occlusion and the controls, cell detection was performed at once and 24 hours after the cell transplantation with a SPECT/CT device. The result showed that as low as 1000 ^{111}In-oxine-labeled cells can be detected by the SPECT/CT device; more importantly, the cell viability was not affected by the agents [29]. Besides ^{111}In-oxine, another radiolabel agent, ^{99}mTc-HMPAO (hexamethylpropylene amine oxime) with a half-life of 6 h, which could avoid the issue of radiation damage, has been used mainly for the stem cell tracking showing low toxicity. In contrast to ^{111}In-oxine, proliferating and differentiating abilities of both human and rat MSCs were not affected by ^{99}mTc-HMPAO labeling. However, in one study of Gleave et al., labeling neural stem and progenitor cells with ^{99}mTc decreased the proliferative capacity of those cells. Clinical studies using ^{99}mTc-HMPAO tracking stem cells are mainly involve in those with chronic ischemic cardiomyopathy or myocardial

infarction at present [30]. A best example of radiotracer used in central nervous system is 2-deoxy-2-[^{18}F]fluoro-D-glucose, or ^{18}F-FDG (half-life: 109 min), which is transported into cells via the GLUT transporter family. It is taken up by metabolically active cells, and once intracellular, ^{18}F-FDG will be phosphorylated to ^{18}F-FDG-6-phosphate by hexokinase. ^{18}F-based tracer has been widely used for tracking neural stem cells (Figure 2(c)) [6]. A novel agent, 3′-deoxy-3′-[^{18}F]fluoro-L-thymidine, has been used for noninvasive imaging of tumor cell and NSC proliferation with PET in the previous studies [31].

However, also some obstacles were involved in the direct imaging, for example, the leakage of radiotracers into tissue cells, dilution of signal due to cell proliferation, and lack of ability to detect cell viability and function. Specially, it is crucial to identify the safe dose of a radiotracer when applying nuclear imaging with a radioisotope to the clinic treatment, taking into account the toxicity of a radiotracer. To overcome these problems is through use of indirect labelling methods. Indirect imaging of stem cells generally involves the so-called "imaging reporter genes" which is introduced into the cell's genome ex vivo. These reporter genes are able to produce the particular protein which will act with radioactive probe so that the probe signal can be detected by PET/SPECT for a long time without being limited to the half-life of the tracer used. The main advantage of reporter gene approaches is that only living cells will be identified, because only viable cells can translate the gene into a particular protein that can be acted with radioactive probe. Unlike direct labelling of cells, the reporter gene in a parent cell will be inherited to daughter cells; therefore, the tracer will not be diluted as cells divide. Additionally, when the transplanted cells die, the imaging signal will be lost, avoiding the false signal [32]. However, the use of reporter genes in human cell therapy still remains limited because whether the introduction of reporter genes into the host cell genome will cause detrimental effects or not is unknown.

1.3. Optical Imaging. Compared with MRI and nuclear imaging for tracking stem cells, optical imaging has advantages of lower cost, rapid acquisition, no radiation toxicity, and relatively high sensitivity (Figure 1) [33]. Fluorescence imaging has been served in the field of cell therapy for CNS disorders for many years, using green fluorescent protein (GFP) and red fluorescent protein (RFP), as well as some fluorescent dyes such as DiD, Dil, and indocyanine Green (ICG) [5]. However, the application of fluorescence-based imaging techniques in cell tracking is limited by the short wavelengths as it is unable to obtain fluorescence signal through the bone and skin [34]. On the other hand, semiconductor nanocrystals, also called quantum dots (QDs), are a novel class of biocompatible fluorescent that are relatively photostable and have narrow luminescence bands used in cell tracking. Near-infrared- (NIR-) emitting QDs may be especially useful to track transplanted cells in the human brain because their longer wavelengths allow easier penetration of tissue such as bone and skin [35]. Many studies demonstrate the safety and efficacy of NIR fluorescence labeling with QDs as a method of identifying and tracking stem cells in a rodent model of cerebral infarction. NIR fluorescence labeling allows noninvasively tracking of transplanted cells engrafted in the infarction region as long as 8 weeks after transplantation [36]. Recently, a study of injecting embryonic stem cells labeled with six different QDs into mice backs showed QD800-labeled cells providing most prominent fluorescence intensity [37]. Those findings suggest that NIR fluorescence imaging is a long-term, noninvasive imaging technology in the field of cell therapy in vivo. Therefore, NIR-emitting tracer may be a potential tool to track the transplanted cells in humans.

However, cell labeling with QDs also could not image transplanted cells for a long time as directly labeling with regard to dilution due to cell proliferation. Additionally, when used for biological imaging and cell therapy, the toxicity of QD limits its wide usefulness. However, thanks to the recent advances in the development of surface coating material, more biocompatible QDs were used in cell tracking. In a recent study by Chen et al., cells labeled with Ag_2S QDs were transplanted into a mice model to visualize cell dynamic migration. The difference of cell viability, proliferation, and the pluripotency-associated transcription factors released by stem cells is negligible between control and labeled hMSCs [38].

Bioluminescence imaging (BLI) has been widely applied in preclinical studies of stem cell imaging to in the brain for years. Bioluminescence involves introducing a reporter gene, which could code for a special luciferase protein, into the target stem cells. The charge-coupled device camera system can detect and quantify the photons emitted through the progress of the luciferase enzyme reacting with its substrate luciferin or coelenterazine [39]. Luciferase transformed d-luciferin into oxy-luciferin and light at the present with ATP and O_2 in order for signals to be detected. In addition, BIL could be used to quantify the number of transplanted cells as the light emission is directly proportional to the number of cells [40]. Bioluminescence can track stem cells for a considerable long term due to the luciferase gene that is stably integrated into the genome of stem cells. Therefore, BLI also was used to study gene expression quantification, tumor development tracking in rats, and stem cell localization in mice (Figure 2(d)) [41]. However, at present, BIL is only confined to small animals, but not to large animals, because BIL can only penetrate a few centimeters of tissue. Moreover, the introduction of a reporter gene runs imponderable risk for the clinic application. Therefore, BLI is limited for a preclinical study.

1.4. Multimodality. As described above, no single imaging technologies can provide all the information required in tracking stem cells and monitoring their biological behavior; therefore, researchers tried to develop multimodality image to overcome the drawbacks of single imaging technology. Multimodality molecular imaging generally combined more than one imaging modality with the purpose of integrating modality-specific strengths [42, 43]. For example, a complementary use of SPECT for high indication of functional activity and CT for anatomic images enables the integration

of structural and functional information, which has been used in clinic for many years.

Multimodality noninvasive imaging reporter genes can now also be developed to be combined with different imaging technologies to obtain sufficient information of the biologic behavior of stem cells. The widely employed strategies of multimodal reporter gene imaging are as follows: incorporate more than one reporter gene into one plasmid; incubate the plasmid and stem cells in order to facilitate plasmid to "enter" the cells; those genes are then transcribed into different proteins which can be imaged by different imaging modalities. In one study of Jackson et al., they used USPIO-MRI and ^{11}C PET to monitor stem cell viability, proliferation, and differentiation in an animal model of Parkinson's disease for the first time. They combined the advantage of high anatomical spatial resolution in MRI and high sensitivity in PET to obtain sufficient information to assess dopaminergic function [43]. Additionally, BIL/PET imaging was deemed feasible by Cao et al. [44] and Waerzeggers et al. [45] using reporter gene technology which BLI served the higher sensitivity for detecting luc-expressing cells and ^{18}F–FHBG-PET served for localization of tk-expressing cells.

Although multimodality noninvasive imaging has been successfully used in many preclinical trials, it also has some limitations. As fusion proteins containing different types of molecular probes or substrates are needed for multimodality imaging, fusion reporter genes generally are difficult to construct with a large size. Additionally, fusion proteins may lose some bioactivity at the process of gene fusion and protein expression. Therefore, it is necessary to develop a signal molecular probe or reporter gene available for multimodal imaging. A single reporter gene, Human TYR, can be detected and imaged by photoacoustic imaging, MRI, and PET in vivo and may overcome some of the aforementioned limitations. This system combines the high sensitivity for both PAI and PET and high spatial resolution for T1-weighted images, which may be a potential tool in biomedical research [32].

Another type of multimodality imaging is based on multimodal contrast agents, which integrate multiple properties in one agent to be detected by several imaging techniques. Magnetic quantum dots which combine fluorescent QDs with magnetic nanoparticles form a novel type of new materials for bioimaging. As the fluorescence and magnetic properties are integrated in a single agent, the advantage of fluorescence image and MRI can be combined to obtain the required information of transplanted cells [46]. As described before, Mn is the common useful T1 contrast agent used for cell tracking. Radiomanganese (^{51}Mn and ^{52}Mn) was ever used as a myocardial perfusion PET agent, with successful studies conducted in humans. Of which, ^{52}Mn (t1/2 = 5.591D) has presented itself as a strong candidate for PET applications. Therefore, ^{52}Mn-based PET not only could offer high sensitivity and reduced manganese dose, but also provides valuable complementary information paired with manganese-enhanced MRI (MEMRI). Importantly, besides cell tracking in the central neural system, this dual-modality manganese-based PET/MRI approach may be used to other aspects, including neuronal tract tracing and brain activation-induced uptake measurement [47].

1.5. Limitation. In spite of these successes and great potentials, many problems exist in these cell tracking technologies, including cytotoxicity, signal dilution, or loss in long-term tracking due to cell proliferation, insufficiency of single imaging technology to attain comprehensive information of cell dynamic state, and limited capability of revealing cell functionality and viability [48]. It is necessary to overcome these problems before cell therapy applied for clinic treatments. Currently, there is no perfect tracking agent approved by FDA to label and track stem cells for the purpose of cell therapy. It is important to understand whether those tracking agent affect the viability, differentiation, migration/homing, distribution, and engraftment of stem cells before their applications in the clinics. Many factors including composition, particulate shape, appropriate size, and surface functional groups are related to the cytotoxicity of tracking agent. Specially, different studies have different views on the cytotoxicity of the signal tracking agent. For example, SPIOs are generally considered as nontoxic in most studies; however, SPIOs coated with poly-L-lysine were reported to partially impair the differentiation function and potentials of some stem cells. As pointed out, for now, manganese and gadolinium are unlikely to be used clinically because of their metal toxicity. One of the most critical issues in stem cell therapy is how to trace and monitor the transplanted cells in vitro for a long enough time. The rapid increase in the number of transplanted stem cells limits the use of MRI agents or radiotracer, which leads to the dilution or deletion of labeled tracers. Additionally, although multiple modalities over single modality may attain more necessary information to reveal the spatial location of transplanted cells, many problems, such as more equipment and cost and higher technical difficulty, must be overcome. As a point before, fusion reporter genes, which can be detected by MRI and PET simultaneously, are usually larger and difficult to construct. Thus, the best solution is to construct a single reporter gene that can be detected by multiple imaging methods.

In the previous studies, the small rodent models are highly useful for stem cell preclinical experiment. However, the small rodent central nervous system and cerebrovasculature are different from those of human, which limits the transformation of the result of animal research into prospective clinic application directly. Large animal models may short the gap between rodent animals and humans to a certain extent, which have been used in lab but were complicated and expensive. Furthermore, clinical imaging has more limitation compared with experimental animal studies, for example, animal MRI scanners can reach16T or higher, whereas high field in human studies is around 7T, as most clinical MRI scanners being less than 3T in the country. More importantly, currently tracking technologies can only provide the certain information of migration routine and final temporal-spatial location of transplanted stem cells. For clinical researchers, it is more meaningful to visualize the viability and differentiation of transplanted stem cells

and even cell functionality. One approach is to design an advance nanoparticle probe, which can detect stimuli associated with stem cell viability or functionality. Those stimuli include growth factors and enzymes expressed by stem cells, chemical secretion during cell differentiation, transgene expression during cell growth, intercellular and extracellular pH changes during cell death, and metal ion level which is essential for cell to play its normal physiological function.

2. Conclusion and Future Prospects

Stem cell therapies based on animal model have provided much evidence of benefits for neurological diseases. However, unless safety and efficacy of the transplanted cells are guaranteed, stem cell therapy can be taken to the clinical trial. Therefore, it is important to track the biological behaviors of transplanted cells in vivo, including proliferation, migration, viability, and functional reconstruction. Currently, every imaging technique used for cell tracking has merits and defects. The selection of imaging methods and tools should accord to the requirements and designs of the study: is high sensitivity or high spatial resolution or low cost needed? Among the various molecular imaging approaches mentioned above, MRI is the most promising tool for use in the clinic, since it is nonradioactive and not hampered by tissue depth. However, more data could be gotten to present a clearer sight of survival, differentiation, and migration routine of the transplanted stem cells in the host, through combining different imaging techniques such as PET, SPECT, and optical imaging. Furthermore, multimodality imaging strategy may overcome the instinctive drawbacks of signal imaging modality, as the combination of two or more imaging modalities may provide more comprehensive information for clinical setting. More importantly, advance imaging modalities which can reveal the viability, differentiation, distribution, and function reconstruction of transplanted cells would greatly promote the clinical application of stem cell therapy in the future.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Electromagnetic Fields for the Regulation of Neural Stem Cells

Mengchu Cui, Hongfei Ge, Hengli Zhao, Yongjie Zou, Yujie Chen, and Hua Feng

Department of Neurosurgery, Southwest Hospital, Third Military Medical University, Chongqing, China

Correspondence should be addressed to Yujie Chen; yujiechen6886@foxmail.com

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Localized magnetic fields (MFs) could easily penetrate the scalp, skull, and meninges, thus inducing an electrical current in both the central and peripheral nervous systems, which is primarily used in transcranial magnetic stimulation (TMS) for inducing specific effects on different regions or cells that play roles in various brain activities. Studies of repetitive transcranial magnetic stimulation (rTMS) have led to novel attractive therapeutic approaches. Neural stem cells (NSCs) in adult human brain are able to self-renew and possess multidifferential ability to maintain homeostasis and repair damage after acute central nervous system. In the present review, we summarized the electrical activity of NSCs and the fundamental mechanism of electromagnetic fields and their effects on regulating NSC proliferation, differentiation, migration, and maturation. Although it was authorized for the rTMS use in resistant depression patients by US FDA, there are still unveiling mechanism and limitations for rTMS in clinical applications of acute central nervous system injury, especially on NSC regulation as a rehabilitation strategy. More in-depth studies should be performed to provide detailed parameters and mechanisms of rTMS in further studies, making it a powerful tool to treat people who are surviving with acute central nervous system injuries.

1. Introduction

In 1985, Barker et al. demonstrated the possibility of noninvasively influencing both the central and peripheral nervous systems via localized magnetic fields (MFs) that could easily penetrate the scalp, skull, and meninges, thus inducing an electrical current in the brain or peripheral nervous system [1]. Now, this technique is primarily used in transcranial magnetic stimulation (TMS), which can be administered in different forms and appears to induce specific effects on different regions or cells that play roles in various brain activities. Studies of repetitive transcranial magnetic stimulation (rTMS) have led to novel and attractive therapeutic approaches [2, 3]. Different rTMS techniques, such as single, paired, or repetitive trains of intermittent theta burst stimulation (iTBS), have been commonly applied to many refractory neuropathy conditions, such as degenerative diseases, malignant tumors, and traumatic diseases, and especially in neurology and psychiatry for both diagnostic and therapeutic purposes [4]. Interestingly, the various means of stimulation exert completely different regulatory effects because high-frequency rTMS (defined as >5 Hz) stimulates enhanced cortical excitability and produces long-term potentiation (LTP). In contrast, low-frequency rTMS (defined as <1 Hz) decreases cortical excitability and induces long-term depression (LTD) [5]. However, the intrinsic cellular and molecular mechanisms underlying rTMS(MF)-based therapies are still elusive.

Neural stem cells (NSCs) are a type of self-renewing stem cell which possess the multidifferential ability to produce neurons and glia in the nervous system during the embryonic period. Some NSCs persist in the adult mature brain, and their capacity to differentiate into multiple cell types allows them to produce neurons throughout the lifespan [6]. They maintain homeostasis and repair damage [7]. Compared to differentiated cells, adult stem cells can proliferate to sustain themselves and differentiate into one or more specialized cell types within a certain cell lineage [8]. As such, for the ultimate purpose to regenerate and recover normal functions, stem cells are a promising tool for tissue or organ repair. In particular, adult stem cells are most often in a quiescent state and can be triggered by intrinsic or extrinsic factors or their complicated combination to initiate self-renewal and differentiation [9, 10]. The current consensus is that a series of

niche signals and cellular intrinsic processes are involved, and several researchers have made great efforts to identify the roles of these signals and processes in brain physiology and explore their potential use in cell-based therapies to treat neurological or neurodegenerative diseases [11, 12]. In the adult mammalian brain, the subgranular zone of the hippocampal dentate gyrus and the subventricular zone of the lateral ventricles are two of the main areas where NSCs reside [13, 14]. The production of new neurons maintains the NSC pool [15], and thus, NSCs or neural progenitor cells (NPCs) are the most important component in brain regeneration and plasticity. The nervous system is perhaps the most difficult system to repair in regenerative medicine. In addition, adult NSCs are located deep in the body and are few in number. Although SCs have been studied for decades, how to influence these cells noninvasively and efficiently for specific applications remains a challenge. The latest research has reported that rTMS(MF) has a variety of effects on adult NSCs, shedding light on possible cures for intractable diseases, cerebral trauma, stroke, depression, dementia, Parkinson's disease, and so forth Therefore, strategies to generate a MF to stimulate endogenous processes of NSCs have gained considerable interest, but the behavior of NSCs in the context of rTMS(MF) therapy needs further elucidation [16]. Focused on the integration of two promising approaches, MFs and NSCs, this review will discuss the effects of MFs on NSCs and the potential mechanisms as well as provide an outlook regarding future directions. Thus, noninvasive MF stimulation, specifically transcranial magnetic stimulation (TMS) on NSCs, may be a promising option.

2. The Electrical Activity of Neural Stem Cells

NSCs are a type of immature, undifferentiated cells. Due to their property of "stemness," their physiological features, especially electrophysiological characteristics, are distinct from well-known neurons, and they realize their functions mainly based on electrical activities. It is widely known that neural cells have specific excitability and that ion channels are the molecular foundation enabling them to generate electrical activities. Neural cells and NSCs participate in intercellular signaling transitions and transmembrane signaling transduction, which are the prerequisites for cells to become physically activated to exert their functions (Figure 1). During the course of NSC differentiation, the expression of ion channels and their states are continually varied to accommodate the microenvironment (niche) of different periods. Although studies of NSCs have become gradually more in-depth, the characteristics of NSCs cultured and differentiated *in vitro* have been primarily explored based on morphology and immunocytochemistry, whereas few studies have performed functional identification of NSCs. Studies indicate that neural cells under different conditions are characterized by different electrophysiological features, and the development and differentiation of ion channels are precise indicators of specialized NSCs. Their unique electrophysiological properties not only provide a new and efficient functional means to better identify NSCs and types of differentiated neural cells but also help to elucidate the mechanisms underlying MF stimulation and NSCs.

2.1. Passive Membrane Properties of NSCs. Resting membrane potential (RMP), membrane input resistance (Rin), and membrane capacitance (Cm) are major parameters of the passive membrane properties of cells. Liu et al. [17] reported that epidermal growth factor/basic fibroblast growth factor- (EGF/bFGF-) reactive neural progenitor cells originate from the subventricular zone (SVZ) or spinal cord of rats and can be classified into three types based on their *I-V* curve: type I exhibits delayed outward currents, type II exhibits no rectification, and type III exhibits outward and inward rectifying currents. Significant differences in the passive parameters have been found among the three types of cells. Compared with other types, type I cells are characterized by a high Rin and a low RMP. Liu hypothesized that immature type II neurons may be glial cells, while type III cells may be undifferentiated NSCs. This hypothesis corresponds with the work of Doetsch et al. [18] on the morphology of NSCs. The major transmembrane channels of glial cells are dense passive K^+ channels, which result in a higher RMP and a lower Rin. As NPC differentiate, RMP increases while Rin decreases due to the addition of passive K^+ channels, but they are still distinct from mature neurons, reflecting immature differentiation. By studying hippocampal slices of nestin promoter-GFP transgenic mice to directly observe fluorescent cells, Fukuda et al. [19] classified neurons into two species according to the levels of Rin and RMP: type I with low Rin and high RMP and type II with high Rin and low RMP. In addition, Fukuda et al. performed a classical morphological identification and found that type I cells are GFAP-positive and polysialic acid neural cell adhesion molecule- (PSA-NCAM-) negative, while type II cells are GFAP-negative and PSA-NCAM-positive, in agreement with the reported functional outcomes of NSCs at different stages.

Certainly, NSCs/NPCs can be distinguished from each other during different periods of life. Compared to adult NSCs, neonatal and embryonic NPCs exhibit a more depolarized RMP between −55 mV and −40 mV [20–24]. The Rin of neonatal NPCs is different under different circumstances; for connected cells, the Rin is 150 MW, whereas for isolated cells, it is 650 MW in the presence of a gap junction blocker [20]. In addition, *in vitro*, the Rin of embryonic NPCs is 1 GW [23]. Thus, adult NSCs/NPCs have a more hyperpolarized RMP and a lower Rin than embryonic and neonatal NSCs/NPCs. This disparity may indicate the morphological and functional changes that the neonatal or embryonic SVZ undergoes during developmental shifts in the neurogenic niche that are distinct from the adult SVZ. Above all, these studies indicate that different types of neural cells have different electrophysiological features of their passive membrane.

2.2. Ability to Generate the Action Potentials (APs). Neural cells transmit excitatory signals via APs, but the ability of NPCs to generate APs significantly differs across stages of life. Neurons can be evaluated based on their action potential duration (APD) 50 and APD 90, namely, the time required for 50% and 90% repolarization of the AP, respectively. A

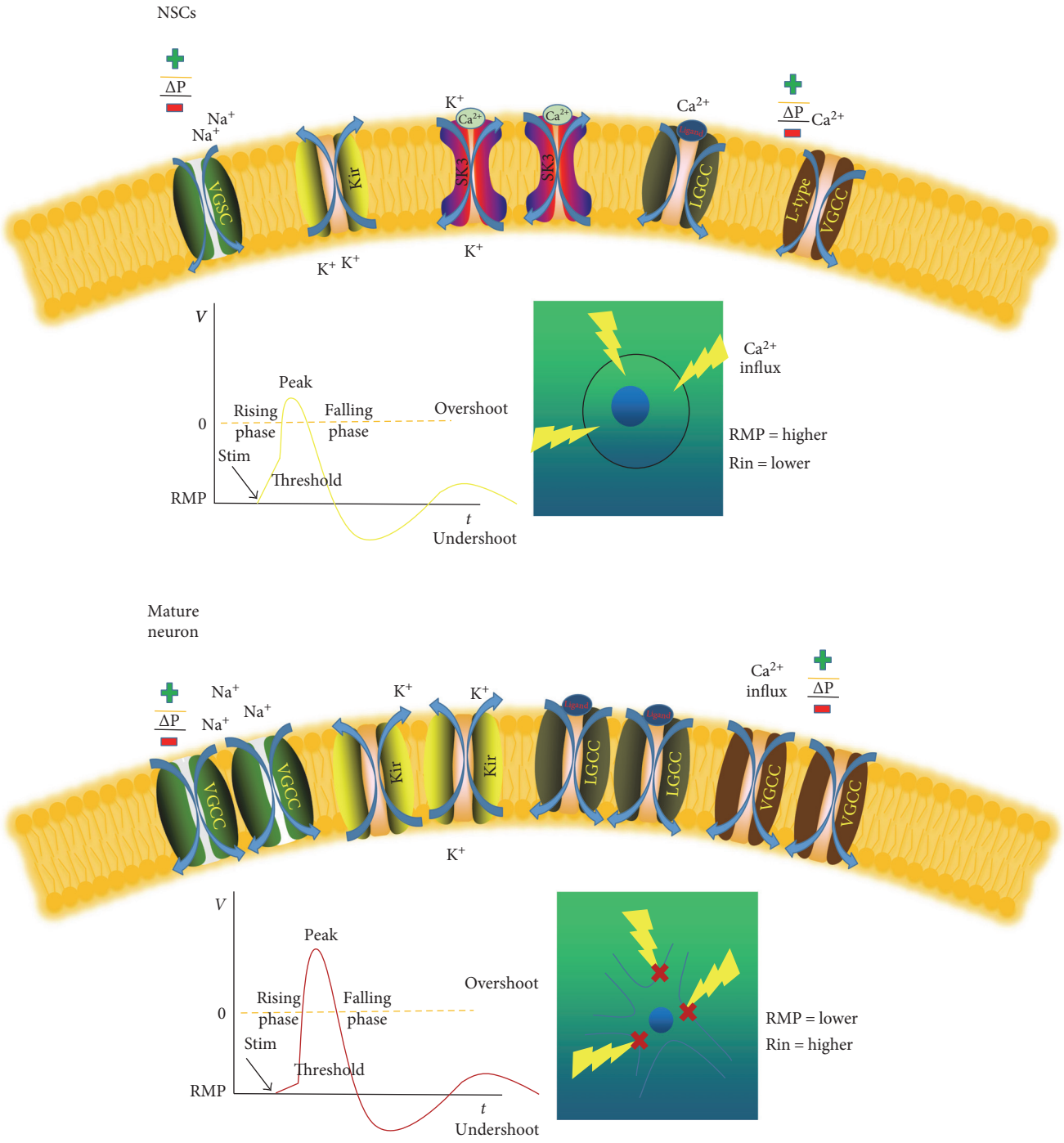

Figure 1: The electrophysiological differences between NSCs and neurons. Compared to mature functional neurons, NSCs exhibit a higher RMP and a lower Rin due to shortage of Kir; moreover, it is not easy for NSCs to generate AP because of a lack of VGSCs. VGCCs, mostly functional L-type and LGCC, are probably the main roles in modulating intracellular Ca^{2+} concentration especially L-type VGCCs, Ca^{2+}-dependent K^+ channel SK3 abundantly expressed in NSCs is responsible for the migration and proliferation.

shortage of Na^+ channels makes it difficult to induce Aps; however, as the expression of ion channels increases, the *I-V* curve begins to show a rectified performance, and transmembrane ionic currents prompt the opening of more channels, resulting in positive feedback. Compared with mature neurons, the majority of studies have shown that there is no spontaneous AP in NSCs, and APs are induced by depolarization of distant mature neurons. Liu et al. [17] found that further differentiated cells hold a greater ability to generate APs, longer duration APs and higher amplitude APs, which are related to a lack of voltage-gated Na^+ channels (VGSCs), particularly tetrodotoxin- (TTX-) sensitive Na^+ channels. In the process of specialization, as the expression of Na^+ channels increases,

the amplitude of APs is augmented. Therefore, the development of Na^+ channels is closely related to shortened durations of APs, indicating that more Na^+ channels are involved for the same level of depolarization stimuli.

2.3. Voltage-Gated Ion Channels of NSCs. There are four major types of voltage-gated ion channels: Ca^{2+}, Na^+, K^+, and Cl^- channels. Some of these channels are opened through membrane depolarization, while some are opened through membrane hyperpolarization. In terms of their voltage sensitivity, researchers most often focus on excitable cells. In neurons, these channels play a key role in neuronal functions, such as in synaptic transmission, the generation of APs, membrane current transmission, long-term potentiation, and the modulation of gene expression. However, the role of VGSCs in NSCs has been ignored because of their inexcitability. It is well known that glial cells are the insulation of the central nervous system and also exhibit VGSC currents. However, the quantity or quality of VGSC currents in glial cells are too small to generate APs, and their functional connectivity is controversial [25]. Furthermore, as an immature cell type, adult NSCs are unable to produce APs in response to depolarizing currents [26, 27]. Similarly, embryonic and neonatal NSCs exhibit neither VGSC currents nor APs upon depolarizing current injection [20, 24, 28, 29]. Interestingly, some (13–55%) cultured NPCs have small transient inward VGSC currents [23, 26, 27, 30]. Given that experiments carried out *in vitro* may not translate to *in vivo* situations, the expression of VGSCs in NSCs/NPCs required confirmation by electrophysiological studies in situ. K^+ channels, whose gene expressed before differentiation corresponds to both voltage- and Ca^{2+}-dependent types, are widely found throughout the brain, regardless of the cell type (neurons or glial cells). K^+ currents can be probed both before and after differentiation. In addition to their primary role in the routine modulation of neuronal excitability, K^+ channels also generally participate in the regulation of critical processes such as membrane potential, proliferation, and apoptosis across a wide range of cellular activities [31–34]. K^+ channel currents are usually recognized as two classes: inward and outward K^+ currents. Outward currents include rapidly activated and inactivated transient currents such as A-type K^+ (KA) channel currents, which are highly sensitive to 4-aminopyridine, and slowly inactivated or non-inactivated currents such as delayed rectifying K^+ (KDR) channel currents, which are rarely inactivated and are tetraethylammonium- (TEA-) sensitive due to their unique properties. Inward currents include inwardly rectifying potassium channel (Kir) currents that are mainly responsible for the RMP. KDR channels play a critical role in repolarization and are rarely found in immature cells. Wang et al. probed KDR and Ca^{2+}-dependent K^+ channels in mouse brain slices 15~25 days after birth. Liebau et al. [35] found hyperexpression of a class of Ca^{2+}-dependent K^+ channel (SK3), which plays a variety of roles in many physiological processes of NSCs.

Voltage-gated Ca^{2+} channels (VGCCs) comprise three major subfamilies: CaV1.x, CaV2.x, and CaV3.x. They are extensively expressed in neurons and are responsible for modulating gene expression, neurotransmission, and various fundamental Ca^{2+}-dependent intracellular events such as differentiation, apoptosis, and proliferation [26, 27, 34]. VGCCs are considered to play functional roles in early neuronal development [36]. In adult NPCs, however, the functional modulation and roles of VGCCs are unclear. Most recent studies have investigated L-type (CaV1.2) and T-type (CaV3.1) VGCCs in adult cultured NSCs and have detected their expression at the transcriptional or translational levels [37]. *In vivo*, ischemia-induced neurogenesis in adult mice can be blocked using L-type VGCC antagonists, but there is no evidence to show that they exert an effect on basal neurogenesis, suggesting a special role of VGCCs in disease-related neurogenesis [38]. Ca^{2+} transients are rarely found in the majority of NSCs, whereas small VGCC currents (Ca^{2+} transients) can be detected using a higher depolarization level induced by high K+ concentration (100 mM) in adult NPCs [37]. Furthermore, no reports have detected a significant VGCC inward current in NPCs using physiological recording methods [27]. However, considering that large outward K^+ currents may mask small Ca^{2+} currents, conditions should be changed to detect such small currents. Previous studies of changes in the concentration of intracellular Ca^{2+} caused by activation of Ca^{2+} channels in NSCs are limited despite the importance of Ca^{2+} in migration, proliferation, and differentiation. One can question the functional role of masked small VGCC currents and the mechanism of membrane depolarization that activates VGCCs. In the future, it is important to determine the clear mechanism by which intracellular Ca^{2+} concentrations can effect cellular activities, particularly those that affect NSC function. In addition to VGCCs, NSCs also express another type of channel at the transcript level. The canonical transient receptor potential channel 1 (TRPC1) channel, a voltage-gated Ca^{2+} channel [37], plays a key role in Ca^{2+} influx and proliferation of NSCs [39]. The above findings may suggest that Ca^{2+} may be the key factor in studying the mechanisms of NSC functional activities. In addition, even the outcomes among studies may differ, and interfering factors such as animal species, tissue origin, tissue parts, immediately extracted NSCs versus cultured NSCs, and single-cell recordings versus brain slice recordings should be noted. In general, it is widely accepted that undifferentiated NSCs exhibit a high RMP, a low Rin, and inward-rectifying potassium currents without inward Na^+ currents.

3. The Fundamental Mechanism of Electromagnetic Fields

Noninvasive transcranial magnetic stimulation (rTMS) produces an electromagnetic field that can easily penetrate the skin and skull to influence the brain with little decay [40]. Electromagnetic fields act on the brain and induce currents based on the Faraday electromagnetic effect. Repeated electromagnetic fields can also affect the refractory period and influence connective horizontal neurons to modulate the balance between excitation and inhibition. In addition, electromagnetic fields induce electric currents whose function in the brain to change the excitability of cells depends on

the intensity and frequency of the stimulation. It is currently accepted that low-frequency rTMS (defined as <1 Hz) diminishes the excitability of neuronal cells, whereas high-frequency rTMS (defined as >5 Hz) enhances neuronal excitability, resulting in the modulation of brain activity [41, 42]. Solid evidences from clinical tests such as positron emission tomography (PET) [43] and functional magnetic resonance imaging (fMRI) [41] also support this fact. Different frequencies of stimulation may exert different effects on brain metabolism; high frequency may increase the metabolism level, while low frequency may decrease the metabolism level and cerebral flow. Furthermore, electromagnetic fields can regulate neurotransmitters both at the transcript level and expression level, which may provide an alternative route to help elucidate potential mechanisms. In studying the activities of cells involved in signal transduction, we should consider physical mechanisms combined with transduction, and ion channels may be the fundamental factors that are initially modulated.

4. Effects of Electromagnetic Fields on Neural Stem Cells

A general survey of the present study shows that a series of different parameters, including intensity, frequency, orientation, and distance, and models, have been extensively applied and studied. Many of these studies are specific to the model; therefore, rTMS and NSC studies are complicated and difficult to classify. Fortunately, the number of studies in the field is small, the details of which are reported above.

Francis et al. found that the exposure of adult mice to ELFEFs *in vivo* produces a significant enhancement in the number of newborn neurons in the GCL of the DG [44–46]. The vast majority of those that survive differentiate into immature neurons and then mature granule cells, which migrate into the GCL. The expression of NeuN (commonly considered a marker of differentiated neurons) was investigated 4 weeks after protocol. Compared with estimates based on DCX labeling right after exposure, the total number of newly generated neurons was markedly reduced. In exposed and control mice, less than half (45% and 48%, resp.) of the newly generated immature neurons (DCX+) had become mature NeuN-expressing cells. These observations are consistent with previous reports showing that later-born granule cells localize predominantly in the inner core of the GCL [47].

The BrdU and nestin-corporation method shows us that EMFs can also increase the number of BrdU and nestin-positive cells within the area between the SVZ and lesion at 7 and 14 days after lesioning, indicating that EMF exerts a positive effect on the proliferation and migration of NSCs [48]. Cuccurazzu et al. [49] showed that extremely low-frequency and low-intensity EMF stimulation promotes adult hippocampal neurogenesis. In addition, Arias-Carrion et al. [50] showed that transcranial magnetic field stimulation promoted neurogenesis by the SVZ cells in nigrostriatal lesions.

Abbasnia et al. [16] found that with both low (1 Hz) and high (30 Hz) frequency rTMS, there is a marked rise in the proliferation of NSCs in the adult murine intact brain 2 weeks after application. An increase in the frequency of neurosphere formation and the size of the neurosphere was also observed in the rTMS-treated animals throughout the experiment. Furthermore, differentiation of the induced neurospheres showed that both NSCs treated with either the one-week or two-week rTMS protocol were more neurogenic than those of the sham-treated group. Moreover, *in vitro*, both 1 Hz and 30 Hz rTMS treatments applied for one week promoted NSC proliferation and neuronal differentiation. Interestingly, their findings also showed that there is no difference between low-frequency rTMS and high-frequency rTMS in terms of promoting NSC proliferation and increasing their neurogenesis. However, a marked increase in the quantity and size of neurospheres was observed for one week following both low- and high-frequency rTMS, indicating that only the discrepancy in neurosphere size (diameter) with low-frequency rTMS reached statistical significance. This implies that even one week of low-frequency rTMS stimulation results in a subtle increase in NSC proliferation. To understand these findings, the authors prolonged the application time (2 weeks) of the low- and high-frequency rTMS application. Given that low-frequency rTMS and high-frequency rTMS are similarly effective, they concluded that compared to high-frequency rTMS, low-frequency rTMS may be a safer and more tolerable therapeutic option with fewer risks [16]. In addition, intermittent theta burst stimulation (iTBS), which is a newly developed rTMS therapeutic protocol, was studied by Luo et al. [51], who compared it to the conventional 20 Hz high-frequency rTMS in an ischemic model. Their results showed iTBS significantly enhanced NSC migration and differentiation in the peri-infarct striatum, indicating that differences among different parameters may exist, and further studies are needed to clarify the effects of rTMS on NSCs.

However, experiments investigating only one parameter while controlling for all other parameters have drawn some useful conclusions. Studies focusing on the effects of high-intensity pulsed electromagnetic stimulation (HIPEMS) on the proliferation and differentiation of neonatal rat NSCs *in vitro* were carried out by Meng et al. [52]. NSCs isolated from neonatal rats were exposed to HIPEMF (0.1 Hz, 0.5–10 Tesla (T), 5 stimuli). A control group was correspondingly included. Given that a high number of stimulations (>30) might exert a suppressive effect on the growth of NSCs, Meng set the stimulus number to a low value—5 times per experiment. After a series of protocols were performed, they found that with 5 0.1 Hz frequency stimulations, rat NSCs showed poor *in vitro* growth in the HIPEMF 6.0–10.0 T peak intensity group, whereas a significant enhancement in the proliferation of rat NSCs was observed in the 0.5–4.0 T peak intensity, HIPEMF-stimulated group. The results showed that NSC proliferation in the 3.0 T and 4.0 T HIPEMS groups were remarkably higher than that of the other groups after 24 to 168 h of stimulation. Therefore, no linear relationship exists between the groups in terms of the proliferation of NSCs; the 6.0 T, 8.0 T, and 10.0 T groups were lower than the control group, indicating that high-intensity stimulation restricts the growth of NSCs. Flow cytometry was applied to detected the rate of neuron-specific enolase-positive

neurons, and the results showed there were no differences between the HPEMS groups and the control group. Therefore, we can conclude that HIPEMF promotes the proliferation of rat NSCs *in vitro* under a certain range of intensities and fixed parameters. Furthermore, there is a window effect, with 4.0 as the critical value, suggesting a linear strength-effect relationship within the peak intensity range of 0.5–4.0 T in promoting the proliferation of NSCs.

There are few related studies of the effects of rTMS on NSCs, few of which have investigated the effects of proliferation and differentiation *in vivo* [53–55]. Ueyama et al. [53] employed a BrdU-labeling method to investigate the effect of high-frequency (25 Hz) rTMS (1000 pulses/day) on neurogenesis after 14 days of application. The results showed increased cell proliferation in the dentate gyrus of the hippocampus, with most cells expressing the neuronal marker. A similar study was carried out by Feng et al. [54] in a chronic rodent model of depression. The author applied high-frequency (15 Hz) rTMS (1000 pulses/day) for a period of approximately 21 days and found an incremental increase in hippocampus cell proliferation, indicating increased neurogenesis. In a rat model of focal cerebral ischemia, 7 days after the application of high-frequency (10 Hz) rTMS (300 pulses/day), Guo et al. [55] observed a significant increase in the proliferation of NSCs in the SVZ of the lateral wall of lateral ventricle.

Although several studies of the effect of TMS(MF) on NSCs have been performed, there is no systematic analysis of MFs and NSCs due to the complexity of NSCs or to the large scale of the MF parameter. Nevertheless, according to the present study, we can conclude that rTMS(MF) is able to promote proliferation, differentiation, migration and inhibit apoptosis of NSCs in a conventional way. We also show that different strengths and different numbers of stimuli can induce different effects of HIPEMF on NSCs, indicating that there exist several potential routes for further exploration.

5. Potential Mechanisms of Electromagnetic Field Regulation on NSCs

Possible mechanisms behind the effects of rTMS NP/SCs have not yet been very well characterized. A thorough understanding of the underlying mechanism may help to optimize the stimulation protocol, characterize how EMF exerts its effect in animal models at the molecular level, and increase the translation of results to humans, thereby increasing their application in the clinic and proving an effective tool for clinicians.

5.1. High-Frequency rTMS Enhances the Expression of BDNF. Several studies have reported that BDNF is a key factor for increased hippocampal cell proliferation and neuronal differentiation after the application of rTMS [54]. In addition, reports show that in several brain areas of rats, including the hippocampal CA1 and CA3 subfields, high-frequency rTMS (20 Hz) stimulates the expression of BDNF [56]. In addition to the increase in BDNF expression, the expression of pERK1/2 was also increased [57], indicating rTMS might activate the BDNF/ERK signaling pathway to upregulate cell proliferation in the hippocampus.

5.2. The miRNA-106b-25 Cluster in a Model of MCAO Stimulated by High-Frequency rTMS. A number of miRNAs play a role in the determination of NSC fate, including NSC differentiation and proliferation [58–60]. Given the significant effects that rTMS exerts on gene expression, it is possible that rTMS also has the potential to modulate miRNAs. Guo et al. found that 10 Hz rTMS stimulation in a rat model of cerebral ischemia resulted in a remarkable enhancement of miR-25. Brett et al. demonstrated that the miRNA-106b-25 cluster could also promote the proliferation of adult NSCs [61, 62]. However, there was a significant decrease in its corresponding factor-target gene p57 [63, 64]. As we previously illustrated, p57, which can be suppressed by mir-25, is a Cdk inhibitor (CKI) that binds to Cdks to modulate transitions between cell cycle phases. Proteins of the Cip/Kip family inhibit the transition from G1 to S, thereby regulating the cell cycle; therefore, they proposed that rTMS might increase the expression of miR-25 in order to repress its target gene p57, thereby, as mentioned above, promoting adult NSC proliferation and inhibiting cell-cycle arrest. Moreover, the researchers also found that when miR-25 is inhibited, the proliferation of NSCs located in SVZ was also blocked. In summary, rTMS mainly activates the miR-25/p57 signaling pathway, which is responsible for the enhancement of adult NSC proliferation after focal cerebral ischemia. However, Liu et al. [65] performed a corresponding experiment for miR-106b and demonstrated that in rats with focal cerebral ischemia, the miR-106b-25 cluster increased NSC proliferation *in vitro* after high-frequency rTMS, the effects of which were dose-dependent [6]. They also showed that the miR-106b/p21/Cdk/cyclin pathway plays as an important role in this process. Interestingly, they also found that the trend for miR-25 after rTMS *in vitro* is completely different compared with those for miR-106b and miR-93. As such, the results of Liu dramatically disagree with Guo's. Taken together, miR-25 may have a more elaborate and complex role in the proliferation of NSCs after rTMS, despite the discrepancy between the two experiments. However, further studies are required to determine how miR-25 is affected after rTMS in NSCs.

5.3. Epigenetics May Be the Central Mechanism of ELFMF. More and more proof suggests that epigenetic mechanisms, particularly chromatin modifications, may act as critical modulators of differentiation and proliferation in NSCs [66, 67]. Leone et al. [68] demonstrated a marked increase in the expression of the proproliferative gene Hes-1 as well as the neuronal determination genes NeuroD1 and Neurogenin1. Several studies have illustrated that Hes1 is a repressive bHLH transcriptional factor that prolongs the stemness of NSCs by repressing proneural gene expression [69]. In contrast, inactivation of Hes1 weakens the repression of proneural genes and correspondingly upregulates the expression of proneural genes (as Mash1, Neurogenin1, and NeuroD1), resulting in acceleration of neuronal differentiation [70–72]. Furthermore, *in vitro* studies have also demonstrated that Hes1

is a switch for NSC proliferation and neuronal differentiation. These results are consistent with the results of previous studies. Interestingly, Hes1 can also repress its own expression by binding to its promoter, leading to the disappearance of Hes1 mRNA and protein. This negative feedback mechanism may mediate the switch between differentiation and proliferation. However, before the initiation of these events, there is an initial increase in the acetylation of H3K9 and binding of the phosphorylated transcription factor cAMP response element-binding protein (CREB) on the regulatory sequence of these genes. In addition, electromagnetic field-dependent epigenetic modifications can be inhibited by the Cav1 channel blocker nifedipine, which also involved increased occupancy of CREB-binding protein (CBP) to the same locus. Leone et al. also found that NSCs isolated from the hippocampus *in vitro* and exposed to ELFEFs showed enhanced proliferation and neuronal fate specification through changes in pCREB levels at specific bHLH neuronal gene promoters and Cav1 channel-dependent modulation of H3K9 acetylation. CBP, a histone acetyltransferase that is recruited by pCREB, is therefore involved in the epigenetic changes. Furthermore, similar results were observed in *in vivo* studies. Piacentini et al. demonstrated that ELFEF applied to cortical NSCs could enhance the quantity and function of voltage-gated Ca^{2+} channels, resulting in an increase in the concentration of intracellular Ca^{2+}, and Ca^{2+}-mediated signaling generated by Cav1 channels plays an important role in several fundamental cellular functions including the proliferation and differentiation of NSCs [73–76]. The potential mechanisms by which Ca^{2+} signaling regulates the transcription of numerous genes include bHLH transcriptional factors and the activation of CREB [77–80]. CREB, as a Ca^{2+}-dependent transcription factor, modulates the initiation of transcriptional programs, thereby exerting an important influence on adult neurogenesis [81, 82]. Furthermore, exposure to ELFEFs also leads to the accumulation of Cav1-dependent CREB phosphorylation at Ser133 in differentiating NSCs. The significance of the phosphorylation of CREB includes effectively promoting the expression of neuronal genes (NeuroD1 and Neurogenin1) and recruiting the histone acetyltransferase CBP, which can be prevented using the Cav1 channel blocker, nifedipine.

To prove this function of histone acetylation and to illustrate how CREB acts as a recruiter of histone acetyltransferases, Leone et al. [68] exposed differentiating NSCs to ELFEF and found increased H3K9 acetylation and pCREB binding to the promoters of proneuronal genes; these events could be significantly inhibited by nifedipine, thereby significantly enhancing the mRNA expression of Cav-1-dependent proneuronal genes. H3K9 is an important type of histone acetylation that loosens the compact structure of chromatin, thereby promoting the binding of regulatory sequences and increasing transcription. CBP cooperates with CREB in several molecular pathways [83], particularly those that regulate embryonic neural differentiation in the central nervous system [84]. Chatterjee et al. [85] recently showed that a CBP activator could enhance neurogenesis in adult mice. Therefore, epigenetic chromatin modifications at specific neuronal gene regulatory sequences may mediate the effect of ELFEFs on adult hippocampal neurogenesis *in vivo*.

5.4. Neurotransmitter Distribution Could Also Be Involved. Alternatively, another contributing factor modulating the proliferation of NSCs in the SVZ could be the variety of neurotransmitters released by axon terminals innervating that region [86]. To our knowledge, several studies have found that nerve endings are intensively distributed in the SVZ, originating either from the local neural circuitry such as GABAergic neurons of the adjacent striatum [87–89] or from distant brain regions such as dopaminergic neurons of the substantia nigra and ventral tegmental area [90, 91], and serotonergic neurons of the raphe nuclei [92]. Importantly, it is well known that GABA is an inhibitory neurotransmitter but it could also preserve the balance in proliferation and regulate the biological states of NSCs in the SVZ [88]. Moreover, dopamine [57, 93] and serotonin [92] have been shown to have a positive influence on NSC proliferation in the SVZ. Therefore, several neurotransmitter systems could be activated by rTMS to modulate the niche of NSCs in the SVZ (or other region with NSCs) to cause an increase in cell proliferation after rTMS treatment. In terms of the previous *in vitro* studies mentioned above, showing that both low- and high-frequency rTMS increase cell proliferation and neuronal differentiation, these findings suggest that electromagnetic fields in the human body itself could be a potential mechanism by which the body regulates cell proliferation and differentiation [94]. In support of this perspective and to further illustrate the mechanisms, findings at the molecular and cellular level are discussed.

5.5. Ca^{2+} Ion Channels Are Proposed as a Link between These Mechanisms. However, based on the present studies and the electrophysiology features of NSCs, some conclusions can be drawn: Ca^{2+} and CREB might be the hinge of effects because of a lack of excitability of NSCs. According to the Faraday effects, a possible mechanism could be that MF facilitates the intracellular and extracellular exchange of ions through long-term opened ion channels and upregulates the expression of voltage-gated Ca^{2+} channels (VGCCs) or TRPC1 channels could result in a current and potential difference of NSCs due to Ca^{2+} that floods from the extracellular matrix or endoplasmic reticulum through voltage-dependent channels or the force of MF itself. On one hand, intracellular Ca^{2+} stimulates phosphorylation of the transcription factor CREB, activating the CREB signaling pathway, and pCREB recruits more CBP to initiate the transcriptional machinery, including histone acetyltransferases. At the same time, histone modifications secondary to electromagnetic field-activated signals, particularly calcium, lead to chromatin unravelling, thereby promoting the binding of pCREB to the promoter region. On the other hand, pCREB is able to bind to the promoter of a series of miRNA to modulate their expression. In addition, miRNA as well as epigenetic mechanisms could affect the expression of BDNF, which plays a critical role in the activities of NSCs.

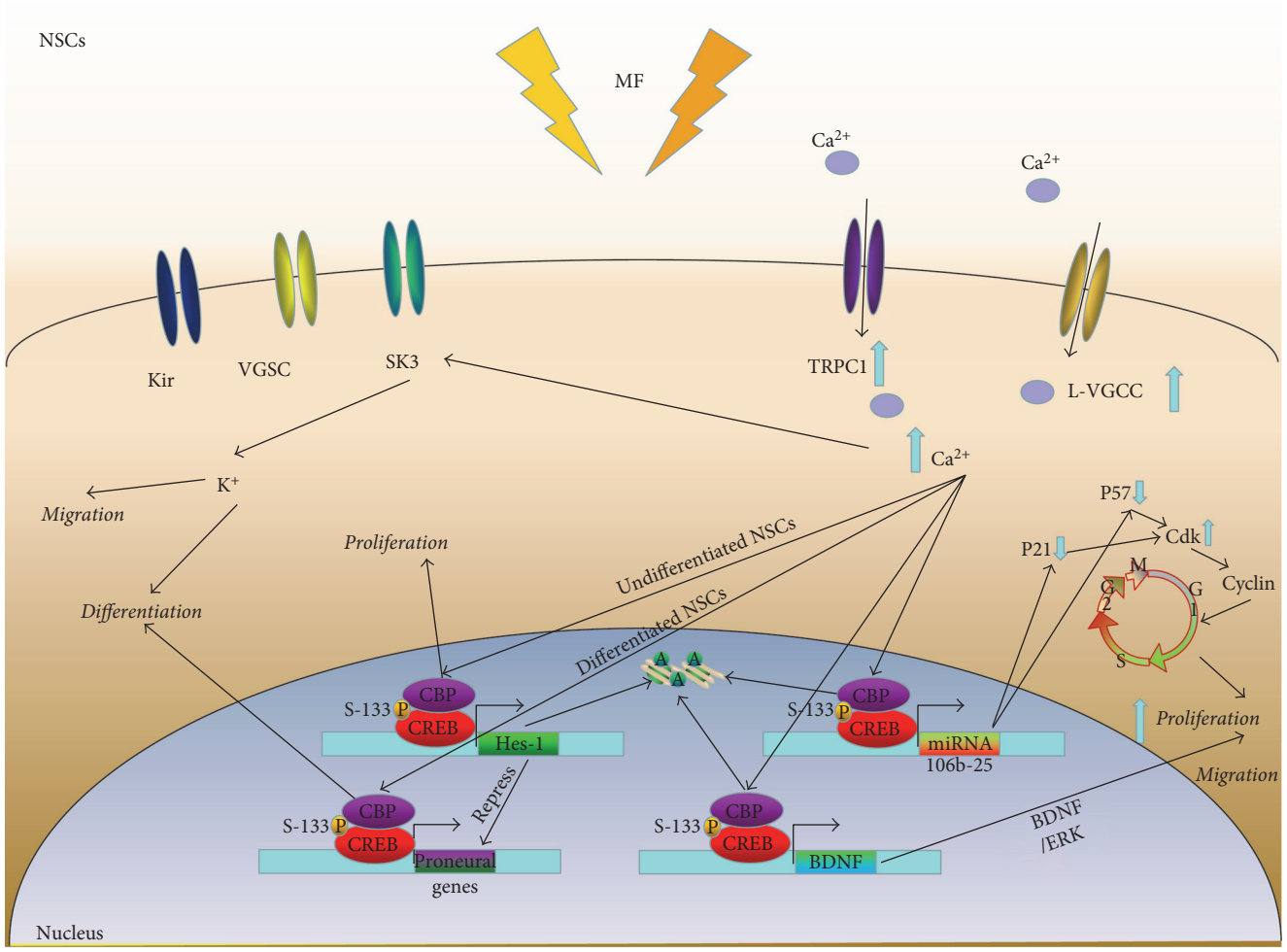

Figure 2: Potential mechanisms of electromagnetic field regulation on neural stem cells. Ca^{2+} and CREB might be the hinge of effects, because of a lack of excitability of NSCs, and according to the Faraday effects, a possible mechanism could be that the MFs facilitate the exchange of intracellular and extracellular ions through these long-term opened ion channels and upregulate the expression of voltage-gated Ca^{2+} channels (VGCCs) or TRPC1 result in a current and potential difference of NSCs, Ca^{2+} flood into from extracellular matrix or endoplasmic reticulum through the voltage-dependent channel or the force of MF itself; on the one hand, intracellular Ca^{2+} stimulates phosphorylation of transcription factor CREB activating the CREB signaling pathway, pCREB recruits more CBP, and p300 initiates the transcriptional machinery, including histone acetyltransferase. Alternatively, calcium or other ELFEF-activated signals could induce histone modifications and chromatin unravelling, leading to the pCREB binding and the start of transcription. On the other hand, the pCREB is able to bind to the promoter of a series of miRNAs to modulate their expression. In addition, CREB itself as well as the epigenetics mechanisms could affect the expression of BDNF which plays a critical role in the activities of NSCs.

Overall, these mechanisms discussed above have the potential to mediate rTMS effects on NSCs (Figure 2). However, there might be some enigmatic affiliation among them, suggesting that further investigations should explore the underlying interactions among these mechanisms in order to fully understand the rTMS effects on NSCs, thereby elucidating the additional pathways involved.

6. Clinical Applications

6.1. Overview of the Current Applications. The growing interest in noninvasive brain stimulation generated by TMS has led to its widespread application in various neurological and psychiatric disorders and to rehabilitation applications for better diagnostic and therapeutic purposes. rTMS has been used for diagnostics and treatments of refractory brain diseases, including depression, Parkinson's disease, multiple sclerosis, dementia, stroke, auditory hallucination, neural tinnitus, anxiety, sleep disorder, obsessive-compulsive disorder, epilepsy, schizophrenia, PSTD, substance addiction, and so forth. In particular, rTMS is a treatment used worldwide, with definite therapeutic effects for depression patients resistant to antidepressant medications, and it was authorized for use in treatment-resistant depression by the US Food and Drug Administration (FDA) in 2008. In addition, Alzheimer's disease (AD) is a widespread degenerative disease whose early diagnosis and prevention is critically important. The diagnosis of early AD has been achieved using TMS coupled with peripheral magnetic stimulation [22]. Additionally, the effective treatment of the cognitive problems

Table 1: Current studies of electromagnetic fields and transcranial magnetic stimulation.

Category	Model	Method of stimulation	Field intensity	Stimulation pattern	Stimulation duration	Post stim assessment	Region assessed	Main effect of TMS	Potential mechanism	Reference
In vitro	Embryonic neural stem cells (eNSCs) from embryonic day 13.5 (E13.5) BALB/c mice	sXc-ELF exposure system (IT'IS Foundation, Zurich, Switzerland)	1 mT	Exposed to ELF-EMF (50 Hz, 1 mT) for 1, 2, and 3 days with 4 hours per day	1/2/3 days	Same day	eNSCs in vitro	The neuronal differentiation ↑ Neurite outgrowth ↑	The expression of TRPC1 and proneural genes (NeuroD and Ngn1) ↑	Ma et al. (2016) [95]
	Embryonic neural stem cells (eNSCs) from embryonic day 13.5 (E13.5) BALB/c mice	sXc-ELF exposure system (IT'IS Foundation, Zurich, Switzerland)	0.5 mT, 1 mT, and 2 mT	At a frequency of 50 Hz sinusoidal waves with magnetic intensities of 0.5 mT, 1 mT, and 2 mT for 3 days or with a magnetic intensity of 2 mT for 1 day, 2 days, and 3 days, with an intermittent cycle of 5 min on/10 min	1/2/3 days	Same day	eNSCs in vitro	Intermittent exposure to ELF-EMF no change in proliferation of eNSCs and percentages of Tuj1-positive cells and GFAP	Sox2↓ Math1, Math3, Ngn1 Tuj1 mRNA levels ↑	Ma et al. (2014) [96]
	NSCs from the hippocampus of neonatal 3-day-old SD rats	rTMS 90 mm figure-of-eight coil (Yirui De, CCY-I, Wuhan, China)	50% of the device's maximum power (peak value 3.5 T)	200/400/600/800/1000 pulses per day, 10 s trains with 10 Hz frequency	3 days	Same day	NSCs in vitro	NSC proliferation in vitro in a dose-dependent manner ↑	miR-106b expression ↑ miR-106b/p21/cdks/cyclin pathway	Liu et al. (2015) [65]
	Neonatal rat neural stem cells	HMF-S20-type pulsed magnetic field device (manufactured by High Magnetic Center of Huazhong University of Science and Technology)	0.5–10 T	0.1 Hz, 0.5–10 Tesla (T) [8 groups of B–I, resp.]	5 stimuli of high-intensity pulsed electromagnetic field (HIPEMF)	24th h, 48th h, 72nd h, and 7th day	NSCs in vitro	6.0–10.0 T peak intensity led to poor growth of rat NSCs in vitro, and in the condition of 0.5–4.0 T peak intensity, HIPEMF stimulated the proliferation of rat NSCs	——	Meng et al. (2009) [52]
	NSCs from postnatal day 0 (P0) CD-1 mice	Solenoid generating alternating EFs	1 mT	50 Hz continuously	Up to 12 days	Same day	NSCs in vitro	Neurogenesis ↑	Cav1 channel activity ↑ intracellular Ca2þ signaling ↑	Piacentini et al. (2008) [73]

Table 1: Continued.

Category	Model	Method of stimulation	Field intensity	Stimulation pattern	Stimulation duration	Post stim assessment	Region assessed	Main effect of TMS	Potential mechanism	Reference
	Normal, healthy mice	rTMS (model 9000 MS, Neurosoft, Ivanovo, Russia) 100 mm circular coil	Maximum output intensity of the device	1 Hz and 30 Hz	1 Hz: received 150 pulses/day (5 second train, 10 second pause) in 450 seconds and 30 Hz group received 150 pulses/day (1 second train, 5 second pause) in 30 seconds. 7 or 14 consecutive days	7 days or 14 days	NSCs from subventricular zone	NS/PC proliferation and neuronal differentiation ↑	BDNF; activation of different neurotransmitter system	Abbasnia et al. (2015) [16]
In vivo	NSCs from the hippocampi of newborn C57bl/6 mice	Solenoid generating alternating EFs characterized by a sinusoidal waveform with amplitudes of 5–1000 μT and frequencies of 1–100 Hz	1 mT	50 Hz; 3.5 h/day	12 days (12 D × 3.5 h)	One month	Hippocampal dentate gyrus	Proliferation and neuronal differentiation of NSCs ↑	pCREB signaling pathway epigenetic modulation	Leone et al. (2014) [68]
	Adult male SD rats model of depression in chronic unpredictable stress	rTMS round coil (inner diameter, 2.5 cm; outer diameter, 5 cm; custom-made YIRD, China)	1.26 T	15 Hz, 15 s trains, 900 pulses daily	7 days	1 day	Hippocampal dentate gyrus	NSPC proliferation ↑	BDNF expression; BDNF/ERK signaling pathway ↑	Chen et al. (2015) [57]
	Ischemic injury in rats	rTMS round prototype coil 6 cm in diameter with 3.5 T peak magnetic welds (YRD-CCI, Wuhan, China)	Stimulation intensity was set at 120% of the average resting motor threshold (RMT), namely, 26% of the maximum output of the stimulator	Stimulation for 3 s followed by rest for 50 s, which was repeated ten times (300 pulses per day) at the rate of 10 Hz	7 days	12 h	The ischemic cortex subventricular zone (SVZ)	Adult NSC proliferation ↑	miR-25 expression ↑ miR-25/p57/cdks/cyclins pathway	Guo et al. (2014) [55]

Table 1: Continued.

Category	Model	Method of stimulation	Field intensity	Stimulation pattern	Stimulation duration	Post stim assessment	Region assessed	Main effect of TMS	Potential mechanism	Reference
	Ischemic injury in male Wistar rats	rTMS figure-of-eight coil (CCY-II, Wuhan Yiruide Medical Equipment, Wuhan, China)	20 Hz group: 120% RMT (24% of the maximum stimulator output) iTBS80% RMT (16% of the maximum stimulator output)	40 trains at 20 Hz for 1 s 15 s interval 20 trains with 600 pulses	10 days	2 days	Ipsilateral SVZ peri-infarct striatum	Improvements of functional recovery ↑ Volume of the infarct area ↓ Migration, differentiation, and proliferation ↑	BDNF/TrkB signaling pathway; ↑ expression levels of BDNF ↑	Luo et al. (2017) [51]
	Normal, healthy mice	Deep brain magnetic stimulation via two coils placed either side of the cage	10 mT peak	Varying pulsed magnetic fields	4 or 7 days	1 day	Dentate gyrus	NSPC proliferation Neurogenesis ↑	c-fos gene; ↑ expression level of fgf1b ↑; stimulate neural activity in certain brain regions by modulating the balance between excitatory and inhibitory neurons	Zhang et al. (2014) [97]
	Nigrostriatal lesion and chromaffin cell transplant in rats	Oscillatory magnetic field via two 7 cm Hemholtz coils positioned dorsal and ventral to the head	0.7 mT	60 Hz, 2 h morning and afternoon	60 Hz, 2 h morning and afternoon	Same day	SVZ	NSPC proliferation ↑	—	Arias-Carrion et al. (2004) [50]
	Normal, healthy rats	rTMS figure-of-eight coil	70% maximum power	25 Hz, 4 × 10 s trains daily	14 days	1 day	Dentate gyrus	NSPC proliferation neurogenesis ↑	—	Ueyama et al. (2011) [53]
	Normal, healthy C57 mice	Solenoid generating alternating EFs	1 mT	1 to 7 h/day for 7 days	7 days	Same day	Dentate gyrus (DG)	NSC proliferation ↑	Expression of Mash1, Hes1 ↑, and NeuroD2 mRNAs; ↑ Ca^{2+} influx ↑	Cuccurazzu et al. (2010) [49]

and language deficits caused by AD has been realized using low- and high-frequency repetitive magnetic stimulation (MS) [9, 12]. Currently, more and more researchers are actively studying rTMS in the clinic with the hope of using it as a promising treatment for several psychiatric and neurological disorders [18, 23].

6.2. Opportunities and Challenges of EMF-Based NSC Therapy. As discussed above, EMF is widely used in different types of diseases due to the diverse effects it exerts on the human body. The interaction between EMF and NSCs were the focus of this article. EMF's positive effects on the natural properties of NSCs, including the ability to self-renew and multidifferentiate, suggests that stem cell therapy is a powerful method to treat refractory diseases. To some degree, it may be the ultimate weapon to fight these diseases. Therefore, we propose that EMF-based NSCs are the future of stem cell therapy and should be the focus of research on the development of biological science technology. However, their use could face barriers such as ethical considerations, the isolation of the stem cells, and the safety or stability of their application. In contrast, the application of EMF, as a noninvasive technique, could easily and remarkably influence NSCs, particularly endogenous NSCs, thereby providing a new way to solve the current issues associated with the use of stem cells as a therapy for neurological diseases. Opportunities come with challenges; for example, (1) a broad range of parameters requires additional clinical tests and animal experiments. (2) The safety of EMF-based NSC therapy is unclear, and there is no doubt that high-intensity EMF can cause damage to cells and may induce the mutation of the cells. (3) The relationship between the biological parameters and the physical parameters should be elaborated in the future.

7. Limitations

rTMS has a definite positive effect on the brain and has been widely exploited in the clinic. However, rTMS is characterized by certain limitations that restrict the use of the technique and its applications. (1) Lack of focus: it is difficult to stimulate precise regions. (2) As an interdisciplinary field requiring extensive knowledge of physiology, its mechanisms are complicated and remain elusive. We lack the requisite understanding of how rTMS regulates biological processes. Therefore, in the future, researchers from different fields are encouraged to cooperate with one another and to combine their studies for a better understanding of the underlying mechanisms. (3) There is essentially little nonhuman experimental data demonstrating how TMS works at the cellular and molecular levels (Table 1) [2]. Thus, a better understanding of rTMS-induced neural plasticity is needed to optimize treatment protocols and to develop new diagnostic and therapeutic strategies using rTMS [3]. Thus, there is an enormous parameter space to explore by conducting appropriate experiments and clinical practices, carefully recording the data, which will provide novel insight into the dose, orientation, frequency, intensity, period, and so forth, offering numerous diverse possible applications. (4) Because of the high voltage and strong currents, the safety of TMS needs further discussion. It is generally recognized that single-pulsed TMS is safe, while high-frequency or high-intensity TMS may cause unexpected side effects, which means that unified clinical guidelines and more tests are required.

8. Perspective and Conclusion

Despite its limitations, TMS is a promising therapeutic tool for many refractory neural diseases. It is noninvasive and has a clear positive influence on different parts of the brain, especially on NSCs. Nevertheless, NSCs are promising for traumatic, degenerative, and psychiatric diseases. All these findings contributed to TMS being deemed as a brain science technology of the 21st century. In the near future, we should perfect the technique of TMS, and more in-depth studies should be performed. Clinical applications must be expanded to collect more data regarding the modality.

All the assumptions made in this review are based on previously reported studies, although there are many discrepancies among reported results. However, we must mention that different research circumstances, for instance, will help guide us toward a more detailed understanding of rTMS. We believe that the efforts of excellent researchers will accelerate the development of TMS applications, making it a powerful tool to treat people who are surviving with painful diseases.

Conflicts of Interest

The authors declare no conflict of interests.

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Review Article

Potential Therapeutic Mechanisms and Tracking of Transplanted Stem Cells: Implications for Stroke Treatment

Yanhong Zhang[1] and Honghong Yao[1,2]

[1]*Department of Pharmacology, School of Medicine, Southeast University, Nanjing, Jiangsu 210009, China*
[2]*Key Laboratory of Developmental Genes and Human Disease, Institute of Life Sciences, Southeast University, Nanjing, Jiangsu 210096, China*

Correspondence should be addressed to Honghong Yao; yaohh@seu.edu.cn

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Stem cell therapy is a promising potential therapeutic strategy to treat cerebral ischemia in preclinical and clinical trials. Currently proposed treatments for stroke employing stem cells include the replacement of lost neurons and integration into the existing host circuitry, the release of growth factors to support and promote endogenous repair processes, and the secretion of extracellular vesicles containing proteins, noncoding RNA, or DNA to regulate gene expression in recipient cells and achieve immunomodulation. Progress has been made to elucidate the precise mechanisms underlying stem cell therapy and the homing, migration, distribution, and differentiation of transplanted stem cells *in vivo* using various imaging modalities. Noninvasive and safe tracer agents with high sensitivity and image resolution must be combined with long-term monitoring using imaging technology to determine the optimal therapy for stroke in terms of administration route, dosage, and timing. This review discusses potential therapeutic mechanisms of stem cell transplantation for the treatment of stroke and the limitations of current therapies. Methods to label transplanted cells and existing imaging systems for stem cell labeling and *in vivo* tracking will also be discussed.

1. Introduction

Stroke is a leading cause of death and long-term disability worldwide [1–5], and current epidemiological data suggest that the economic and social burdens of this disease will progressively increase over the next few decades. Approximately 795,000 individuals in the United States experience a stroke from 2003 to 2013 [6, 7]. Pathological subtypes comprise ischemic stroke and hemorrhagic stroke [8, 9]. In the Western world, ischemic stroke accounts for 87% of all stroke cases, and the remainder are hemorrhagic (intracerebral hemorrhage and subarachnoid hemorrhage) [6]. In ischemic stroke, an embolus or thrombus occludes a blood vessel, causing a reduction in blood blow to the brain and triggering a cascade of pathological responses associated with energy failure, excessive intracellular calcium, excessive excitatory amino acid release, the generation of reactive free oxygen species, and inflammation, ultimately causing irreversible brain impairment [10–12]. In the present study, numerous experiment animal models are used for the study of ischemic stroke, which are mainly divided into two broad categories: focal and global ischemia [13]. Focal ischemia is commonly used in basic research to mimic human stroke condition, which can be classified as transient or permanent occlusions. Among them, the middle cerebral artery occlusion (MCAO) model is widely accepted. Thread embolism is advanced through the external carotid artery to block the MCA resulting in consequent ischemic damage mainly in the corpus striatum and cortex brain regions [14].

To date, intravenous tissue plasminogen activator (tPA), which is only administered within 4.5 h of ischemic stroke, is effective [8, 15]. For patients who are unable to be treated within that therapeutic window, tPA is largely inadequate. Additionally, intravenous tPA enhances the risk of cerebral hemorrhage which limits its clinical application [16]. In recent year, another promising strategy for treatment of acute ischemic stroke is endovascular blood clot removal in large cerebral arteries with a stent retrieve [17, 18].

Numerous randomized trials have suggested that patients with a proximal cerebral arterial occlusion treated with rapid endovascular treatment could improve reperfusion and functional neurologic outcomes better than systemic tPA [19–21]. Numerous neuroprotective drugs targeting excitotoxicity, inflammation, or oxidative stress have proven unsuccessful [12, 22]. Conversely, emerging evidence indicates that stem cells may be a promising therapeutic avenue for cerebral ischemia. Stem cells possess self-renewal and multidirectional differentiation abilities [23]. At present, different types of stem cells are under investigation to determine their efficacy for the treatment of stroke, including mesenchymal stem cells (MSCs) [24], human umbilical cord blood mononuclear cells [25], neural stem cells (NSCs) [26], and adipose-derived progenitor cells [27]. Stem cell therapy has received considerable attention and is under extensive study, but the precise stem cell-mediated mechanisms governing improved outcomes after stroke remain unclear. Preclinical data suggest that stem cell therapy is a promising regenerative medical treatment given the limited capacity of the central nervous system (CNS) for self-repairs after ischemic stroke. Stem cells appear to release neurotrophic and growth factors to induce innate repair mechanisms, such as angiogenesis and neurogenesis [28, 29], in the adult brain and modulate the inflammatory response [30]. Additionally, stem cells secrete exosomes, which cross the blood-brain barrier (BBB) [31] to transfer certain proteins, noncoding RNA, and lipids to regulate recipient cells [32–34].

It is important to observe the survival, migration, distribution, and clearance of implanted stem cells to better understand their therapeutic mechanisms. *In vivo* imaging modalities for cell tracking are crucial tools for the development and optimization of stem cell therapy. Optical imaging, magnetic resonance imaging (MRI), magnetic particle imaging (MPI), and nuclear imaging, including single photon emission computerized tomography (SPECT) and positron emission tomography (PET), are generally used for cell tracking. Tracker agents must be safe, nontoxic, and biocompatible in clinical trials. Nanoparticles, particularly those labeled with superparamagnetic iron oxide (SPIO), are widely used in preclinical and clinical trials [35–37]. SPIO-labeled cells are tracked using MRI or MPI. SPECT and PET are used to track cells labeled with radioisotopes such as In-111-oxine [38] and ^{125}iodine [39].

To further enhance the therapeutic effects of stem cells for the treatment of stroke and to determine an optimized therapeutic strategy, proper methods for cell labeling and appropriate imaging modalities must be employed. In this review, the potential therapeutic mechanisms of stem cell transplantation for the treatment of stroke and the limitations of current therapies will be discussed. We will also discuss methods for labeling transplanted cells and existing imaging systems for stem cell labeling and tracking *in vivo*.

2. Mechanisms of Stem Cell Transplantation to Treat Ischemic Stroke

2.1. Cell Replacement and Differentiation. Stem cell differentiation and appropriate incorporation into the existing neural network to replace the functions of lost neurons after transplantation represent critical aspects of cell-based therapy. Accumulating evidence suggests that transplanted stem cells have the ability to replace lost neurons via migration to damaged regions and promote neural differentiation, which contributes to behavioral improvements in different stroke models [40, 41]. Choi et al. [42] transplanted human bone marrow-derived mesenchymal stem cells (BM-MSCs) after photothrombotic ischemia and observed the elevated expression of neural and synaptic-relative proteins; additionally, the cells not only integrated well into the existing host circuitry but also enhanced endogenous neural differentiation in MSC-treated groups. At 7 days after transplantation, significant behavioral improvements appeared in the BM-MSC-treated group. Another study reported the utility of transplanting human embryonic stem cell- (hESC-) derived neural precursor cells (hNPCs) into the cortex to replace dying brain cells after permanent distal middle cerebral artery occlusion in rats, resulting in improved functional outcomes. The majority of transplanted hNPCs were positive for nestin, a marker of neural precursor cells. Approximately 10% of the cells differentiated into neuronal phenotypes 2 months after transplantation, and very few cells expressed astroglial or oligodendrocyte markers [43]. Other preclinical studies have reported the ability of NSCs from the human fetal striatum and cortex to survive, migrate, and differentiate into neurons in the stroke-damaged rat striatum [44]. Furthermore, homogenous populations of human neural stem cells (hNSCs) not only possess a remarkable ability to migrate into damaged regions and differentiate into neurons, astrocytes, and oligodendrocytes but also exhibit lower tumorigenicity *in vivo* [45]. Cheng et al. demonstrated the ability of intravenously delivered NSCs to traverse the BBB and migrate into the ischemic brain. Approximately 86% of transplanted NSCs maintained proliferative capability and enhanced the proliferation of endogenous cells. The intravenous administration of NSCs 24 h after stroke significantly improves functional deficits, but a reduction in cerebral infraction volume was not detected by TTC staining [46].

2.2. Endogenous Repair Mechanisms. Mounting evidence indicates that implanted stem cells accelerate long-term functional recovery by migrating toward the ischemic zone to enhance endogenous repair mechanisms via the secretion of growth factors [47, 48]. The adult mammalian brain contains a population of NSCs in the subventricular zone (SVZ) of the lateral ventricle that migrates to the olfactory bulb and generates new neurons [49, 50] and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) [51, 52]. Brain injury, such as stroke, induces neurogenesis and angiogenesis [53–55] and promotes the proliferation and migration of neuroblasts or neural progenitor cells derived from the SVZ toward the injured site [56–58]. Angiogenesis is observed immediately after stroke because new blood vessels significantly increase by 3 days postinjury, and the proliferation of endothelial cells increases as early as 1 day postinjury [59]. Under ischemic conditions, SVZ multipotent NSCs derived from the stroke-injured cortex are capable of neurosphere formation and give rise to a subpopulation of reactive

astrocytes in the cortex that contribute to astrogliosis and scar formation. Expression of the transcription factor *Ascl1* converts SVZ-derived reactive astrocytes into neurons *in vivo* [60]. However, the brain possesses a limited ability to form new neurons after injury, and endogenous regeneration mechanisms are insufficient to replace lost neurons [58]. Thus, there is a need to develop novel methods to enhance stroke-induced neurogenesis. Chromatin-modifying agents, which have previously been used as novel biological probes as well as for the treatment of cerebral ischemia, represent a viable method to stimulate endogenous NSCs and enhance NSC-mediated endogenous brain repair mechanisms [61]. Interestingly, channelrhodopsin-2 (ChR2) transgenic mice that undergo the optogenetic stimulation of glutamatergic activity in the striatum after stroke release glutamate into the SVZ, causing SVZ neuroblast proliferation and migration to the peri-infarct cortex via activation of the AMPA receptor. The stimulation of striatal glutamatergic activity may increase the survival and neuronal differentiation of recruited neuroblasts, thus improving functional recovery [62].

2.3. Secretion of Trophic Factors and Regulation of the Ischemic Microenvironment. Stem cells may regulate the neurovascular microenvironment to promote tissue repair and regeneration via autocrine or paracrine activity involving the release of cytokines, growth factors, or secreted extracellular vesicles. A recent study demonstrated the ability of extracellular vesicles released from stem cells to elicit biological functions similar to the stem cells themselves [63, 64], which represents a novel mechanism of intercellular communication, by delivering their cargo consisting of synaptic proteins, noncoding RNA, DNA, and lipids to acceptor cells, thus altering their gene expression under physiological and pathophysiological conditions [65–68]. Extracellular vesicles primarily include exosomes and microvesicles [69]. Exosomes are small (30–100 nm) membrane vesicles formed by the fusion of multivesicular bodies (MVBs) with the cell plasma membrane, are secreted by diverse cell types, and are present in body fluid such as blood, saliva, urine, and cerebrospinal fluid (CSF) [70, 71]. Exosomes are involved in cell communication, migration, angiogenesis, and cell growth processes in tumors and are considered natural carriers for applications in clinical trials. The systemic administration of exosomes released from mesenchymal stromal cells resulted in significant functional enhancement in the footfault test and a modified neurological severity score starting 2 weeks after treatment, as well as increased neurite remodeling, neurogenesis, and angiogenesis in the ischemic boundary zone after stroke in rats [72]. Further study demonstrated that exosomes harvested from microRNA 133b-overexpressing multipotent mesenchymal stromal cells improved neurological outcomes post-MCAO in rats beyond those elicited by naive exosomes because the exosomes indirectly downregulated the expression of Rab9 effector protein with kelch motifs (RABEPK) to further stimulate the release of exosomes from cultured primary astrocytes and then promote neurite outgrowth and elongation *in vitro* [73]. MRI suggested that the intravenous injection of xenogenic (from minipig) adipose-derived mesenchymal stem cells (ADMSC) and ADMSC-derived exosomes reduced brain infarct size 28 days after acute ischemic stroke, and neurological function underwent a significant improvement on day 14 following stroke. Moreover, in the xenogenic ADMSC/ADMSC-derived exosome treatment group, immune reactions and damage to major organs (brain, heart, lung, liver, and kidney) were not observed [74]. Exosomes generated from glioma stem cells promote the angiogenic capacity of endothelial cells by transferring miR-21 to downregulate the expression of vascular endothelial growth factor (VEGF) [32]. Stem cells enhance the endogenous repair capacity of the brain [32] and attenuate inflammatory reactions [75] though the secretion of trophic or growth factors. The majority of transplanted brain-derived neurotrophic factor- (BDNF-) overexpressing human NSCs express C-X-C chemokine receptor 4 (CXCR4), a chemokine receptor that is associated with inflammation [76]. Pretreatment of NSCs with BDNF causes the secretion of VEGF and macrophage colony-stimulating factor (M-CSF), CXCR4, and vascular cell adhesion molecule-1 (VCAM-1) expression and differentiation into mature neurons [77].

2.4. Alleviation of the Inflammatory Response. It is critical to alleviate the inflammatory response given its contribution to secondary brain injury after cerebral ischemia and experimental subarachnoid hemorrhage (eSAH) [78]. During cerebral ischemia, damaged tissue releases damage-associated molecular patterns (DAMPs) [79], which lead to a series of inflammatory responses such as the activation of microglia and the production of proinflammatory factors, followed by neutrophil recruitment and infiltration, which increase the permeability of the BBB [80, 81] and activate the complement system [82]. A variety of inflammatory factors regulate inflammation in the brain, such as tumor necrosis factor (TNF-α) and interleukin 1 (IL-1). MSCs possess the ability to orchestrate other cells to exert anti-inflammatory effects. Microglia cells incubated with IL-1-primed MSC conditioned medium increase their expression of anti-inflammatory, neurotrophic mediators and decrease their secretion of inflammatory markers such as interleukin 6 (IL-6), granulocyte colony-stimulating factor (G-CSF), and TNF-α [30]. MSCs and extracellular vesicles derived from MSCs intravenously injected after focal cerebral ischemia in mice were shown to modulate immune responses and attenuate postischemic immunosuppression in the peripheral blood [64]. Hypoxia-inducible factor 1-α- (Hif-1α-) modified MSCs implanted in a rat MCAO stroke model promote neurotrophin secretion while inhibiting the generation of proinflammatory cytokines [83]. According to Shichita et al., the efficient internalization of DAMPs, such as high-mobility-group box 1 (HMGB1), peroxiredoxins (PRXs), S100A8, and S100A9, is mediated by macrophage scavenger receptor 1 (MSR1) and macrophage receptor with collagenous structure (MARCO) in a murine model of ischemic stroke. Musculoaponeurotic fibrosarcoma bZIP transcription factor B (MAFB), a critical modulator of myeloid cell differentiation and proliferation [84, 85], enhances the expression of MSR1 in infiltrating myeloid cells. MSR1, MARCO, and MAFB deficiency causes the impaired clearance of DAMPs with consequent severe inflammation and neuronal injury [86].

2.5. Neuroprotective Effects and the Promotion of Axon Growth. Occlusion of a blood vessel by an embolus or thrombus causes a reduction in blood flow to the brain, which induces the disruption of the mitochondrial electron transport chain and the failure of oxidative phosphorylation. ATP supply fails, and excessive intracellular calcium is present in cells, ultimately causing neuronal damage [87]. Spermine and spermidine, which are free radical scavengers, have the ability to reduce lipid peroxidation [88] and modulate ion channels, receptor, and calcium trafficking [89]. In ischemic stroke, spermine significantly reduces infarction and neurological deficit [90]. Human mesenchymal stem cell treatment has a limited ability to restore cellular polyamine homeostasis, while levels of its metabolic products putrescine and spermidine significantly increase [91]. After CNS injuries such as ischemia and trauma, energy failure causing intracellular signaling disruption and several deleterious cascades are activated resulting in axonal degeneration and neuron death [92, 93]. In one systemic study, miR-133b overexpression in multipotent MSCs was systemically induced in rats subjected to MCAO. MiR-133b released from MSCs was transferred into astrocytes and neurons via exosomes both *in vitro* and *in vivo*, thus regulating connective tissue growth factor (CTGF) and *ras* homolog gene family member A (RhoA) expression and increasing axonal plasticity and neurite remodeling in the ischemic boundary zone (IBZ), subsequently promoting functional recovery after stroke [94, 95]. The transplantation of human neural progenitor cells 1 week after stroke significantly increases dendritic plasticity, promotes axonal rewiring, reduces the impairment of axonal transport, and enhances stem cell-induced functional recovery [47].

3. Limitations of Stem Cell Therapies

The clinical effectiveness of stem cell therapy is controversial, although accumulating evidence suggests that stem cell therapy has the potential to improve behavior and neurological function after experimental cerebral ischemia. Steinberg et al. [96] stereotactically implanted modified BM-MSCs into the brains of 18 patients with stroke. The surgical procedure and cell treatment were generally safe, and a significant improvement in neurological function was achieved after 12 months, which is consistent with a meta-analysis of preclinical studies indicating that stereotactic intracranial administration of MSCs significantly improves stroke outcomes [97]. Furthermore, human neuronal cells intracerebrally implanted into stroke patients with subcortical motor deficits measurably improved function in some patients, although a significant benefit in motor function was not observed [98]. A phase 2 trial comprising 58 patients with subacute ischemic stroke reported the safety of the intravenous administration of autologous bone marrow-derived mononuclear cells, but no beneficial improvements to neurological function were observed [99]. Another clinical trial suggested that the intra-arterial infusion of autologous bone marrow mononuclear stem cells results in minimal adverse reactions and may improve locomotion and language skills and decrease infarction volume, although these benefits were not significant compared with the nontreated group [100].

Stem cells represent an effective strategy to treat brain injury, but the precise mechanisms underlying stem cell therapy remain elusive due to the lack of appropriate cell tracking technology. Furthermore, the cell type, timing, dosage, and route of administration as well as the safety and biocompatibility of the tracker agents must all be considered. Stem cell therapy for the treatment of stroke improves functional recovery and offers the benefit of extending the intervention window via both intracerebral/intracranial (IC) transplantation and peripheral implantation routes, such as intravenous (IV), intra-arterial (IA), and intranasal administration [101]. IC transplantation is a more invasive procedure that allows precise injection into a chosen location, such as the penumbra and the ischemic core, to guarantee minimal cell delivery to untargeted areas [102, 103]. IV and IA systemic administration are less invasive and convenient approaches that results in the wide distribution of injected cells, but very low levels of cells migrate to the site of injury [38, 104]. Intranasal delivery of stem cells is noninvasive and targets the brain [105, 106]. Different administration routes cause the differential biodistribution of transplanted cells, although all routes improve functional recovery of the brain. Thus, it is critical to understand the homing of transplanted stem cells to sites of injury and to monitor transplant dynamic processes, including cell proliferation, migration, and biodistribution. To obtain optimal therapeutic effects and enhance our understanding of the mechanism by which stem cells promote functional recovery in neurological disorders, it is essential to develop noninvasive, reproducible, and quantitative *in vivo* imaging approaches to track stem cell fate. In recent year, the methods for *in vivo* labeling and tracking of implanted stem cells consist of MRI [39, 107], optical imaging (fluorescence and bioluminescence imaging) [108, 109], and nuclear imaging including SPECT [110] and PET [111].

4. *In Vivo* Imaging Systems and Tracker Agents for Transplanted Stem Cells

4.1. SPIO Nanoparticles. Extensive work has been done to synthesize and make surface modifications to SPIOs. Iron oxide nanoparticles are roughly divided into SPIO, ultrasmall SPIO (USPIO), monocrystalline iron oxide nanoparticles (MION), and micron-sized superparamagnetic iron oxide (MPIOs) based on size. SPIO contrast agents are particles composed of an iron-oxide core coated with dextran (ferumoxide) or carboxydextran (ferucarbotran) [112] and protamine sulfate (Pro), which are FDA-approved agents. SPIO nanoparticles are capable of labeling the vast majority of mammalian cells and are imageable during animal experiments and clinical trials. MRI is used to determine the homing, migration, and differentiation of stem cells labeled with SPIO [113, 114]. This image modality possesses high spatial resolution, which facilitates long-term and single-cell detection, and is noninvasive and utilizes nonionizing radiation. Cells labeled with SPIO exhibit low-intensity signals during T2 and T2* MRI imaging [113, 115]. MION labels stem cells without requiring the use of a transfection agent [116] and

does not affect cell viability, phenotype, and *in vitro* differentiation capacity [112]. Many measures have been taken to improve labeling efficiency and enhance MRI detection sensitivity. Compounding fluorescent mesoporous silica-coated SPIO for stem cell MRI is used to enhance the detection sensitivity and efficiency for cell labeling with no adverse reactions [117, 118]. It is also useful to combine MRI with other noninvasive imaging modalities such as reporter gene-based molecular techniques to overcome any deficiencies and obtain more information on the behavior of implanted cells. hNSCs stably expressing enhanced green fluorescence protein (eGFP) and firefly luciferase (fLuc) reporter genes were labeled with SPIO for MRI and grafted into an experimental stroke model. The survival, tumorigenicity, and immunogenicity of grafted cells were efficiently tracked in real time and investigated for 2 months using multimodal MRI and bioluminescence imaging (BLI) techniques [41]. MSCs labeled with SPIO synthesized in the laboratory were intra-arterially injected in a canine stroke model, given its similarity to the human brain, and were tracked using *in vivo* 3.0 T MRI imaging for at least four weeks [119]. SPIO (448 μg/mL) had no adverse effects on the viability of adipose-derived canine MSCs [120]. However, exact cell quantification using an MRI imaging system may result in errors because MRI possesses large background signals from subject interfaces, and certain pathological conditions such as hemorrhage cause similar MRI signals, resulting in mistakes during the measurement of iron-containing contrast agent accumulation.

Magnetic particle imaging (MPI) is a novel molecular imaging technique that is limited to magnetic tracers and directly images SPIO nanoparticle-tagged cells [121, 122]. SPIO tracers introduced into the body generate MPI signals, while animals themselves neither generate nor reduce MPI signals [123, 124]. Thus, MPI provides accurate quantification, high image contrast, and longitudinal observation to monitor the distribution and location of stem cells. MPI is very suitable for preclinical and clinical applications to evaluate functional brain physiology during pulmonary perfusion [125] and traumatic brain injury [126], and there are few background tissue signals using optimized long-circulation SPIO trackers [127]. MPI is applicable to track transplanted cell redistribution and localization *in vivo*. In a recent study, the intravenous administration and dynamic distribution of SPIO-labeled MSCs in rats were monitored using MPI. Tracer clearance from the body can also be quantified using longitudinal MPI [128]. In other studies, MPI is able to track the long-term fate of exogenously labeled human stem cells with high image contrast in the murine brain and whole body for weeks to months [129].

4.2. Radiopharmaceuticals. Cells labeled with radioisotopes are generally tracked more accurately using SPECT and PET given their extraordinary sensitivity and tissue penetration, minimal background signals, and capacity to scan an entire body to investigate cell distribution to other organs. Radiotracers lacking toxicity and effects on cell viability are urgently needed. ^{111}In causes damage to labeled cells due to its radioactivity and toxicity, although it has a half-life of 67 h, thus allowing long-term monitoring of up to 14 days [130, 131]. Cells labeled with indium-111-oxine (^{111}In oxine) exert low negative effects on cell viability [38]. However, the radioactive decay of usable tracers is not suitable for long-term tracking and limits the development of nuclear medicine techniques. Radioactive technetium-99m (^{99m}Tc) and ^{18}F-fluorideoxyglucose (^{18}FDG), a glucose analogue, are not suitable for long-term monitoring due to their short half-lives. It is necessary to combine two imaging modalities to address this defect. In recent study, an MRI/SPECT/fluorescent tri-modal probe (^{125}I-fSiO4@SPIOs) was synthesized by labeling fluorescent silica-coated SPIO with ^{125}iodine to quantitatively track MSCs transplanted intracerebrally or intravenously into stroke rats, and the therapeutic efficacy of different injection routes and possible therapeutic mechanisms were evaluated. Neurobehavioral outcomes were significantly improved due to the upregulation of VEGF, basic fibroblast growth factor (bFGF), and tissue inhibitor of matrix metalloproteinase-3 (TIMP-3), although IC-infused MSCs migrated to the lesion site along the corpus callosum and IV-injected MSCs were primarily entrapped in the lung [39].

4.3. Fluorophore and Reporter Gene Expression Labeling Techniques. Optical imaging systems incorporating fluorescent imaging (FLI) and bioluminescence imaging (BLI) are used for whole-body imaging but with lower resolution and sensitivity. Fluorescent nanoparticles are suitable for stem cell long-term monitoring [132] and do not affect cell viability and proliferation. Luciferase produces a natural form of chemiluminescence during substrate oxidation. Stem cells transfected with the luciferase reporter gene are detectable using BLI, which is both noninvasive and quantitative. In one study, endothelial colony-forming cells (ECFC) were infected with a lentivirus containing eGFP and fLuc grafted into a photothrombotic (PT) stroke model. Strong BLI signals suggested that ECFCs migrate into the ischemic region [133]; overall, it was possible to monitor endogenous neural stem cells (eNSCs) in a PT stroke model using BLI *in vivo*. The stereotactic injection of conditional lentiviral vectors (Cre-Flex LVs) encoding fLuc and eGFP in the SVZ of nestin-Cre transgenic mice generates specifically labeled eNSCs. This results in significant increases in BLI signals, indicating the proliferation of eNSCs. Additionally, BLI signals relocalize from the SVZ toward the infarct region during the 2 weeks following stroke, demonstrating that nestin-positive eNSCs originating from the SVZ promote proliferation, migration toward the infarct region, and differentiation into both astrocytes and neurons during ischemic stroke [134]. In another study, labeled umbilical cord-derived mesenchymal stem cells (UMSCs) with multi-gold nanorod (multi-GNR) crystal-seeded magnetic mesoporous silica nanobeads (GRMNBs) were further transfected with lentivirus-luciferase protein (Luc-GRMNBs-UMSC). Photoacoustic PA signals suggested Luc-GRMNBs-UMSC homing to the infarcted area with the aid of a magnet and 7T MRI were suitable for the long-time tracking of transplanted stem cells. MRI revealed multiple low signals located inside the damage site, indicating Luc-GRMNBs-

Table 1: Tracer agents currently available for tracking stem cells in stroke.

Tracer agent	Imaging modality	Labeled cell type	Route of administration	Results
Radiotracer				
^{111}In oxine	SPECT	hUTC	IV	Approximately 1% of transplanted cells migrate to the site of injury, increasing vascular and synaptic densities in the IBZ [38]
Nanoparticles				
SPIOs	3.0T MRI	MSCs	IA	Safe and feasible; ipsilateral MCA conditions and infarction volume affected the number of cells grafted [119]
	4.7T MRI	NSCs	IC	The majority of contralaterally grafted NSCs migrated to the peri-infarct area [76]
	MRI, BLI	hNSCs	IC	Tracking the fate and function of implanted cells in real time for 2 months [41]
MPIOs	MRI	eNSCs/ NPCs	IC	Immediate, cell-independent MPIO accumulation at the site of injury [136]
	MRI	hMSCs	IC	Good label stability, did not affect hMSC viability [112]
FMNC	MRI	MSCs	IC	Safe and high efficiency for cell labeling, migration, and accumulation in the ischemic region [118]
fmSIO$_4$@SPIONs	3.0T MRI	NPCs	IC/IA	High MR sensitivity and cell labeling efficiency [117]
AIE NPs	FLI	BMSCs	IC	Low cytotoxicity and feasible [137]
GRMNBs	PA, 7.0T MRI, IVIS	MSCs	IV	Enhanced stem cell homing and reduced infarct volume, allowed short- and long-term monitoring [135]
MGIO	1.5T MRI	hfMSCs	IV	Low toxicity and feasible [138]
Gd-DTPA	MRI	BMSCs	IC	Safe and high efficiency [139]
Report gene				
D-luciferin	BLI	BMSC	IP	Higher signal intensity of luciferase-expressing BMSCs 2 h after transplantation and migration to the IBZ [140]
Fluc and eGFP condition lentiviral vectors (Cre-Flex-LVs)	BLI, MRI	eNSCs	IC	A significant increase in eNSC proliferation and migration, and 21% of cells differentiated into astrocytes and neurons [134]
GFP and Luc2 double fusion reporter gene	BLI	ECFC	IA	Functional recovery, improved angiogenesis, neurogenesis, and increased apoptosis [133]

SPECT: single photon emission computed tomography; hUTC: human umbilical tissue-derived cells; IV: intravenous; IBZ: the ischemic boundary zone; SPIOs: superparamagnetic iron oxide; MRI: magnetic resonance imaging; MSCs: mesenchymal stem cells; IA: intra-arterial; MCA: middle cerebral artery; NSCs: endogenous neural stem cells; IC: intracerebral; BLI: bioluminescence imaging; hMSCs: human MSCs; MPIO: micron-sized superparamagnetic iron oxide; eNSCs: endogenous NSCs; NPS: neural progenitor cell; FMNC: fluorescent magnetite nano cluster; fmSIO$_4$@SPIONs: fluorescent mesoporous silica-coated superparamagnetic iron oxide nanoparticles; AIE NPs: fluorescent nanoparticles with aggregation-induced emission; FLI: fluorescent imaging; BMSCs: bone marrow-derived MSCs; GRMNBs: multigold nanorod (multiGNR) crystal-seeded magnetic mesoporous silica nanobeads; PAI: photoacoustic imaging; IVIS: interactive video information system; MGIO: microgel iron oxide; hfMSCs: human fetal MSCs; IP: intraperitoneal; Fluc: firefly luciferase; eGFP: enhanced green fluorescent protein; eNSCs: endogenous NSCs; ECFC: endothelial colony-forming cell.

UMSCs migrated to the stroke region [135]. It is necessary to understand the primary distribution and homing of eNSCs *in vivo* because stroke affects neurogenesis in the adult mammalian brain.

Recent studies investigating tracer agents that are currently available for stem cell tracking in stroke are displayed in Table 1.

5. Conclusion

Several preclinical and clinical trials have shown that stem cell therapy for cerebral ischemia is safe and feasible and has the ability to promote neurologic functional recovery. However, the precise mechanisms underlying the benefits of stem cell transplantation have not yet been fully elucidated. To achieve optimal therapeutic effects and enhance our understanding of the mechanisms by which stem cells promote functional recovery in neurological disorders, it is essential that we develop noninvasive, reproducible, and quantitative *in vivo* imaging approaches to track stem cell fate. Additionally, the combination of different labeling agents facilitates better and long-term stem cell tracking *in vivo* with appropriate safety and feasibility. Each imaging modality has advantages and disadvantages, and the combined use of different imaging modalities strengthens their respective advantages, allowing us to gain a better understanding of the homing, distribution, and differentiation of implanted cells *in vivo*.

Conflicts of Interest

The authors declare no potential conflict of interests.

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Review Article

Three-Dimensional Organoid System Transplantation Technologies in Future Treatment of Central Nervous System Diseases

NaiLi Wei,[1,2] ZiFang Quan,[3] Hailiang Tang,[1] and JianHong Zhu[1]

[1]*Department of Neurosurgery, Fudan University Huashan Hospital, National Key Laboratory of Medical Neurobiology, The Institutes of Brain Science and The Collaborative Innovation Center for Brain Science, Shanghai Medical College, Fudan University, Shanghai 200040, China*
[2]*Department of Neurosurgery, The Second Hospital of Lanzhou University, Lanzhou, Gansu 730030, China*
[3]*Department of Neurology, The First Affiliated Hospital, University of South China, Hengyang, Hunan 421001, China*

Correspondence should be addressed to Hailiang Tang; tangtang052105192@gmail.com and JianHong Zhu; jzhu@fudan.edu.cn

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In recent years, scientists have made great achievements in understanding the development of human brain and elucidating critical elements of stepwise spatiotemporal control strategies in neural stem cell specification lineage, which facilitates successful induction of neural organoid in vitro including the cerebral cortex, cerebellar, neural tube, hippocampus cortex, pituitary, and optic cup. Besides, emerging researches on neural organogenesis promote the application of 3D organoid system transplantation in treating central nervous system (CNS) diseases. Present review will categorize current researches on organogenesis into three approaches: (a) stepwise, direct organization of region-specific or population-enriched neural organoid; (b) assemble and direct distinct organ-specific progenitor cells or stem cells to form specific morphogenesis organoid; and (c) assemble embryoid bodies for induction of multilayer organoid. However, the majority of these researches focus on elucidating cellular and molecular mechanisms involving in brain organogenesis or disease development and only a few of them conducted for treating diseases. In this work, we will compare three approaches and also analyze their possible indications for diseases in future treatment on the basis of their distinct characteristics.

1. Introduction

Stem cell therapy provides with an alternative and the last resort for curing many diseases in an extensive CNS spectrum of disease. However, poor clinical efficiency casts a showdown for stem cell therapy. In general, if neural stem cells take action, they should undergo three steps: proliferate and differentiate into due neural cells, migrate and distribute to accurate location, and integrate into host tissue and form synapse connection [1]. Unfortunately, this process usually takes several weeks [2]. Only a fringe of them finally survives and takes action. Currently, researchers and scientists devote themselves to improving the efficiency through optimizing various parameters such as engineering ideal matrices, suitable delivery approaches, and improving differentiation efficacy. However, stem cells are poorly manipulated in vivo. Once they are engrafted in vivo, they lose our control. Therefore, high-survival rate and stable environment in vivo are critical for stem cell transplantation. In order to solve these problems, organoid-like tissue might provide us with a promising approach.

Organoid is defined as a multicellular formation that spontaneously develops and self-organizes from stem cells or organ progenitors, resembling the structure and function of an organ in vivo [3]. Organoid system recapitulates the process of organogenesis in vivo and harbors stable hemostasis and architecture. Different from traditional stem cell therapy which always concentrated on specific populations of stem cells or progenitor cells, organoids provide with a complete set of cell types of an organ [3–6]. This novel

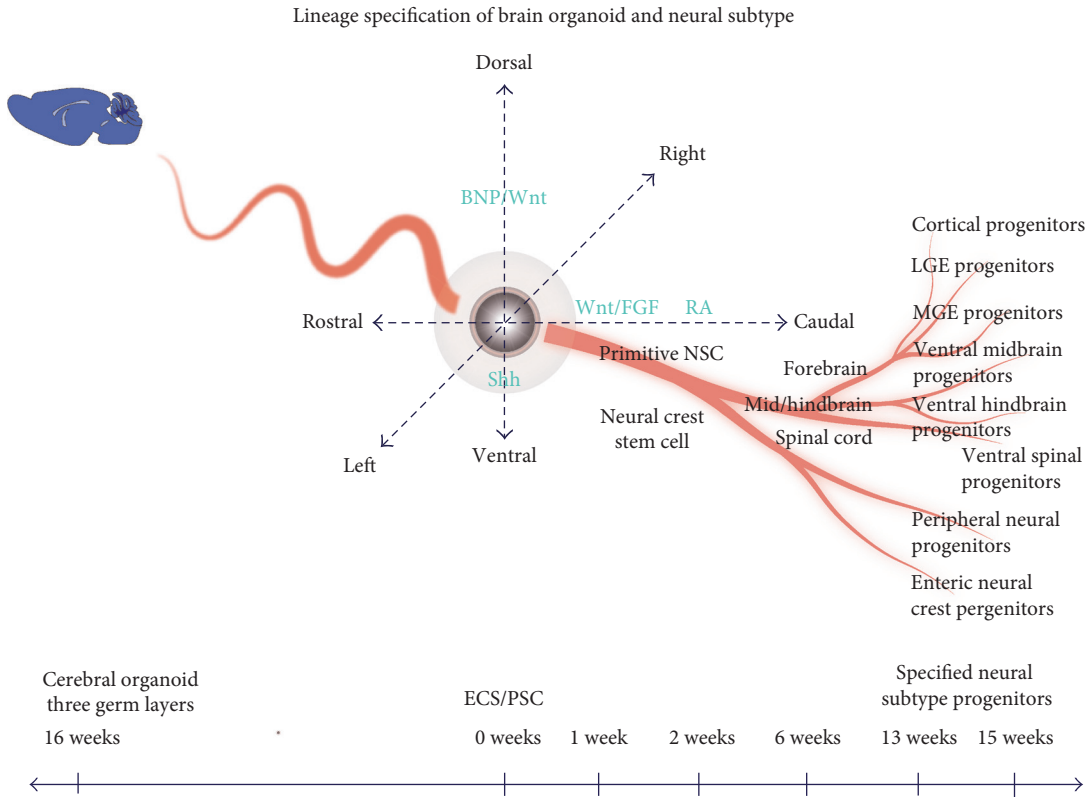

Figure 1: Steered with the specific spatiotemporal control strategies, a single embryonic stem cell (ESC) or pluripotent stem cell (PSC) can develop to a three germ layer brain and pure neural type. The outline has been depicted in Tao's paper (Tao et al.). BNP: bone morphogenetic protein; ESC: embryonic stem cell; FGF: fibroblast growth factor; LGE: lateral ganglionic eminence; MGE: medial ganglionic eminence; NSC: neural stem cell; PSC: pluripotent stem cell; RA: retinoic acid; Shh: sonic hedgehog.

therapy renders an obvious advantage over traditional stem cell therapy. Besides, this method focuses on full-functional organ-like tissue transplantation rather than purified neural cell type treatments. After engrafting into the host, they still have a stable environment in situ and can support themselves for self-renew and self-organize to integrate with host tissue. Thus, stem cells in the organoids have a higher survival rate and form functional connections with the surrounding tissue in the host [4, 7–9].

In recent decades, neural organoid has entered into and captured our eyes. Lancaster and his team successfully established a protocol for culturing pluripotent stem cell- (PSC-) derived "cerebral organoids" that recapitulated the developing human brain's cellular organization segregates into distinct brain regions [10]. Although cerebral organoids could not fully model the organization of the brain, the method still shed a light for future treatment of diseases through organoid system transplantation which can be established in vitro culturing. In addition to Lancaster's team, several other teams developed region-specific neural organoid such as the neocortex [11], telencephalon [12], cerebellar [13], neural tube [14], pituitary gland [15], hippocampus cortex [16], optic cup [17], neural retina [18], and inner ear sensory epithelial tissue. Single embryonic stem cells (ESCs) or PSCs can be self-organized to form three-layer cerebral organoid but can also be directed to develop a region-specific neural organoid. Furthermore, they can also be manipulated and assembled to form specific morphogenesis organ (Figure 1). In specific spatiotemporal control conditions, scientists have directed ES or PSC to differentiate into both neuronal subtypes and glial subtypes. Neuronal progenitors can be specified into GABAergic, glutamatergic neurons, dopaminergic neurons, interneurons, and motoneurons [19–26], while glial progenitors can be specified into astrocytes, oligodentrocytes, and other glial subtypes [19, 27–29]. It is worthwhile mentioning that special signals can also be utilized to enhance the acquisition of the transmitter phenotype [19, 23]. These findings stretch a promising panorama for clinical treatment by distinct organoid system transplantation. Over the past decades, scientists have devoted themselves to elucidating critical element brain development and spatiotemporal control of the processes, which are extensively and fully reviewed in several perfect papers [5, 19–21, 27, 30–35]. These findings provide us with rationale and logistical feasibility to steer organogenesis to specific region. In addition, we can also design and assemble organoid to form specific morphology or function through manipulating numbers of specific stem cell types, neural network composition, numbers of receptors, and ligands. These organoids could be applied to treat central nervous system (CNS) diseases.

Although the classification of organoid was reviewed in previous papers [3], the authors focused on the purpose of

organoid researches rather than the approaches to organoid formatting. Based on methods and application orientations, present review categorizes the organogenesis into three approaches. According to distinct procedure in the induction of organoid organization, we categorize these researches into three approaches (Table 1): (a) stepwise, direct organization of region-specific or population-enriched organoids [12, 18, 36–47]; (b) assemble and coculture distinct organ-specific progenitor cells or stem cells to form specific morphogenesis organoid [7, 8, 13, 17, 26, 48]; and (c) assemble embryoid bodies for induction of full layer organoid [14, 49, 50]. Besides, it will also provide with details of the examples and discuss on the rationale and logistical feasibility. In the following parts, we also compare distinct approaches and analyze for their possible indications for diseases.

2. Neural Organogenesis Approach

2.1. Stepwise, Direct Organization of Region-Specific or Population-Enriched Organoids. Currently, most researches on neural organogenesis adopt stepwise spatiotemporal control strategies to acquire neural organoids from ESCs or IPSCs [12, 18, 36–47]. In the initial stage, ESCs or IPSs are allowed to reaggregate in low-adhesion condition, namely, serum-free EB-like protocol (SFEBq), which ensures that they have enough time to proliferate and expand [47, 51]. In this stage, ESCs or IPSCs maintain pluripotency and EB-like masses harbor three germ layers (ectoderm, mesoderm, and endoderm). In the stage of neural induction, referring to neuroectodermal formation, EB-like masses are transferred to N2 medium to induce neural germ layers. Treating with exogenous signal inhibitor of BMP, Wnt, and nodal inhibitor, they can efficiently form neuroepithelial tissue, neural tube construct [14], or neocortex [42]. The early neural organoids usually display initial structure and morphology with apical-basal polarity and dorsal-ventral polarity. Further induction can promote region identity and acquire region-specific organoids. Human cerebral cortex is well structured with six layer neurons. Deep and superficial layers of neurons are distinct populations, which are connected with each other and have distinct projections and functional fate [33, 52]. As a result, with a good master of region-specific neural organoid induction technology, we can prepare specific population of progenitor cells which we want to perform cell replacement therapy. Although we cannot get purified cell population, high number of specific neural population can be acquired through using this method [13, 23, 39, 42]. Thus, this organogenesis approach will be neural type population orientated.

With the support of specific spatiotemporal control strategies, this approach efficiently directs ESCs or PSCs differentiate into and self-organize region-specific neural organoids with a high number of specific progenitor cells (Figure 2(a)). Both intrinsic and extrinsic signals are involved in the regulations. During embryonic days 9 and 10, corticogenesis in mice takes places in a polarized epithelium with its apical surface forming the lumen of the tube (future ventricles). Early cortical neural stem cells (NSCs) divide symmetrically. At E11, NSCs begin to divide asymmetrically. One daughter cell retains its NSC identity while the other becomes a neuron. Early-born neurons form the deep layers of the cortical plate (layers 5 and 6), and later-born neurons migrate outward past the deep layers to establish the superficial or upper layers (layers 2–4). Although this neural induction process seems to be a cell fate program, it could be manipulated by this approach. For example, Muguruma et al. [13] reported that they acquired polarized cerebellar plate in 3D culture with a stepwise spatiotemporal control strategy. Firstly, they dissociated ESCs at day 0. In order to promote neuroectodermal differentiation, they inhibit mesenchymal differentiation by addition of the transforming growth factor β- (TGF-β-) receptor blocker. On days 2–14, ESCs were treated with FGF2 and insulin with the aim to be steered to differentiate into cerebellar progenitors. On day 14, additional FGF19 and SDF1 treatments induced progenitors to self-form cerebellar plate neuroepithelial structures with dorsal-ventral polarity. After these treatments, neuroepithelial rosettes had transformed into large and continuous flat-oval structures with the apical side inward regarding the ova. Admittedly, major portion of the cerebellar plate neuroepithelium generates Purkinje cells and interneurons and they finally acquired those electrophysiologically functional Purkinje cells.

Muguruma et al.'s success displays a good example which shows how scientists manipulate lineage of organogenesis. However, it will be the tip of an iceberg in the future. Recently, considerable excellent review papers have mapped neural subtype specification lineage and fundamental developmental principles [19–21, 28, 30, 32, 53]; based on which, we can briefly conclude as following: (1) Early cortical neural stem cells (NSCs) residing in a polarized epithelium divide symmetrically at their early expansion. At E11 in mice, NSCs begin to divide asymmetrically, generating one neuronal progenitors and the other continuing maintaining NSC identity. In this stage, apical surface forming the lumen of the tube (future ventricles), early neural progenitors migrate up and down within the ventricular zone (VZ) of the neuroepithelium. Neuronal daughters detach and migrate to subventricular zone (SVZ). (2) Although neocortical excitatory or inhibitory neurons can be generated in both VZ and SVZ, different regulator factors still determine their subtypes. Cux2 and Cux2+ excitatory progenitors, respectively, generate distinct subtypes of upper-layer and deep-layer neurons. SST+ or PV+ progenitors result in inhibitory neurons in all layers except layer I whereas CR+ or VIP+ cells give rise to inhibitory neurons particularly abundant in layers IV, III, and II. NPY+-derived cells could be found in all cortical layers; transcription factor FOXA2 is critical for midbrain DA neuron development while coexpressions of the floor plate (FP) marker FOXA2 and the roof plate marker LMX1A are as well required. (3) Astrocytes in the cerebral cortex are produced from the cortical ventricular zone (VZ) or from the ventral forebrain. In addition, glia of the cerebral cortex is also produced from the postnatal SVZ, a specialized reservoir of glial and neuronal progenitors. Almost all of

Table 1: Related research.

Author	Published year	Organoid induction designs	Organoid type	Induction condition factors	Culture medium	SFEBq procedure
Approach 1: Stepwise, direct organization of region-specific or population-enriched organoids						
Lakshmi Subramanian	2017	Neural tissue samples between GW8 and GW10; coronal vibratome sections were transferred to culture medium.	Forebrain	Wnt inhibitor; TGF-β inhibitor; high O_2 penetration; concentration of matrigel	Cortical slice culture medium	No
Marina Bershteyn	2017	hIPSCs were cultured in using cortical differentiation medium.	Cerebral organoids	Rho kinase inhibitor; Wnt inhibitor; TGF-β inhibitor	Cortical differentiation medium; N2 culture medium	No
Adrian Ranga	2016	ESCs were cultured in high-throughput combinatorial screening of 3D microenvironments; stepwise induced.	Cyst-like structure neural tube with apical-basal polarity	RA; sonic hedgehog; synthetic nondegradable materials	Neurobasal medium	No
Lixiong Gao	2016	hESCs were cultured with SFEBq procedure; H1 cells were selected to induce organoids.	Neural retinal tissue	Wnt signal inhibitor; SAG; CHIR99021; N2; retinoic acid; high oxygen concentrate (40%)	N2 culture medium	Yes (3 days)
Hideya Sakaguchi	2015	hESCs were cultured in SFEBq culture for 73–84 days and dissociated with neural tissue dissociation kit and then cultured in neurobasal medium.	Hippocampal tissue	Wnt inhibitor; TGF-β inhibitor; N2 supplement; chemically defined lipid; neurobasal medium; bone morphogenetic protein; Wnt	N2; neurobasal medium	Yes (3 days)
Anca M. Paşca	2015	hCSs were dissociated to culture to induce organoids.	Cortical tissue with functional neural network	BMP inhibitor and TGF-β inhibitor; NT3; BDNF; FGF2; EGF; neurobasal; dorsomorphin; SB-431542	Neural medium	No
Atsushi Kuwahara	2015	Elective NR differentiation from hESCs.	Neural retina	BMP4-inhibiting GSK3 and FGFR; Y-27632; gfCDM; CHIR99021; SU5402	N2; neural retina medium; retinosphere medium	No
Karl R. Koehler	2013	ESCs were dissociated to induce organoids.	Inner ear sensory epithelial tissue	BMP4; TGF-β inhibitor FGF2 and LDN; N2B27 medium; N2 medium; SU5402; SB-431542	N2B27 medium	Yes (3 days)
Taisuke Kadoshima	2013	Stepwise induction of neocortex with high numbers of pyramidal neurons.	Neocortex	Rho kinase inhibitor, TGF-β inhibitor, and Wnt inhibitor; B27, N2, and chemically defined lipid concentration; hedgehog signals	N2 and chemically defined lipid concentration	Yes (3 days)
Lucy A. Crompton	2013	Neurospheres were dissociated cholinergic neurons to culture in NEM.	Forebrain cholinergic neurons	Nodal/TGF-β signaling inhibitor, ROCK inhibitor Y-27632, FGF2, and EGF	Modified chemically defined media, NEM	Yes (4 days)

Table 1: Continued.

Author	Published year	Organoid induction designs	Organoid type	Induction condition factors	Culture medium	SFEBq procedure
Yichen Shi	2012	Neuroepithelial cells were dissociated to induce organoids.	Cerebral cortex with projection neurons and neural networks	SB431542, FGF2, noggin, neurobasal, Y-27632	N2B27 (N3) medium; N2	No
Jessica Mariani	2012	Undifferentiated PGP1-1 and colonies were dissociated into single cells to induce organoids.	Early forebrain	N2 supplement, Y-27632, FGF2, Wnt inhibitor DKK1, BMP inhibitor BMPRIA-Fc, TGF-β/activin/nodal inhibitor SB431542	N2	Yes (3 days)
Teruko Danjo	2011	Foxg1: venus^{+} cells were sorted to stepwise, induce to ventral telencephalic tissues.	Ventral telencephalic tissues	BDNF, vibratome, Shh	Neuron culture medium; NT3; neurobasal/B27	Yes (3 days)
Mototsugu Eiraku	2008	ESCs were sorted to induce of polarized cortical neuroepithelia.	Cortical tissues	FGF, Wnt, and BMP; N2 medium, KSR	Cortical slice culture medium	No
Kiichi Watanabe	2005	Stepwise induction of neocortex with high number of telencephalic precursors.	Cortical organoids with high number of telencephalic precursors	Dkk1, Bf1. LeftyA, Wnt3at, Wnt, and nodal	Cortical slice culture medium	Yes (3 days)
Approach 2: Assemble and direct disctinct organ-specific progenitor cells or stem cells to form specific morphogenesis organ						
Chikafumi Ozone	2016	Coculture specific ventral hypothalamic NE tissue and nonneural ectoderm formation.	Anterior pituitary	KSR; FGF2; BMP4	gfCDM	Yes (3 days)
Keiko Muguruma	2015	hESCs were cultured to form neural tube-like NE structures and dissociated with anti-KIRREL2 antibody and then cocultured with RL tissues to generate Purkinje cells.	Polarized cerebellar tissue and Purkinje cells	SDF1 and FGF19; FGF2	gfCDM; N2	Yes (3 days)
Andrea Meinhardt	2014	Assembled R1 mESCs, IB10 mESCs, and 46C in 3D matrigel matrix and cocultured to induce the formation of neuroepithelial cyst.	Neuroepithelial cyst	RA, SAG, cyclopamine	N2B27 medium	No
Hidetaka Suga	2011	Assembled nonneural ectoderm and hypothalamic neuroectoderm cells in three-dimensional culture and cocultured to induce of adenohypophysis like tissue.	Adenohypophysis	SAG, DAPT, BIO, Wnt4, and Wnt5, FGF8, Nodal, IWP2, FGF10	CDM medium; DAPT-free medium	Yes (10 days)
Mototsugu Eiraku	2011	Assembled neuroectodermal epithelium tissue and nonretinal neuroectodermal epithelium in three-dimensional culture and cocultured to induce of optic cup.	Optic cup	40%-O2/5%-CO2, N2, RA, CUY21 generator	N2	Yes (3 days)
Xue-Jun Li	2008	Cultured neuroepithelial cells to generate motoneurons and then coculture of motoneurons and myocytes.	Ventral spinal tissue	RA and Shh, BDNF, GDNF, IGF1, Shh, B27	Neural differentiation medium; N2B27	No

Table 1: Continued.

Author	Published year	Organoid induction designs	Organoid type	Induction condition factors	Culture medium	SFEBq procedure
Approach 2: 2.3 Assemble embryoid bodies for induction of multilayer organoids						
Yun Li	2017	Differentiation of hESCs to EBs. EBs were embedded in droplets of matrigel. Embedded EBs were subsequently cultured to induce cerebral organoids.	Cerebral organoids	bFGF; ROCK inhibitor; orbital shaker; WIBR3; BDNF	Neurobasal; N2	Yes (6 days)
Madeline A. Lancaster	2013	Assembled EBs in the droplets of matrigel and cocultured to induce the formation of cerebral organoids.	Cerebral organoids	Retinoic acid, protein kinase (ROCK) inhibitor, neurobasal, N2, insulin, B27	N2B27	Yes (6 days)

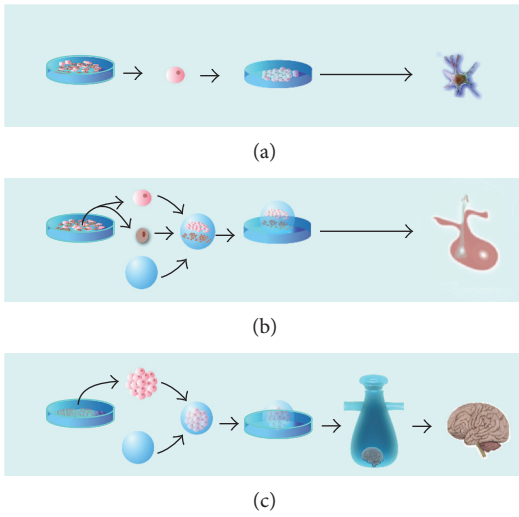

Figure 2: Schematic of three neural organogenesis approaches in vitro. (a) Stepwise, direct organization of region-specific or population-enriched organoids; (b) assemble and direct distinct organ-specific progenitor cells or stem cells to form specific morphogenesis organ; (c) assemble embryoid bodies for induction of multilayer organoids.

the neural subtype specification can be mapped in recent year researches [19] and could be manipulated to induce what we want (Figure 1).

Neural organogenesis via this approach is always neural population orientated. Also, it is expected to efficiently acquire specific neural progenitors or stem cells after region-specific differentiation induction. The region-specific identity, regulated by cell surface signals, is important for neural network reconstruction [54] in the process of neuronal self-recognition and non-self-discrimination. Differentiation is directed by series of distinct epigenetic mechanisms [55–57]. In the stage of stepwise induction, ESCs or IPSs gradually lose their differentiation pluripotency due to DNA methylation and losing GC [56]. Distinct region specificity may have distinct cell identity. Although these specific neural stem cells are not purified, they still maintain their region identity ability [55]. The neurons with the same specific region identity easily connected with each other [58]. After they are engrafted to the specific region in vivo, they might easily establish neural network with local neurons. By contrast, neurons without an additional common factor have to take time to reconstitute their neural network. Compared with purified stem cell population transplantation, this population-enriched organoids may have a high-survival rate and efficiency to form mature synapse connections.

However, the organoids lost the potentiality to form multiple germ layer structure because stepwise differentiation induction steers the series of transcriptional regulators and DNA methylation to specific germ layer structure. Although stepwise strategy can induce initial neural cystic formation, this method might not construct sophisticated morphology efficiently [48]. Majority of these organoids can only form simple structures.

2.2. Assemble and Direct Distinct Organ-Specific Progenitor Cells or Stem Cells to Form Specific Morphogenesis Organ. This approach has been extensively applied to generate organoids with complex morphology, such as the pituitary [7, 8], optic cup [17], feather buds [59], salivary gland [60], hair follicle [61, 62], gingival tissues [63], and tooth [64]. These tissues usually locate in the transition region among distinct structure layers, and their organoid formation requires communication among the distinct region tissues. However, one induction condition only steers specific region identity. In order to acquire these organoids with complex architecture, they assemble two or more distinct populations of progenitors or stem cells in the 3D matrix and coculture under a specific differentiation induction condition [7, 8, 13, 17, 26, 48]. This approach is defined as a term of "self-assembly" by Sasai [65]. Briefly, it refers to the spontaneous formation of a patterned organ with multistructure and multicellular by selective aggregation of cells or by rearrangement of the relative positions of cells within the structure [65]. Through assembling two or more cell types in a 3D culture, this method is sought to recapitulate an interactive microenvironment and mimic multicellular or multistructure level in the vivo organogenesis. For example, pituitary gland consists of neurohypophysis and adenohypophysis (Figure 2(b)). Adenohypophysis (anterior and intermediate lobes of the pituitary gland) contains several types of endocrine cells while neurohypophysis (posterior pituitary) consists of the axons and secretory termini of hypothalamic vasopressin and oxytocin neurons. In order to resemble structure of the pituitary gland, Suga et al. [8] detached outer component of epithelium cells of day 6 aggregates and cocultured them with inner neuroepithelial cells treating with hedgehog signaling. They found that this synthetic approach could successfully generate pituitary endocrine cells. At the interface of these two epithelia, Rathke's-pouch-like three-dimensional structures generated earlier. Functional organ bud was constructed in vitro, which was proved by the evidence that various endocrine cells efficiently secreted hormone in response to corticotrophin-releasing hormone after grafted in vivo. Based on the same approach, Eiraku's team achieved another success by reconstructing functional optic cup in vitro [17] and mimicking the multistructures of the optic cup consisting of the outer (pigmented) and inner (neurosensory) layers of the retina.

In addition to the pituitary gland and optic cup, the cortex in the central nervous system (CNS) also illustrates a prime example of an organ with extreme neuronal diversity and multilayer structures. Cell types of the cortex are broadly classified into excitatory projection neurons (PNs) and inhibitory interneurons (INs). This approaches might be applied to assemble PNs and INs in ratio, mimicking the vivo structures so as to allow enhanced cortical plasticity in the corticogenesis. Moreover, the process of spontaneous formation of ordered patterns and structures from a population of elements promotes functional connection with each other. Through bridging connections among neurons, glial cells and the vasculature, astrocytes provide with microenvironment and homeostatic processes for neuronal regeneration. Coculture astrocyte progenitors and neural stem cell

might promote neurogenesis and synaptic connections [66–68]. Pouchelon et al. [69] found that functional differentiation of postsynaptic L4 neurons and cognate intracortical circuits were associated with TC-input-type-specific control. In addition, the finding also instructs the development of modality-specific neuronal and circuit properties during corticogenesis and shows another example of interactive communications among cellular levels. Due to these evidences, assembling multiprogenitors or differentiated PSCs or ESCs facilitates neurogenesis and functional connections. The approach appears more suitable for the organogenesis, requiring multicellular or multistructure interactive communications.

Assembling neural subtypes in the neural organoids also plays an important role because also it is important to reprogram the subtype diversity so as to promote the generation of functional neural circuit in the self-organization tissues. Distinct projecting neurons choose highly selective synaptic connectivity, both pre- and postsynaptic, within the same local circuits [70]. Both postsynaptic target of inhibitory interneurons and the properties of their synaptic connections depend on the identity of their projection partners [70]. Emerging data demonstrate that projection neurons and interneurons might "chemical match" for the development of excitatory and inhibitory cell assemblies [20]. Meanwhile, synaptic input also has the capability to affect specific neuron subtype differentiation during cortical circuit assembly [69]. Astrocytes comprise up to 40% of all CNS cells, which not only provide support to neurons but also actively regulate synapse formation and maturation [71]. Consequently, it appears a critical role of the assembling way of specific neural subtypes in establishing the neural circuit in the organoid.

2.3. Assemble Embryoid Bodies for Induction of Multilayer Organoids. Figure 2(c) illustrates procedures of this approach. After 4-day suspension culture, ESCs or PSCs aggregate and form embryoid/embryoid-like bodies. The procedure in vitro culture recapitulates the key events of embryogenesis in vivo to obtain the three developmental germ layers from which all cell types arise [4, 17, 50, 72]. The cell pellets are entrapped in a droplet of matrigel or collagen to coculture for and differentiate to develop a specific organ in a specifying differentiation strategy. Through manipulating extrinsic signal modulation, scientists can germ layer specification and cell differentiation [73]. In addition, embryoid bodies with three germ layers could also differentiate into functional tissue-specific cells with three germ layers. Takashi Tsuji's team designed a clustering-dependent embryoid body transplantation plan to develop a 3D integumentary organ system. In the system, formation involves three germ layers of cell types, respectively, dermis, hair follicles and sebaceous glands. After transplantation, hair follicles successfully generated with fine connections with the surrounding tissues such as the epidermis, arrector pili muscles, and nerve fibers, without tumorigenesis. Takagi et al.'s work provides not only a good example for assembling embryoid body approach but also an example for future application orientation that it appears to be suitable for the organogenesis involving more than one germ layer.

Different from other organoid induction approaches, this approach resorts to acquire specific organoid with full layer structure. Researchers adopt this approach to investigate natural organ development procedures or mechanisms involving diseases [4, 17, 50, 72]. Unlike EB-like aggregation in SFEBq procedure, this approach prolongs the culturing time of EB-like population. Additionally, they assemble and coculture EB-like populations in 3D matrix in order to induce self-organization and morphogenesis.

At present, only a few neurological scientists focus on neural organogenesis or cerebral organoid with multiple germ layers [50] because the overwhelming majority of them hold the theory that neural organ induction starts in neuroectodermal stage. Lancaster et al. developed a cerebral organoid in vitro based on this method. Cerebral organoids showed recapitulate features of human cortical development, namely, characteristic progenitor zone organization with abundant outer radial glial stem cells. Most brain tissues derived from neuroectodermal layer whereas mesoderm and endoderm germ layers involve neural organogenesis. Formation of three germ layers cannot be isolated from each other. The germ layers are defined by their position at the stage of late gastrula. At the late stage of embryogenesis, their regional divisions are no longer distinct [74]. Cardiovascular and cerebrovascular derived from mesoderm germ layer stretch throughout the body including the brain and transport blood and energy. Nervous system originated from neuroectoderm forms parasympathetic and sympathetic nervous systems and governs the function of the cardiovascular system [75]. Therefore, they are supported by each other and connected with each other. Mesenchymal stem cells derived from mesoderm germ layer could also be applied in the degenerative neurological diseases [76–78]. It was found that human mesenchymal stem cells (hMSCs) in culture could provide humoral signals that selectively promote the genesis of neurons and oligodendrocytes from NSCs [68]. In addition, MSC could differentiate into neuron-like cells as well as by a competence to generate a "neuroprotective" environment [79]. This approach may facilitate local reconstitution of vascular networks. Considering the above discussion, we can make a speculation that the assembling embryoid body approach might be applied to generate a cerebral organoid with multiple germ layers. The organoid is more probably suitable for treating patients with multilayer brain tissue loss including traumatic brain injury, stroke [80], hemispherectomy, or lobotomy because of tumor, epilepsy, and intracranial hematoma.

3. Similarities and Differences

3.1. Similarities. Neural organogenesis is regulated by a series of epigenetic regulators. In order to develop to an organoid, a single cell in all approaches has to undergo spatiotemporal steering process. Neurons differentiate and migrate to specific regions and layers along anterior-posterior (AP) and dorsal-ventral (DV) axis [5] and are

regulated by various regulator factors. Wnt, FGF, and retinoic acid (RA) are responsible for their caudalizing activity in the embryological context; Shh signaling for ventralization of embryonic neural tissue; BMP and Wnt signaling for dorsalization [5]. Besides, time order also determines the locations of neurons. Pioneer neurons are the earliest-born neurons in the cortex and then followed by deep cortical layers VI and V, then by upper layers IV, and lastly layers II/III [81]. Late-born neurons tend to localize more basally to early-born neurons [11]. All the three approaches possess the common epigenetic regulating factors. Even though neural organoids in the three approaches are different from each other in composition and structure in the organoids, they have a common neuroectodermal induction process.

In addition, all approaches adopt 3D culture to mimic in vivo microenvironment to provide a scaffold and niche for stem cells to aggregate, attach, and form organoids. Biochemical and biophysical signals are also involved to steer organogenesis in all three approaches. These signals determine organogenesis microenvironments consisting of a complex array of signaling mechanisms from niche support cells, the ECM, and mechanical forces, as well as systemic and physiochemical conditions such as oxygen and pH levels [82]. For example, the identity of PSCs is associated with local oxygen concentration and hypoxia inducible factor-1α (HIF-α) plays a distinct and stage-specific roles in reprogramming human cells to PSCs [83] and involves in angiogenesis and stem cell maintenance. NSCs within the SVZ maintain the integrity of their vascular niche through HIF-1-mediated signaling mechanisms [84]. Relief of hypoxia in developing the cerebral cortex by growth of blood vessels temporospatially coincided with NSC differentiation [85]. Considerable biophysical factors such as adhesion and viscoelastic and stress relaxation of extracellular matrices take impact not only on cell spreading and proliferation but also on the differentiation to specific cell types [86–89]. Biophysical cues also generate a change in protein conformation in response to tension or compression and thus to take effect on the cell formation [87]. All of these signals could be manipulated for lineage of specific organ. Currently, a three-dimensional culture is widely applied in organogenesis. In 3D organoid culture system, it allows the formation of brain tissues through either self-assembly or active induction. Some scientists attempt to display several subtype stem cells in ratio or in multilayer in order to mimic the ratio or structures in vivo and finally acquired full functional organ [4, 61]. With the support of 3D organoid culture, scientists have the possibility and opportunity to rewrite the structure or composition of organogenesis program in vitro.

3.2. Differences. Neural organoids via the first approach are specific region orientated. ESCs or IPSCs can be stepwise, induced to differentiate into an organoid with high number of neural populations. These populations of neural stem cells are not purified cells. Instead, the organoids consist of several region-specific neural populations with special cell surface marker. These cells can form specific morphology and structure [18, 39, 40]. Additionally, they can organize local neural connections among distinct populations [13, 40]. By treating with specific markers, neural organoids could be dissociated to collect purified stem cells or progenitor cells. Therefore, we can efficiently acquire purified neural cell populations with region identity via the first approach induction.

Via the second approach, neural organoids are specific morphology orientated. The organoids usually consist of several anatomic parts. Anatomically, the morphogenetic self-organization locates in the cross-connection area among distinct regions and requires coculture of distinct populations of neural cell populations. Coculture can steer these parts to generate functional and morphologic connections. In order to promote the morphogenesis, distinct populations with different region identity were assembled in 3D droplets. As a result, the ratios among distinct populations, the matrix composition, biophysical, and biomechanical parameters need to be designed precisely mimicking in vivo process. This approach has made higher requirements for assembling protocol. However, this neural organoids have specific indications for diseases. The organoids could be engrafted into the brain as an integral preorgan. However, size and morphology of these artificial organoids have to match with host tissue. Otherwise, they are being potential occupying lesion.

Neural organoids via the third approach are full-germ layer orientated. They have more complex and full structure and morphology. At present, researches focus on the development of brain structure [14, 49, 50] and few of them have successfully mimicked the brain structure generation in vivo although Li et al. reported a folded cerebral organoid with simple structure [49]. This approach aims to acquire not only full function but also both integral structure and morphology. Cell populations in the neural organoids involve not only neural populations but also cell populations derived from other germ layers such as vessels and the immune system. However, how to reconstruct the cerebrovascular and immune system in the organoids still remains to be solved.

4. Clinical Treatment Consideration

4.1. Region-Specific or Population-Enriched Organoids. Stem cell therapy is a promising approach to replace damaged cells in the brain or replenish losing cells in the nucleus [90–92]. In a variety of neuronal degenerative diseases, patients have specific neural population loss or damaged. In Parkinson's disease (PD), midbrain dopamine (DA) neurons, especially innervating motor neurons, are degenerated at least at an early stage. However, Huntington's patients gradually lose their medium spiny GABA (γ-aminobutyric acid) neurons in the striatum. Motor neuron loss could also be observed in spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS) patients [19]. The specific neural subtypes are preferentially affected and degenerated, so few pharmacy drugs could curb the pathological insult progress. Neural stem cell therapy improves these diseases not only in animal models but also in clinical trials [77, 93–98]. Traditional purified cell therapy has low clinical efficiency. However, these neural populations generated by the first neural organoid induction approach could improve its treatment

efficiency. These populations in the neural organoids have specific region identity. After engrafting in to the host brain, they could connect with local neurons in an efficient way. Thus, neural organoids via the first approaches are more suitable for these diseases with specific neural type loss or damage.

4.2. Assembled Specific Morphogenesis Organoids. These neural organoids are specific morphology orientated and theoretically suitable for treating these diseases with specific neural structure damaged or atrophy. For example, Sheehan's syndrome always follows after pituitary atrophy which results from postpartum bleeding and pituitary tumor or surgery [99, 100]. Traditional treatment with pharmaceutical drug has several adverse effects. These patients might have another alternative treatment by transplantation of artificial pituitary induced by assembling and coculturing hypothalamic as well as oral ectoderm stem cells [7]. Other similar diseases can be optic atrophy [17], retinal diseases [18], and so on. In addition, peripheral nerves are other potential indications for the second neural organoids. Schwann cell in the peripheral nervous system is derived from the neural crest. Maturity of Schwann cell requires interaction among the Schwann cell and peripheral tissues [101]. Skin-derived precursor cells facilitate the regeneration process of peripheral nerve [102]. In addition, coculture of progenitor cells of peripheral tissues and neural stem cells might promote the generation of peripheral nerves.

4.3. Assembled Multilayer Organoids. Actually, these neural organoids are a preorgan with integral structure and function and can treat these diseases with structure loss or damage. These patients might have an integral structure loss of brain region because of traumatic brain injury, stroke, hemispherectomy, or lobotomy caused by tumor, epilepsy, and intracranial hematoma. There are no niches for stem cells to attach. Therefore, organoids have to support by themselves. Before engrafted to the host brain, the neural organoids must generate a preorgan with full structure.

5. Conclusion

To conclude, 3D organoid system transplantation renders obvious advantage over traditional approaches which probably focus on pure populations of particular stem cell-derived cell types. Instead, 3D organoid system resembles natural self-formation process of specific organ through assembling cell subtypes, layers, cell subtype proportion, and manipulating biophysical signals. These strategies promote correct connections among multilayer and multicellular synapses and establishment of local neural circuits. In comparison with conventional therapy, 3D organoid system transplantation promotes stem cell survival and functional connection after grafting in vivo [4, 8, 17, 61, 64]. Although 3D organoid system transplantation was reported to treat CNS diseases only in a few papers [4, 8, 17, 61, 63, 64], it still appears to be promising in the future treatment. There are three approaches in neural organoid which could be applied, choices of which can be determined depending upon due diseases. The first organogenesis approach is the region-specific or population-enriched organoids which refer to the fundamental method. We could acquire specific neural subtypes or specific organ, which could be applied to treat neuronal degenerative diseases, such as Parkinson's disease, Huntington disease, ALS, and SMA. The second synthetic approach designed in the multicellular level or multiculture level can generate functional self-formation tissue to treat neural organ-associating functional disorders such as pituitary gland atrophy and optic cup loss. Peripheral nerve damage could also be treated by this organoids. The organogenesis approach by assembling embryoid bodies for specific organ is theoretically more suitable for patients with total layer tissue loss, such as traumatic brain injury, stroke [80, 103], hemispherectomy, and lobotomy because of tumor, epilepsy, and intracranial hematoma. In order to promote local functional connections, scientists should design the neural subtype diversity in the process of in vitro organoid induction, matching the ratio between excitatory and inhibitory neurons, neurons and astrocyte, and input synapses and output synapses.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

NaiLi Wei and ZiFang Quan contributed equally to this work.

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Review Article

Endothelial Progenitor Cells for Ischemic Stroke: Update on Basic Research and Application

Shaohua Liao,[1,2] Chunxia Luo,[2] Bingzhen Cao,[3] Huaiqiang Hu,[3] Suxia Wang,[2] Huili Yue,[1] Lin Chen,[2] and Zhenhua Zhou[2]

[1]*Department of Neurology, People's Liberation Army 152 Hospital, Pingdingshan, Henan 467000, China*
[2]*Department of Neurology, Southwest Hospital, Third Military Medical University, Chongqing 400038, China*
[3]*Department of Neurology, General Hospital of Jinan Military Region, Jinan, Shandong 250031, China*

Correspondence should be addressed to Zhenhua Zhou; exploiter001@126.com

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Ischemic stroke is one of the leading causes of human death and disability worldwide. So far, ultra-early thrombolytic therapy is the most effective treatment. However, most patients still live with varying degrees of neurological dysfunction due to its narrow therapeutic time window. It has been confirmed in many studies that endothelial progenitor cells (EPCs), as a kind of adult stem cells, can protect the neurovascular unit by repairing the vascular endothelium and its secretory function, which contribute to the recovery of neurological function after an ischemic stroke. This paper reviews the basic researches and clinical trials of EPCs especially in the field of ischemic stroke and addresses the combination of EPC application with new technologies, including neurovascular intervention, synthetic particles, cytokines, and EPC modification, with the aim of shedding some light on the application of EPCs in treating ischemic stroke in the future.

1. Introduction

In the world, stroke is the second cause of death and the leading cause of adult disability [1]. It is also the fifth cause of death and the leading cause of disabilities among American adults [2], of which 87% is ischemic stroke [3]. Hospitalized patients with ischemic stroke in China have a 3.3–5.2% mortality rate and a 34.5–37.1% death/disability rate 3 months after onset [4–6]. In the pathological process of ischemic stroke, the blood supply is interrupted after cerebral vascular occlusion, together with energy failure, acidosis, excitatory amino acid release, intracellular calcium overload, and generation of free radicals, which eventually lead to brain parenchymal damages composed of necrosis, apoptosis, and autophagy [7–11]. However, the treatment of ischemic stroke is still very limited. Clinical trials on neuroprotective drugs have not been successful [12], and the only FDA-approved treatment of acute stroke is to apply t-PA within 4.5 hours after onset. The emerging intravenous rt-PA thrombolysis prior to intravascular therapy in recent years requires that the femoral artery puncture be performed 120–212.5 minutes after the onset of symptoms [13]. As such, there are only about 2%–5% of stroke patients who meet the criteria for intravenous t-PA with or without bridging therapy due to its narrow therapeutic time window [14, 15]. Most patients still live with varying degrees of neurological dysfunctions. Therefore, a new effective treatment is badly needed to change this situation.

EPCs are regarded as immature endothelial cells which circulate in the peripheral blood. In 1997, Asahara et al. [16] isolated CD34 and Flk1-positive mononuclear cells from the peripheral blood, and these cells were named EPCs because of endothelial cell characteristics in culture medium. It is now believed that EPCs are precursor cells of mature vascular endothelial cells, which belong to stem cell populations with self-renewal capacity that can differentiate into mature endothelial cells (ECs). EPCs are confirmed to insert into the endothelium of newly formed vessels in the ischemic area, which play an important role in the process of endothelial repair and angiogenesis after injury. Studies also verify

that EPCs have the potency of secreting a variety of cytokines and growth factors, which provide nutritional and antiapoptotic support for the circulating and resident EPCs and other cells (ECs, cardiomyocytes, neurons, neural stem cells, and so forth). Circulating human EPCs injected into nude mice after transient middle cerebral artery occlusion (tMCAO) can protect the neurovascular unit and contribute considerably to the recovery of neurological function [17], which has made itself an important candidate for stem cell therapy. In this review, we discuss the current development of EPC research in ischemic cerebrovascular diseases. In the first section of this review, we describe the basic research in the field of EPCs, including the effect on blood vessels and secreting function of EPCs. In the second part, the clinical application of EPCs is introduced, specially emphasizing the combination of EPC application with new technologies. This review is ended with the consideration of the safety of EPC application, which needs to be carefully concerned in future clinical trials.

2. Basic Research

2.1. Dynamic Changes of EPCs under Pathophysiological Conditions. Under physiological conditions, a small poll of hematopoietic stem cells (HSCs) in the bone marrow niche were differentiated and released into circulation, which are bone marrow-derived EPCs marked with KDR+, CD34+, and CD133+, and the level of EPCs in the peripheral circulation is low [18–20]. The supplementation of some food, such as onion peel, black raspberry, fish oil, and red wine, may be helpful in increasing the number of circulating EPCs [21–24]. Multiple factors (cytokines released by target tissue, growth factors, sex hormones, etc.) mobilize EPCs to migrate from the bone marrow stroma into the blood circulation. This process relies on the activation by endothelial nitric oxide synthase (eNOS). Upregulation of vascular endothelial growth factor (VEGF) may mobilize EPCs to migrate into the blood circulation [25], and the release of EPCs from the bone marrow may also be promoted by upregulating granulocyte colony-stimulating factor (G-CSF) [26, 27]. EPC level and G-CSF level are elevated after acute myocardial infarction [28]. Parathyroid hormone (PTH) can also facilitate bone marrow stem cell (BMSCs) and/or progenitor cell release into circulation [29, 30]. Under hypoxic or inflammatory conditions, endothelial cells (ECs) can upregulate the expression of stromal cell-derived factor-1α (SDF-1α) [31] and interact with EPCs that highly express C-X-C chemokine receptor type 4 (CXCR4) [32, 33], which not only promotes EPC mobilization from the bone marrow but also stimulates EPC recruitment and adherence to the ischemic regional vascular endothelium [20, 34, 35]. Nitric oxide (NO) and erythropoietin (EPO) are currently considered to be key factors for EPC mobilization. EPCs themselves can also promote the aggregation of more circulating EPCs by releasing VEGF and SDF-1α [36].

In the process of EPCs migrating to ischemic or damaged areas, CXCR4/SDF-1 plays an important role in directing EPCs to migrate to the damaged vascular endothelium [31, 37]. The binding of interleukin-6 (IL-6) and glycoprotein (gp80 or gp130) expressed by EPCs promotes the proliferation and migration of EPCs [38]. Some drugs, such as statins, can promote EPC migration and proliferation and reduce EPC apoptosis by activating the Akt/NOS pathway and upregulating matrix metalloproteinase-2 (MMP-2) and MMP-9 expression [39], which enhance EPC function.

In the ischemic area, EPC homing to the damaged vessels is considered as an essential step in the interaction with ECs of many cytokines and their receptors. The interaction of P-selectin expressed by platelet and P-selectin glycoprotein ligand-1 (PSGL-1) expressed by EPCs plays a key role in the process of EPC adherence to neovascularization [40, 41]. In addition, the interaction of $\beta1/\beta2$ integrins with the ligands, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), expressed in ischemic vessel endothelium, high-mobility group box 1 (HMGB1) and gpIIb-dependent platelet aggregates, and $\alpha4$ integrins also participate in and promote EPC adhesion and homing [41–46].

The interaction of VEGF and EPCs is complicated and lies in many steps. In the process of dynamic change, VEGF is one of the critical factors and plays an essential role for EPCs. VEGF has effects on mobilization and migration of EPCs through the receptor KDR [47]. In hypoxia circumstance, HIF-1α is activated in the damaged tissue, which leads to increased levels of VEGF. Then, the VEGF prompts a migration of EPCs and hematopoietic cells [48], and the migratory effects have been documented by several studies [49, 50]. The protection of neurovascular unit of VEGF secreted by EPCs is illustrated in the "Secreting Function of EPCs" section.

For the dynamic changes in the function and number of EPCs under ischemic or inflammatory conditions [51], the use of microbeads and Q-dot-based nanoparticle is superior to conventional flow cytometry in analyzing the microvesicles released from EPCs. Other studies used Dex-DOTA-$Gd3^+$ as a magnetic resonance imaging (MRI) contrast agent to observe the survival period of transplanted EPCs in the rat hind limb ischemic model [52] or used DiI-Ac-LDL staining or ^{111}In-oxine radioactive markers to track transplanted EPCs [17, 53, 54]. These methods can be used to monitor or track EPCs transplanted in the body, providing evidence for EPC-based clinical or preclinical trials.

2.2. The Effect of EPCs on Blood Vessels. EPCs display three fundamental activities within the vascular systems, which include secretion, repairing endothelial damage, and formatting new blood vessels in ischemic tissues [18]. The secreting function of EPCs is mainly described in the next paragraph. In the process of atherosclerosis, focal arterial lesions contain cholesterol, fibrosis, and inflammatory cell infiltrates [55, 56], which substantially indicate the destruction of a balance between endothelial damage and repair. EPCs homing into the artery wall may assist to repair the endothelial injury [57], although the mechanisms involved are still unclear. In the ischemic or inflammation condition, the damaged tissue may release a variety of factors and induce the mobilization of EPCs from the bone marrow to the peripheral blood

[58]. Bone marrow-derived EPCs can home to the neovascularization site, proliferating and differentiating into ECs [59, 60] and participating in angiogenesis. The transplanted EPCs may also appear in the newly formed vascular endothelium of the ischemic site, participating in postischemic angiogenesis [16, 61]. It has been demonstrated that MMP9 plays a key role in poststroke EPC-induced angiogenesis [62]. Some factors including VEGF, SDF-1, platelet-derived growth factor (PDGF), and microparticles secreted by EPCs can stimulate tip and stalk cells [63], to promote angiogenesis and local EC proliferation and migration [64]. EPCs can also differentiate into ECs, replacing or directly integrating with the damaged endothelial layer [65–68] to repair the vascular endothelium. However, it is also argued that circulating EPCs may not directly replenish ECs, but activate resident ECs [69] by secreting VEGF, hepatocyte growth factor (HGF), and other factors, or releasing microvesicles from the cell membrane to transmit mRNA to ECs that promote EC proliferation, form microtubules, and reduce apoptosis [70]. EPCs also contribute to the recovery of vascular ECs by secreting exosomes, a nanoscale vesicle encapsulated by lipid membrane structures [71]. This new approach may play a dominant role in the working mechanism of EPCs.

2.3. Secreting Function of EPCs. The neurovascular unit is a complex network of interactions, including neurons, astrocytes, microglias, microvascular ECs, and pericytes [72]. EPCs interact with the neurovascular unit by secreting multiple factors [36, 73–75]. Moreover, EPCs secrete SDF-1α and VEGF, creating a microenvironment for neuronal survival and regeneration [76, 77].

Further studies have shown that EPCs secrete multiple growth factors such as VEGF, SDF-1α, and insulin-like growth factor-1 (IGF-1), which can not only recruit more circulating EPCs and maintain their survival but also protect the existing collateral circulation and neurovascular unit [78]. VEGF may also promote angiogenesis and stimulate the proliferation and migration of new neurons [79].

Wang et al. [80] confirmed that cocultured EPCs and neural progenitor cells (NPCs) may secrete VEGF and brain-derived neurotrophic factor (BDNF) and provide synergistic protection through activating the PI3K/Akt pathway and minimizing cerebral vascular EC ischemia/reperfusion injury. It has also been found that intravenous combined transplantation of bone marrow stromal cells (BMSCs) and EPCs contributes to the recovery of neurological function in the rat cerebral ischemia model, which may be achieved by high expression of basic fibroblast growth factor (bFGF), BDNF, and VEGF [81] and may be associated with the eNOS/BDNF pathway [82].

In short, complex interactions between EPCs and the neurovascular unit take place in the ischemic area. In the progress, EPCs and the factors they secrete jointly contribute to poststroke angiogenesis and neurogenesis, reconstructing the functions and structures of vascular and neural networks, which promote the recovery of neurological function after ischemic stroke [78] (Figure 1).

3. Application

3.1. Clinical Trial. Clinical trials for EPCs used as a marker of prognosis or transplanting therapies have been or are being carried out, primarily targeting the limbs and the cardiovascular and cerebrovascular ischemia. The number of EPCs can be used as a marker of endothelial dysfunction in cardiovascular diseases [83–85]. In the case of acute coronary events or myocardial infarction, the growing number of EPCs indicates that EPC-mediated repair is a physiological response to severe cardiovascular events [86–88]. In the observation of 122 patients with coronary heart disease and normal control group, the number of circulating EPCs was significantly decreased in patients with coronary heart disease [89–91]. Adams et al. verify that mobilization of lin-2/Sca-1+/c/kit + cells into the peripheral blood could be motivated in a long-term treatment of PTH followed by G-CSF administration in mice [92]. PTH treatment mobilizes endothelial stem cells (ESCs)/EPCs from the bone marrow into the peripheral blood in mice of MCAO, which enhances tissue repair and function recovery and reduces adverse immune response [93]. Some trials applied patients' own EPCs mobilized and recruited by G-CSF [94] to the site of myocardial infarction; some used EPCs from the bone marrow in the coronary artery of patients with myocardial infarction [95–97] and successfully recovered the function of the left ventricle; some have started the second phase trials [98–100]; and some trials conducted direct endocardial injection of unfractionated bone marrow cells [101] or injection of mononuclear cells from patients' own bone marrow in critical limb ischemia [102], both of which have improved ischemic symptoms.

It has been proven that the level of circulating EPCs is an independent predictor of the prognosis of patients with acute ischemic stroke [103]. High levels of EPCs in these patients indicate that the infarct volume is smaller and less likely to develop, which may be a marker for the severity of acute stroke [104]. Clinical observational trials have shown that the number of circulating EPCs significantly decreased in patients with cerebrovascular disease than control subjects [105], and the absence of circulating EPCs is associated with increased risk of future vascular events, but not indicating recurrence of stroke [106]. In the ten cases of acute middle cerebral artery infarction, it has been proven to be viable and safe to conduct intravenous injection of patients' own mononuclear cells within 72 hours after onset [107]. Several studies have been conducted or are still undergoing, but with no available results reported, with the purpose of assessing the safety and efficacy of autologous stem cell administration to treat ischemic stroke. Most clinical trials are focusing on bone marrow- or adipose tissue-derived mesenchymal cell transplantation (NCT02378974; NCT01091701; NCT01461720; NCT01678534; NCT01716481; NCT01922908; NCT01297413; NCT00875654; NCT02580019;NCT01714167; NCT02580019; NCT01714167; NCT02580019; and NCT02564328). The remaining studies use peripheral blood- or umbilical cord blood-derived hematopoietic stem cells intracerebrally or infused into the middle cerebral artery of patients (NCT01518231; NCT01249287; NCT00761982;

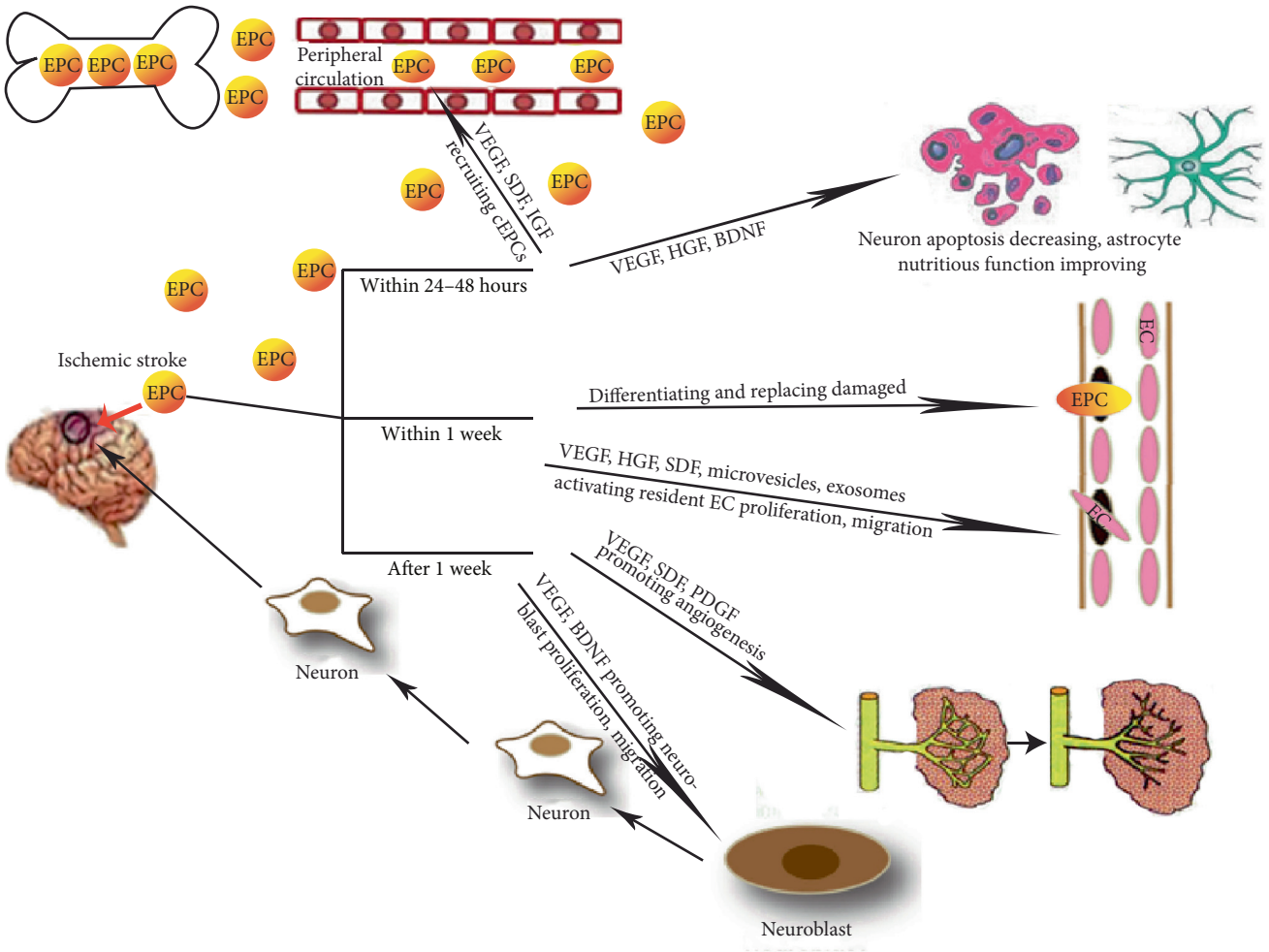

Figure 1: EPCs interact with the neurovascular unit. In the early stage (within 24 to 48 hours), EPCs provide nutritional support for glial cells and reduce neuronal apoptosis through secreting cytokines; during the acute phase (within 1 week), EPCs repair the blood-brain barrier (BBB) and reduce cerebral edema by replacing and repairing the vascular endothelium or promoting the proliferation and migration of resident ECs, thereby reducing nerve cell injury in the ischemic penumbra; in the late acute phase (after 1 week), EPCs recover and reconstruct the neurological functions of nerve cells in the necrotic region by promoting angiogenesis, blood supply, and proliferation and migration of neuroblasts. The figure partly refer to Li et al. [63].

Table 1: Clinical trials for ischemic stroke with endothelial progenitor cells.

References	Study type	Estimated enrollment	Recruitment status	Start date	Investigator
NCT01289795	Observational	30	Unknown status	2010.7	Matthias Endres
NCT01468064	Interventional	20	Recruiting	2011.11	Zhenzhou Chen
NCT02157896	Observational	30	Completed	2013.5	Hao Chen
NCT02605707	Interventional	30	Recruiting	2014.11	ZhenZhou Chen
NCT02980354	Observational	200	Recruiting	2017.2	Ulvi Bayraktutan

NCT01438593; and NCT00950521) [108]. In this review, we have also queried clinical trials of EPC application in ischemic stroke in ClinicalTrials.gov (Table 1), which have no available results reported.

3.2. Time and Methods in Clinical Application. Due to continuous changes in the microenvironment of the stroke site, the timing of stem cell transplantation is a factor that must be considered. However, current animal and clinical trials have not identified a perfect timing for transplantation. Transplantation within 24 hours of stroke has been partially demonstrated to have neuroprotective effects [109, 110]. In some trials, neural stem cell (NSCs) transplantation was used to treat stroke, and it was found that when the

transplantation was conducted on the second day after onset, the number of surviving cells was greater compared with the transplantation done in the sixth week [111]. Taking into account the excitotoxicity, brain edema, inflammatory response, and the expression of nutritional factors and other factors, most researchers believe that 7 days after the onset of stroke is a better time for transplantation, because at this time the brain microenvironment has entered the stage of promoting regeneration [112].

Stem cells can be transplanted in the following ways: intracerebral or intracerebroventricular injection, intravascular infusion, and intranasal delivery [113]. Transplanted stem cells may appear in the damaged core and surrounding areas [114]. Different transplantation methods will affect the cell migration, distribution, and number of cells in the target area [115]. In addition, it is also necessary to take into account the type of disease, the dose of transplanted cells, and the timing of transplantation [113]. In the clinical trial in ischemic stroke, intravascular infusion of EPCs, especially super selective injecting into the ischemic area, maybe a feasible and effective approach.

3.3. Combination of EPCs and New Technologies

3.3.1. Combination of EPCs and Neurovascular Intervention. The experiment of using EPCs to be implanted on several different scaffolds to form microvascular networks [116] or using stents of collagen-coupled CD34 antibody seeded with EPCs transfected with the A20 gene [117] has become a very promising approach. Blindt et al. has designed an EPC-capturing stent, instead of an EPC-covering stent [118], and a short-term result using such a stent is feasible and effective in a clinical trial [119–121], which is helpful to lead to further development of tissue-engineered stent. Another approach is to design clinical trials, in which intra-arterial EPC perfusion is conducted before or after the intravenous t-PA with mechanical thrombectomy bridging therapy or stent implantation in the intracranial and extracranial artery, so that a high concentration of EPCs is formed; then observe the indicators of postoperative brain edema, vascular reendothelialization, postoperative restenosis rate, and neurological function recovery, so as to find out whether the combination of EPCs transplantation and neurovascular intervention technology is better in protecting the neurovascular unit. In the process, patients of the selective operation implant stent in the intracranial and extracranial artery, and EPCs from the periphery blood or bone marrow are perfused through a hyperselective catheter during the operation. The cell number for implantation is referred to the paper [122, 123]: 20×10^6 or 3×10^6. The clinical trial is not perfect and the detail is not completed now. With the rapid development of neurovascular intervention, the combined application may be a direct and effective way to utilize EPCs and also overcome side effects of the stent treatment and provide expansive prospect in clinical therapy in ischemic stroke.

3.3.2. Combined Transplantation of EPCs and Cytokines. The combined transplantation of FGF-2/PDGF-BB and EPCs has been proven to promote EPC migration [124]. SDF-1α and VEGF alone decreased apoptosis, and they may play synergistic role in promoting cell survival and the angiogenesis of EPCs [125]. There is also a study in combined therapy of FGF-2 and G-CSF with EPCs to improve the angiogenic effect in mouse hind limb ischemia models [126]. In the poststroke local acidic environment (pH 6.5), the biological activity of EPCs is impaired, and TPO, stem cell factor (SCF), and IL-3 each could reduce the exposure of EPCs to acid-induced apoptosis. The combined transplantation of the three factors and EPCs can stimulate EPC proliferation and reduce apoptosis, which may be a better choice for vascular endothelial repair and angiogenesis [125]. In the future, growth factor analogues that are more stable in low pH condition may provide better therapeutic strategies with combined transplantation of EPCs.

3.3.3. Combination of EPCs and Synthetic Particles. A nanoparticle is an ideal carrier whose shape, size, surface charge, composition, and coating can be highly customized. It can also protect its carriers and may be released in a controlled manner [127–130]. Nanoparticles can be implanted in molecules, such as VEGF, FGF-2, transforming growth factor-β (TGF-β), G-CSF, and PDGF [108], that promote EPC function and coated the surface with the amino acid sequence LQNAPRS, which has recently been shown to recognize CD133 [131] and anti-CD34 antibodies that are used to recognize EPC [132], which is a type of nanoparticle that contributes to EPC survival and promote angiogenesis. Experiments were carried out using a synthetic pH-sensitive polymer (urethane spherical sulfamethazine) to load SDF-1α and release it in the local acidic environment of the cerebral infarction [133]; other experiments used computer to redesign SDF peptide analogues, which would more effectively induce EPC migration [134] and enhance neurogenesis and angiogenesis. This process may be related to SDF-1α/CXCR4 interaction and recruitment of more EPCs, MSCs, and NSCs.

3.3.4. EPC Modification and Pretreatment. To enhance the therapeutic effect, EPCs can also be used for its modification, mainly gene transduction. Experiments have been conducted to use transduced EPCs to overexpress CXCR4, VEGF, IGF-1, hypoxia-inducible factor-1 (HIF-1), eNOS, and other genes, and the transplantation has achieved positive results [135–138]. Other studies used virus-transduced EPCs to overexpress VEGF, which enhanced EPC proliferation and promoted angiogenesis [139]. Compared with conventional EPCs, using EPCs to overexpress anticoagulant and vascular protection genes more effectively reduce pathological vascular remodeling [140, 141]. Due to the fact that stem cells can secret a variety of factors, it is also possible to overexpress antiapoptotic or angiogenic factors through gene manipulation before transplantation, such as kit ligands, VEGF, and FGF2 [142–144]. These gene modification strategies are likely to enhance the therapeutic effect of EPCs [145]. Another method of enhancing the function of EPCs is ischemic preconditioning, which can increase the expression of VEGFR2 on EPCs, thereby promoting the angiogenic effect

Table 2: Combination of EPCs and cytokines or pretreatment.

Cytokine or pretreatment	Approach	Effect
FGF-2/PDGF-BB	Combined transplantation	EPC migration ↑
SDF-1α/VEGF	Combined transplantation	EPC apoptosis ↓
SDF-1α + VEGF	Combined transplantation	EPC survival ↑, angiogenesis ↑
FGF-2/G-CSF	Combined transplantation	Angiogenesis ↑
TPO + SCF + IL-3	Combined transplantation	EPC proliferation ↑, apoptosis ↓
SDF-1α	Coincubation	Angiogenesis ↑
Nanoparticle	Carrying cytokines	EPC function ↑
Gene transduction	Overexpressing cytokines	EPC function ↑
Ischemic preconditioning	Increasing VEGFR2 expression	Angiogenesis ↑

of EPCs after application [146]. Other preconditioning triggers have been tried out in stem cells or progenitor cells including hypoxia, hydrogen sulfide, hydrogen dioxide, carbon monoxide, and some cytokines and pharmacological agents. The preconditioned stem/progenitor cells show enhanced paracrine effects and better cell survival, which promote functional recovery much better [147]. Alternative test of EPCs is to coincubate with SDF-1α, which has also promoted angiogenesis in the limb ischemia models [148] (Table 2). These studies suggest that enhancing the EPC function through modification techniques and pretreatment may have a greater advantage in the treatment of ischemic stroke.

3.4. Safety. The safety and potential risks of EPC transplantation are also validated in some studies. The impact of EPC effect on formation and progression of atherosclerotic plaques still remains controversial [149], which may be involved with a more accurate phenotypic characterization of EPCs [145]. It has been found that bone marrow-derived EPCs are associated with early angiogenesis in tumors, and in later tumors, these neovessels are diluted by vessels from the periphery [150], which indicate that EPCs are involved in the earliest phases of tumor angiogenesis and therefore EPCs transplantation should not be applied to tumor patients [78]. EPCs may also increase ischemia-induced inflammatory factors, including IL8, monocyte chemotactic protein-1 (MCP-1), and recruit mononuclear-macrophages, thereby aggravating ischemic injury [53, 151, 152]. After EPC transplantation, the connection between nascent capillary endothelial cells is not tight enough and the permeability is high, which may aggravate brain edema [153] and increase the risk of bleeding. EPCs and paracrine VEGF promote angiogenesis, which may lead to uncontrolled growth of local capillaries, developing into hemangioma or capillary groups. Other possible side effects include epilepsy, direct injection-induced injury, and transplantation failure caused by allotransplantation-induced immune responses [154, 155]. In the current clinical trials, there are some limitations which include lack of appropriate controls, randomization, blinding, and a small number of patients followed up for short periods [145]. However, transplantation of EPCs in patients with acute myocardial infarction did not affect plasma C-reactive protein and leukocyte levels [96] and did not lead to tumor angiogenesis in the 5-year follow-up [99]. More experimental animal studies of EPC-based therapy, especially in ischemic cerebrovascular disease, and systemic designed clinical trials should be carried out to interpret the safety issues of EPC application in the future.

4. Conclusion

As a kind of adult stem cells, EPCs' biological characteristics have been determined to repair BBB, improve microcirculation, reduce neuronal apoptosis, and promote the proliferation and migration of neural stem cells through replacing and repairing vascular endothelial cells, promoting angiogenesis, and secreting cytokines and growth factors, which have enabled it to protect the neurological vascular unit. The combination of EPC transplantation with neurovascular intervention, synthetic particles, gene modification, and other technologies will further enhance the therapeutic effect of EPCs and play a more significant role in the treatment of ischemic stroke. There may be a promising approach of EPC application although some safety issues need to be solved.

Conflicts of Interest

The authors declare that there are no competing interests regarding the publication of this paper.

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Research Article

The Effect of Pyrroloquinoline Quinone on the Expression of WISP1 in Traumatic Brain Injury

Yongqi Ye, Pengju Zhang, Yuhang Qian, Baoxin Yin, and Meijuan Yan

The Jiangsu Key Laboratory of Neuroregeneration, Nantong University, 19 Qixiu Road, Nantong 226001, China

Correspondence should be addressed to Meijuan Yan; ymz@ntu.edu.cn

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WISP1, as a member of the CCN4 protein family, has cell protective effects of promoting cell proliferation and inhibiting cell apoptosis. Although some studies have confirmed that WISP1 is concerned with colon cancer and lung cancer, there is little report about the influence of WISP1 in traumatic brain injury. Here, we found that the expression of WISP1 mRNA and protein decreased at 3 d and then increased at 5 d after traumatic brain injury (TBI). Meanwhile, immunofluorescence demonstrated that there was little colocation of WISP1 with GFAP, Iba1, and WISP1 colocalized with NeuN partly. WISP1 colocalized with LC3, but there was little of colocation about WISP1 with cleaved caspase-3. Subsequent study displayed that the expression of β-catenin protein was identical to that of WISP1 after TBI. WISP1 was mainly located in cytoplasm of PC12 or SHSY5Y cells. Compared with the negative control group, WISP1 expression reduced obviously in SHSY5Y cells transfected with WISP1 si-RNA. CCK-8 assay showed that pyrroloquinoline quinone (PQQ) had little influence on viability of PC12 and SHSY5Y cells. These results suggested that WISP1 played a protective role after traumatic brain injury in rats, and this effect might be relative to autophagy caused by traumatic brain injury.

1. Introduction

Traumatic brain injury (TBI), also known as brain injury, is mainly caused by external mechanical forces. There were a series of pathological, physiological, and biochemical changes, such as subarachnoid hemorrhage, cerebral blood tube spasms, disturbance of cerebral circulation, and cerebral edema. All these secondary and primary brain injuries led to higher mortality rates. In the present study, Feeney et al.'s [1] method was used to establish a traumatic brain injury (TBI) model in rats.

WISP1 (Wnt1 inducible signaling pathway protein 1) is a CCN family member, which is more broadly identified with development and tumorigenesis [2]. CCN protein family consists of 6 family members, including cysteine-rich protein 61 (CYR61/CNN1) and connective tissue growth factor (CTGF/CCN2), as well as nephroblastoma-overexpressed secreted protein (NOV/CCN3), WISP1 (CCN4), WISP2 (CCN5), and WISP3 (CCN6) [3]. The CCN family is characterized by four cysteine-rich modular domains that include insulin-like growth factor-binding domain, von Willebrand factor type C module, thrombospondin domain, and C-terminal cysteine knot-like domain. In the extracellular matrix, WISP1 combinates leucine-rich proteoglycans and affects ability of the cell to anchor the extracellular matrix [4].

WISP1 could express in a variety of tissues, and there is no tissue specialty. Pennica et al. discovered that WISP1 was expressed in many tissues including the adult heart, lung, kidney, small intestine, spleen, pancreas, ovaries, and brain, but there were significance changes about express levels among different tissues [5]. WISP1 could block p53-mediated DNA damage and apoptosis [6] and promote cell proliferation and cell adhesion [7]. WISP1 was associated with neoplastic growth [8]. WISP1 has a relationship with the formation and evolution of lung cancer, renal cell carcinoma, colorectal cancer, and other tumors. In the recent years, study showed that WISP1 could promote cardiac remodeling following myocardial infarction [7] and lung

tissue repair and regrowth [9]. In addition, WISP1 may play a vital role in bone formation and fracture repair [10, 11] and can limit neuronal cell injury during oxidative stress [12].

β-Catenin is a soluble protein located in the cytoplasm which is first found in 1980 by German scientists [13]. Subsequent studies show that β-catenin is homology analogs of armadillo gene of Drosophila in mammals [14]. Intracellular β-catenin mainly exists in the cell membrane, cytoplasm, or nucleus in complex forms. The location of β-catenin in the cell is related to biological functions. For β-catenin in cell membrane, β-catenin protein in normal mature cells mediated cell adhesion and migration and affected the polarity and integrity of the epithelium [15]. β-Catenin in the cytoplasm passes through into the nuclear, leading to gene transcription, which is closely related to the development and progression of many diseases [16]. β-Catenin in the nucleus promotes transcription of downstream target genes, which accelerate the cell cycle, promote cell proliferation, produce abnormal protein, and eventually lead to the occurrence of tumor [17].

WISP1, as a CCN family member, utilizes protective pathways that include the traditional wingless canonical and noncanonical signaling of Wnt1. In addition, WISP1 can increase the nuclear expression of β-catenin [4]. Interestingly, study finds that β-catenin can promote the expression of WISP1 by Wnt1 signaling [18]. WISP1 and its signaling pathways with β-catenin represent a novel target that has the potential ability to promote tissue proliferation, repair, and regeneration in multiple cell systems [4, 19, 20].

Pyrroloquinoline quinone (PQQ) is a new oxidoreductase coenzyme which was discovered in the late 1970 of the 20th century. And it is an anionic water soluble compound which exists in almost all biological tissues [21]. PQQ has also been attended broadly in nutrition and pharmacology as an important antioxidant or nutrient. Numerous studies have shown that PQQ has many other pharmacological effects, such as anti-inflammatory and liver and heart protection [22]. It is reported that PQQ-deficient diet cause impaired growth, immunological defects, and decreased fertility in mice [23].

Recent studies have shown that PQQ alters intracellular signaling pathways. For example, when the phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathways are blocked, the protective effects of PQQ will be lost, which implicates that PQQ could regulate neuronal cell survival by (PI3K)/AKT signaling pathway [24]. Similarly, WISP1 has been shown to rely on PI3K and AKT to provide cytoprotection in neurons [4, 12]. WISP1 ultimately modulates apoptotic pathways of Bad, glycogen synthase kinase-3β (GSK-3β), Bim, Bcl-xL, mitochondrial membrane permeability, cytochrome c release, and caspase activation to prevent cell injury [12]. PI3K and AKT are critical pathways to foster cellular proliferation and block apoptotic injury. However, whether WISP1 can be affected by the activation of PQQ by PI3K/AKT has not been reported.

Although many experiments have confirmed that WISP1 has obvious effective on tissue proliferation, repair, and regeneration, there is little report about the effect of WISP1 in traumatic brain injury. In the present study, we try to explore the effect of WISP1 protein on cell expression, which is necessary to treat brain injury by understanding brain injury repair processes. Therefore, we have established a rat model of TBI to try to further research the function of WISP1 in traumatic brain injury.

2. Materials and Methods

2.1. Animals and the TBI Model. Sprague-Dawley (SD) rats (200–250 g) were obtained from the Experimental Animal Center of Nantong University (Nantong, China). All animals ($n = 117$) were divided into thirteen groups: sham, 1 d, 3 d, 5 d, and 7 d post-TBI and 1 d, 3 d, 5 d, and 7 d post-TBI + 1 mM PQQ or 2 mM PQQ. Referring to Feeney et al.'s [1] TBI method model, briefly, the rats were deeply anesthetized with chloral hydrate (10% solution), and the heads were fixed in the stereotactic frame, and a 10 mm diameter craniotomy were performed adjacent to the central suture, midway between the lambda and the bregma. The dura was kept intact over the cortex. Injury was delivered by impacting the right cortex with a fluid percussion brain injury device (AmScien Instruments, Richmond, USA). Sham rats ($n = 9$) were craniotomized only. After all the procedures, animals were returned to their cages and allowed freely to get the food and water. Animals were housed under a 12 h light/dark cycle, and room temperature was kept at $25 \pm 0.5^{\circ}$C.

2.2. Real-Time PCR Analysis. Total RNA was extracted from the frozen cortex brain tissues with TRIZOL Reagent (Sigma) according to the manufacturer's recommendations. The RNA was reverse-transcribed to cDNA using reverse transcription kit (Thermo) with oligo$(dT)_{18}$ primers. Primers of WISP1 and GAPDH were designed with primer 5 software and synthesized by biotech company (GENEray, Shanghai, China), WISP1 sense primer: 5′-GCCCGAGGTACGCAATAGGAGT-3′ and antisense primer: 5′-CCCACGGTGCCATCAATACAGG-3′; GAPDH sense primer was also designed in same way, GAPDH sense primer: 5′-CAACGGGAAACCCATCACCA-3′ and antisense primer: 5′-ACGCCAGTAGACTCCACGACAT-3′. Quantitative real-time PCR analysis was performed using the LightCycler 96 (Roche), applying real-time SYBR Green PCR technology (Roche). The reaction mixtures contained 5 μl SYBR Master Mix, 0.1 μl of each PCR forward and reverse primer (10 μM), 1 μl cDNA, and 3.9 μl nuclease-free water for a final volume of 10 μl. After one cycle of 95°C for 10 min, 45 PCR cycles were performed, each consists of a denaturation step (95°C, 10 s) and an annealing step (60°C, 30 s). Total RNA concentrations from each sample were normalized by the quantity of GAPDH mRNA. All experiments were repeated at least three times.

2.3. Western Blot. The brain tissues were stored in −80°C; tissue samples were lysed with extraction buffer. Concentration of the protein was tested using bicinchoninic acid assay kit (Beyotime, Jiangsu, China), followed by electrophoresis separation on SDS-PAGE, and then transferred to a PVDF membrane (Millipore, Bedford, MA, USA) for 120 min at 100 V. The membranes were blocked with 5% skim milk

and then incubated overnight with WISP1 antibody (diluted 1 : 500 in TBS; Santa Cruz, Cell Signaling Technology), β-actin (1 : 4000, Sigma), and β-catenin (1 : 500, Santa Cruz). The PVDF membrane was washed with TBST (TBS with 0.1% Tween 20) for 10 min at least three times and incubated with corresponding HRP-conjugated secondary antibody for 2 h at room temperature. After washing the PVDF membrane for 10 min at least 3 times, the protein was visualized using Beyo ECL Star (Beyotime, Jiangsu, China).

2.4. Immunofluorescence. The brain tissue was fixed with 4% paraformaldehyde at 4°C, after the brain tissues sink to the bottom of the bottle and after dehydrating by concentration of gradient (10%, 20%, 30%, and 5%), and then, 12 μm frozen sections were prepared and examined. All sections were blocked with blocking solution (10% goat serum, 3% BSA, and 0.1% Triton X-100) for 1 h at 37°C, incubating overnight with antibody WISP1 (diluted 1 : 500; Santa Cruz), anti-GFAP (1 : 400; BD Pharmingen), anti-NeuN (1 : 1000; Abcam), β-catenin (1 : 500; Santa Cruz), anti-cleaved caspase-3 (1 : 400; Cell Signaling Technology), anti-LC3 (1 : 500; Cell Signaling Technology), and anti-Iba1 (1 : 1000; Wako) and then washing them with 0.01 M PBS for 10 min at 3 times and followed by incubating with a mixture of FITC- or Cy3-conjugated secondary antibodies for 2 h at room temperature and then being washed again in PBS for 10 min at 3 times. The stained sections were examined with a Leica fluorescence microscope (Leica DM 5000B, Germany).

2.5. EdU Assay. Cell proliferation was detected by Cell-Light EdU DNA cell proliferation kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. Astrocytes were cultured into 96-well plates at 4×10^4 cells/well. After being incubated with 50 μM EdU for 24 h, cells were washed with PBS, followed by fixation in 4% formaldehyde for 30 min, then being permeabilizated in 0.5% Triton X-100 for 10 min. After extensive washing with PBS, cells were incubated with Apollo for 30 min and Hoechst for 30 min. Proliferative cells was calculated as the percentage of EdU-positive cells relative to the total number of cells.

2.6. Transwell Migration Assay. Astrocytes were cultured at 4×10^3 cells/well with DMEM in the upper chamber of a 24-well transwell chamber with 8 μm pore size polycarbonate filters (Costar). 10% fibronectin was added in the lower chamber as chemoattractant. Astrocytes migrate for 24 h and then followed by fixation in 4% formaldehyde for 1 h and stained with 0.1% crystal violet. Images were taken with a microscope and five fields were counted.

2.7. Cell Culture. The SHSY5Y and PC12 cells were obtained from the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM with 10% FBS at 37°C in an incubator containing 5% CO_2.

2.8. Transfection. To silence the expression of WISP1 gene, WISP1 si-RNA was obtained (Invitrogen), WISP1 sense primer: 5′-GGACAUCCAUACACUCAUUTT-3′ and antisense primer: 5′-AAUGAGUGUAUGGAUGUCCTT-3′. si-RNA transfection was performed with lipofectamine 3000 (Invitrogen) according to the manufacturer's guidelines. All data were obtained after being transfected for 72 h.

2.9. CCK-8 Test. SHSY5Y cells and PC12 cells were seeded onto 96-well plate ahead of time and then treated with different concentrations of PQQ for 24 h. Subsequently, cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C for 2 h after adding CCK-8 test solution (Dojindo, Japan), and the cell viability were detected by using microplate reader at 450 nm.

2.10. Statistical Analysis. For each experiment, the mean and standard error were determined. The data were analyzed by means of analysis of variance (one-way ANOVA). Statistical significance was determined at the level of $P < 0.05$.

3. Results

3.1. Expression and Localization of WISP1 after TBI. In order to observe the WISP1 mRNA expression after TBI, qRT-PCR was performed. The results showed that, compared with the sham group, WISP1 mRNA slightly decreased at 1 d, reached the minimum at 3 d after TBI, and then recovered at 5 d and 7 d after TBI. Meanwhile, 1 mM or 2 mM PQQ increased WISP1 mRNA expression at 3 d, 5 d, and 7 d post-TBI, but the effect of 1 mM PQQ was obvious to the 2 mM PQQ (Figure 1).

To investigate the cell type that WISP1 located, double-labeling immunofluorescence was performed. The colocalizations of WISP1 with astrocyte marker GFAP, neuronal marker NeuN, microglia marker Iba1 were examined respectively. Compared with the sham group, WISP1-positive signals decreased slightly in the control lateral brain and reduced significantly in the ipsilateral brain. However, GFAP positive signals increased slightly in the control lateral brain and had a dramatic increase in the ipsilateral brain. There was a little amount colocalization between WISP1 and GFAP in the sham group. However, obvious colocalizations were observed in the brain at 3 d after TBI including ipsilateral and control lateral brain (Figure 2).

NeuN-positive signals obviously decreased in the ipsilateral brain and decreased slightly in the control lateral brain compared with the sham group (Figure 3). Double-labeling immunofluorescence demonstrated that there were obvious colocations of WISP1 and NeuN either in ipsilateral or in control lateral brain after TBI at 3 d compared with the sham group.

At the same time, there was a modicum colocation of WISP1 and Iba1. Compared with the sham group, Iba1-positive signals increased substantially in the ipsilateral brain (Figure 4).

3.2. WISP1 and the Relationship between Autophagy and Apoptosis. Brain injury was often accompanied by the occurrence of apoptosis and autophagy. In the present study, double immunofluorescence was used to detect the colocalization of WISP1 and autophagy marker LC3 and apoptosis marker cleaved caspase-3. Compared with the sham group, LC3-positive signals had a substantial increase in the ipsilateral brain and had no significant change in the control lateral

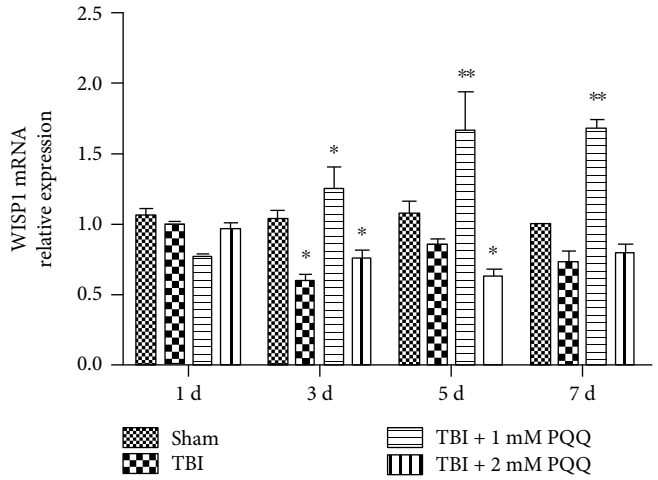

Figure 1: The expression of WISP1 mRNA after TBI ($^{*}P < 0.05$, $^{**}I < 0.01$ versus sham). 1 d: ipsilateral 1 d post-TBI group; 3 d: ipsilateral 3 d post-TBI group; 5 d: ipsilateral 5 d post-TBI group; 7 d: ipsilateral 7 d post-TBI group.

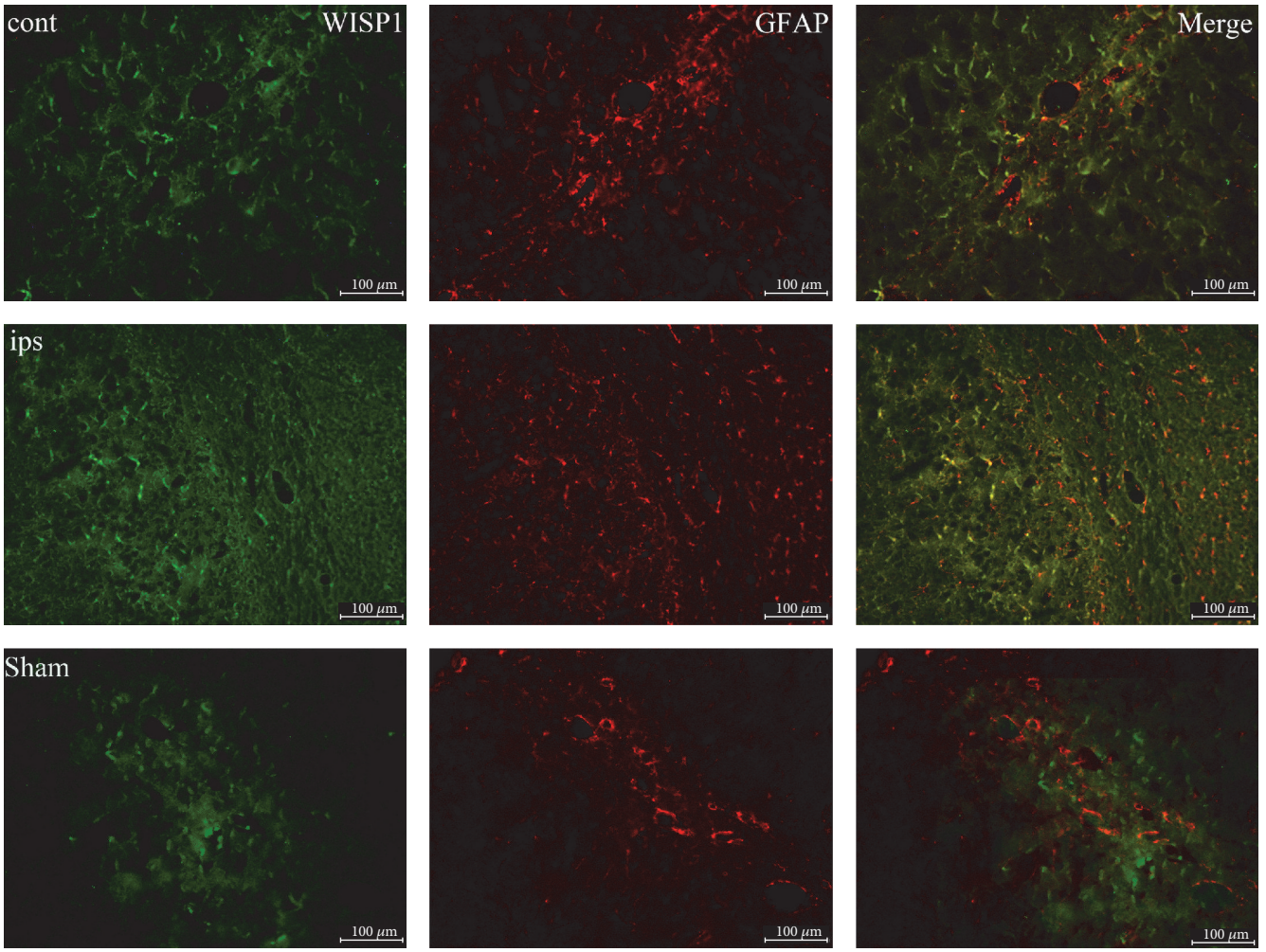

Figure 2: Double-labeling immunofluorescence of WISP1 and GFAP at 3 d after TBI. Green: WISP1; red: GFAP; cont: the control of ipsilateral 3 d post-TBI group; ips: ipsilateral 3 d post-TBI group; sham: sham group; bar = 100 μm.

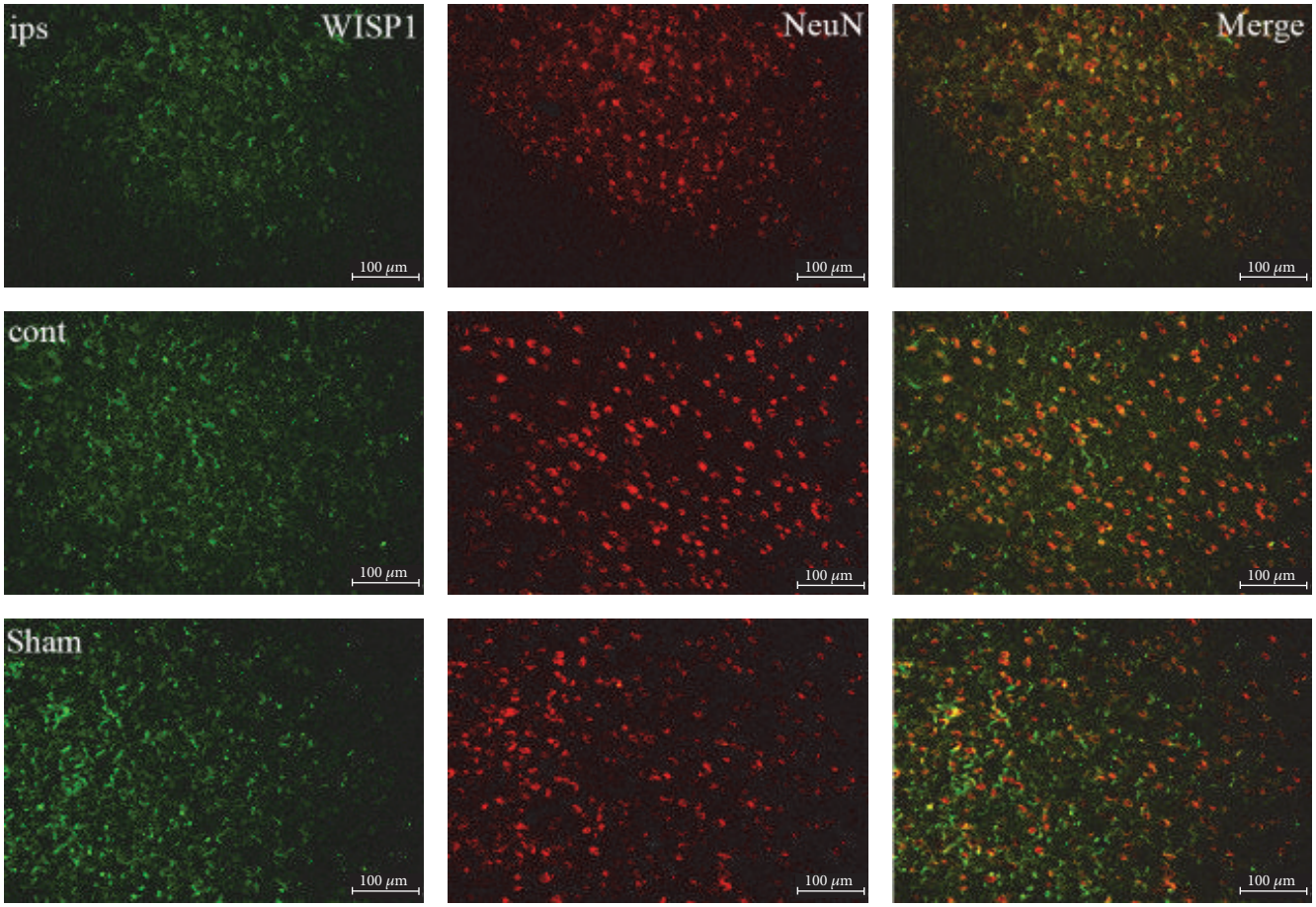

Figure 3: Double-labeling immunofluorescence of WISP1 and NeuN at 3 d after TBI. Green: WISP1; red: NeuN; cont: the control of ipsilateral 3 d post-TBI group; ips: ipsilateral 3 d post-TBI group; sham: sham group; bar = 100 μm.

brain after 3 d post-TBI. After treatment with 1 mM PQQ at 3 d post-TBI, WISP1-positive signals rose obviously; LC3 positive signals declined markedly in the ipsilateral brain. Double-labeling immunofluorescence showed that there were a lot of colocalization of WISP1 and LC3 (Figure 5).

After 3 d post-TBI, compared with the sham group, cleaved caspase-3 expression was obviously upregulated in the ipsilateral brain and had no significant change in the control lateral brain. After treatment with 1 mM PQQ at 3 d post-TBI, cleaved caspase-3-positive signals declined obviously in the ipsilateral brain. Double-labeling immunofluorescence showed that there was a little colocalization of WISP1 and cleaved caspase-3 (Figure 6).

3.3. The Relationship of WISP1 and β-Catenin. The expression changes of WISP1 and β-catenin protein were performed by Western blot, which indicated that the protein expression trend of WISP1 was similar to that of β-catenin at different time points after TBI. The protein expression of WISP1 and β-catenin decreased slightly at 1 d after TBI, minimized at 3 d post-TBI and rose again at 5 d after TBI, and slightly reduced at 7 d after TBI compared with the sham group (Figure 7(a)). Meanwhile, the treatment with 1 mM PQQ increased the protein expression of WISP1 and β-catenin at 3 d, 5 d, and 7 d post-TBI (Figure 7(b)). When 2 mM PQQ was treated, there was no obvious change trend (Figure 7(c)).

After 3 d post-TBI, as far as the sham group, WISP1 expression significantly reduced in the ipsilateral brain and slightly reduced in the control brain. Moreover, β-catenin-positive signal change trend was similar to that of WISP1. Double-labeling immunofluorescence showed that there was a lot colocalization of WISP1 with β-catenin (Figure 8).

The PI3K inhibitor LY294002 (10 μM) and GSK-3β inhibitor SB21673 (5 μM) with WISP1 (10 ng/ml) were administrated. The experiments showed that the expression of WISP1 reduced with the administration of glutamate and then upregulated after using 50 μM PQQ as well as WISP1 protein. Interestingly, the expression of WISP1 did not increase under the glutamate after blocking the PI3K/GSK-3β signaling pathway, just as the β-catenin did (Figure 9).

Double-labeling immunofluorescence showed that there were a lot of colocalization of WISP1 and neuron in TBI, so SHSY5Y cells and PC12 cells were chosen to study further. Immunofluorescence displayed that WISP1 distributed in the cytoplasm either SHSY5Y cells or PC12 cells (Figure 10).

The colocalization of WISP1 and β-catenin were detected in SHSY5Y cells or PC12 cells. Immunofluorescence showed that β-catenin was distributed in the cytoplasm of SHSY5Y

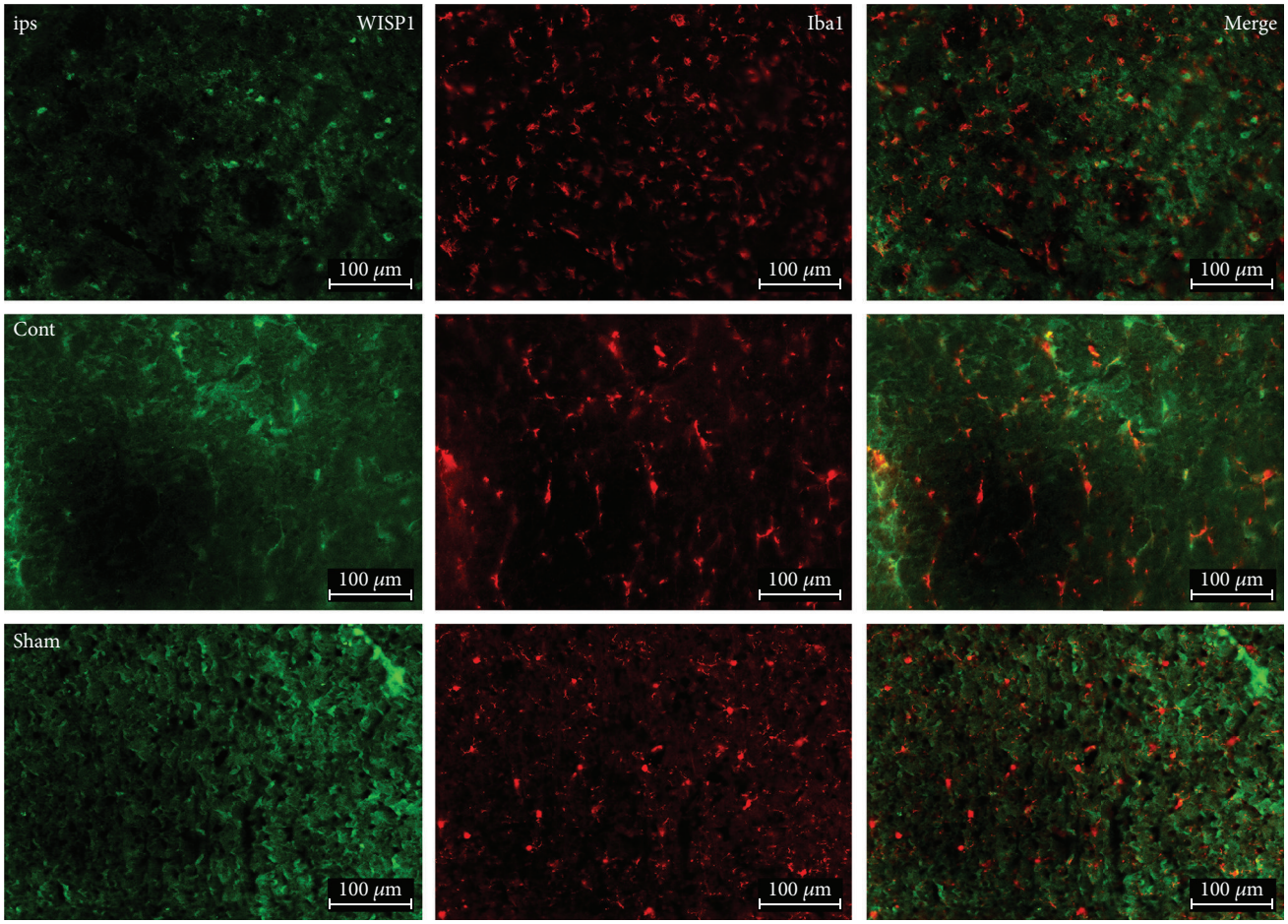

Figure 4: Double-labeling immunofluorescence of WISP1 and Iba1 at 3 d after TBI. Green: WISP1; red: Iba1; cont: the control of ipsilateral 3 d post-TBI group; ips: ipsilateral 3 d post-TBI group; sham: sham group; bar = 100 μm.

cells or PC12 cells, and there was obvious expression in nuclear of SHSY5Y cells. Double-labeling immunofluorescence showed that WISP1 colocalizated with β-catenin in the cytoplasm of SHSY5Y or PC12 cells (Figure 11).

3.4. The Effect of PQQ on the Viability of Cells. The effects of different concentrations or time of PQQ on the activity of PC12 and SHSY5Y cells were performed with CCK-8 assay. The results showed, incubating PC12 cells for 24 h with 10, 20, 30, 40, and 50 μM PQQ, that there was no distinct effect on PC12 cell activity compared with the control group (Figure 12(a)). Meanwhile, the similar results were obtained in SHSY5Y cells. That is, there was no obvious change of cell viability in SHSY5Y cells treated with or without 10, 20, 30, 40, and 50 μM PQQ for 24 h (Figure 12(b)). When 50 μM PQQ was incubated for 6 h, 12 h, 24 h, and 48 h in PC12 cells, there was slight influence on PC12 cell activity at 48 h (Figure 12(c)). At the same time, adopting the same approach in SHSY5Y cells, the activity of SHSY5Y cells was consistent with PC12 cell activity (Figure 12(d)).

Western blot was used to detect the protein expression of WISP1 in PC12 or SHSY5Y cells that were treated with different concentrations of PQQ for 24 h. Compared with the control group, the expression of WISP1 increased slightly with 10 μM PQQ, reached the peak in PC12 cells treated with 20 μM PQQ for 24 h, and then other groups decreased gradually. Moreover, β-catenin expression in PC12 cells peaked with 20 μM PQQ for 24 h. There was no obvious difference with 10, 30, 40, and 50 μM PQQ (Figure 13(a)). Meanwhile, the similar results were obtained in SHSY5Y cells (Figure 13(b)).

50 μM PQQ was used to detect the protein expression of WISP1 in PC12 or SHSY5Y cells at 6, 12, 24, and 48 h. Compared with the control group, results showed that the expression of WISP1 reduced gradually in a time-dependent manner and reached the minimum in PC12 cells treated with 50 μM PQQ for 48 h. β-Catenin protein expression in PC12 cells reached the minimum at 48 h and increased slightly at 6 h, 12 h, and 24 h compared with the control group, but it was not significant ($P > 0.05$) (Figure 14(a)). At the same time, the similar results were obtained in SHSY5Y cells. The expression of WISP1 in SHSY5Y cells was similar to that of β-catenin in PC12 cells; β-catenin protein expression in SHSY5Y cells reached the minimum at 48 h and increased slightly at 6 h and 12 h, but there was no significant difference compared with the control group ($P > 0.05$) (Figure 14(b)).

Prior to adding the glutamate, 25 μM, 50 μM, and 100 μM PQQ were added in the cell medium, respectively.

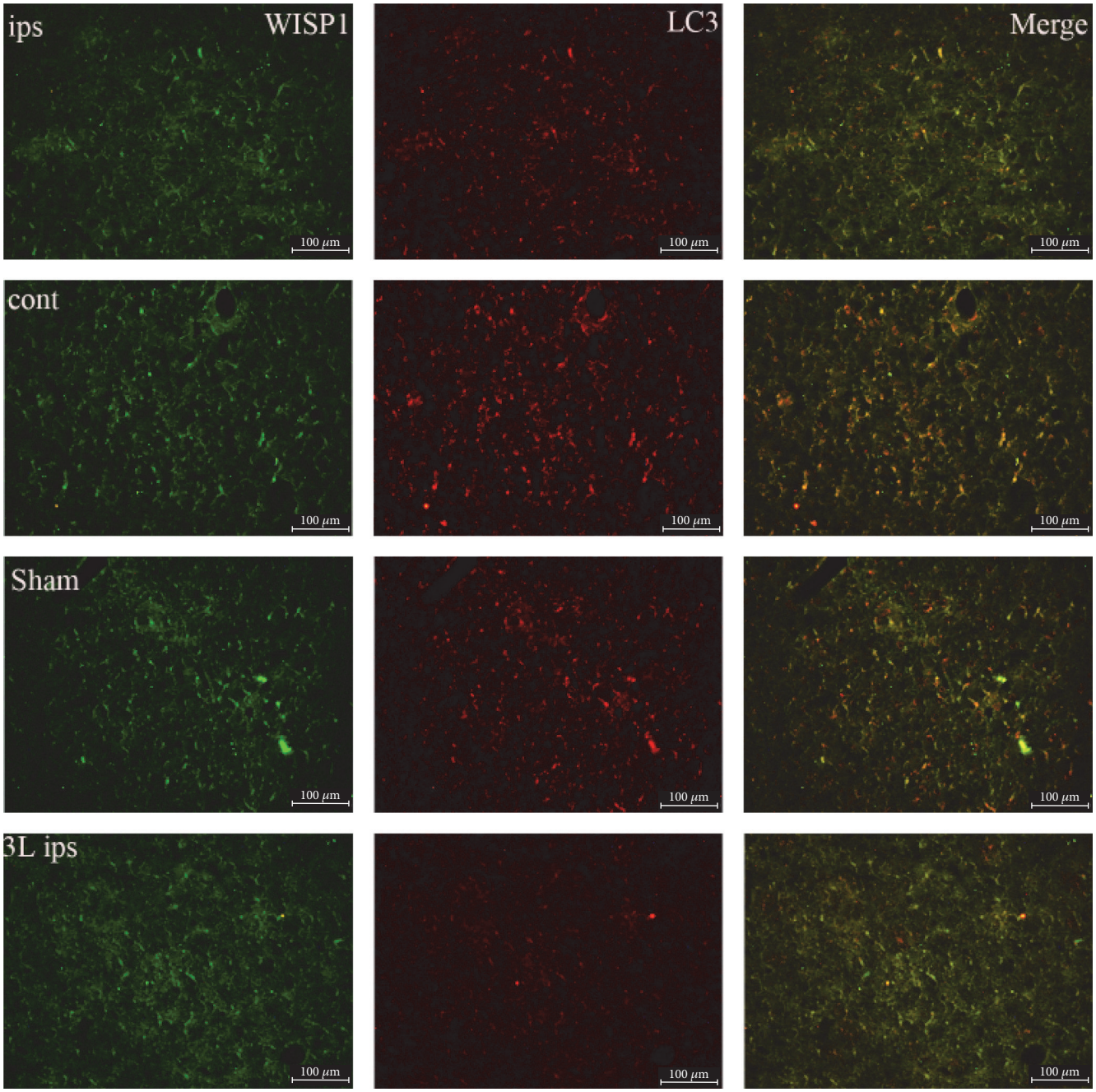

Figure 5: Double-labeling immunofluorescence of WISP1 and LC3 at 3 d after TBI. Green: WISP1; red: LC3; cont: the control of ipsilateral 3 d post-TBI group; ips: ipsilateral 3 d post-TBI group; sham: sham group; 3 L ips: 3 d post-TBI + 1 mM PQQ group; cont: the control of ipsilateral 3 d post-TBI + 1 mM PQQ group. Bar = 100 μm.

EdU assay results showed that PQQ could promote the proliferation of astrocytes inhibited by glutamate ($^{*}P < 0.05$, $^{**}P < 0.01$) (Figure 15).

Transwell migration assay demonstrated that 5 μM, 50 μM, and 100 μM PQQ could reverse the astrocyte migration inhibited by glutamate ($^{*}P < 0.05$, $^{**}P < 0.01$) (Figure 16).

To observe the function of WISP1, WISP1 si-RNA was transfected in the astrocytes. Compared with negative control group, the expression of WISP1 obviously declined after being transfected with WISP1 si-RNA for 48 h, which suggested that WISP1 si-RNA was efficient ($^{*}P < 0.05$, $^{**}P < 0.01$) (Figures 17(a), 17(b), and 17(c)).

The expression of WISP1 was silenced, and the EdU assay was performed. WISP1 could increase the positive signals of astrocytes ($^{**}P < 0.01$) (Figures 17(d) and 17(e)). Similarly, to determine whether WISP1 plays an important role in cell migration, transwell migration assay was used to detect the migration of astrocytes; the results show that migrated cell numbers were significantly increased following interference expression of WISP1 ($^{**}P < 0.01$) (Figures 17(f) and 17(g)).

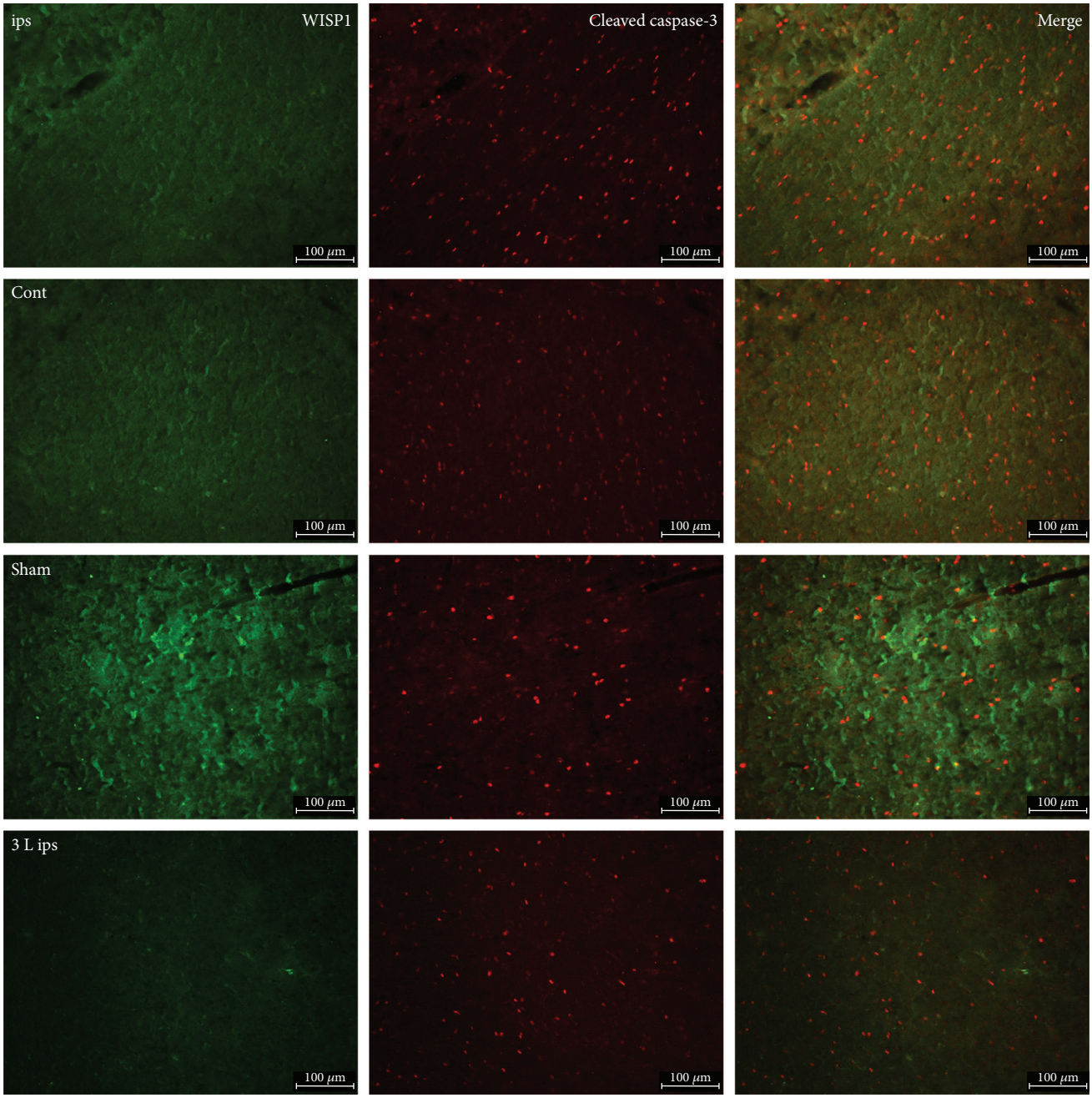

Figure 6: Double-labeling immunofluorescence of WISP1 and cleaved caspase-3 at 3 d after TBI. Green: WISP1; red: cleaved caspase-3; cont: the control of ipsilateral 3 d post-TBI group; ips: ipsilateral 3 d post-TBI group; sham: sham group; 3 L ips: 3 d post-TBI + 1 mM PQQ group; cont: the control of ipsilateral 3 d post-TBI + 1 mM PQQ group. Bar = 100 μm.

4. Discussion

Traumatic brain injury (TBI) is mainly caused by an external mechanical force, which can be divided into primary and secondary damage. TBI can be classified based on the severity or mechanism of injury, as well as other features. In most cases, TBI leads to higher mortality rates and disability; moreover, TBI could cause physical, pathological, and behavioral effects. Therefore, more and more people attracted general attention. Protecting neurons are the main methods to treat cerebral trauma, and the treatment goals are focused in the cell death program targets. However, there is not an effective method to treat TBI so far.

WISP1 is a family of secreted proteins and regulate various developmental processes. Wnt1 proteins combine the cell membrane receptor by autocrine or paracrine effects, activate intracellular signaling pathways, regulate target gene expression, and play an important role on cell proliferation, polarity and differentiation, and migration. WISP1 secreted Wnt1-induced protein 1, also known as CCN4, cognating connective tissue growth factor (CTGF) [2]. WISP1 was later associated with neoplastic growth

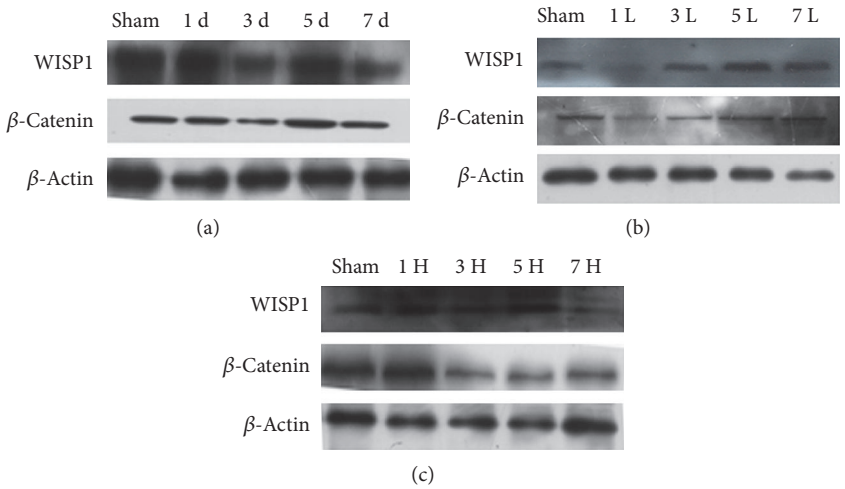

Figure 7: WISP1 and β-catenin protein expression ($P < 0.05$, $P < 0.01$). Sham: sham group; 1 d: ipsilateral 1 d post-TBI group; 3 d: ipsilateral 3 d post-TBI group; 5 d: ipsilateral 5 d post-TBI group; 7 d: ipsilateral 7 d post-TBI group; 1 L: 1 d post-TBI + 1 mM PQQ group; 3 L: 3 d post-TBI + 1 mM PQQ group; 5 L: 5 d post-TBI + 1 mM PQQ group; 7 L: 7 d post-TBI + 1 mM PQQ group; 1 H: 1 d post-TBI + 2 mM PQQ group; 3 H: 3 d post-TBI + 2 mM PQQ group; 5 H: 5d post-TBI + 2 mM PQQ group; 7 H: 7 d post-TBI + 2 mM PQQ group.

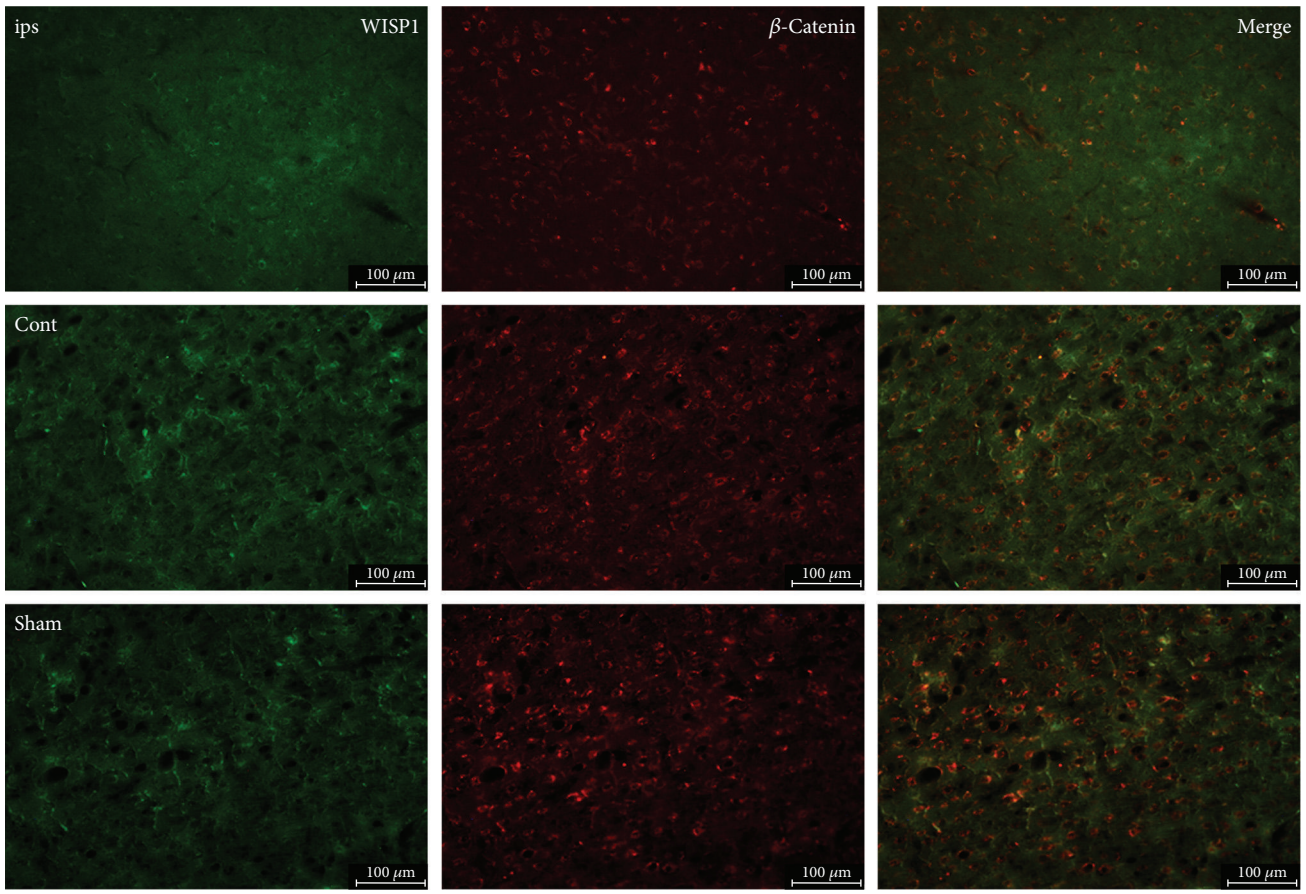

Figure 8: Double-labeling immunofluorescence of WISP1 and β-catenin at 3 d after TBI. Green: WISP1; red: β-catenin; cont: the control of ipsilateral 3 d post-TBI group; ips: ipsilateral 3 d post-TBI group; sham: sham group; bar = 100 μm.

in the gastrointestinal tract [25]. In the present study, WISP1 mRNA expression was detected by qRT-PCR after brain injury. Compared with the sham group, WISP1 mRNA expression reached to the minimum at 3 d and then recovered at 5 d after TBI. Meanwhile, the expression of WISP1 mRNA increased with 1 mM or 2 mM PQQ at

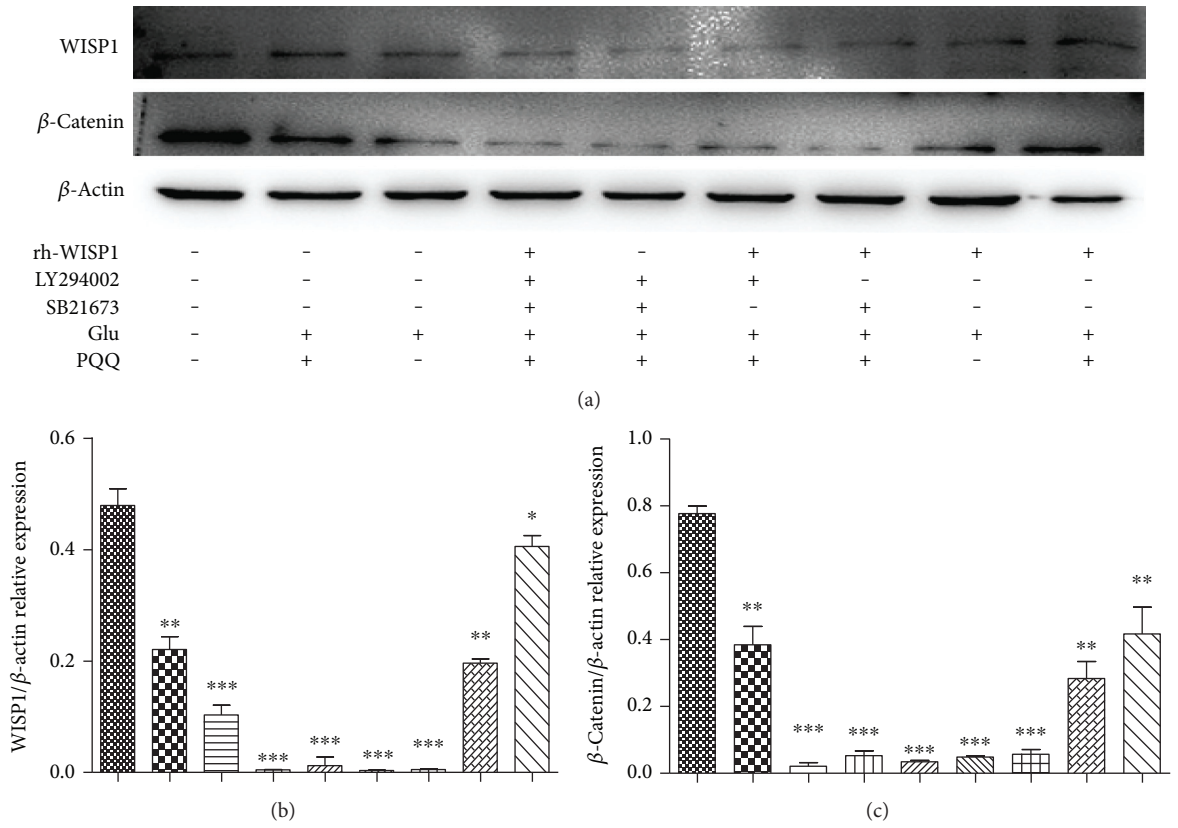

Figure 9: WISP1 and β-catenin protein expression after blocking PI3K/GSK-3β signaling pathway ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus sham).

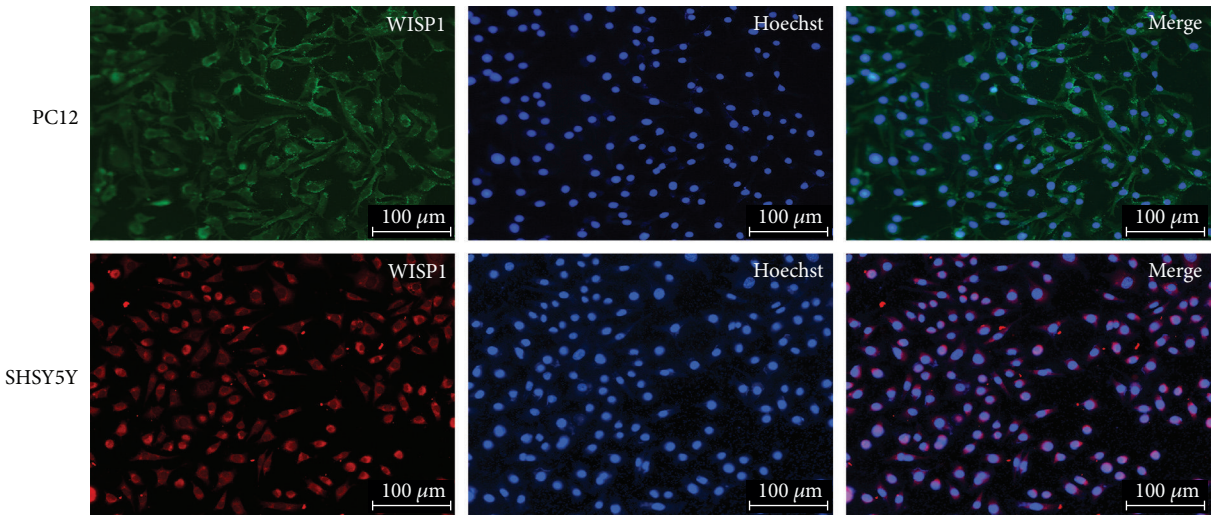

Figure 10: Immunofluorescence of WISP1 in SHSY5Y with PC12 cells. Red or green: WISP1; blue: Hoechst; bar = 100 μm.

different time post-TBI, but the effect of 1 mM PQQ was obvious to the 2 mM PQQ, which might be the concentration of 1 mM PQQ in animals that can reach the optimum serum concentration in TBI.

Astrocyte activation is a common reaction in the central nervous system under many pathophysiological situations, which assume astrocyte cell hypertrophy, swelling, protuberance extension, and glial fibrillary acidic protein expression increase [26, 27]. Astrocytes began to activate after TBI and may even cause several neurodegenerative diseases. In the paper, double-labeling immunofluorescence was performed to detect the cell type that WISP1 located in the brain. Results indicated WISP1 located mainly in the neuron of the brain through the colocalization of

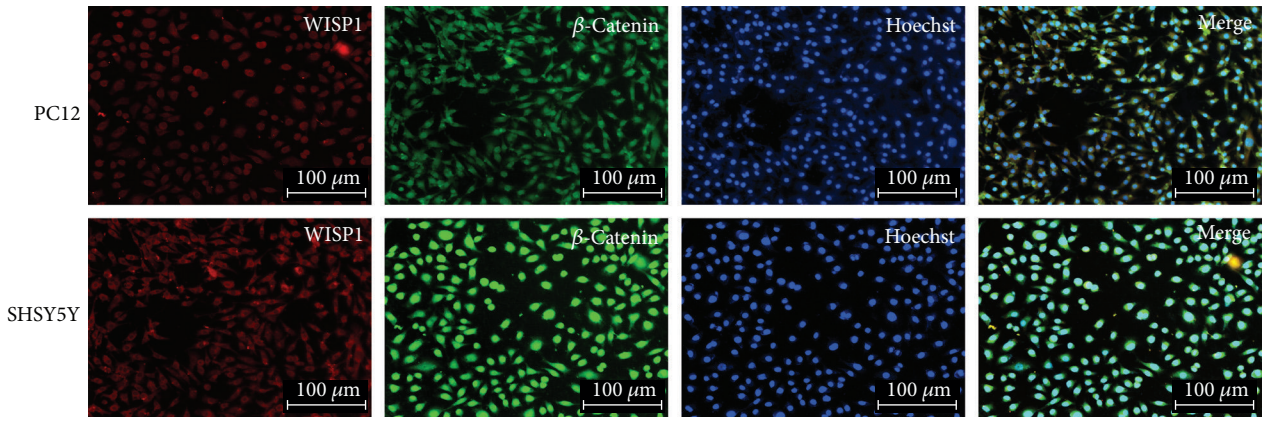

Figure 11: Double-labeling immunofluorescence of WISP1 in SHSY5Y with PC12 cells. Red: WISP1; green: β-catenin; blue: Hoechst; bar = 100 μm.

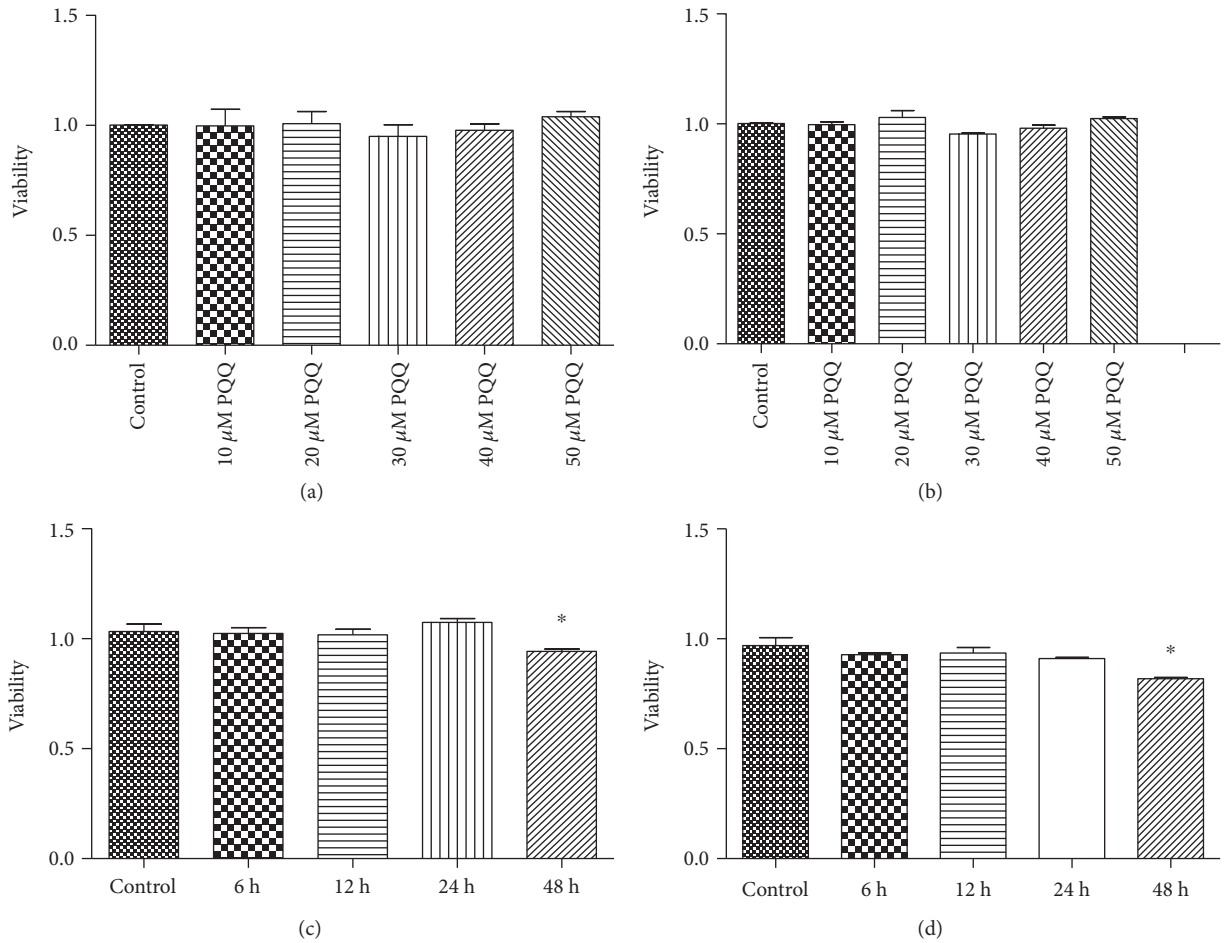

Figure 12: The effect of PQQ on the viability of PC12 and SHSY5Y cells. (a) Control: PC12 cells without PQQ; (b) control: SHSY5Y cells without PQQ; (c) control: PC12 cells without PQQ, $^*P < 0.05$ versus control; (d) control: SHSY5Y cells without PQQ, $^*P < 0.05$ versus control.

WISP1 with astrocyte marker GFAP, neuronal marker NeuN, and microglia marker Iba1, respectively.

After traumatic brain injury, there would be different levels of autophagy. Autophagy has three different categories termed microautophagy, macroautophagy, and chaperone-mediated autophagy [28]. Autophagy is a eukaryotic cell metabolic process, autophagy with disease-linked, is gradually increasing in the recent years. Autophagy is involved in

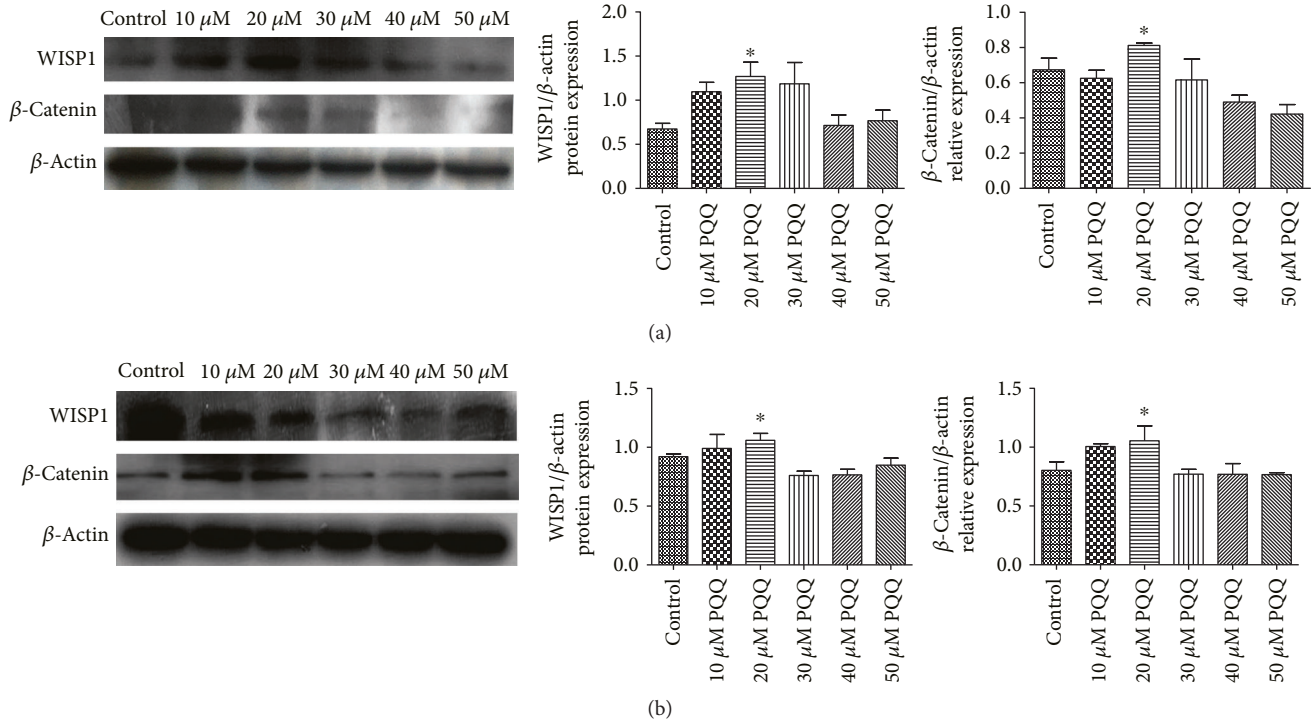

Figure 13: WISP1 and β-catenin protein expressions in PC12 cells and SHSY5Y cells with the treatment of different concentrations of PQQ. (a) Control: PC12 cells without PQQ; 10, 20, 30, 40, and 50 μM: different concentrations of PQQ for 24 h; (b) control: SHSY5Y cells without PQQ ($^*P < 0.05$).

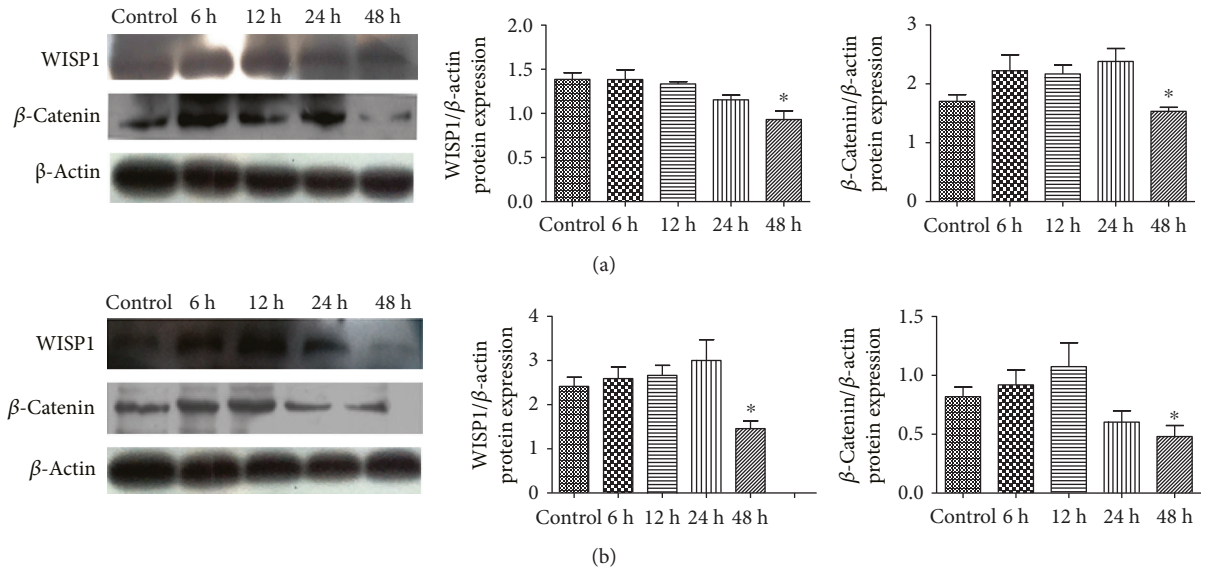

Figure 14: WISP1, β-catenin protein expression in PC12 cells, and SHSY5Y cells with the treatment of 50 μM PQQ at different time. (a) Control: PC12 cells without PQQ; 6 h, 12 h, 24 h, and 48 h: different time points; (b) control: SHSY5Y cells without PQQ ($^*P < 0.05$).

pathogenesis of neuronal injury and death including mitochondrial damage, activating inflammation, oxidative free radicals, and caspase activation [29]. Apart from cell death which was caused by TBI, autophagic cell death and apoptosis occupy a considerable proportion, autophagy protects damaged cells and also exacerbates cell damage, which mainly depend on the role of autophagy after injury and stage. Autophagy is involved in regulation of cell survival after death in traumatic brain injury, and as a result, autophagy is important to brain injury and nerve injury and repair [30]. Double immunofluorescence of WISP1 and autophagy marker LC3 showed that WISP1 and LC3 had a lot of colocalization at 3 d post-TBI. After treatment with 1 mM PQQ, WISP1-positive signals rose obviously; LC3-positive signals declined markedly in the ipsilateral brain. All these data demonstrated that PQQ may have a protective role on autophagy.

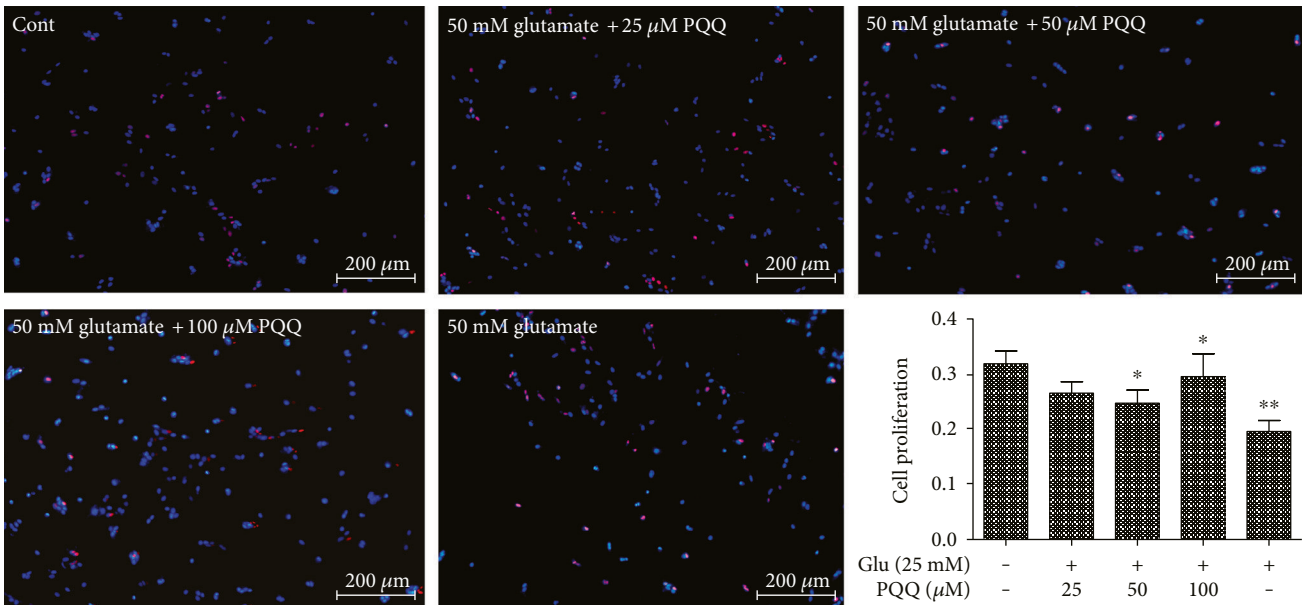

Figure 15: The proliferation of astrocytes with glutamate and PQQ ($^{*}P < 0.05$, $^{**}P < 0.01$).

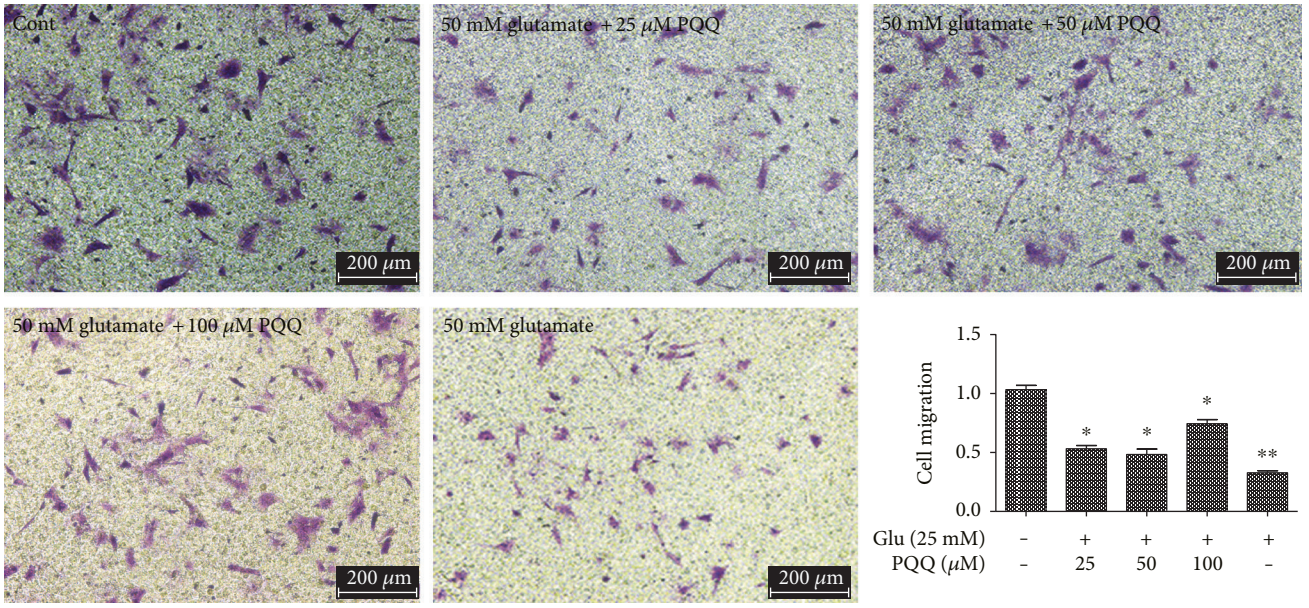

Figure 16: The migration of astrocytes after glutamate and PQQ ($^{*}P < 0.05$, $^{**}P < 0.01$).

In addition, apoptosis is believed to be a significant contributor to the pathogenesis of a variety of disorders after TBI. For example, in the brains of patients with Alzheimer's disease, apoptotic DNA fragmentation [31] and caspase activation have been observed [32]. Caspase-3 activation is relevant to the apoptosis of neurons after various types of damage. In this study, double-labeling immunofluorescence showed that WISP1 colocalizated with cleaved caspase partly. Cleaved caspase-3 expression was obviously upregulated in the ipsilateral brain after 3 d post-TBI. After treatment with 1 mM PQQ, cleaved caspase-3-positive signals declined obviously, which reminded that WISP1 might not be involved directly in the process of apoptosis.

WISP1 and β-catenin act as new targets investigated in recent years, which can promote tissues proliferation as well as various cell repair. In regard to the canonical pathways of Wnt1 [33], WISP1 can block phosphorylation of β-catenin in neurons that may be mediated through the inhibition of GSK-3β which prevent β-catenin phosphorylation [33]. WISP1 also can block GSK-3β activity in other cell systems such as cardiac cells [12, 34]. In neurons, WISP1 through a PI3K-mediated pathway promotes the nucleus expression of

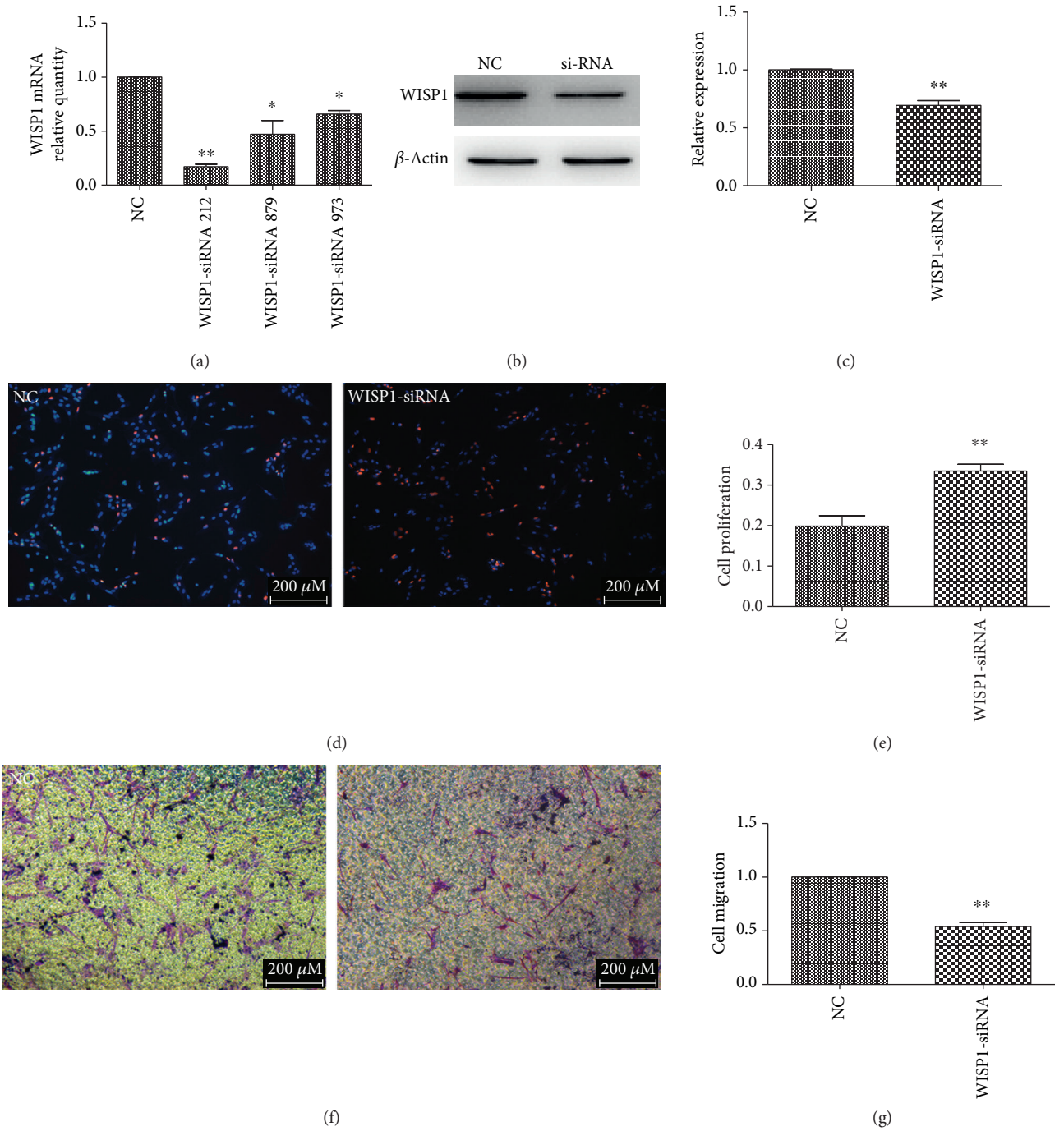

Figure 17: The proliferation and migration of astrocytes after transfected by WISP1 si-RNA. NC: negative control group ($^{*}P < 0.05$). (a) qRT-PCR showed that the expression of WISP1 in astrocytes transfected with WISP1 si-RNA. (b) Western blot showed that WISP1 si-RNA could decreased the expression of WISP1 protein. (c) The statistic graph of (b). (d) Proliferation of astrocytes transfected with WISP1 si-RNA compared with the normal group. (e) The statistic graph of (d) ($^{**}P < 0.01$). (f) The migration of astrocytes transfected with WISP1 si-RNA compared with the normal group. (g) The statistic graph of (f) ($^{**}P < 0.01$).

β-catenin [4]. In addition, WISP1 expression is governed by β-catenin activity and WISP1 regulates its own expression through the ability of WISP1 to control β-catenin phosphorylation and nuclear translocation [4]. Western blot showed that the protein expression of WISP1 and β-catenin minimized at 3 d and rose again at 5 d after TBI compared with the sham group. Meanwhile, the treatment with 1 mM or 2 mM PQQ increased the protein expression of WISP1 and β-catenin correspondingly. All these results demonstrated that WISP1 may modulate the protein expression of β-catenin.

PQQ, also known as methoxatin, has been detected in a wide variety of foods and other sources. PQQ, as an important antioxidant and nutrient, has also begun to focus on nutriology and pharmacology. PQQ belongs to vitamin B

[21]. PQQ has many other pharmacological effects, including anti-inflammatory, hepatoprotective, heart protection, and antioxidizing effect [22, 35, 36]. PQQ has a neuroprotective function and a very good effect on the epilepsy model by PI3K/AKT signaling pathway [37]. Although the role of PQQ as a vitamin in animal or human nutrition is controversial, accumulating evidence suggest that PQQ plays important roles on cell protection. In the experiment, we used different concentrations of PQQ to treat SHSY5Y or PC12 cells; results showed that PQQ had no toxicity on the cell lines. Similarly, PQQ had no significant effect on the animal without TBI. In subsequent experiments, in addition, PQQ may protect cells through influencing the expression of WISP1.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Review Article

Traumatic Brain Injury and Stem Cell: Pathophysiology and Update on Recent Treatment Modalities

Cesar Reis,[1] Vadim Gospodarev,[1] Haley Reis,[2] Michael Wilkinson,[2] Josileide Gaio,[3] Camila Araujo,[1] Sheng Chen,[4] and John H. Zhang[1,5]

[1]*Department of Physiology and Pharmacology, Loma Linda University School of Medicine, 11041 Campus Street, Risley Hall, Room 219, Loma Linda, CA 92354, USA*
[2]*Loma Linda University School of Medicine, Loma Linda, CA 92354, USA*
[3]*Department of Epidemiology & Biostatistics, Loma Linda School of Public Health, Loma Linda, CA 92350, USA*
[4]*Department of Neurosurgery, Second Affiliated Hospital School of Medicine, Zhejiang University, Hangzhou, China*
[5]*Department of Neurosurgery, Loma Linda University School of Medicine, Loma Linda, CA 92354, USA*

Correspondence should be addressed to John H. Zhang; johnzhang3910@yahoo.com

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Traumatic brain injury (TBI) is a complex condition that presents with a wide spectrum of clinical symptoms caused by an initial insult to the brain through an external mechanical force to the skull. In the United States alone, TBI accounts for more than 50,000 deaths per year and is one of the leading causes of mortality among young adults in the developed world. Pathophysiology of TBI is complex and consists of acute and delayed injury. In the acute phase, brain tissue destroyed upon impact includes neurons, glia, and endothelial cells, the latter of which makes up the blood-brain barrier. In the delayed phase, "toxins" released from damaged cells set off cascades in neighboring cells eventually leading to exacerbation of primary injury. As researches further explore pathophysiology and molecular mechanisms underlying this debilitating condition, numerous potential therapeutic strategies, especially those involving stem cells, are emerging to improve recovery and possibly reverse damage. In addition to elucidating the most recent advances in the understanding of TBI pathophysiology, this review explores two primary pathways currently under investigation and are thought to yield the most viable therapeutic approach for treatment of TBI: manipulation of endogenous neural cell response and administration of exogenous stem cell therapy.

1. Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability, affecting approximately 1.7 million Americans annually [1]. According to the World Health Organization, TBI will continue to be a major health problem and primary reason for disability leading into 2020 [2]. The proportion of TBI-related hospitalizations due to motor vehicle accidents increases through age 44 before decreasing beginning at ages 45–64, when falls become the leading cause of TBI-related hospitalization [3]. Traumatic brain injury pathophysiology includes blood-brain barrier breakdown, widespread neuroinflammation, diffuse axonal injury, and subsequent neurodegeneration [4]. Several treatment options to date include hyperbaric oxygen therapy, noninvasive brain stimulation, task-oriented functional electrical stimulation, and behavioral therapies [5]. There is an emerging treatment option for brain injury, which entails the use of stem cells for neuroregeneration and repair. Exogenous stem cell transplantation has been shown to increase endogenous cellular proliferation and promote immature neural differentiation in the injured region of the brain [6]. Understanding regenerative capacities of endogenous neural stem cells, as well as the impact of exogenous neural stem cells on proliferation and differentiation, will further elucidate how to improve functional recovery and brain repair after TBI.

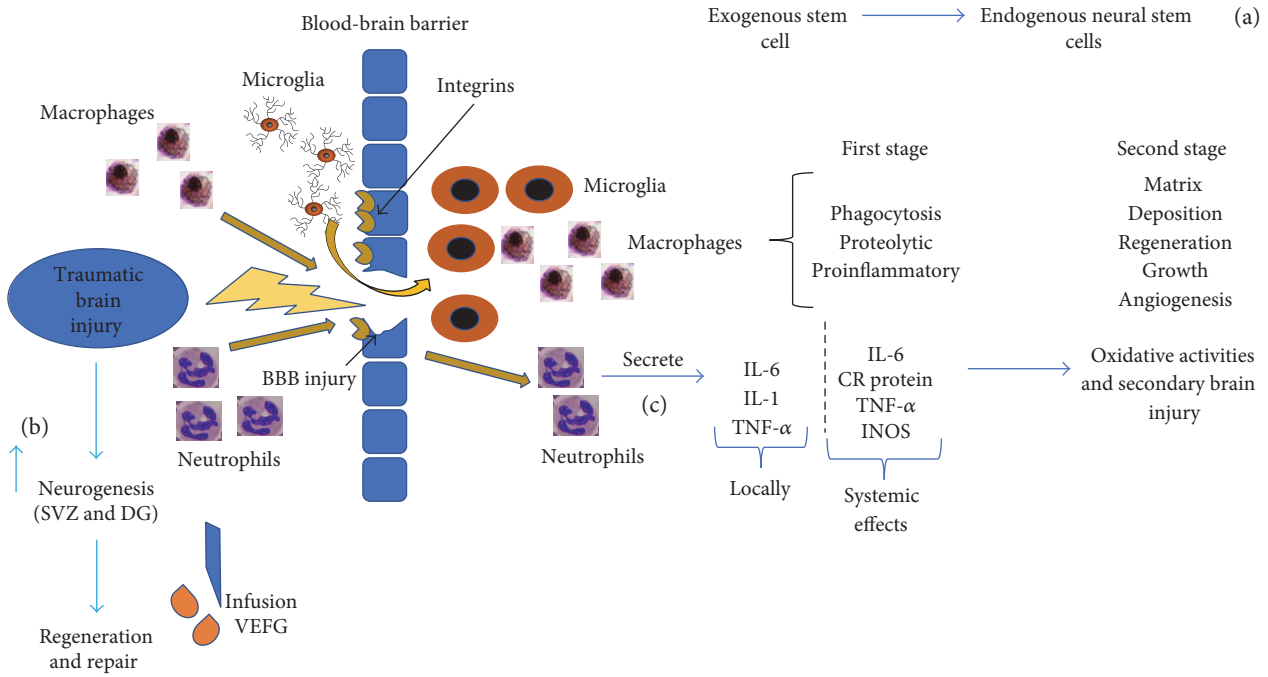

Figure 1: After TBI, exogenous stem stimulate proliferation of endogenous neural stem cells (a). After TBI, there is induction of neurogenesis (b) post-TBI infusion of VEGF. After TBI and rupture of the blood-brain barrier, macrophages will stimulate an initial phase of phagocytic, proteolytic, and proinflammatory functions, while the second phase is characterized by anti-inflammatory functions, which includes regeneration, growth, angiogenesis, and matrix deposition. Microglia initiate inflammatory events. This figure also demonstrates neutrophil invasion and their impact on pathological processes of brain trauma, which includes alteration of vascular permeability and contribution to oxidative damage via secretion of lysosomal enzymes, and changes in cerebral blood flow. In addition, neutrophils act by releasing inflammatory cytokines such as IL-6, IL-1, and tumor necrosis factor alpha (TNF-α) (c). Microglia, monocytes, macrophages, and neutrophils invade areas exhibiting blood-brain barrier damage, and there is extensive upregulation of neutrophil adhesion factors including integrin receptors. Gerry Shaw, microglia and neurons, 25 July 2005, by Creative Commons; hematologist, segmented neutrophils, 31 August 2009, Creative Commons; microphages by Patho via Wikimedia Commons; microglia by Frontier in Cellular Neuroscience, 30 January 2013, Creative Commons.

2. Pathophysiology

In direct contrast to other tissues in the mammalian body, the brain is unable to properly regenerate and reconnect the injured areas to the uninjured areas of the brain [7]. The detailed pathophysiology of traumatic brain injury remains to be fully elucidated, but there are several components that have been studied and widely accepted as normal sequela following brain injury.

After brain injury, there are blood-brain barrier breakdown and neurodegeneration in areas including the injured cortex, hippocampus, and a portion of the diencephalon. Microglia, monocytes, macrophages, and neutrophils invade areas exhibiting blood-brain barrier damage (Figure 1) [8], which is also associated with extensive upregulation of neutrophil adhesion factors [9], including integrin receptors (Figure 1) and immunoglobulin superfamily members [10–12]. This process is carried out through innate signaling pathways and, in the case of TBI, through release of damage-associated molecular pattern molecules (DAMPs), better known as danger signals [13]. This response is an effort to restore normal homeostasis, but, if the extent of injury is too great, maladaptive immune responses can ensue. This inflammatory response can persist for years and eventually contributes to neurodegeneration. However, the use of anti-inflammatory medications shortly after TBI has not been shown to be an effective treatment, which suggests that inflammation may play a beneficial role, particularly in the acute phase of TBI [14].

3. Microglia

Microglia are the first responders in TBI and initiate inflammatory events. These cells have been shown to remain in the injured area more than one year following brain injury. It is unclear if microglia are responding to the degenerative process or are active players in the prolonged white matter degeneration [15]. Studies have revealed that in the acute phase response to cell death, microglia play a neuroprotective role by transforming into highly mobile phagocytic cells as they insert themselves into the damaged glial limitans, connecting to form a phagocytic barrier. Prevention of this response by blocking purinergic receptor signaling or connexin hemichannels results in exacerbation of pathological processes, including increased leaking of material into brain parenchyma [8]. Microglia are capable of different polarization states known as M1 and M2. The M1 macrophage response is rapidly induced and maintained at the site of injury and typically overwhelms the smaller and shorter-lived anti-inflammatory response of M2 cells. TNF-α and

IFNγ promote differentiation into M1, which is capable of producing oxidative metabolites and proinflammatory cytokines. This response is essential for host defense but can lead to secondary damage in healthy cells and tissue [16–18]. M2 cells are activated in the presence of IL-4 [19] or IL-1β [20], promote angiogenesis and matrix remodeling, and regulate the immune system. A recent study found that cycle AMP functions synergistically with IL-4 to drive the M1 to M2 conversion after experimental spinal cord injury. The presence of M2-converted microglia ameliorated production of proinflammatory cytokines, including TNF-α [21]. Kigerl and colleagues found further evidence supporting M1 macrophages to be neurotoxic and M2 cells to be neuroprotective. Finding a way to shift infiltrating blood monocytes towards the M2 phenotype after TBI may promote repair and regeneration and decrease secondary injury caused by proinflammatory events [22].

The use of stem cells in inducing earlier and longer-lasting effects of M2 was studied using mesenchymal stem cells (MSCs) after TBI. They found upregulation of M2 expression markers in mice 3 and 7 days after TBI, as well as reduction of lysosomal activity in microglia 7 days after TBI. Intracerebroventricular infusion of MSCs promoted proregenerative activity and reduced phagocytosis, effectively reversing the M1 proinflammatory phenotype typically acquired by microglia after brain injury [23]. A study investigating multipotent adult progenitor cells (MAPCs) to modulate the microglia phenotype found a systemic reaction in response to the stem cells, particularly with T regulatory cells in the spleen and blood. In order to increase the M2/M1 ratio and increase M1 macrophage apoptosis using MAPCs, direct contact between the stem cells and splenocytes was required [24]. In the case of MSCs, they act locally in the brain at the lesion site and control the polarization of microglia through the release of active molecules instead of cell-cell contact [23, 25]. A study using MSCs in a mice model found they were able to alter the ratio of IL-10 and TNF-α in favor of IL-10, further supporting MSCs' ability to shift microglia to an anti-inflammatory phenotype. In addition, they found enhanced proliferation of T lymphocytes in microglia-MSC cocultures, indicating increased antigen-presenting ability of microglia in the presence of MSCs [26]. Investigating stem cells that can induce early and persistent M2 phenotypes to foster growth and tissue repair is an important topic in stem cell translational research.

4. Neutrophils, Monocytes, and Macrophages

Neutrophil invasion has a significant impact on pathological processes of brain trauma, which includes alteration of vascular permeability [27], contribution to oxidative damage via secretion of lysosomal enzymes, and changes in cerebral blood flow. Neutrophils act by releasing inflammatory cytokines such as IL-6, IL-1, and tumor necrosis factor alpha (TNF-α) (Figure 1(c)) [28]. Shortly following TBI in human subjects, researchers found a systemic inflammatory response as evidenced by an increase in circulating leukocyte counts, elevating expression of TNF-α, IL-6, C-reactive protein, and iNOS. This increase in oxidative activity not only can lead to systemic damage but can further exacerbate secondary local damage at the initial site of TBI [29]. However, since neutrophils can recruit monocyte-derived macrophages, it was interesting to find that when this ability was blocked, mice were found to inadequately repair and recover motor skills, according to a study by Shechter and colleagues [30].

Macrophages exhibit an initial phase of phagocytic, proteolytic, and proinflammatory functions, while the second phase is characterized by anti-inflammatory functions, which include regeneration, growth, angiogenesis, and matrix deposition (Figure 1) [31, 32]. In direct contrast, another study by Hsieh in 2014 demonstrated improved hippocampal neuronal survival and functional recovery by reducing the number of macrophages following cortical injury 2–4 weeks after injury. This study revealed an association between the C-C chemokine receptor 2, which guides monocytes to inflamed tissues, and pathological processes in chronic stages of TBI [33].

5. Axonal Degeneration

In addition to ongoing inflammation following TBI, a study of the porcine brain injury model by Chen and colleagues revealed that axonal degeneration continues up to 6 months following initial brain trauma, which causes continued impaired axonal transport and, in effect, accumulation of amyloid precursor proteins (APP) and amyloid-B (Aβ) peptides. These results are very important since Aβ is a hallmark in pathology of Alzheimer's disease, and many studies have linked brain trauma with an increased risk of developing Alzheimer's disease [34, 35]. Thus, accumulation of proteins, particularly in the discrete swellings at the terminal ends of disconnected axons, may be linked to lysis or leakage of swollen axons, causing protein release into surrounding tissue and cerebrospinal fluid. Another possible cause of APP proteolysis is caspase-3 activation through a cleavage process that interrupts normal intracellular processing of APP [36]. Nikolaev and colleagues found that activation of the APP/death receptor 6/caspase 6 apoptotic pathway leads to axonal destruction following the loss of proteins important for neuronal survival, including brain-derived neurotrophic factor, neurotrophin 3, and nerve growth factor (NGF) activation [37]. A study used this pathway to examine whether it represents a common mechanism for axonal degeneration in response to multiple insults. They found that inhibition of APP cleavage prevented axonal degeneration triggered by NGF withdrawal. However, blocking this pathway did not protect against degeneration caused by mechanical or chemical insults [38]. Though the precise mechanism behind APP proteolysis and subsequent accumulation of Aβ peptides following TBI is unclear, long-term formation of Aβ peptides may play a role in the link between a history of brain trauma and increased risk of developing Alzheimer's dementia.

In summary, the role of specific cellular responses that are associated with inflammation and neurodegeneration in pathophysiology of TBI is quite complex [39]. The natural inflammatory response is crucial to foster healing and regeneration following TBI, but the intricate balance between

inflammations, which are thought to promote regeneration and maladaptive chronic neuroinflammation, requires further exploration. Currently, it is with these challenges in mind that novel treatment options have been studied to improve outcomes following TBI, particularly those involving the use of stem cell therapy. It is thought that endogenous adult neural stem cells, as well as progenitor cells residing in the neurogenic regions of the brain, may provide regenerative and reparative function to CNS injuries, such as TBI [40]. More specifically, there is an increased neurogenic response following TBI, especially in the subventricular zone (SVZ) and dentate gyrus (DG) of the hippocampus; eliciting this endogenous response could provide improved regeneration and repair following TBI (Figure 1) [41]. Exogenous stem cell therapy is also an area of interest for treatment of TBI, as it is thought to not only provide repair mechanisms but also stimulate proliferation of endogenous neural stem cells [42] (Figure 1).

6. Endogenous Neural Stem Cells

Traumatic brain injury places significant stress on the human brain, making it very difficult to maintain appropriate cognitive abilities. Although other organs within the body, such as the skin, possess the capability to self-renew after injury, the brain cannot simply regenerate. Much of the focus within the last 10 years has been spent on discovering the impact of neural stem cells on the regenerative efforts of the brain. Since the 1960s, it has been suggested that new adult brain cells are capable of regenerating; however, it was not until the late 1990s that confocal microscopy revealed that newborn brain cells can differentiate into neurons upon maturation [43, 44].

Neural stem cells have been localized to two regions of the adult brain, namely, the SVZ of the lateral ventricles, which generate neuroblasts that travel via a rostral migratory stream (RMS) to the olfactory bulb and the subgranular zone (SGZ) of the hippocampal DG, which integrate within the DG and become fully mature within a few weeks in a process called adult hippocampal neurogenesis [45]. It remains unclear whether these NSC regions can replace the lost neurons after damage or injury. Most recently, it has been suggested that the neurogenic system within the SVZ of adults is inactive [46]. Although this system has been shown to be present in other animal models such as rodent studies, it remains unclear how much migration or neuroblast development occurs in the olfactory bulb [47]. However, repeated evidence suggests that NSCs are found in the SVZ of adults and extensive migration to the olfactory bulb occurs in infants up to 18 months [48].

There has been some evidence to support self-renewal of progenitor cells in the DG occurring throughout life suggesting that some newly regenerated cells are morphologically and phenotypically similar to hippocampal neurons [49]. Just how much hippocampal self-regeneration occurs and to what functional significance it possesses have led to further investigation. It has been shown that up to one-third of hippocampal cells turn over during adulthood while also exhibiting a 4-fold decline in the amount of neuroblasts resulting in a net loss of hippocampal neurons including the DG despite some degree of neurogenesis. Functionality of hippocampal neurogenesis has not been adequately assessed; however, comparison of neurogenesis in humans with rodent studies may suggest that similar functionality is dependent on similar regeneration rates. Furthermore, because the DG acts as a control mechanism in neuronal circuitry, small amounts of neurogenesis here could have substantial influence on pattern separation in the process of new memory formation [50].

7. Response of NSC to TBI

Increasing evidence suggests that TBI induces neurogenesis in animal models via ipsilateral NSC maturation and integration into functionally active brain cells of peridamaged regions of the hippocampus [51]. Interestingly, both human and animal cerebral cortex and regions of white matter exhibit TBI-induced neurogenesis either from proliferation of cells from neurogenic regions such as the SVC or from locally born cells [52, 53]. It is well accepted that neurogenesis is induced following events of TBI (Figure 1), but more recently efforts to quantify recovery from TBI have shown variable results. One animal study revealed that cognitive recovery does occur in rats [54]. In humans, several adult brain maladies have been shown to induce neurogenesis, such as that associated with Huntington's disease [55], ischemic stroke [56], Alzheimer's disease [57], epilepsy [58], and hemorrhage [59]. Neurogenesis following TBI has been studied in humans to see whether newborn cells have the capacity to replace those that are damaged, thereby restoring brain function. Recent studies revealed cognitive deficit recovery in human models following TBI with increased recovery of cognitive function seen in children compared to that in adults [54, 60].

8. Manipulation of NSC in Response to TBI

The regenerative capacity of NSCs found in the adult brain is important for responding to TBI-related injuries. Recently, manipulation of various growth factors has shown significant efficacy in promotion of neurogenesis. In adult animal studies, it has been shown that intraventricular infusion of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) enhances cell proliferation in the hippocampus and SVZ leading to improved cognitive function [61, 62]. In addition, post-TBI infusion of recombinant VEGF improved neurogenesis in the SVZ, thereby promoting recovery and aid in survival of neurons produced in the DG (Figure 1) [63, 64]. Besides growth factors, pharmaceutical agents like statins [65], erythropoietin [66], and even antidepressants, such as imipramine [67], have shown to enhance endogenous neurogenesis and lead to improved cognitive recovery.

Compared to animal brain models, simply promoting neurogenesis is significantly more complex in humans. Concerns over possible treatment-related injuries from stimulation of endogenous neurogenesis following TBI are still under investigation and are associated with worrisome results. It has been suggested that post-TBI–induced neurogenesis has contributed to the onset of post-TBI epilepsy

[68]. Specifically, increased neurogenesis within the hippocampus has been known to result in development of epilepsy and other seizure-like symptoms. However, ablation of aberrant seizure-induced hippocampal neurogenesis can reduce incidence of seizures and essentially cure epilepsy. The technical challenges surrounding human models in manipulating NSC in response to TBI remain a burgeoning area of focus [69].

9. Exogenous Stem Cells

9.1. Embryonic Stem Cells. The use of embryonic stem cells (ESCs) in the treatment of traumatic brain injury remains a growing field of research. Three notable clinical trials have used embryonic stem cells in TBI rodent models with promising results. Across the board, using ESCs is associated with better outcomes, such as recovery of motor function [70], improved cognitive function, and high survival of transplanted cells [71].

Ikeda and colleagues were one of the first investigators to use nonhuman primate stem cells, which they used to study embryonic stem cells' capacity to restore function to damaged neural tissue. In this experiment, Cynomolgus monkey embryonic stem cells were first treated with retinoic acid before being transplanted into the periventricular area of mice brains. Some of the treated cells later developed into Islet1+ motoneurons. These cells were then transplanted into mice that had undergone an experimental stroke model of brain injury. Approximately one month after the transplant, mice in the experimental arm had greater recovery of motor function than that in the control mice [70].

The team of Peruzzaro and colleagues aimed to determine if an enriched postsurgical environment will have a beneficial effect on three key factors of embryonic stem cell transplants in terms of their integration, migration, and survival. In a rodent TBI model, the medial frontal cortex was injured via cortical impact. One group of rats was placed into an enriched environment, while the other was placed in a standard environment. In each group, two arms of the study emerged; the rats were treated with either murine cortical embryonic stem cells or did not receive such treatment. The enriched environment animals performed statistically as well as the sham group, compared to groups with just embryonic stem cells or only an enriched environment [72]. This trial highlighted the importance of an enriched postsurgical environment in the healing process. Although the enriched environment groups were not outperforming the other groups by a statistically significant measure, these results are promising because they represent building blocks for future studies. Further research could incorporate the use of enriched environments in stem cell and TBI studies to investigate this phenomenon.

A preclinical trial performed by Haus and colleagues aimed to study the long-term benefits of treating TBI with neural stem cells. Rats underwent an impact immunodeficient model of TBI and were found to demonstrate hippocampal-dependent spatial memory impairment for at least two months. The experimental group, treated with transplanted human neural stem cells, had better long-term consequences, including an increased survival in the host hippocampal cells, improved cognitive function, and no change in scar tissue. The investigators concluded that although no change was observed in the volume of scar tissue, beneficial effects were still seen, suggesting that measure of recovery should focus more on percentage of surviving cells rather than a decrease in lesion volume. This study also observed a small number of improved behavioral components correlating with a 9–25% survival rate of the transplanted cells [71]. This correlation of cell survival with improvement in behavioral measures is an encouraging revelation, which provides a foundation and direction for future studies.

9.2. Multipotent Adult Progenitor Cells. MAPCs are a population of bone marrow-derived adherent progenitor cells [73]. First isolated in 2002, MAPCs are able to differentiate in vitro into MSCs but also cells with visceral mesoderm, neuroectoderm, and endoderm characteristics and proliferate extensively with little loss of differentiation potential or senescence [74]. MAPCs can be considered a distinct in vitro cell population from MSCs, as they express different surface proteins, have lower MHC Class I expression, and display more robust endothelial differentiation [75]. Bedi and colleagues investigated the long-term effects of MAPC treatment after TBI on microglia phenotype as well as cognitive and motor function. They found a localized reduction in activated microglia 120 days after injury in the DG of the hippocampus, contributing to reduction in the prolonged neuroinflammatory response and preservation of normal neuronal responses. MAPC therapy improved spatial learning, information retention, and memory retrieval as well as motor deficits, demonstrating the utility of intravenous administration of MAPCs to improve long-term cognitive function following TBI [76].

9.3. Adult Neural Stem Cells. Neural stem cells (NSCs) are multipotent cells that can differentiate into neural cells; however, their differentiation into other tissue types is limited [77]. NSCs are found in the SVZ of the lateral ventricle and the SGZ of the hippocampal dentate gyrus, as well as other parts on the brain, such as cerebral cortex, amygdala, hypothalamus, and substantia nigra. These cells can be isolated, grown in culture, and generate multiple neural lineages, which can be used in neurological disorders as an essential component of cell-replacement therapy [78].

In a TBI rat model, adult NSCs were transplanted into injured areas of the brain. They survived the transplantation process and migrated to injured sites while expressing markers for mature astrocytes and oligodendrocytes [79]. In other rat model studies, two weeks after the NSCs were transplanted in the cortex, it was revealed that approximately 1-2% of the cells became engrafted and were able to improve motor function [80]. Park and colleagues reported that injured rats experienced an improved cognitive function after NCS were transplanted into the hippocampal region [81]. Moreover, Lee et al. revealed that hemorrhagic stroke model rats, when injected with NCSs intravenously or intracerebrally, had a decrease in the initial neurologic deterioration

and less edema due to the anti-inflammatory and antiapoptotic properties of NSCs [82].

The optimal time window for transplantation is 7–14 days [77], and beyond that, the glial scar forms, inhibiting perfusion and graft survival [83]. A significant challenge with NSC transplantation is the ability to deliver cells to the area of interest. Routes of administration include intrathecal, intravenous, and intra-arterial infusion. Unfortunately, the engraftment rates are low and there is a constant risk for embolus formation during intravascular infusions. However, a nanofiber scaffold implantation was suggested by Walker et al. as a novel method to be used to provide the support necessary for cell proliferation, which gives direction to future studies [84].

9.4. Inducible Pluripotent Stem Cells. To observe functional aspects of transplanted induced pluripotent stem cells (iPSCs) compared to those of ESCs, Wang and colleagues used a rodent model of ischemia and three different treatment options, which were comprised of pluripotent stem cells, embryonic stem cells, and phosphate-buffered saline for the control. The animals received injections into the left lateral ventricle stereotactically. At the two-week period, it was found that the embryonic stem cell treatment group animals exhibited a marked recovery in their glucose metabolism, which was then followed by a decrease. Imaging tests were performed approximately one month after treatment. Both stem cell treatment groups had better neurologic scores than the control group, signifying that the experimental groups experienced greater recovery of their cognitive function. Further analysis revealed that the transplanted cells survived and migrated to the region of ischemia. However, in the conclusion of their study, the investigators endorsed that iPSCs may be a preferable option to ESCs [85].

In a similar study conducted by Perruzzano, the team of Dunkerson and colleagues explored the impact of an enriched environment in stem cell transplants. Since treatment with iPSC therapy had previously produced promising results, it was time to evaluate the efficacy of the two therapies combined. An animal TBI model of impact to the medial frontal cortex was used. As in previously mentioned experiments, two groups were formed: one in an enhanced environment and the other in a standard environment. Approximately one week following injury, the rats underwent either an iPSC transplant or media control. Notably, the rats that were exposed to both an enriched environment and iPSCs performed as well as the sham and enriched environment group. Across the board, the combined therapy group demonstrated the best cognitive recovery in comparison to that of other groups. Investigators concluded that combined therapy must be strongly considered in future studies that attempt to explore treatment options for TBI [86].

Although iPSC therapy is promising, like many treatments, it is not free of potential side effects. Early animal model studies of iPSCs showed a tendency for embryonic-derived cells to form benign tumors from the embryonic germ layers. Some of the cells transplanted into animal models have led the animals to alternatively develop conditions such as neurodegenerative disease. However, assays have been developed to target problem cells and decrease these events [87]. One of such assays encourages the differentiation of iPSCs into MSCs (iPSCs-MSCs). The traditional protocol used for differentiation of human iPSCs was modified by initially inhibiting Smad2/3 signaling in iPSCs cultured with mTeSR1 medium followed by passaging cells by trypsinization in 7.5% CO_2 using plastic culture dishes to improve the differentiation into MSCs. The modified protocol achieved enrichment of iPSCs-MSCs, as they expanded more rapidly and to a greater extent, but eventually underwent senescence. This method did not result in teratomas in murine models [88]. Another tactic for increasing the safety of iPSCs includes the addition of inducible caspase-9 (iC-9) suicide gene. An in vitro mouse model demonstrated the possibility of inducing apoptosis in tumors grown from iPSC transplants without interfering with the differentiation of the stem cells into neurons [89]. An in vivo study has similarly used caspase-9 to decrease tumor size. Following a subcutaneous transplantation of iPSCs with iC-9, teratoma formation occurred. A chemical inducer of dimerization was then administered, and the teratomas had drastically reduced [90]. Since using this gene has no detrimental effects on the differentiation of the iPSCs into neurons, it seems like a promising treatment for use in TBI.

9.5. Mesenchymal Stem Cells. MSCs are multipotent stromal cells derived from a variety of tissues [91] and have the capacity to differentiate into mesenchymal and nonmesenchymal tissue, including neural cells [92]. The ease of access and abundance of sources, as well as their potential to differentiate, have brought these cells to attention of investigators conducting studies in regenerative medicine.

The ability of MSCs to differentiate into neural cells was evidenced by Sanchez-Ramos and colleagues. They demonstrated that when human and mouse MSCs are exposed to specific experimental culture conditions, human and mouse MSCs have the ability to differentiate into neuron and glia-like cells [92]. In addition, MSCs have also been shown to cause an increase in proliferation and differentiation of native NSCs; the mechanism of which may be directly related to chemokines released by MSCs or indirectly via activation of surrounding astrocytes [93].

Besides their ability to differentiate, MSCs selectively migrate to injured tissues in TBI rat models, with subsequent differentiation in neurons and astrocytes and subsequent improvement in motor function [94]. The proposed mechanism through which this occurs is once again related to chemokines, growth factors [95], and adhesion molecules, such as the vascular cell adhesion molecule (VCAM-1), which allows MSCs to adhere to the endothelium of injured tissue [96]. Zhang et al. investigated the anti-inflammatory and immunomodulatory characteristics of MSCs using a TBI rat model. Neurological function was improved in the MSC group from days 3–28 compared to that in controls. MSC treatment group also had a significant decrease in brain water content, to the point where there was no significant difference between MSC and sham group 72 hours after TBI. MSC treatment reduced the number of microglia/

macrophages, neutrophils, CD3 lymphocytes, and apoptotic cells in the injured cortex, as well as proinflammatory cytokines [97].

Another noteworthy fact regarding MSCs is their ability to suppress lymphocytes. This characteristic was observed in vitro and in vivo models. A study conducted by Bartholomew and colleagues made it evident that MSCs reduced the proliferative response of lymphocytes [98], which could be useful in reducing the secondary effects of injury in TBI [99]. MSCs have also been noted to upregulate the expression of TIMP3, a metalloprotease inhibitor, which decreases the permeability of BBB in TBI mouse models, thereby enhancing their ability to recover from TBI [100]. Using MSCs, Li et al. detected significantly reduced areas of hypoperfusion in both remote regions and regions adjacent to brain lesions in TBI rat model. Since perfusion is linked to functional deficits, the authors found that this reduction in hypoperfusion was associated with a greater functional recovery as demonstrated by the modified neurological severity score (mNSS score) [101].

One appeal for use of MSCs as a TBI treatment method is their ability to cross the BBB through paracellular pathways [102]. Given that MSCs can migrate across the BBB and the endothelial cell layers of injured tissue, route of administration can be intravenous, as demonstrated in rats that were intravenously transplanted with MSCs following induced cerebral ischemia or received MSCs directly via injection into the brain lesion [103]. Moreover, research using genetically modified MSCs solely for production of growth factors, cytokines, and chemokines that enhance neuronal cells after injury highlights a novel possibility of TBI treatment without actual MSC transplantation [104].

MSCs provide an opportunity for clinical translation, as evidenced by recent clinical trials. A significant challenge with use of MSCs for TBI treatment remains to be the long-term possibility of brain tumor development due to the MSC's capacity of antitumor response suppression [105]. Two clinical trials aimed to test the feasibility and safety of using MSCs in patients with TBI. In 2008, seven TBI patients received a MSC transplant during a cranial operation and then received a second dose administered intravenously. At the six-month follow-up, patients had improved neurological function with no signs of toxicity [106]. Limitations of this study include a small sample size and lack of a control group.

From 2012-2013, 10 patients with severe TBI were recruited for a phase I clinical trial. MSCs were administered intravenously or intrathecally. Individuals had improvement in neurological function as measured by the NIHSS (National Institutes of Health Stroke Scale), GCS (Glasgow Coma Scale), and GOS (Glasgow Outcome Scale). No mortality or adverse events occurred, bringing support for the safety and feasibility of this treatment. Similar to the previous study mentioned, limitations include a small sample size as well as no control group [107]. Although MSC therapy for TBI appears to be safe and feasible, continued research is needed to better assess the efficacy of treatment compared to controls.

9.6. Bone Marrow Stromal Cells. Human bone marrow contains hematopoietic and nonhematopoietic stem cells with multipotent characteristics. Bone marrow stromal cells (BMSCs) differentiate into mesenchymal stem cells and, which when exposed to appropriate conditions, possess capacity to differentiate into numerous cell types [108], such as neuron and glial-like cells [92].

Shen et al. investigated outcomes in TBI rat models that were transplanted with BMSCs. The cultured BMSCs were implanted into the injured area of the brain followed by evaluation of neurologic function. Data analysis revealed an increase in expression of glial cell line-derived neurotrophic factor (GDNF), which is thought to be a potent promoter of neuronal survival, as well as genes of other neurotrophic factors. The investigators also noted that a great number of BMSCs that survived and migrated around the site of injury had done so 14 days following transplantation. Furthermore, the TBI rats with transplanted BMSCs presented with less apoptotic cells when compared to those of the control group and had improved neurologic outcomes [109].

In 2016, Cox et al. conducted an investigation utilizing BMSC treatment in human adults with TBI. This trial enrolled patients that were admitted to trauma or neurotrauma ICU and were assigned to one of the dosage treatment arms. The investigators were able to demonstrate the safety of this treatment modality and downregulation of inflammatory cytokines, as well as its ability to preserve critical regions of the brain, which correlate with an increase in functional outcomes [110].

Local and intravenous administration of BMSCs has been investigated for treatment of neurological injury and other neurological diseases [111]. A study protocol by Weiss et al. proposed to use intranasal tissue as a route of BMSC administration to the CNS through the trigeminal nerve. In their initial results, a Parkinson patient reported improvement in many sensory, as well as motor abilities. The authors anticipate at least a 10% improvement in neurological function [112].

10. Stem Cell-Derived Exosomes

MSCs have an important role in improving functional outcome after experimentally induced traumatic brain injury (TBI). Exosomes, which are of endosomal origin, are secreted by all cells, including MSCs [113]. These microvesicles are currently being investigated as another potential therapeutic agent for treatment of TBI. Utilization of exosomes for TBI grew from studies that revealed an improvement in post-stroke neuroregeneration, functional recovery, and neurovascular plasticity in animal models [114, 115]. Exosomes are thought to function as vesicular carriers to promote intercellular communication, specifically through transfer of microRNA (miRNA), which is one of the mechanisms that Xin and colleagues revealed to be responsible for neurite outgrowth [116].

In 2015, Zhang and colleagues administered MSC-derived exosomes to TBI rats via tail vein injections. Compared to saline-treated controls, exosome-treated TBI rats exhibited significant functional recovery as well as an increase in newly formed endothelial cells in both the lesion boundary zone and the dentate gyrus. The mechanisms

involved may be related to an increase in brain vascular density and angiogenesis in those that received MSC exosome administration. Additionally, exosome treatment significantly reduced brain inflammation by reducing the number of CD68+ microglia/macrophages and GFAP+ astrocytes [117]. As a follow-up, in their most recent investigation, Zhang and colleagues intravenously introduced exosomes generated from MSCs that were cultured in 2-dimensional (2D) versus 3-dimentional (3D) collagen scaffolds into experimentally induced TBI rats, which revealed that exosomes derived from 3D scaffolds were associated with a better outcome, in terms of spatial learning, compared to exosomes derived from MSCs that were cultured in traditional, 2D conditions [118]. The aforementioned investigations reveal a potential mechanism through which MSCs have an important role in improving functional outcome after TBI, namely, through exosomes, and that these microvesicles could hold the key to a more refined therapy for TBI and other neurologically devastating conditions, such as stroke.

11. Systemic Anti-Inflammatory and Immune Responses

Important areas to consider when working towards clinical translation of stem cell therapy are choosing which type of stem cells to use and the systemic effects of stem cell therapy. MSCs have been found to reduce the persistent inflammatory response following TBI by decreasing lymphocytes [75]. A study investigating MSCs and how they regulate macrophages found that by secreting secretin tumor cell line-1 (STC-1), MSCs can inhibit inflammasome activation in macrophages and prevent maturation and secretion of proinflammatory cytokines, including IL-1β [119]. Kim and colleagues investigated extracellular vesicles (EVs) produced by BM-MSCs as an effective therapy for TBI. The EVs were able to decrease inflammation 12 hours after TBI and improve pattern separation and spatial learning impairments 1 month later [120]. Additionally, the authors note that a limitation to using BM-MSCs for producing EVs is they senesce after undergoing expansion in culture [121], but in preparing this experiment, researchers used a standardized protocol during cell expansion in culture to reduce variation and retain progenitor features and preselected a preparation that could expand well beyond the amount required for experimentation [120, 122]. Understanding how to improve efficacy with cell expansion and differentiation contributes to the effectiveness of using stem cells to promote recovery following TBI.

As mentioned previously, Zhao and colleagues found that iPSC-MSCs were less tumorigenic than BM-MSCs, readily expandable, and homogenous, thereby offering more uniform biological activities [88]. In response, Yun and colleagues investigated the anti-inflammatory effects of iPSC-MSCs in the cornea after chemical and mechanical injury. iPSC-MSCs were able to reduce corneal inflammation, thereby offering an alternative therapy for inflammatory diseases [123].

The proinflammatory environment leads to BBB breakdown and worsens neurological deficits following injury. MAPCs have been shown to bypass the pulmonary capillary bed compared to larger MSCs after intravenous injection, leading to more cells contacting splenocytes [124]. Investigator found that MAPC therapy preserved splenic mass and attenuated BBB permeability secondary to their interaction with splenocytes. MAPCs increased the proliferative rate of CD4+ T cells, IL-4, and IL-10 in stimulated splenocytes and stabilized the vascular environment in the perilesional area [125]. Targeting the inflammatory response following acute TBI is an important aspect of stem cell therapy, as a majority of neurological deficits following TBI is caused by both the initial insult and secondary inflammatory responses. MSCs and utilization of exosomes are indicated for clinical translation, as animal studies continue to support their ability to modulate inflammation and promote neuroregeneration.

12. Clinical Translation in Diffuse Axonal Injury

Direct implantation of stem cells promotes neuronal regeneration, improved neurological scores, and anti-inflammatory effects in animal models studying responses after controlled cortical impact injury. However, diffuse axonal injury has not been as thoroughly studied in animal models and present in humans from blast injuries [126] and even mild head injuries [127]. Xu and colleagues used an impact acceleration rat model to study the effects of human-derived oligodendrocyte progenitor cells (hOPCs) from a human ESC line in remodeling myelin and axonal regeneration following diffuse axonal injury. hOPCs were able to survive in the deep sensorimotor cortex and migrate with near exclusive affinity to white matter tracts. In the area surrounding the transplantation site, the percentage of myelin basic protein (+) oligodendrocytes, those that ensheath axons, was significantly higher at 3 months compared to that at 6 weeks and compared to that of shams. This supports not only the notion that human ESCs and NSCs can be guided to specific fates after transplantation but also, because rapid proliferation was not observed, there is a lower possibility of overgrowth or tumors. This study provides a TBI model that targets myelin remodeling as a regenerative strategy following diffuse axonal injury [128].

13. Conclusion

Clinical trials have shown that MSC transplantation may decrease TBI patients' sequela and has the potential to become an effective treatment modality [129]. MSC safety and efficacy have been investigated in patients with complications following TBI [130] and determined that earlier interventions lend themselves to better results.

Stem cells used as a marker for TBI recovery and improved Glasgow Coma Scale are the focus of another ongoing investigation. Endothelial progenitor cells, which play an active role in vascular repair and revascularization, have been shown to increase 48 hours after TBI and were correlated to clinical outcomes. In the same study, patients with

low circulatory levels of endothelial progenitor cells were more likely to present with poor clinical outcomes [131]. Even though long-term follow-up is pending, utilization of endothelial progenitor stem cell transplantation is another potential therapeutic strategy for future interventions, which may enhance vascular repair in patients with TBI and contribute to an improvement in their neurologic outcome.

Although significant research has been conducted in the area of traumatic brain injury, both in terms of complexity underlying the pathophysiology and utilization of stem cell therapy in its treatment, a lot remains to be understood in order to determine the best method to promote recovery of functional brain tissue. Unfortunately, compared to other mammalian tissues, the brain does not have sufficient capacity to regenerate itself and theoretically requires assistance to do so after TBI. Use of neural stem cell therapy, whether through manipulation of endogenous or transplantation of exogenous NSCs, is an approach that numerous studies revealed to have significant potential to promote recovery of brain function in individuals suffering from TBI-associated disability. However, significant amount of research remains to be done in use of stem cells for treatment of TBI due to our limited understanding of potential complications, unexplored ethical implications, routes of administration, and use of combination/cotransplantation therapy. Combination and cotransplantation therapy that utilize NSCs and other cells, such as astrocytes and endothelial cells, which make the central nervous system's microenvironment more optimal for NSC grafting, likely hold the key to the best approach for treatment of aftermath of TBI, especially when considering the multifaceted nature of its underlying pathophysiology. In summary, all studies and future interventions that may impact treatment of TBI require multicenter and randomized prospective trials with long-term follow-up; this will allow future investigators to define the role and impact that each treatment may have on a given patient population.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Cesar Reis and Vadim Gospodarev contributed equally to this work.

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Research Article

Electroacupuncture Improved Hippocampal Neurogenesis following Traumatic Brain Injury in Mice through Inhibition of TLR4 Signaling Pathway

Yuqin Ye,[1,2] Yongxiang Yang,[1,3] Chen Chen,[4] Ze Li,[5] Yanfeng Jia,[5] Xinhong Su,[1] Chaoxian Wang,[1] and Xiaosheng He[1]

[1]*Department of Neurosurgery, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, China*
[2]*Department of Neurosurgery, PLA 163rd Hospital (Second Affiliated Hospital of Hunan Normal University), Changsha 410000, China*
[3]*Department of Neurology, PLA 422nd Hospital, Zhanjiang 524005, China*
[4]*Institute of Psychology, Fourth Military Medical University, Xi'an 710032, China*
[5]*Department of Neurosurgery, The Fourth People's Hospital of Shaanxi Province, Xi'an 710034, China*

Correspondence should be addressed to Xiaosheng He; hexiaos@fmmu.edu.cn

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The protective role of electroacupuncture (EA) treatment in diverse neurological diseases such as ischemic stroke is well acknowledged. However, whether and how EA act on hippocampal neurogenesis following traumatic brain injury (TBI) remains poorly understood. This study aims to investigate the effect of EA on hippocampal neurogenesis and neurological functions, as well as its underlying association with toll-like receptor 4 (TLR4) signaling in TBI mice. BrdU/NeuN immunofluorescence was performed to label newborn neurons in the hippocampus after EA treatment. Water maze test and neurological severity score were used to evaluate neurological function posttrauma. The hippocampal level of TLR4 and downstream molecules and inflammatory cytokines were, respectively, detected by Western blot and enzyme-linked immunosorbent assay. EA enhanced hippocampal neurogenesis and inhibited TLR4 expression at 21, 28, and 35 days after TBI, but the beneficial effects of EA on posttraumatic neurogenesis and neurological functions were attenuated by lipopolysaccharide-induced TLR4 activation. In addition, EA exerted an inhibitory effect on both TLR4/Myd88/NF-κB and TLR4/TRIF/NF-κB pathways, as well as the inflammatory cytokine expression in the hippocampus following TBI. In conclusion, EA promoted hippocampal neurogenesis and neurological recovery through inhibition of TLR4 signaling pathway posttrauma, which may be a potential approach to improve the outcome of TBI.

1. Introduction

As one of the leading life-threatening diseases worldwide, traumatic brain injury (TBI) often causes high mortality or a range of severe neurological deficits and long-term disability due to an inadequate self-repair capacity of the central nervous system (CNS) [1]. For a long time, neural stem cells (NSCs) have been identified in the subgranular zone (SGZ) of hippocampal dentate gyrus (DG), which contributed to endogenous neurogenesis throughout life [2, 3]. The remarkable characteristics of hippocampal NSCs include self-renew, production of newborn neurons, and integration into the damaged neural network following CNS injury, which makes intervention of endogenous neurogenesis as an attracting strategy for the rehabilitation of injured brain. However, accumulating evidence indicated that posttraumatic neurogenesis in the hippocampus was insufficient to overcome the neural damage caused by

TBI [4, 5]. Therefore, it is required to discover an approach to enhance hippocampal neurogenesis for brain reconstruction and rehabilitation after TBI.

Acupuncture, an ancient curing skill, has been applied in relieving pain and boosting body energy since about 2500 BC in China [6]. Electroacupuncture (EA), originating from traditional acupuncture around the 1930s, has been verified to significantly improve the therapeutic effects of the traditional acupuncture in a variety of diseases [7, 8]. In recent years, increasing studies have indicated that EA was conducive to the improvement of neurological function following CNS damage via stimulation of certain acupuncture points, whereas the detailed mechanism was still not fully understood [8–10]. Furthermore, a lot of researches up to now have emphasized the neuroprotection of EA in brain stroke such as cerebral ischemia and intracerebral hemorrhage, but few studies focused on the role of EA in brain trauma, especially concerning the effect of EA on posttraumatic neurogenesis and its potential linkage with the pathophysiological process in the hippocampus after TBI [11–13]. Hence, a better understanding of EA in traumatic brain might provide new clues to promote the inadequate neurogenesis in the hippocampus following TBI.

Previous studies have demonstrated that the therapeutic mechanisms of EA in neurological diseases involved a series of molecules and pathophysiological processes, such as angiopoietin 1- and 2-mediated angiogenesis, mammalian target of rapamycin-associated neuronal autophagy, and $\alpha 7$ nicotinic acetylcholine receptor-related inflammatory responses [9, 12, 14, 15]. As an important member of pattern-recognition receptor family, toll-like receptor 4 (TLR4) has been found in diverse cell types including microglia, astrocyte, and neuron in the CNS [16]. A growing body of evidence suggested that TLR4 signaling pathway played a key role in inflammation of CNS diseases, such as cerebral stroke, Alzheimer's disease, and spinal cord injury [17–20]. Therefore, it has been proposed as a therapeutic target. TLR4 recognized not only pathogen-associated molecular patterns (PAMP) but also damage-associated molecular patterns (DAMP), which could induce intracellular cascade activation and release inflammatory cytokines in response to the pathological condition of injured brain [19, 21, 22]. In addition, several groups have provided evidence to support the crucial role of TLR4 signaling pathway in governing NSC proliferation and differentiation [23–26]. Our previous study also revealed that the expression of hippocampal TLR4 increased significantly and varied in a similar temporal pattern to posttraumatic NSC proliferation in SGZ [27]. However, whether there is any involvement of TLR4 pathway in the mechanism by which EA works in hippocampal neurogenesis after TBI remains unclear.

Therefore, the current study was performed to investigate the effect of EA treatment on neurogenesis in the hippocampus of experimental TBI mice. Moreover, the involvement of TLR4 and its downstream cascade in the potential mechanism of EA-related neurogenesis were also explored. The finding might be helpful for novel insight into neural reparation and functional recovery after TBI.

2. Materials and Methods

2.1. Experimental Design. Male C57BL/6 mice, weighing 18–20 g, were provided by the Experimental Animal Center of Fourth Military Medical University (Xi'an, China). Animals were housed in a standard laboratory bedding environment ($22.0 \pm 2^{\circ}$C) and maintained on a controlled 12-hour light-dark cycle (light on 08:00–20:00). Enough food and water were available for all animals. The present experimental protocols and animal procedures complied with the National Experimental Animal Guidelines and were approved by the Fourth Military Medical University Ethic Committee (FMMUEC). All efforts were taken to minimize animal suffering throughout the experimental duration.

The first experiment was designed to investigate the effect of EA treatment on hippocampal neurogenesis and TLR4 expression after TBI. Seventy-two mice were randomly divided into four groups: sham, sham + EA, TBI, and TBI + EA groups ($n = 18$ in each). The sham group received sham injury operation; the TBI group was subjected to TBI treatment; the TBI + EA group was treated with EA postinjury. Immunofluorescence (IF) staining, water maze test (WMT), and neurological severity score (NSS) test were performed to evaluate the neurogenesis, neurocognitive, and neurobehavioral functions at 21, 28, and 35 days after TBI. The protein and mRNA level of TLR4 were, respectively, detected by Western blot (WB) and real-time PCR.

In the second experiment, TLR4 ligand lipopolysaccharide (LPS) was used to activate TLR4 in the hippocampus. The effects of TLR4 activation on EA-related neurogenesis, neurocognitive, and neurobehavioral functions following TBI were explored. Twenty-seven mice were randomly divided into three groups: TBI + EA, TBI + EA + LPS, and TBI + EA + vehicle (Veh) groups ($n = 9$ in each). The TBI + EA group underwent the same treatment as above; the TBI + EA + LPS group was subjected to EA treatment and LPS administration posttrauma; the TBI + EA + Veh group received EA treatment and vehicle endotoxin-free water (solvent of LPS) injection posttrauma. The neurogenesis, neurocognitive, and neurobehavioral functions were, respectively, assessed by IF staining, WMT, and NSS test as described above.

In the third experiment, downstream molecules and inflammatory cytokines of TLR4 pathway were determined to further disclose the potential mechanism of EA-related neurogenesis in the hippocampus posttrauma. Thirty mice were randomly divided into six groups: sham, sham + EA, TBI, TBI + EA, TBI + EA + LPS, and TBI + EA + Veh groups ($n = 6$ in each). Each group was subjected to the same treatment as above, respectively. The expression of downstream molecules in TLR4 pathway was examined with WB, and the level of inflammatory cytokines was detected by enzyme-linked immunosorbent assay (ELISA) at 35 days after TBI.

2.2. Establishment of TBI Mouse Model. Following intraperitoneal (i.p.) chloralhydrate (400 mg/kg) anesthesia, controlled cortex injury (CCI) was produced in mice to establish TBI model. The mice were secured in a

stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) by an incisor bar and two lateral ear pins. An incision was made at the midline on the scalp, and the fascia was reflected to expose the skull for craniotomy. The drilling site was between the lambda and bregma and 2.5 mm lateral to the sagittal suture in the right hemisphere. After the skull flap (4.0 mm diameter) was removed, brain contusion was produced on the exposed dura using a CCI device (Hatteras Instruments, Cary, NC, USA). According to our previous study [28], the impact parameters were set at 1.0 mm for cortical impact depth, 3.0 m/s for impact velocity, and 100.0 ms for contact time. Briefly, a piston rod with an impact tip of 3.0 mm diameter was centered at craniotomy site and impacted dura perpendicularly to contuse the underlying cortex. Then, the skull flap was reset, the scalp was sutured with nylon threads, and incision was cleaned with sterile alcohol. The mice in the control group were treated only with craniotomy but not cortical impact. Animal core temperature was maintained at $37.0 \pm 0.5^{\circ}$C with a heating pad during surgical operation and postsurgical recovery period.

2.3. Electroacupuncture Treatment. After animals were anesthetized, ST36 acupoint ("Zusanli", locating at 5.0 mm distal to the head of the fibula under the knee joint and 2.0 mm lateral to the tubercle of the anterior tibia) and GV40 acupoint ("Dazhui", locating at the posterior midline and the depression below the spinous process of the seventh cervical vertebra) were selected for EA. Each of two stainless steel needles of 0.3 mm diameter was inserted at a depth of 3.0 mm into the acupoints, respectively, with its end connecting to the output terminal of an EA instrument (Model SDZ-V, SMACL, Suzhou, China). The stimulation parameters were modified from previous studies taken by the Anesthesiology Department of our hospital [29, 30]. EA treatment started at the next day after TBI and continued for 35 consecutive days in accordance with the parameters: alternating dense-sparse wave; 2/15 Hz for frequency; 1.0 mA for current intensity; 30 min per day. Mouse body temperature was maintained at $37.0 \pm 0.5^{\circ}$C by a heating pad during EA treatment.

2.4. Drug Administration. Thymidine analog bromodeoxyuridine (BrdU) (Sigma-Aldrich, B9285, St. Louis, MO, USA) was used to label endogenous NSCs in SGZ for neurogenesis evaluation. BrdU was dissolved in sterile saline solution to a concentration of 10.0 mg/ml before i.p. injection. The mice received a pulse of BrdU (100 mg/kg) injection once per day at 1–7 days posttrauma and were sacrificed at 28 days after the last injection (namely, at the 35th day following TBI).

Evidence showed that ultrapure LPS from *E. coli* serotype 0111:B4 (Invivogen, tlrl-3pelps, San Diego, CA, USA) was extracted with special steps and only activated TLR4 signaling pathway [31]. At 30 minutes before onset of EA, LPS was dissolved in endotoxin-free water to a final concentration of 0.5 mg/ml and was injected into the right lateral ventricle with a dosage of 250 μg/kg using a microliter syringe (Hamilton, Reno, NV, USA) at the coordinates: 2.0 mm posterior to the bregma, 1.5 mm lateral to midsagittal line, and 2.5 mm ventral from the skull surface [32]. For mice in the Veh group, identical volume injection of endotoxin-free water was given at the same coordinates.

2.5. Immunofluorescence Staining. Mouse was anesthetized and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 1 hour. The brain tissue was removed and immersed in 4% paraformaldehyde at 4°C overnight and then dehydrated by alcohol and embedded in paraffin. Next, 5 μm thick coronal sections from −1.50 to −3.56 mm of the bregma (covering the DG of the hippocampus) were prepared in a microtome (Leica, Nussloch, Germany) and dried at 94°C overnight. Ten sections (100 μm apart) from each mouse brain were selected and deparaffinized by alcohol and dimethylbenzene. For DNA denaturation, the sections were incubated in citric acid antigen retrieval buffer (pH = 6.0) at 95°C for 10 min. To block nonspecific signals, the sections were then incubated in PBS with 1% donkey serum albumin and 0.3% Triton X-100 at room temperature for 30 min.

Newly generated neurons in the hippocampus were double-labeled by BrdU/NeuN to assess the neurogenesis level after TBI. Briefly, brain sections were incubated for 12 hours at 4°C with primary antibodies: anti-BrdU sheep polyclonal antibody (1 : 200, GeneTex, GTX21893, Irvine, CA, USA) and anti-NeuN rabbit monoclonal antibody (1 : 100, CST, 24307, Beverly, MA, USA). After being washed three times with PBS, sections were incubated for 1 hour at room temperature with the following secondary antibodies: Alexa Fluor 488-labeled donkey anti-sheep antibody (1 : 1000, Invitrogen, A-11015, Eugene, OR, USA) and Alexa Fluor 594-labeled donkey anti-rabbit IgG antibody (1 : 1000, Invitrogen, R-37117, Eugene, OR, USA). After three times washing with PBS, an antifade mounting medium (Electron Microscopy Sciences, CAT17895-01, Hatfield, PA, USA) was used to mount section before cover slipping. Negative controls were set to verify the immunolabeling specificity.

Images were captured under a confocal laser scanning microscope (FV1000, Olympus, Tokyo, Japan) with a FLUOVIEW image system (v.1.4a, Olympus, Tokyo, Japan), assembled in Photoshop 7.0 software (Adobe Systems, San Jose, CA, USA). BrdU/NeuN double-positive cells at SGZ of five consecutive visual fields (400x) in each section were counted. The average number of positive cells in the five visual fields was viewed as the number of positive cells for each section, and the average number of positive cells in five sections was regard as the final number of newborn neurons in each mouse brain.

2.6. Western Blot. Hippocampal tissues were isolated from the brain on ice and stored in −80°C. Samples were homogenized and digested in a homogenizer on ice for 15 minutes with a lysis buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris (pH = 7.4), 1% Triton X-100, 0.5 mM EDTA, 1 mg/ml aprotinin, 1% deoxycholate, 10 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 12,000 rpm for 20 minutes at 4°C, and protein concentration was examined with bicinchoninic acid Protein Assay kit (Beyotime, P0011, Shanghai, China). Equivalent

Table 1: Animal physiological parameters.

Group	BT (°C)	HR (/min)	BP (mmHg)	PG (mmol/l)	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	pH
Sham	37.6 ± 1.3	372.2 ± 28.5	133.4 ± 9.7	7.6 ± 0.3	97.4 ± 10.1	42.0 ± 5.9	7.37 ± 0.03
TBI	37.3 ± 1.6	369.5 ± 17.4	126.7 ± 14.4	7.0 ± 0.4	98.9 ± 13.5	39.3 ± 4.2	7.40 ± 0.06
TBI + EA	37.0 ± 1.9	365.3 ± 29.9	134.6 ± 11.3	7.4 ± 0.6	95.4 ± 17.8	43.6 ± 5.4	7.42 ± 0.03
TBI + EA + LPS	36.7 ± 1.1	373.5 ± 22.3	130.8 ± 14.9	7.7 ± 0.4	95.8 ± 13.5	40.5 ± 4.9	7.41 ± 0.06
TBI + EA + Veh	37.2 ± 0.7	371.9 ± 15.2	134.1 ± 7.2	7.4 ± 0.2	96.6 ± 10.8	44.7 ± 6.5	7.36 ± 0.04
Sham + EA	36.9 ± 1.5	370.4 ± 13.8	131.6 ± 10.2	7.5 ± 0.9	96.5 ± 9.2	44.2 ± 4.1	7.33 ± 0.02

Data are expressed as mean ± SD and no statistical difference between all groups.

amount of protein (40 μg) was loaded and separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane 4°C for 50 minutes. Membranes were blocked with 5% nonfat milk solution in tris-buffered saline with 0.1% Triton X-100 (TBST) for 1 hour and then incubated overnight at 4°C with appropriate primary antibodies as below: rabbit anti-mouse TLR4 antibody (1 : 1000, Thermo Fisher, PA5-23125, Rockford, IL, USA), rabbit anti-mouse myeloid differentiation factor 88 (Myd88) antibody (1 : 500, Santa Cruz, sc-17320, Dallas, TX, USA), rabbit anti-mouse TNFR-associated factor (TRAF6) antibody (1 : 1000, Novus Biological, NB100-56179, Littleton, CO, USA), rabbit anti-mouse toll/IL-1 receptor domain-containing adapter-induced interferon-β (TRIF) antibody (1 : 1000, Enzo Life Sciences, ALX-215-016, Farmingdale, NY, USA), rabbit anti-mouse TRIF-related adaptor molecule (TRAM) antibody (1 : 1000, OriGene, TA-306163, Rockville, MD, USA), rabbit anti-mouse nuclear factor-κB (NF-κB) p65 antibody (1 : 1000, GeneTex, GTX21893, Irvine, CA, USA), and rabbit anti-β-actin antibody (1 : 2000, Proteintech, 20536-1-AP, Rosemont, IL, USA). Following three washes in TBST, the membranes were incubated with the second antibody: horse radish peroxidase- (HRP-) conjugated goat anti-rabbit IgG antibody (1 : 20,000, Cell Signaling Technology, 7074, Boston, MA, USA) for 1 hour at room temperature. Immunoreactivity was detected by WesternBright Enhanced chemiluminescence reagents (K12045-d20, Advansta, Menlo Park, CA, USA), and optical densities of the bands were analyzed by Gel-Pro Analyzer software (version 6.0, Media Cybernetics, Rockville, MD, USA).

2.7. Enzyme-Linked Immunosorbent Assay. Hippocampal tissues were isolated from the brain and pulverized in a homogenizer under liquid nitrogen. The lysates were incubated in a lysis buffer containing 150 mM NaCl, 10 mM Tris pH 8.0, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5 μl/ml of protease inhibitor (Sigma-Aldrich, P8340, St. Louis, MO, USA) for 1 hour at 4°C and then centrifugalized at 3000 rpm for 20 minutes. Supernatants were collected for TNF-α, IL-1β, and IL-6 level measurement with standard ELISA kits (R&D, Minneapolis, MN, USA). The whole experiments were performed under the manufacturer's instructions. Absorbance of samples was measured in a microplate reader, and data was determined in accordance with the standard provided in the kits.

2.8. Neurological Severity Score. At 21, 28, and 35 days posttrauma, NSS test was performed to assess the neurobehavioral status of mice by an investigator in a blinded manner. As previously described, the NSS consisted of 10 individual parameters for alertness measurement, balancing examine, and motor ability evaluation [33, 34]. Mouse was awarded one score point for the lack of a tested reflex or the failure to complete a task. The accumulated scores increased with the severity of neurobehavioral deficit. The total score was graded on a scale of 0 to 10, with 0 suggesting a normal behavior status and 10 indicating the maximal neurobehavioral dysfunction.

2.9. Water Maze Test. At 31–35 days after TBI, WMT was used to assess neurocognitive function of mice with a 160 cm diameter and 50 cm depth circular tank with a black inner wall filled with water (30 cm depth and 25°C). In accordance with previous studies [35, 36], hidden platform trials were performed to evaluate the learning ability and probe trials were conducted to measure the memory function of mice. For hidden platform trial, the tank was divided into four equal quadrants and a 12 cm diameter black circular platform was hidden 2 cm under water surface in the center of one quadrant. From 31 days posttrauma, each mouse performed four hidden platform trials per day for four days. Briefly, each mouse was allowed to swim freely in the maze and had a maximum of 120 seconds to find the platform. The mouse that failed to reach the platform within 120 seconds was taken out of water and remained on it for 30 seconds. Escape latency referred to the interval between animals was placed into the water and reached the platform. At 35 days posttrauma, hidden platform was removed from the quadrant for probe test. Each mouse was placed into the water to swim freely to find the removed platform. Mouse's trace in quadrant at which the platform was previously located was considered as the route in target quadrant. The times that a mouse swam over the previous platform location were viewed as its platform crossing times. All the parameters in above trails were recorded by a tracking system (DigBehMR, Shanghai Auspicious Software Technology Company Limited, China), and the average data was used to analyze the mouse neurocognitive function in different groups.

2.10. Statistical Analysis. Data were expressed as means ± SD. One-way analysis of variance (ANOVA) and Tukey HSD post hoc test were applied to analyze statistical significance in GraphPad Prism v.5.0 (GraphPad software, San Diego,

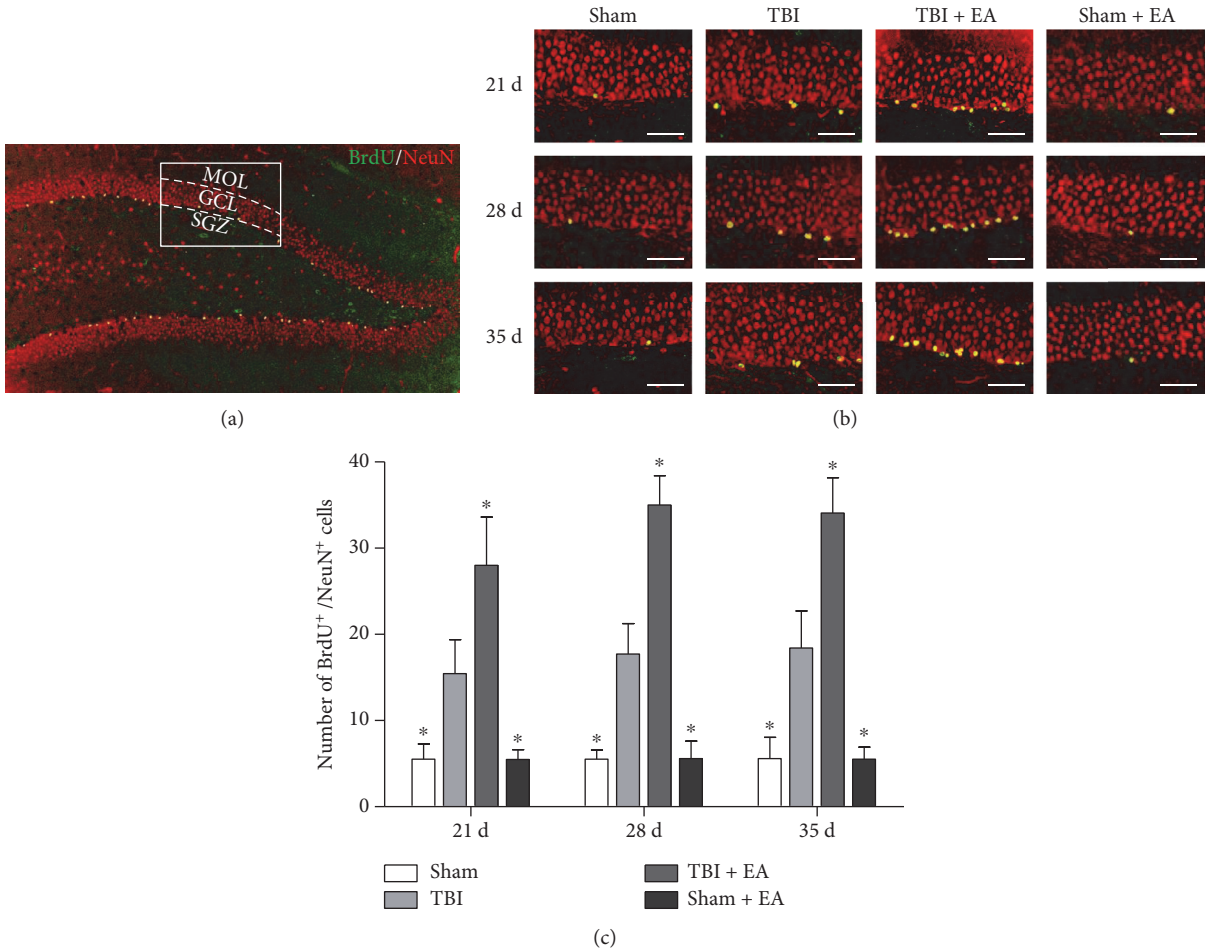

Figure 1: Electroacupuncture (EA) treatment enhanced TBI-induced neurogenesis in the hippocampus after traumatic brain injury (TBI). (a) Coronal section of hippocampal dentate gyrus (DG), stained with BrdU (green fluorescence)/NeuN (red fluorescence). MOL, GCL, and SGZ referred, respectively, to molecular layer, granular cell layer, and subgranular zone in DG. The white pane representing one visual field under confocal laser scanning microscope was shown in (b). (b) Representative immunofluorescence (IF) microphotographs of SGZ in the sham, TBI, TBI + EA, and sham + EA groups ($n = 9$ in each group) at 21, 28, and 35 days postinjury. The newly generated neurons were double labeled with BrdU/NeuN and merged into yellow. (c) Quantitation analysis revealed that, compared with the sham group, the number of BrdU/NeuN-positive cells was notably increased in the TBI group and EA treatment induced much more double-positive cells in the TBI + EA group than in the TBI and sham + EA groups at the three examined time points. Scale bar: 50 μm. $^*P < 0.05$ versus the TBI group.

CA, USA). Difference of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Physiological Parameters. Body temperature (BT), heart rate (HR), mean arterial blood pressure (BP), plasma glucose (PG), and arterial blood gas analysis (pH, PaO_2 and $PaCO_2$) of mice were, respectively, determined during the experimental period. No statistical differences of these physiological parameters among groups were observed ($P > 0.05$) (Table 1).

3.2. EA Treatment Enhanced Hippocampal Neurogenesis after TBI. As shown in Figure 1, the newborn neurons in the hippocampus were double labeled with BrdU (green fluorescence)/NeuN (red fluorescence), which were mainly located in the SGZ of DG. Compared with the sham group, the number of BrdU/NeuN-positive cells in the TBI group was increased at 21, 28, and 35 days posttrauma ($P < 0.05$). Importantly, there were more double-labeled cells in SGZ of the TBI + EA group than the TBI and sham + EA groups ($P < 0.05$), indicating that EA treatment further enhanced the TBI-induced neurogenesis in the hippocampus after TBI. Additionally, the data showed no significant change of BrdU/NeuN-positive cells between the sham group and the sham + EA group.

3.3. EA Treatment Inhibited the Expression of TLR4 in the Hippocampus after TBI. As shown in Figure 2, the TLR4 protein was increased in the TBI group compared to the sham group ($P < 0.05$), and the TLR4 level in the TBI + EA group was significantly decreased compared with that in the TBI and sham + EA groups, respectively, at 21, 28, and 35 days posttrauma ($P < 0.05$). However, there was no statistical difference of TLR4 expression between the sham and sham + EA groups. These results indicated that EA treatment caused an inhibition of TLR4 expression in the hippocampus after TBI.

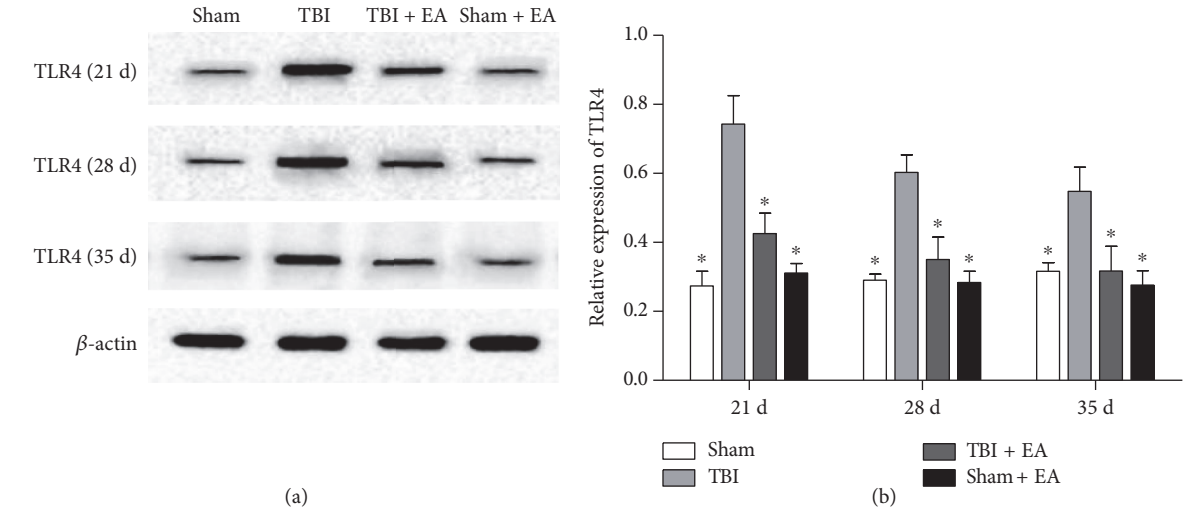

Figure 2: EA treatment inhibited the level of toll-like receptor 4 (TLR4) in the hippocampus after TBI. (a) Representative Western blot (WB) bands of hippocampal TLR4 expression in the sham, TBI, TBI + EA, and sham + EA groups ($n = 9$ in each group) at 21, 28, and 35 days posttrauma. (b) Quantitative analysis suggested that hippocampal TLR4 protein significantly increased in the TBI group compared with the sham group. And EA treatment caused an evident suppression of TBI-induced TLR4 upregulation in the TBI + EA group compared with the TBI and sham + EA groups. $^{*}P < 0.05$ versus the TBI group.

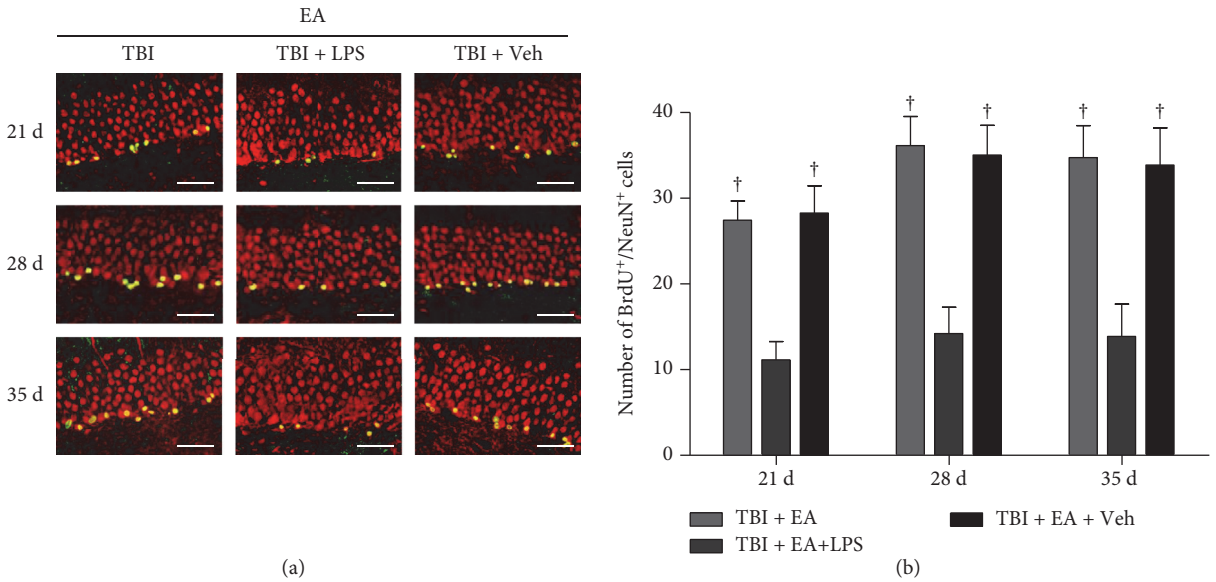

Figure 3: Lipopolysaccharide (LPS) was administrated in lateral ventricle of the brain to activate TLR4 signaling pathway. TLR4 activation attenuated the EA-induced neurogenesis in SGZ of the hippocampus after TBI. (a) Representative BrdU/NeuN double-labeled microphotographs of SGZ in TBI + EA, TBI + EA + LPS, and TBI + EA + Veh groups ($n = 9$ in each group) at 21, 28, and 35 days posttrauma. (b) Statistical data showed that, compared with TBI + EA group, BrdU/NeuN positive cells were significantly decreased in TBI + EA + LPS group and maintained at the same level in TBI + EA + Veh group at the three examined time points. Scale bar: 50 μm. $^{\dagger}P < 0.05$ versus the TBI + EA + LPS group.

3.4. Activation of TLR4 Blocked the Enhancement of Hippocampal Neurogenesis Induced by EA Treatment after TBI. Then, the effect of TLR4 activation on EA-induced hippocampal neurogenesis was evaluated. As shown in Figure 3, LPS administration significantly decreased the number of BrdU/NeuN-positive cells in the TBI + EA + LPS group compared with the TBI + EA group at 21, 28, and 35 days posttrauma ($P < 0.05$), while there was no significant difference between the TBI + EA + Veh group and the TBI + EA group ($P > 0.05$). According to the data of this section and the results of above sections (Sections 3.2 and 3.3), it can be seen that activation of TLR4 abolished the favorable effect of EA on hippocampal neurogenesis posttrauma.

3.5. Activation of TLR4 Eliminated the Improvement of Neurocognitive and Neurobehavioral Recovery Elicited by EA Treatment after TBI. WMT and NSS were conducted to investigate the effect of TLR4 activation on neurocognitive

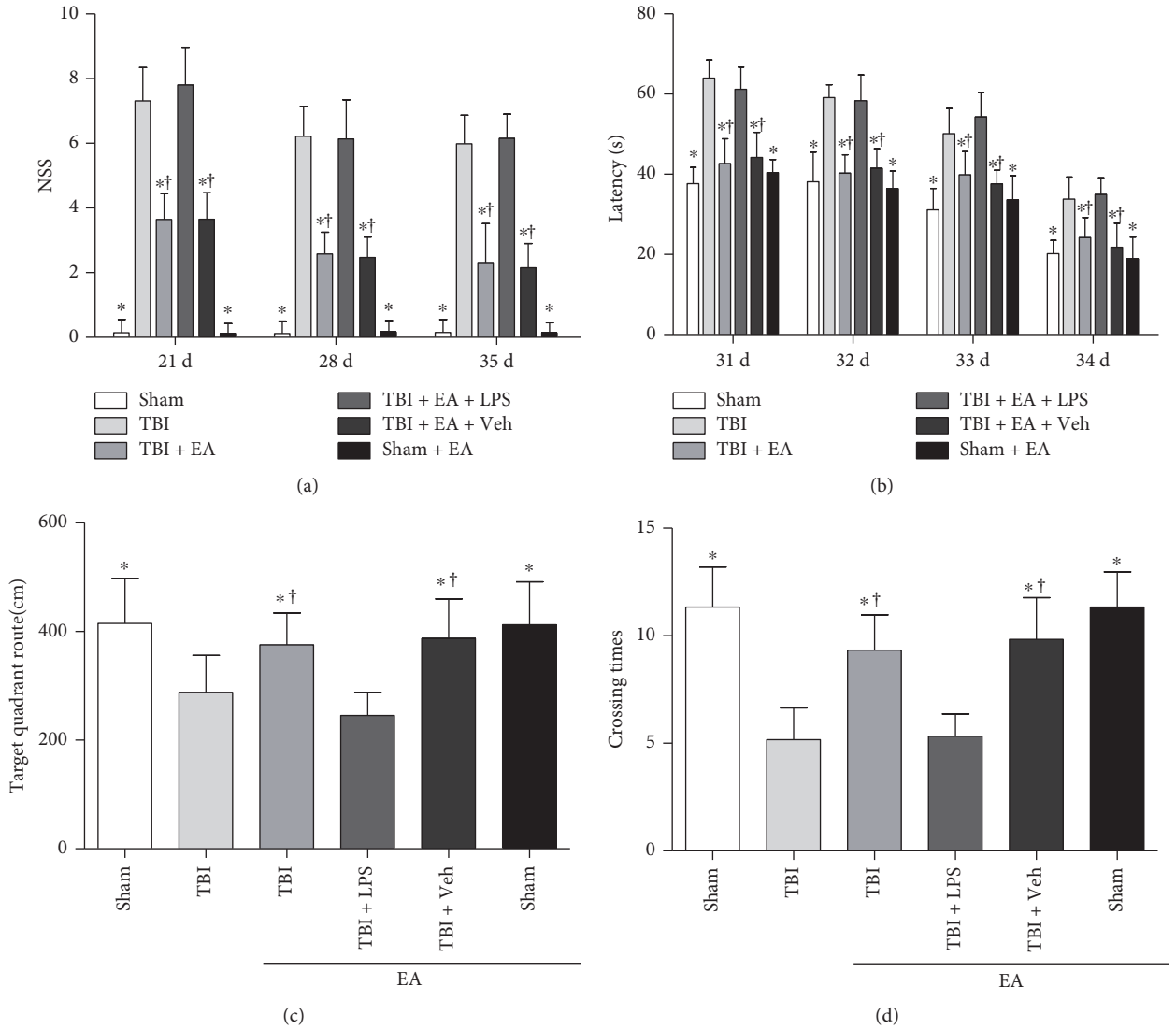

Figure 4: Activation of TLR4 eliminated the promotion of neurological functional recovery induced by EA treatment after TBI. Neurobehavioral and neurocognitive functions of mice in the sham, TBI, TBI + EA, TBI + EA + LPS, TBI + EA + Veh, and sham + EA groups ($n = 6$ in each group) were evaluated by neurological severity score (NSS) and water maze test (WMT). (a) Statistical analysis showed that the TBI group presented higher NSS than the sham group and EA treatment gave rise to an obvious reduction of the severity score in the TBI + EA group. However, LPS administration in TBI + EA + LPS group induced much poorer performance of mice in NSS test compared with the TBI + EA group. (b) Compared with the sham group, mice in the TBI group spent longer escape latency to find the hidden platform at 31–35 days after TBI. EA treatment markedly shortened the latency in the TBI + EA treatment, but the effect was abolished by administration of LPS in the TBI + EA + LPS group. (c, d) The TBI group exhibited less target quadrant route and shorter platform crossing times than the sham group at 35 days posttrauma. EA treatment remarkably increased the route and crossing times of the TBI + EA group, but the favorable role was eliminated by LPS-induced TLR4 activation in the TBI + EA + LPS group. Nevertheless, difference of the above four indexes between the TBI + EA + Veh and TBI + EA groups did not reach the statistically significant level ($P > 0.05$). $^{*}P < 0.05$ versus the TBI group, $^{\dagger}P < 0.05$ versus the TBI + EA + LPS group.

and neurobehavioral functions induced by EA treatment (Figure 4). In cognition assessment, mice in the TBI group spent longer escape latency to find the hidden platform the than sham group, but the mice in the TBI + EA group exhibited shorter latency compared with the TBI and sham + EA groups ($P < 0.05$). Platform crossing times and target quadrant route of the TBI group were less than those of the sham group, but the two indexes were significantly increased in the TBI + EA group compared with the TBI and sham + EA groups ($P < 0.05$). In neurological evaluation, severity score of the TBI group was higher than those of the sham group, but a notable improvement was observed in the TBI + EA group compared with the TBI and sham + EA groups ($P < 0.05$). These results showed that EA treatment rescued the neurocognitive and neurobehavioral deficits caused by TBI.

Compared with the TBI + EA group, LPS administration in the TBI + EA + LPS group caused much poorer performance of mice in neurocognitive and neurobehavioral tests, such as longer escape latency, less crossing times and route,

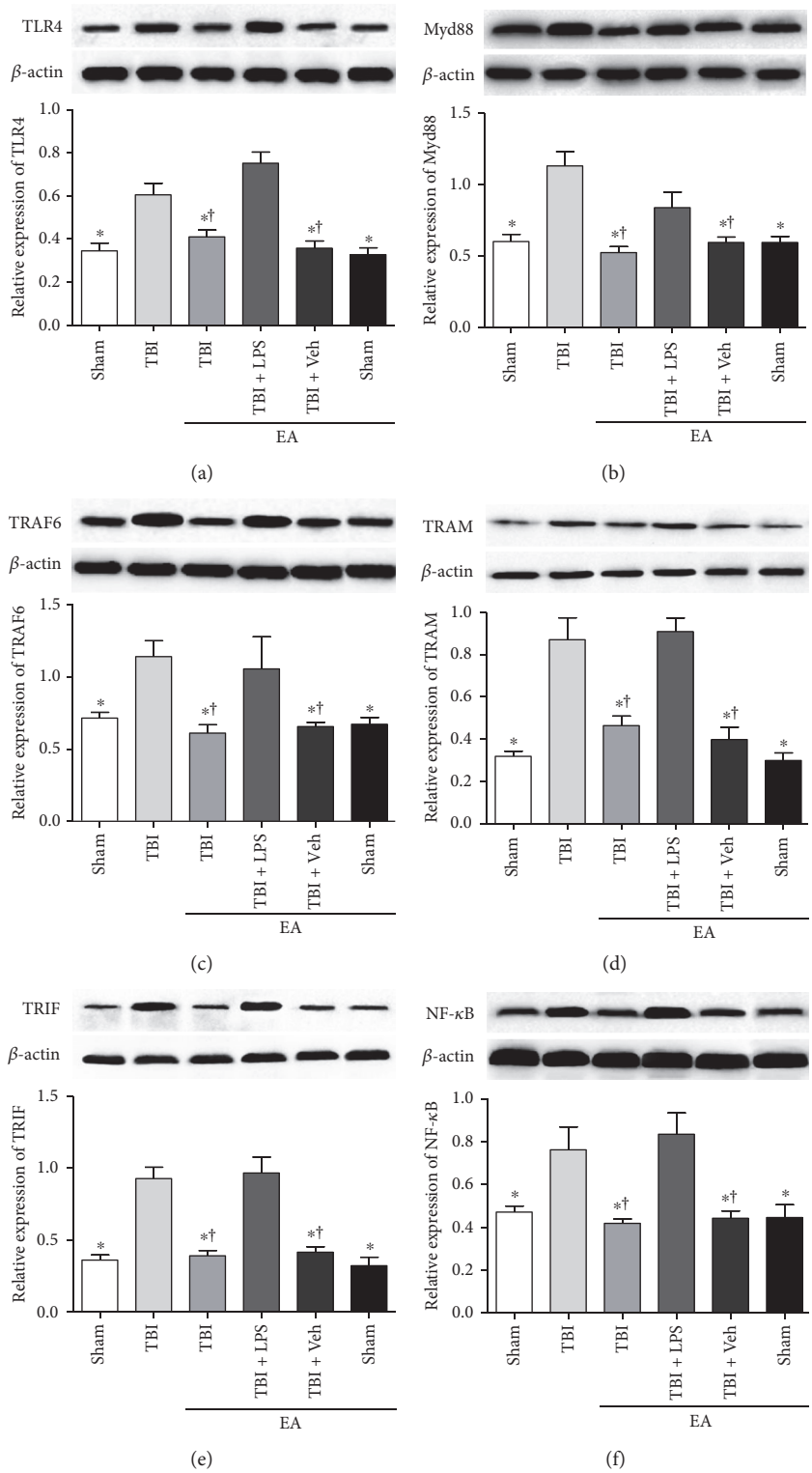

Figure 5: EA treatment exerted an inhibitory effect on both of the two downstream pathways of TLR4 in the hippocampus posttrauma. Hippocampal TLR4, Myd88, TRAF6, TRAM, TRIF, and NF-κB expression of the sham, TBI, TBI + EA, TBI + EA + LPS, TBI + EA + Veh, and sham + EA groups ($n = 3$ in each group) were detected by WB at 35 days after TBI. (a–f) The six kinds of proteins in TLR4/Myd88κB and TLR4/TRIF/κB signaling pathways were upregualted in the TBI group compared with the sham group and the sham + EA group. EA treatment resulted in a significant decrease in the TBI + EA group, but the effects were attenuated by LPS administration in the TBI + EA + LPS group. No difference of the proteins between the TBI + EA and TBI + EA + Veh groups was observed. $^{*}P < 0.05$ versus the TBI group, $^{\dagger}P < 0.05$ versus the TBI + EA + LPS group.

and higher severity scores ($P < 0.05$). Additionally, differences of the above four indexes between the TBI + EA + Veh and TBI + EA groups, as well as between the sham and sham + EA groups, did not reach the statistically significant level ($P > 0.05$). Taken together, it can be inferred that LPS induced TLR4 activation and eliminated the protective effect of EA on neurocognitive and neurobehavioral functions posttrauma.

3.6. EA Treatment Suppressed the Activity of TLR4 Downstream Cascade in the Hippocampus following TBI. After understanding the association between EA treatment and TLR4 in hippocampal neurogenesis, we further examined how the activity of TLR4 downstream signaling pathway in response to EA treatment at 35 days after TBI. WB was performed to determine the level of Myd88 and TRAF6 in TLR4/Myd88-dependent pathway; TRIF and TRAM in TLR4/TRIF-dependent pathway; as well as the two pathways' common target molecule NF-κB.

As shown in Figure 5, the expression of TLR4, Myd88, TRAF6, TRAM, TRIF, and NF-κB p65 significantly increased in the TBI group compared to the sham group ($P < 0.05$). EA treatment in the TBI + EA group produced an evident decrease of the six molecule levels in comparison with the TBI and sham + EA groups, but the effect was relieved by administration of LPS in the TBI + EA + LPS group ($P < 0.05$). The difference between the sham and sham + EA group has no statistical significance. These data indicated that TLR4/Myd88/NF-κB and TLR4/TRIF/NF-κB axles were activated posttrauma, and EA treatment exerted a restraining influence on both of the two pathways.

3.7. EA Treatment Alleviated the Expression of TLR4 Cascade-Induced Inflammatory Cytokines in the Hippocampus after TBI. We further investigated the effect of EA treatment on inflammatory cytokine expression in the downstream of TLR4/Myd88/NF-κB and TLR4/TRIF/NF-κB axles at 35 days after TBI. ELISA was performed to determine the level of inflammatory cytokines including TNF-α, IL-1β, and IL-6 in the hippocampus (Figure 6). Comparison with the sham and sham + EA groups showed that the level of these inflammatory cytokines was markedly elevated in the TBI group but was significantly decreased in the TBI + EA group ($P < 0.05$). However, administration of LPS in the TBI + EA + LPS group induced an evident increase of the inflammatory cytokines in comparison with the TBI + EA group ($P < 0.05$). Significant difference of cytokine expression between the TBI + EA + Veh and TBI + EA groups, as well as between the sham and sham + EA groups, was not observed ($P > 0.05$). Taken together, the data suggested that brain trauma triggered the inflammatory response mediated by TLR4 cascade in the hippocampus, and EA treatment mitigated the inflammation through downregulation of inflammatory cytokines, which might be helpful to posttraumatic neurogenesis.

4. Discussion

In the present study, these above three parts of experiments were performed with a purpose to investigate the effect and potential mechanisms of EA treatment on hippocampal neurogenesis following TBI. It was explored in the first part that how neurogenesis and TLR4 expression in the hippocampus responded to EA intervention in experimental TBI mice. Second, the role of TLR4 in the EA-induced neurogenesis and functional recovery following TBI was investigated in order to discover the potential mechanism of EA in inducing posttraumatic neurogenesis. Last, the influence of EA treatment upon TLR4 downstream pathways and inflammatory cytokines was examined. The corresponding results suggested that EA treatment produced a beneficial effect on neurogenesis and an inhibitory action on the level of TLR4 in the hippocampus after TBI. TLR4 activation induced by LPS eliminated the promotion of EA on hippocampal neurogenesis and neurological functions. In addition, it is interesting to note that EA treatment caused a repression on the activity of TLR4/Myd88- and TLR4/TRIF-dependent pathways, as well as the expression of downstream inflammatory cytokines posttrauma.

As one of the most studied complementary and alternative medicines, acupuncture has been applied to treat pathological conditions of the CNS since thousands of years ago in China [6, 9]. Increasing studies suggest that the easily operated and economical EA is a promising therapy for hypoxic-ischemic brain injury. Pretreatment or treatment of EA worked against cerebral ischemia and reperfusion damage through regulation of neuronal excitotoxicity, apoptosis, oxidative stress, and inflammation in ischemic stroke [37–39]. It had also been shown that EA took definite benefits on the improvement of neural repairing after stroke [40–42]. In addition, the study by Chuang et al. stated that EA intervention at the acute stage of TBI resulted in a decrease of transforming growth-interacting factor in injured area and an increase of the regional cerebral blood flow in rats, which contributed to reduce neuronal apoptosis and improve neurological outcomes posttrauma [43]. Zhou and his colleagues reported that EA at ST36 acupoint improved neurological function recovery by upregulating angiopoietin 1 and 2 expression in the injured cortex of cerebral hemorrhagic rat [12]. However, little is known about the efficacy of EA in neural rehabilitation and functional recovery following TBI. In the current study, we found that EA stimulation at ST36 and GV40 acupoints produced a beneficial effect on hippocampal neurogenesis at 7, 21, and 35 days posttrauma. Although Wong et al. have tried to assess the efficacy and safety of acupuncture for TBI patients in a series of systematic reviews published from 2011 to 2013 [44–46], they could not draw any conclusion because of the insufficient clinical random controlled trials and low methodological quality. In some extent, this finding is controversial with the above experimental findings, suggesting that further large amount and well-designed studies are required to elucidate the exact role of EA in clinical TBI patients.

It has been documented that various molecules and signals are involved in the protective effect of EA on neurological disorders although the precise mechanism is not yet definitely elaborated. Several previous studies suggested that TLR4 might be implicated in the neuroprotective effect of EA in cerebral ischemic injury tolerance [13, 47]. In the

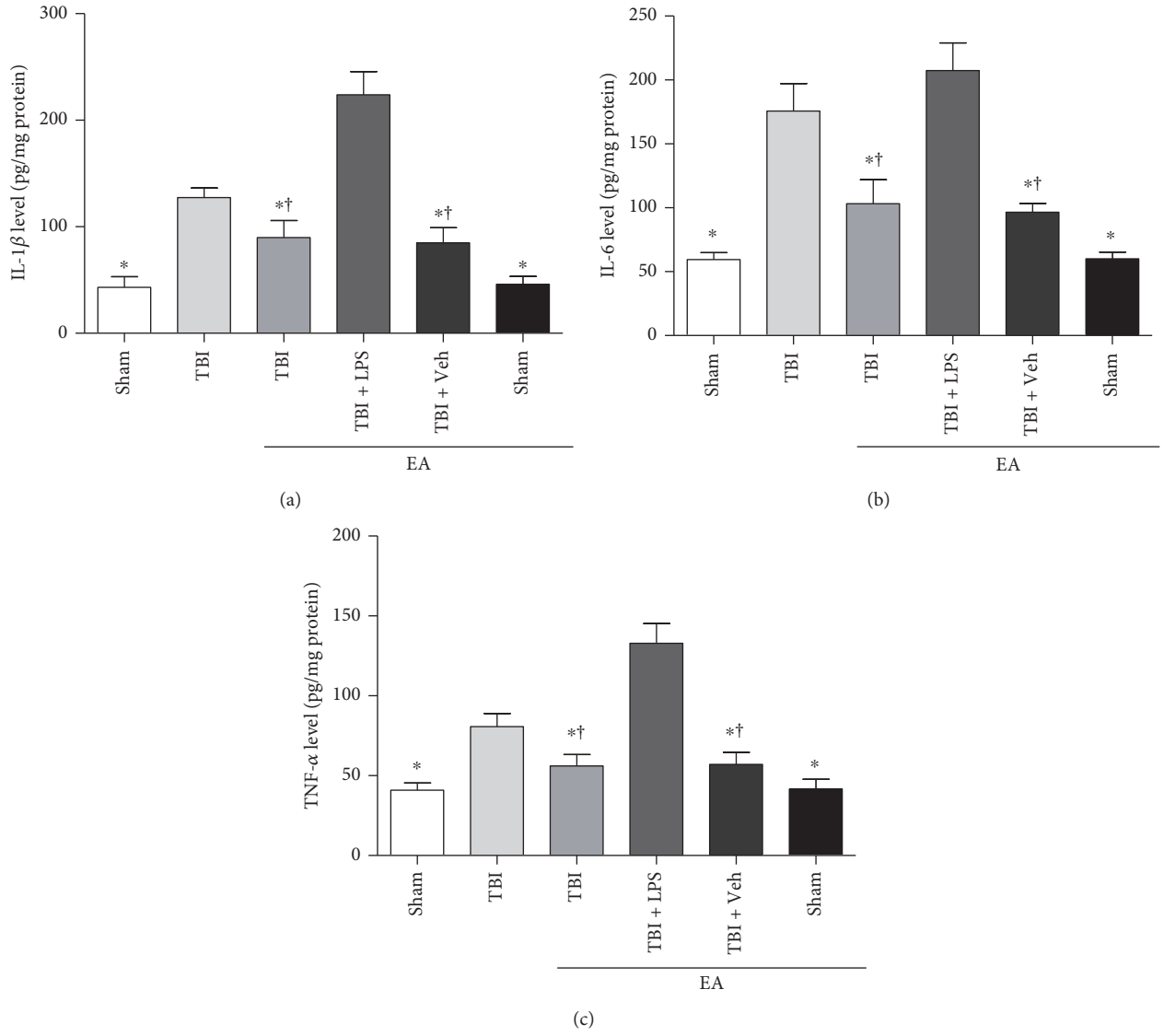

Figure 6: EA treatment suppressed the level of inflammatory cytokines in the downstream of TLR4 signaling pathway posttrauma. TNF-α, IL-1β, and IL-6 expression in the hippocampus were determined by ELISA. (a–c) Quantitative analysis showed that three inflammatory cytokines were elevated in the TBI group compared with the sham and sham + EA groups. EA treatment induced significant abatement of these cytokines in the TBI + EA treatment. Reversely, LPS administration abrogated the inhibitory effect of EA on TLR4-mediated inflammation in the TBI + EA + LPS group. There was no significant difference in these inflammatory cytokine expression between the TBI + EA + Veh and TBI + EA groups. $^{*}P < 0.05$ versus the TBI group, $^{\dagger}P < 0.05$ versus the TBI + EA + LPS group.

present study, the possible involvement of TLR4 in EA-induced hippocampal neurogenesis was investigated. It was observed that both protein and mRNA level of TLR4 in the hippocampus were augmented at 7, 21, and 35 days after TBI, which consisted with the data in our previous study [27]. Importantly, EA treatment reversed the increase of hippocampal TLR4 expression posttrauma. TLR4 activation blocked the promotion of NSC neuronal differentiation elicited by EA treatment in SGZ. Similarly, EA stimulation improved neurological deficits and spatial learning and memory function, which was closely associated with neural repairing posttrauma. But the favorable effects were abrogated by LPS-induced TLR4 activation. Therefore, it could be inferred that the inhibition of TLR4 might be an essential mediator for the effect of EA on hippocampal neurogenesis after TBI.

TLR4 has been identified to play a fundamental role in the regulation of innate immune response and adaptive immune system via TLR4/Myd88- and TLR4/TRIF-dependent pathways [48]. Previous studies showed that the level of TLR4 downstream adaptor protein was upregulated in traumatic brain and inhibition of Myd88 could remarkably improve neuronal survival and neurological function [49, 50]. In addition, preinjury antagonism of TLR4 gave rise to a notable decrease of MyD88 and TRIF expression in the hippocampus of a TBI rat model, which might be related to the neurocognitive function posttrauma [51]. Recently, quite a few studies discovered that TLR4 and

its downstream signaling molecules were responsible for the modulation of adult hippocampal neurogenesis and neural plasticity [24, 52, 53]. Our present work revealed that EA treatment possessed an inhibitory effect on the upregulation of TLR4 downstream signaling molecules posttrauma, including Myd88 and TRAF6 in TLR4/Myd88-dependent pathway and TRAM and TRIF in TLR4/TRIF-dependent pathway. As noted previously, NF-κB was one of the most important effectors in the signaling transduction of both Myd88- and TRIF-dependent pathways [16, 54]. Accordingly, we analyzed whether the NF-κB expression in the hippocampus responds to EA treatment after TBI. Our results showed that the TBI-induced hippocampal NF-κB elevation was significantly inhibited by EA treatment, which varied in the similar pattern to Myd88, TRAF6, TRAM, and TRIF. Taken together, these results indicated that EA treatment could repress the activities of both TLR4/Myd88- and TLR4/TRIF-dependent pathways in the hippocampus following TBI, and this may be one of the mechanisms by which EA inhibits posttraumatic NF-κB expression.

It is well known that neuroinflammation, one of the prominent pathological responses in injured brain, is a double-edged sword for neural plasticity depending on the balance between neurotoxic and neuroprotective effect in the hippocampus [55–57]. On the one hand, inflammatory response is propitious to the initiation of NSC proliferation and differentiation in pathological status of the CNS [58, 59]; on the other hand, overproduced inflammatory cytokines in neurogenic niche are detrimental to NSC survival and fate [25, 60, 61]. Studies have noted that TLR4-mediated inflammation is critical for the property of endogenous NSCs during the process of neurogenesis [23, 62]. Myd88- and TRIF-dependent pathways have been identified as contributing factors for activating NF-κB to trigger the release of inflammatory cytokine and chemokine in TLR4 downstream cascade [63–65]. In the present experiment, it was observed that the level of TNF-α, IL-1β, and IL-6 in the hippocampus was amplified at 35 days after TBI. EA treatment led to an evident downregulation of these proinflammatory cytokines, and this efficacy was eliminated by LPS administration. In view of previous findings and our present results, it can be speculated that, under the given parameters of this study, EA improved endogenous neurogenesis depending on its inhibition of TLR4/Myd88/NF-κB and TLR4/TRIF/NF-κB axle-induced inflammatory cytokines in the hippocampus after TBI. Although we cloud not exclude the possibility that this conclusion may be much associated with the selected time window and ways of EA intervention on the employed TBI model in the present study, our obtained results are consistent with previous literatures which described the efficacy of EA treatment in other neurological disorders, including ischemia stroke and surgical traumatic stress [47, 66].

Indeed, there are some limitations in this study since the experimental design was *in vivo*. It is hard to illustrate that the posttraumatic expression of TLR4 downstream mediators and inflammatory cytokines results from which type of cells in the neurogenesis niche. Therefore, further *in vitro* study is required to recognize the exact role of TLR4 signaling pathway in NSC proliferation and differentiation, as well as in the activation of hippocampal microglia and astrocyte after TBI. In addition, it is beyond the scope of this study to differentiate individual or synergistic effects of the inflammatory cytokines on hippocampal endogenous neurogenesis under the treatment of EA *in vivo*. Thus, such analysis needs to be performed in future *in vitro* investigation.

5. Conclusion

In summary, it is observed for the first time that EA treatment promoted hippocampal neurogenesis and neurological function recovery in TBI mice, at least in part, by suppressing TLR4 signaling pathway and its downstream proinflammatory response. Although preliminary, this present study paved a valuable way for further study and indicated that EA intervention might be one of the promising strategies to improve neurogenesis and neurological function restoration after TBI.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Yuqin Ye, Yongxiang Yang, Chen Chen, and Ze Li contributed equally to this work.

Acknowledgments

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Review Article

Targeting Adult Neurogenesis for Poststroke Therapy

Jianfei Lu,[1,2] Anatol Manaenko,[3] and Qin Hu[1]

[1]*Discipline of Neuroscience, Department of Anatomy, Histology and Embryology, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China*
[2]*Department of Anatomy and Embryology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China*
[3]*Departments of Neurology, University of Erlangen-Nuremberg, Erlangen, Germany*

Correspondence should be addressed to Qin Hu; huqinle20010709@126.com

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Adult neurogenesis mainly occurs at the subventricular zone (SVZ) on the walls of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus (DG). However, the majority of newborn neurons undergo programmed cell death (PCD) during the period of proliferation, migration, and integration. Stroke activates neural stem cells (NSCs) in both SVZ and SGZ. This process is regulated by a wide variety of signaling pathways. However, the newborn neurons derived from adult neurogenesis are insufficient for tissue repair and function recovery. Thus, enhancing the endogenous neurogenesis driven by ischemia and promoting the survival of newborn neurons can be promising therapeutic interventions for stroke. Here, we present an overview of the process of adult neurogenesis and the potential of stroke-induced neurogenesis on brain repair.

1. Introduction

Stroke is one of the leading causes of morbidity and mortality worldwide. In addition, about two-thirds patients had neurologic impairment and disability, based on a population study of follow-up of stroke survivors at five years [1]. Therefore, poststroke rehabilitation becomes a major therapeutic focus for most poststroke patients. Unfortunately, the currently available therapies are only rarely successful in improving recovery from neurological deficits. It is well established that de novo neurogenesis mainly occurs at two distinct regions in the adult brain: the SGZ of the dentate gyrus of the hippocampus and the SVZ adjacent to the lateral ventricle [2, 3]. In pathological conditions such as stroke, increased neurogenesis has been reported in adult animal models and even in stroke patients [4]. The proliferated neural progenitor cells migrate to the injured striatum and cortex; however, most of them failed to survive and rewire the brain. Taking advantage of the neurogenic capacity of the brain and improving the survival of endogenous neuroprogenitor cells shed light on the restorative therapies for stroke and other brain insults. Here, we review adult neurogenesis from a comprehensive perspective and summarize the current status of research on neurogenesis in poststroke therapy.

2. Adult Neurogenesis

Adult neurogenesis (AN) is a process that is continuously producing new neurons which integrate into existing circuits in adult age and have different mechanisms compared with fetal and early postnatal development [5]. AN was first demonstrated by Altman and Das in a rat brain in 1965 [6]. They injected thymidine-H^3 into adult rats and cats to tag the newborn cells and found that the labeled glia cells and neurons are present in various regions of the normal adult mammalian brain. In the 1990s, bromodeoxyuridine (BrdU) was applied to label newborn cells in neurogenesis research. With the application of this new technique, two areas of the adult neurogenesis were found: the DG and the SVZ. In the DG, new neurons continue to be generated from NSCs in the SGZ. NSCs also reside and proliferate in the SVZ and differentiate into neuroblasts. These neuroblasts migrate through the rostral migratory stream (RMS) to the olfactory bulb (OB) and integrate into OB circuits. Recently, some noncanonical

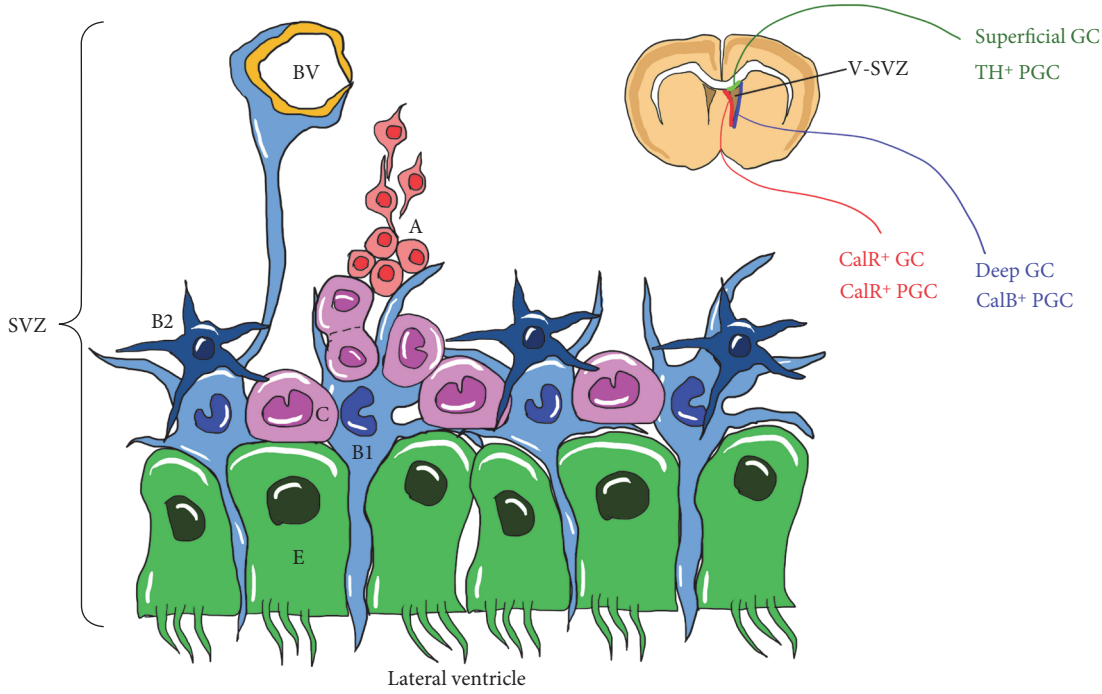

Figure 1: Neurogenesis in SVZ. The SVZ is shown in the left. Type B1 cells (B1, blue) lie atop ependymal cells (E, green) and extend their processes to blood capillary (BV). Type B1 cells divide to produce type C cells (C, pink). Type C cells then give rise to type A cells (A, red). Type B2 cells (B2, dark blue) also reside in the SVZ. The coronal section in the upper right is shown the diversity of newborn OB interneurons. Deep GCs and CalB$^+$ PGCs are derived from ventral NSCs, whereas superficial GCs and TH$^+$ PGCs are derived dorsal NSCs. NSCs from the medial wall produce CalR$^+$ GCs and CalR$^+$ PGCs.

sites of adult neurogenesis, such as neocortex, striatum, corpus callosum, amygdala, and hypothalamus, have been found in different species [7].

The process of maturation of new neurons encompasses the proliferation of resident NSCs and their subsequent differentiation, migration, survival, and functional integration into the preexisting circuitry [8]. AN is mediated by a series of physiological and pathological processes at all these stages. Moreover, programmed cell death (PCD) plays critical roles in regulating the process from NSC proliferation to the integration of neural circuits. We focus on current knowledge of the main neurogenic sites (SVZ and SGZ) of AN with their specificities and address the potential roles of PCD as a regulatory strategy.

2.1. AN in SVZ and SGZ

2.1.1. SVZ. In mammalian animals, new OB neurons are derived from SVZ, on the walls of the lateral ventricles. The SVZ have five main cell types: B1 astrocytes (type B1 cells), B2 astrocytes (type B2 cells), transit-amplifying cells (type C cells), neuroblasts (type A cells), and ependymal cells (type E cells) (Figure 1). Microglia and oligodendrocyte precursor cells (OPCs) also reside in the SVZ. Type B2 cells and ependymal cells are important for maintaining and regulating the niche of SVZ. Type B1 cells lie atop ependymal cells and extend their processes further to blood capillary [9]. Besides, most B1 astrocytes contact the ventricle by extending a thin cellular process between ependymal cells [2]. Type C cells are shaped like a smooth ellipse and have large nuclei with deep invaginations [2]. Type A cells have an elongated cell body with smooth contours. They have one or two processes and join to other type A cells by small junctional complexes. Nestin, SRY-box 2 (Sox2), and brain lipid-binding protein (BLBP) have been considered as NSC markers. Distal-less homeobox 2 (DLX2), epidermal growth factor receptor (EGFR), and mammalian achaete-scute homolog 1 (MASH1) are mainly expressed on type C cells [10]. Doublecortin (DCX), β-III-tubulin (TuJ1), and polysialylated neural cell adhesion molecule (PSA-NCAM) are the unique markers of type A cells [2].

NSCs in the SVZ correspond to type B1 cells. Asymmetric division of type B1 cells produce self-renewed type B1 cells and type C cells [11, 12], which symmetrically divide into type A cells [13]. After birth in SVZ, type A cells form elongated, chain-like aggregates, which are ensheathed by astrocytes [14–16]. These neuroblasts migrate through RMS at the anterior SVZ [17]. The migration of neuroblasts follows a salutatory manner: first, a leading process extended; then, swelling formation and centrosome migration; and last, somal translocation [16, 18–20]. The RMS carries the neuroblasts into the OB where these neuroblasts detach from the RMS and then migrate radially to the outer layer and differentiate into various subtypes of olfactory neurons.

There are two principal types of adult-born OB neurons: periglomerular cells (PGCs) in the glomerular layer (GL) and granule cells (GCs) in the granule cell layer (GCL). Deep GCs and calbindin (CalB)$^+$ PGCs are derived from ventral

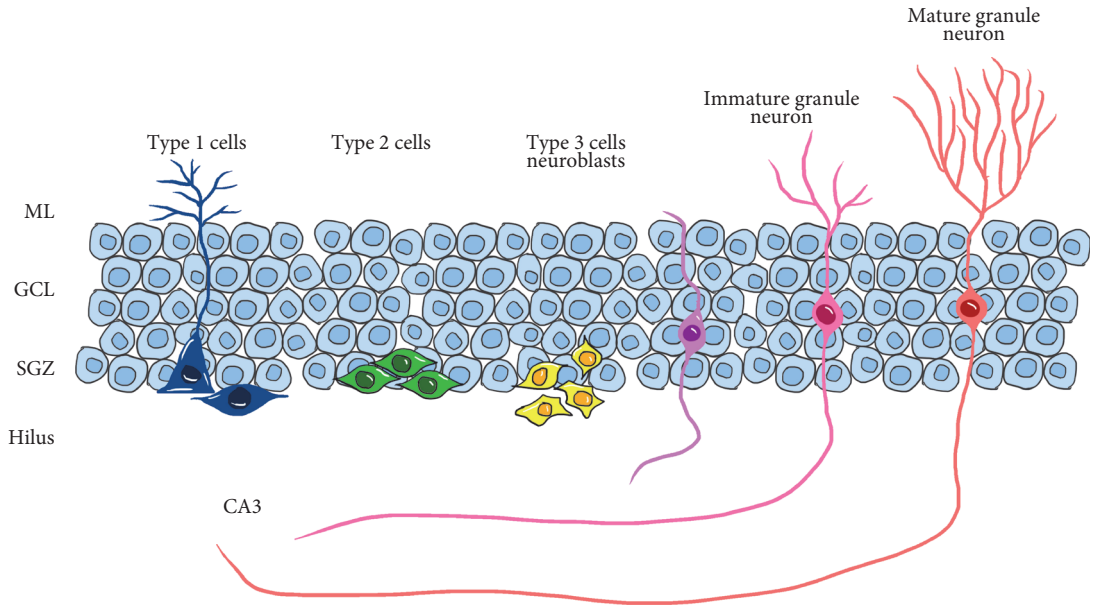

Figure 2: Neurogenesis in SGZ. The SGZ is a thin band of tissue that lies between the granule cell layer (GCL) and the hilus cells in the DG. Type 1 cells are triangular-shaped NSCs and usually extend a strong apical process into the molecular layer (ML). Type 1 cells (blue) generate type 2 cells (green). Type 2 cells are immature neuroblasts that can be further differentiating into type 3 cells (yellow). Type 3 cells progressively acquire characteristics of granule neurons. During the stage of immature (pink) to mature (red), large parts of the dendritic tree and axon elongate toward CA3.

NSCs, whereas superficial GCs and tyrosine hydroxylase $(TH)^+$ PGCs are derived from dorsal NSCs. NSCs from medial wall produce calretinin $(CalR)^+$ GCs and $CalR^+$ PGCs [21] (Figure 1).

2.1.2. SGZ. The adult neurogenic niche of the hippocampus resides in the SGZ, a thin band of cells lying between the hilus cells and the granule cell layer in the DG. NSCs first develop into radical astrocytes (type 1 cells) that, in turn, generate intermediate neural progenitors (type 2 cells). These cells are immature neuroblasts that can be further differentiated into neuroblasts. Neuroblasts can be further divided into more differentiated cells (type 3 cells) [22, 23]. Type 3 cells progressively acquire characteristics of neurons. During the stage of immature to mature, elaborate dendritic arborization grows to the middle of the molecular layer and axon elongate toward CA3 [23] (Figure 2).

Type 1 cells are located in the SGZ and have a triangular-shaped soma. A strong apical process extended into the molecular layer of DG is the typical characteristic of type 1 cells. Type 1 cells have some astrocyte features that may contact blood vessels through the end-feet [22]. Recently, another class of type 1 cells has been identified. These newly identified type 1 cells are characterized by short, horizontal processes [24]. Type 2 cells have a unique morphology that is distinct from type 1 cells: they lack the strong apical process and have a round or ovoid nucleus. Type 3 cells have variable morphologies. The processes of type 3 cells are short and the orientations alter from horizontal to vertical (Figure 2). Type 1 cells express GFAP, nestin [25, 26], BLBP, and Sox2. Type 2 cells have both neural and glial features that express neuronal (DCX and PSA-NCAM) and glial marker (nestin, BLBP) [27]. DCX, PSA-NCAM, NeuroD, and Prox1 are mainly expressed on type 3 cells.

2.2. PCD for the Regulation of Adult Neurogenesis. PCD is the death of a cell in any form, mediated by an intracellular program that mainly occurs during embryo/adult development and in some pathologic conditions [28]. The majority of adult-born neurons are eliminated by apoptosis. There are three main functions of PCD during adult neurogenesis: (1) regulate of the size of the NSC pool, (2) correct the errors during proliferation and migration, and (3) form correct synaptic contacts. The roles of all three functions are to optimize the neural system.

Neuroblasts from SVZ migrate through RMS to the OB and differentiate into GCs and PGCs. GCs are mature at 15–30 days and PGCs at 4 weeks after birth. There are around 30,000 newborn interneurons integrated into OB neural circuits daily in adult mice [10, 14, 29, 30]. However, 50% of NSCs, neuroblasts, and newborn interneurons undergo apoptosis to eliminate redundant and false connected cells. The survivals integrate into neural circuits and persist up to 19 months [30, 31]. Hippocampal NSCs proliferate and differentiate into granule neurons in the DG. In addition, about 30–70% of the newborn cells die of PCD in the first 2 weeks after birth. The remaining forms functional synapses on CA3 pyramidal neurons at 2 weeks after birth, and this projection becomes stable at 4 weeks [32]. About 4 weeks after birth, dendritic processes of newborn neurons extend toward and into the molecular layer and an axon project into the hilar area [33]. At 2 months, the number of DG neurons in $bax^{-/-}$ mice has no difference compared with that in wild-type (WT) mice, whereas at 12 months, the number of DG

neurons is doubled in $bax^{-/-}$ mice [34]. In adult humans, 700 new neurons are added to the hippocampus per day and with a continuous decline during aging [35]. These data indicate that PCD is important for the renewal of neural circuits and occurs at all stages during adult neurogenesis.

2.2.1. PCD of NSCs. Growth factors secreted in the SVZ niche are essential for the survival of NSCs. Thus, NSCs that lack neurotrophic signals are more sensitive to apoptosis stimuli. NSCs from adult $bax^{-/-}bak^{-/-}$ mice show resistance to a series of apoptotic stimuli and are accumulated in the SVZ and SGZ. *Bax* single-deficient NSCs are resistant to apoptosis induced by staurosporine [36]. While in *Mcl1* conditional knockout mice, NSCs in the SVZ are more vulnerable to apoptotic cell death. However, overexpression of Mcl-1 reduces the apoptotic rate by about 50% in NSCs from the SVZ [37]. Regarding all above results, Bcl-2 family proteins play an essential role for the apoptosis of NSCs and regulate the size of the NSC pool. In the DG, *bim* or *puma* deficiency significantly enhanced the survival of adult-born cells but have no change on NSC differentiation [38]. *Puma* deficiency also increases the survival of SVZ NSCs. Puma is required for p53-induced apoptosis in NSCs of DG [39, 40]. Besides, loss of *Trp53* enhances slow and fast proliferation in SVZ populations and associates with their differentiation toward neuronal and glial cell lines [41]. However, opposite results are found in the mice knockout *Trp53* and p53 deficiency induces apoptotic brain lesion. These *p53*-deficient mice have thinner isocotex and enlarged ventricle compared with wild-type mice [42]. Therefore, the exact role and mechanism of p53 in regulating the PCD of NSCs remain unclear. Adult hippocampal NSCs undergo autophagic cell death instead of apoptosis on deprivation of insulin [43–45].

2.2.2. PCD during Migration and Integration. Errors during migration also induce apoptosis in the adult-born neuroblasts. In *bax*-deficient mice, a large number of abnormal neuroblasts accumulate in the RMS [10]. A similar result exists in newborn cells with increased mTOR activity. Heterotopia and ectopic neuroblasts are observed in the RMS and the OB. Moreover, these heterotopia cells survive and integrate to the OB network. They have increased dendritic complexity, altered membrane biophysics, and increased frequency of GABAergic synaptic inputs [46]. However, the effects and functions of these heterotopia and ectopic survived interneurons are still unknown.

The most extensive apoptosis of newborn neurons occurs during the integration into neural circuits. 30–70% of immature neurons are eliminated by apoptosis during the formation of synaptic contacts [29, 31, 47]. This phenomenon can be interpreted by a neurotrophic hypothesis that the neurotrophic substance released for the survival of neurons is limited; thus, newborn neurons need to compete for these trophic signals [48, 49]. Competition for neurotrophic signals not only occurs between homogeneous neuroblasts and immature neurons but also is observed between immature neuron and preexisted mature neuron for new synaptic connections. Using fluorescent retrograde tracers and BrdU-labeling techniques, it is proved that newborn neurons in the DG extend axons into CA3 of hippocampus and may influence the normal hippocampal function [23, 31, 50, 51]. In $bax^{-/-}$ mice, apoptosis is inhibited in immature and mature neurons of DG, and the size of DG neurons enlarges continuously with age [34]. Synaptic connections with efferent and afferent neurons are both observed in this the DG [10]. All these results proved that the immature neurons can extend axons to mature neurons and make contacts. Thus, the apoptosis of adult-born cells is to keep the balance of mature and immature neurons and maintain the integrity of neuronal circuits [52]. However, some opposite data have shown that in *bax*-deficient mice, the pattern separation function of the hippocampus is enhanced. However, knockout *bax* seems to have no effects on other major hippocampal functions [53]. It seems that pattern separation is regulated by immature DG neurons. Other hippocampal functions, such as aligning internal spatial representation to external landmarks, are mediated by mature DG neurons [54]. A similar phenomenon has also been found in the cell replacement of OB. In $bax^{-/-}$ mice, the normal olfactory learning behavior is improved, and the perturbations of newborn cell migration result in imbalance of neural circuits that destroy the olfactory learning ability. Besides, the *bax*-deficient mice show no significant changes on olfactory sensation [55, 56]. Based on above results, we may conclude that the immature neurons and mature neurons have different roles in neural circuits, and apoptosis is the key regulator that keeps the balance of adult-born neurons and the preexisting ones.

2.2.3. PCD of Mature Neurons. Although the majority of cell types that undergo PCD are immature neurons and neuroblasts, mature neurons also have lower levels of PCD in the OB and DG. The purpose of PCD in mature neurons is to renew the preexisting neural circuits [30, 57]. At about 15–30 days after birth, newborn cells differentiate into mature neurons in the OB. Thereafter, about 50% newborn neurons undergo apoptosis. Cells that survive the first 3 months persist up to 19 months [31]. One fourth of the DG neurons born at the peak of DG development on postnatal day 6 died in the first 1 to 6 months [58]. The production of adult-born neurons and elimination of mature neurons are critical for the maintenance of a constant number of neurons in DG and for the regulation of hippocampal functions [34].

3. AN and Stroke Recovery

Adult NSCs in neurogenic regions can be activated by different stimuli such as learning [59] and running [60] and also can be activated in the disease processes including seizure [61], mechanical lesions [62], and ischemic insult [63]. These results raised the possibility that functional deficits induced by stroke may be cured through neuronal replacement by endogenous NSCs. In well-studied rodent models of stroke, cerebral ischemia and hemorrhage have been shown to stimulate proliferation of endogenous progenitor cells and differentiate into neural system cells, including neurons, astrocytes, oligodendrocytes, and ependymal cells [64]. Evidence for stroke-activated neurogenesis has also been reported in the stroke patients [65]. Accumulating evidence

has convincingly demonstrated that stroke-induced neurogenesis in SVZ and SGZ and other noncanonical stem cell niches have also been confirmed in the adult brain.

3.1. Classical Neurogenic Niches after Stroke: SGZ and SVZ. In models of transient global cerebral ischemia, cerebral blood flow is reduced throughout the whole brain [66]. The hippocampus CA1 area plays an important role in cognitive processes such as learning and memory and is more sensitive to hypoxia-ischemia insults than other areas of the brain [67, 68]. Remarkable increased progenitor proliferation in hippocampal SGZ has been observed in many species, such as mice, rats, gerbils, and monkeys, after global cerebral ischemia [69–71]. Liu et al. first reported increased hippocampal neurogenesis after transient global ischemia in gerbils in 1998 [63]. Newborn cells with neuronal features were first seen 26 days after ischemia, migrated from the SVZ to the granule cell layer, and survived for at least 7 months [63]. Since this initial publication, many follow-up studies have confirmed stimulation of neurogenesis in the SGZ across various species of global ischemia [69–71]. Nakatomi et al. further revealed that ischemia-induced adult neural progenitors in DG can replace CA1 pyramidal neurons form functional synapses and integrated into the existing brain circuitry [72]. Tanaka and his colleagues visualized that the neuronal progenitor cells in the DG proliferated, migrated, and differentiated into mature neurons by retroviral vector expressing enhanced green fluorescent protein (EGFP) [73]. Increased NSC proliferation has also been reported in the SVZ following global ischemia [74]. Promoting endogenous neurogenesis in SGZ may contribute to replace the CA1 neuron loss and improve function recovery after global ischemia. However, it is also worth to point that some studies cannot reproduce the evidence that SGZ neural stem cell migrate into CA1 as previously reported by Nakatomi and coworkers. In contrast, the CA1 area merely displays gliogenesis [71, 75].

In focal brain ischemia, middle cerebral artery occlusion (MCAO) is the most frequently used focal brain ischemia model, which produces consistent infarcts in the ipsilateral hemisphere of the cerebral cortex, hippocampus, and striatum [76]. Neurogenesis was increased bilaterally in both SVZ and SGZ after unilateral MCAO, indicating that endogenous neuronal precursors might be in response to contralateral ischemia as well [77]. The vast majority of adult neurogenesis in mammalian species occurs within the SVZ. SVZ are a paired brain structure situated throughout the lateral walls of the lateral ventricles. Significant enhanced proliferations of NSCs in the SVZ were observed in the first 7–14 days after MCAO in mice [78] and rats [77, 79, 80]. A fluorescent tracing of proliferating cells in the SVZ showed that these cells directly migrate from birth site to striatum in the post-MCAO rat brain [81]. In the normal brain, most of the SVZ neuroblasts migrate through the RMS into the OB and differentiate into interneurons. Ischemia may revoke the normal migratory pattern of SVZ NSCs and lead these cells to migrate toward the injured areas and aid in spontaneous recovery [82]. In the damaged striatum, neuroblasts were continuously generated from SVZ precursors as early as 1 week and last to 16 weeks after insult [82]. The SVZ was the principal source of the neuroblasts migrated laterally toward the injured striatal regions and integrated into neuronal networks receiving synaptic input and firing action potentials after MCAO [83, 84]. Inspiringly, Kreuzberg and his colleagues found MCAO also induced SVZ-derived neuroblasts migrated to the cortex, differentiated into mature neurons, and survived for at least 35 days [78]. These results highlight the role of the SVZ NSCs in neuronal regeneration after focal cerebral ischemia and its potential as a new therapeutic target for various neurological disorders.

3.2. Adult Neurogenesis from Noncanonical Sites. Above studies have shown convincing evidence of neuroblasts migrating from the SVZ or SGZ to the ischemic areas. However, several studies have proposed the possibility that there exist other stem cell niches in the adult brain [85, 86]. In fact, this noncanonical site of adult NSCs has been found in rodent striatum [87], hypothalamus [88], neocortex [87], amygdala [87], substantia nigra [89], and brainstem [90]. In addition, endogenous brain repair occurs in, but not restricted to, neurogenic regions. It is reported that astrocytes surrounding the infarct core lesion can be activated to generate neurons [91, 92]. Pericytes and OPCs have also been reported to differentiate into neurons following brain injury [93]. These data indicate that stem cell niches are much extensive. These existed multiple neurogenesis sites may be important for brain repair after injury [94].

4. Targeting AN as Therapeutic Strategy for Stroke

AN has arisen great interest as it can be applied for new therapies to replace damaged neurons and treat severe neurological deficits after stroke and other neurological diseases. However, the endogenous neurogenesis failed in producing adequate amounts of newborn neurons that can survive and integrate to restore the function recovery. Stimulating or enhancing the endogenous neurogenesis driven by ischemia can be a promising therapeutic intervention for stroke. The vast majority of the newborn neurons die between 2 and 5 weeks, which may be caused by the unfavorable environment that is exposed to the detrimental injury niche, lacking appropriate trophic support and failed connections with other neurons after stroke [95]. Reducing endogenous toxic substances, inhibiting inflammatory responses, and promoting the release of growth factors and neurotrophic signals have been suggested as effective manipulations to improve AN.

A wide variety of signaling pathways are related to NSC activity during proliferation, migration, differentiation, and their maintenance in the adult neurogenic regions. As survival is the first factor of newborn neurons, neuroprotective agents or manipulations attempt to benefit from neuronal survival preserving the property to support neurogenesis in long term [96]. Administration of EPO (5 U/g) significantly preserves hemispheric brain volume 6 weeks after stroke and directs cell fate toward neurogenesis and away from gliogenesis [97]. The PI3 kinase/Akt pathway showed to play an

important role in neuronal survival as well as adult neurogenesis. Mutation that produces constitutive activation of the Akt pathway through PTEN deletion induces a robust increase in poststroke neurogenesis [98]. Studies that targeted NOTCH, WNT, and sonic hedgehog (SHH) signaling showed the great potential of these approaches in stroke treatment [99–102]. In addition, many growth factors have been identified to protect NSCs and enhance neurogenesis after stroke. Basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), BDNF, bone morphogenetic protein (BMP), glial cell-derived neurotrophic factor (GDNF), transforming growth factor- (TGF-) α, ciliary neurotrophic factor (CNTF), and platelet-derived growth factor (PDGF) have all been proposed to play essential roles in the adult neurogenesis response to ischemia stroke [103–111]. Recently, some cytokines and hormones such as chemokine, complement, estrogen, and granulocyte-colony stimulating factor (G-CSF) have been proved to benefit against a stroke-induced brain and behavioral pathology [112–116]. The chemokine stromal-derived factor 1 (SDF1) is induced in peri-infarct blood vessels and serves as a tropic signal for migrating neuroblasts to localize to the ischemic area. Administration of SDF1 improves poststroke neuroblast migration and behavioral recovery [114]. Though great progress has been made, the search for strategies and pharmacological agents to enhance endogenous neurogenesis despite a detrimental milieu remains a challenge and is the focus of intense investigation.

5. Concluding Remarks

Majority of stroke patients suffer from serious morbidity and never regain full functional independence. The limited result of stroke treatment has driven the search for stem cell therapies directed at restoring neurological function. However, both technical and ethical issues limit the development of exogenous stem cell therapy [4]. The finding of endogenous neural stem cells in the mammalian brain is a breakthrough and provides a promising approach to repair the damaged lesion after stroke. Great efforts have been made to augment the innate neurogenic capacity of the adult brain, including increasing the survival of NSCs in the neurogenic regions, strengthening their mobilization, and integrating into damaged neural circuits. However, there are issues raised about the AN after stroke. First, little is known regarding the intrinsic properties and the modulation of the NSC fate. Additional research is needed to identify the NSC fate determinants, which modulate the differentiation of NSCs toward specific cell types. Second, the integration of newborn neurons into preexisting neural circuits and the related functional recovery should be studied and improved in future researches [117]. Third, most animal studies of stroke are performed in young adult animals; however, human stroke most frequently occurs in aged patients. Neurogenesis both in the SVZ and SGZ drops precipitously with age, and the effects of age on AN should be considered. Forth, for efficient repair, it may be necessary to provide endogenous and/or graft new cells to form synthetic extracellular matrix so that they can reform appropriate brain structure [118]. The discoveries reported in this review may pave the way for targeting AN as future therapeutic interventions for stroke as well as other central nervous system diseases.

Conflicts of Interest

The authors declare there is no conflict of interest regarding the publication of this paper.

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Review Article

Neuroprotective Effects of Stem Cells in Ischemic Stroke

Weilin Xu,[1] Jingwei Zheng,[1] Liansheng Gao,[1] Tao Li,[1] Jianmin Zhang,[1,2,3] and Anwen Shao[1]

[1]*Department of Neurosurgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China*
[2]*Brain Research Institute, Zhejiang University, Hangzhou, Zhejiang, China*
[3]*Collaborative Innovation Center for Brain Science, Zhejiang University, Hangzhou, Zhejiang, China*

Correspondence should be addressed to Jianmin Zhang; zjm135@vip.sina.com and Anwen Shao; anwenshao@sina.com

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Ischemic stroke, the most common subtype of stroke, has been one of the leading causes of mobility and mortality worldwide. However, it is still lacking of efficient agents. Stem cell therapy, with its vigorous advantages, has attracted researchers around the world. Numerous experimental researches in animal models of stroke have demonstrated the promising efficacy in treating ischemic stroke. The underlying mechanism involved antiapoptosis, anti-inflammation, promotion of angiogenesis and neurogenesis, formation of new neural cells and neuronal circuitry, antioxidation, and blood-brain barrier (BBB) protection. This review would focus on the types and neuroprotective actions of stem cells and its potential mechanisms for ischemic stroke.

1. Introduction

Stroke has been the second most common disease to cause death and disability in adults around the world [1]. Ischemic stroke, which accounts for about 87% of cases, is the most common subtype of stroke [2]. Ischemic stroke is the result of insufficient blood and oxygen supply to the brain. The cell in the central portion of the ischemic tissue, known as infarct core, is afflicted with irreversible damage, and the area around the infarct core, called penumbra, is at risk of infarction and can be reverted [3]. Various causes could contribute to this pathophysiology process, such as cerebral artery stenosis, occlusion, and rupture, and eventually induced acute cerebral blood circulation disorders. It was reported that females were less likely to suffer from ischemic stroke for both the reasons of sex steroids and biologic sex [4].

The pathological mechanism in the process of cerebral ischemia and neuroprotective effects of various drugs have been comprehensively studied throughout these years, which consists of cellular apoptosis, inflammation, oxidative stress, brain edema, and BBB interruption [5, 6]. Currently, the most common strategy used for studying the pathophysiology process and selecting potential efficacious drugs was the occlusion model of the middle cerebral artery (MCAO) in mice or rats [7]. However, treatment options to date are very limited. Stem cell therapy, with its vigorous advantages, has attracted researchers around the world.

Stem cells are defined as clonogenic cells that own the capacity to self-renew and differentiate into multiple cell lineages [8]. The application of stem cells in treating multiple diseases has been present for decades, and human stem cell transplantation therapy is now a well-established treatment for various malignant and nonmalignant hematological diseases and some autoimmune disorders [9, 10]. In the past decade, the benefits of stem cells in treating ischemic stroke have been experimentally demonstrated [11]. Low incidence of adverse effects and vast therapeutic value earn great attractions.

The basic principle of stem cell therapy of cerebral ischemia is to replace ischemic tissues in an organotypic appropriate manner. Replacement of lost neurons could rebuild the neuronal circuitry. Replacement of glial cells like astroglia or oligodendroglia could regain proper nerve conduction. Moreover, the transplantation of exogenous stem cells could also provide trophic support to tissue at risk in the penumbra surrounding the infarct area [12, 13]. Moreover, the stem cells could exert its neuroprotective effects through anti-inflammation, antiapoptosis, antioxidative, blood-brain barrier protection, promotion of angiogenesis, and promotion of neurogenesis [14, 15].

This review would focus on the types of stem cells and neuroprotective actions of stem cells and its potential mechanisms for ischemic stroke.

2. Stem Cell Types for Treating Ischemic Stroke

2.1. Exogenous Stem Cells

2.1.1. Embryonic Stem Cells (ESCs). ESCs are obtained from blastocysts in the early stage with the capacity of totipotent and unlimited self-renew. They could translate into various types of cells in the central nervous system (CNS), which makes the ESCs one of the most promising stem cells in treating ischemic stroke. The ESCs were firstly isolated and reported by Evans and Kaufman and Bremnes et al. in vivo and vitro, respectively [16, 17]. Numerous studies have demonstrated that neurons stemmed from ESCs could harmonize with the cells of receptors, which also verified the underlying efficacy of ESCs. However, some factors limited the widespread application of ESCs: (1) the availability of ESCs due to ethical concerns about the use of unwarranted embryos, (2) the risk of tumorigenicity, such as teratoma, (3) cell conservation, and (4) immune reaction after transplantation.

2.1.2. Hematopoietic Stem Cells (HSCs). HSCs could be isolated from bone marrow or umbilical blood. It has been widely studied in the treatment of ischemic stroke. Taguchi and his colleagues demonstrated that administration of CD34+ cells could enhance neovascularization in the ischemic zone and thus promote neurogenesis in mice 48 hours after ischemic stroke, and reduce the infarct area [18]. Besides, peripheral blood hematopoietic stem cell (CD34+), which was directly intracerebral implanted, was observed to differentiate into glial cells, neurons, and vascular endothelial cells. They could also enhance the angiogenesis and neurogenesis [19]. In addition, the HSCs could be used in both autologous and allogeneic transplantations without ethical problems. However, the application of HSCs owns its disadvantages of consistency of number and potency of HSCs, especially obtained from umbilical cord blood.

2.1.3. Neural Stem Cells (NSCs). Exogenous NSCs were mainly obtained from the embryo or the fetus. The NSCs have the capability to differentiate into glial and neurons, thus exert its neuroprotective effects for the patients. Besides, the NSCs have no risks of tumor tumorigenicity [20, 21]. Mack demonstrated that transplantation of exogenous NSCs could significantly improve the neurological functions with little transplantation-related toxicity [22]. However, most of the studies regarding the exogenous NSCs were restricted to experimental stroke models due to the potential ethical issues. Another severe limitation of exogenous NSCs was that only a small number of cells could survive after transplantation.

2.1.4. Mesenchymal Stem Cells (MSCs). The MSCs could be isolated from bone marrow, adipose tissue, umbilical cord blood, and peripheral blood. They could also be used both in autologous and allogeneic transplantation. Many studies have shown that transplanted MSCs could secrete cytokines and growth factors, which could enhance the process of angiogenesis and neurogenesis, and subsequently improve the neurological functions [23–26]. Besides, the MSCs were reported to reduce the cellular apoptosis by downregulating the expression of caspase-3 [27]. Moreover, autologous application could avoid immune reactions without ethical problems. All the advantages abovementioned lead to the wide study of MSCs. However, the cell culture of MSCs to generate sufficient numbers requires several weeks, which limited its use in acute phage of ischemic stroke.

2.1.5. Others. Induced pluripotent stem cells (iPSCs) has been widely studied since the technique was developed by Yamanaka and his colleagues in 2006. The iPSCs were observed to differentiate into several types of neural cells and could express neuronal specific markers. Besides, the iPSCs could also reduce the infarct areas and improve neurological functions in experimental models [28, 29]. What is more, the iPSCs have no concerns regarding immune reaction, ethical issues, and the source of the stem cells. However, some critical techniques remain to be resolved before it could be extensively used, such as low efficiency of reprogramming and underlying tumorigenicity. In addition, NT2N immortal cell lines have also been reported in treating ischemic stroke.

2.2. Endogenous Neural Stem Cells (NSCs).

Mature neurons were once considered to lose the capability of regeneration. However, more and more studies have shown that NSCs existed in the subventricular zone (SVZ) and the subgranular zone of the dentate gyrus [30–32]. In normal conditions, the endogenous NSCs are under dormant state. They could be activated by ischemic attack and could migrate to the infarct areas to replenish the lost cells. Sharp et al. found that the NSCs in bilateral dentate gyrus increase after experimental cerebral ischemic models [33, 34]. However, the number of endogenous NSCs activated by ischemic stroke was so limited that their neuroprotective role was always unsatisfied.

3. Neuroprotective Properties of Stem Cells in Ischemic Stroke

The neuroprotective effects of stem cells in ischemic stroke have been widely studied, and the mechanism involved in this effect includes antiapoptosis, anti-inflammation, promotion of angiogenesis and neurogenesis, formation of new neural cells and neuronal circuitry, antioxidation, and BBB protection. The following review will particularly probe into these molecular mechanisms.

3.1. Replacement of Damage Tissues and Formation of New Neuronal Circuitry. The most direct way in restoring neurological functions is to replace damaged tissues with new differentiated neural cells from stem cells. The MSCs could be induced to differentiate into neural cells by epithelial growth factor and BDNF in vivo. Besides, Ishibashi and his colleagues demonstrated that the totipotent stem cells could transfer to the ischemic areas, differentiate into mature neurons, and form new neural circuity, which could thus improve the neurological functions [35]. However, some

researchers doubted the efficacy of this effect. In their opinion, the stem cells differentiated into new neural cells were mainly in vivo in specific cultures. The cells transplanted to the brain may exert its neuroprotective effects in other mechanisms because only a small number of cells were tracked surrounding the ischemic areas [36, 37]. Therefore, more studies regarding the roles of stem cells in replacing damaged tissues and forming new neuronal circuitry should be launched.

3.2. Promotion of Angiogenesis. For treating ischemic stroke, focus should not only be placed on the regeneration of neural cells, but should also be placed on the supporting tissues, such as blood vessels. Angiogenesis was augmented after stroke. Zhang and his colleagues studied the structural changes after stroke, and they found that vascular volume was increased from 3% prior to stroke to 6% at 90 days after stroke [38]. Angiogenesis has also been observed after transplantation of stem cells surrounding infarcted areas. The main mechanisms of action were the increase of vascular endothelial growth factor (VEGF) or the level of other endogenous factors, like brain-derived neurotrophic factor and fibroblast growth factor. In addition, stem cells could also exert its role in angiogenesis by regulating the expression of Notch 1, angiopoietin-1, angiopoietin-2, and so on [39, 40].

3.3. Promotion of Neurogenesis. Endogenous neurogenesis, the process of self-repairing, is increased after ischemic attack. Neurogenesis is necessary for the neurological recovery for patients with cerebral ischemia [41]. Jeong and his colleagues showed that mesenchymal stem cells not only reduced the apoptosis cells but also assisted in enhancing the endogenous neurogenesis by expressing the brain-derived neurotrophic factor (BDNF) [42]. Besides, Zhao et al. found that electroacupuncture (EA) treatment could activate endogenous NSC and neurogenesis in the dentate gyrus of rats [43]. In addition, MSCs were reported to ameliorate neurological deficit if rats by modifying cerebral plasticity through neurotrophic effect and forming new synapses with host brain [44].

3.4. Anti-Inflammatory Effects of Stem Cells. Inflammation is a complex immune response of organisms to the injury. Under normal condition, the inflammation could help to scavenge the necrotic cells or tissues and initiate the tissue repair process [45]. However, excessive activation of immune responses is harmful to the organisms and can cause injury [46]. Stem cells had exerted its double roles in regulating inflammation by upregulating anti-inflammatory cytokines and attenuating the expression of proinflammatory cytokines. Many studies have demonstrated that stem cells could reduce the expression of IL-1β, IL-6, and TNF-α in the early stage of cerebral ischemic attack. Some researchers also suggested that the delivery of stem cells should be in the early stage as the inflammation reactions would gradually attenuate with the time going on. Besides, Zhu et al. demonstrated that human umbilical cord blood mesenchymal stem cells (hUCB-MSCs) could upregulate the expression of IL-10 and downregulate the IL-1β, IL-6, and TNF-α in the peri-ischemic brain tissues at the same time [47]. In addition, allotransplantation could suppress the immune reaction of the receptors. Vendrame and his colleagues showed that HUCBCs decreased inflammation in the brain after stroke partly by suppressing T cells [48].

3.5. Antiapoptotic Effects of Stem Cells. Apoptosis is one type of cell death characterized by energy dependence and programmed cell death [49]. The term "apoptosis" was first described by Kerr et al. [50]. Apoptosis is of vital importance to the normal physiological metabolism, growth, and development, keeping hemostasis by scavenging the aging or damaged cells, shaping of organs, or regulating the immune system by the removal of defective and excessive cells [51, 52]. However, uncontrolled apoptosis may result in various pathological processes of different diseases, like cancers, Alzheimer's disease, and stroke [53, 54]. The effect of antiapoptosis of stem cells for ischemic stroke has been extensively verified in experimental models. This effect may be mediated by modulating the expression of some trophins, like fibroblast growth factor, brain-derived neurotrophic factor, VEGF, glial cell line-derived factor, and nerve growth factor. Zhang and his colleagues showed that NSC transplantation could significantly reduce the number of apoptotic cells in the penumbra at 7 days by upregulating the expression of Bcl-2 [55]. Besides, Zhu and his colleagues also verified the antiapoptotic effect of hUCB-MSC for ischemic stroke around the ischemic region [47]. In addition, Li demonstrated that BMSCs transplantation could upregulate Livin protein, downregulating caspase-3 protein, thus reducing the apoptosis of neural cells [56]. The antiapoptotic effect was associated with the neurological recovery in experimental models [57, 58].

3.6. Others. Except for the common mechanisms abovementioned, several studies have also suggested other effects of stem cells in ischemic stroke. Tang and his colleagues showed that MSCs could protect BBB integrity by downregulation of aquaporin-4 expression via p38 signaling pathway after ischemic attack [59, 60]. Borlongan et al. found that the rats that received bone marrow stromal cells transplantation were more likely and earlier to restore the BBB and CBF [61]. Besides, Calió and his colleagues also demonstrated that transplantation of bone marrow MSCs could significantly decrease oxidative stress [62].

4. Some Issues in Controversy

4.1. Optimum Timing of Treatment. Stem cells therapy has been shown to be efficacious in treating ischemic stroke. However, the optimum timing for cells delivery has not been determined. The patients could benefit from early delivery of stem cells if the aim was to activate endogenous repair mechanism and inhibit apoptosis as this process mainly occurred during the first weeks after stroke. Park and his colleagues found that the active substances secreted by the transplanted cells exerted their neuroprotective effects only in the early 3–7 days postischemia. After that, the stem cells would keep silence functionally [63]. The results from de Vasconcelos

Dos Santos et al. also suggested that transplantation of bone marrow MSCs was observed to exert its function in the first week with utmost efficacy on the first day [64]. However, patients would benefit from a later timescale if the aim of transplantation was to directly replace the infarcted tissues and rebuild new neuronal circuitry as the oxidative stress and inflammation have faded away. The optimum timing of stem cell delivery is in controversy all the time. Several studies in comparison of delivery in the acute stage and chronic stage have gained conflicting results [65, 66]. So, more studies regarding the optimum timing of treatment are warranted.

4.2. Cell Numbers to Be Given. The cell number to be given has not been determined. It varied according to different types of stem cells, routes of delivery, and the characteristics of the patients. Excessive amounts of cells could increase the risks of tumorigenicity and thrombosis of blood vessels. No therapeutic effect could be achieved with too small number of cells. There were few clinical studies regarding the number to be given currently. Further researches should focus on this issue before stem cell therapy could be extensively clinically used.

4.3. Optimum Routes of Delivery. The route of stem cells delivery varied across different studies, including intracerebral implantation, intravenous route, and intra-arterial route [67]. Intracerebral implantation could directly accumulate stem cells in the infarct areas and achieve more vigorous neuroprotective effects for the patients with ischemic stroke. However, the disadvantages of intracerebral implantation are obvious that invasive operations would inevitably disrupt normal brain tissues, which could do more damages to patients [68]. The intravenous route is the easiest and safest way for the treatment and do little secondary injury to patients [69]. However, only a small number of cells could reach the brain, and thrombosis may occur after intravenous delivery of stem cells. The intra-arterial route could delivery stem cells more directly than the intravenous route and do less injury to the patients than intracerebral implantation. However, the occlusion of the artery may affect the delivery of stem cells with the route of intracarotid administration [24]. The best route of delivery has not been determined. The decision regarding the route of delivery should take several issues into account, such as safety, practicality, cell type, and the aim of treatment.

4.4. The Safety of Stem Cells. As numerous studies have demonstrated the efficacy of stem cells in treating ischemic stroke, some issues regarding the safety could not be ignored. A rare but serious side effect of stem cell treatment was its potential of tumorigenicity [23]. Miura and his colleagues found that murine bone marrow-derived mesenchymal stem cells (BMMSCs), after numerous passages, obtained unlimited population doublings and proceeded to a malignant transformation state, resulting in fibrosarcoma formation in vivo. Its potential mechanism involved accumulated chromosomal abnormalities, gradual elevation in telomerase activity, and increased c-Myc expression [70]. Besides, the mechanism of extracting, preparation and administration of the cells could also cause some risks of diseases, such as immune reactions and vascular thrombosis [71]. A meta-analysis regarding the safety of stem cell therapy was performed by Jeong et al. in 2014, the results of the pooled safety analysis showed that the incidence rates of death, seizure, and infection were 13%, 15%, and 15%, respectively [72]. So, the trials in the future should not only focus on the efficacy but also on the reduction of the incidence of adverse effects.

5. Conclusions and Perspective

Overall, numerous experimental researches in animal models of stroke have demonstrated the promising efficacy effects of varied types of exogenous and endogenous stem cells in treating ischemic stroke. The underlying mechanism involved antiapoptosis, anti-inflammation, promotion of angiogenesis and neurogenesis, formation of new neural cells and neuronal circuitry, antioxidation, and BBB protection. Significant efficacy with low incidence of adverse effects indicates the vast potential therapeutic value of stem cells in the treatment of ischemic stroke. However, some important issues, such as optimum timing of treatment, dosage, optimum routes, and some rare but serious adverse effects, have not been resolved. Further studies, both preclinical and clinical studies are warranted for an effective, feasible, and safe cell-based therapy.

Conflicts of Interest

The authors declare no competing financial interests.

Authors' Contributions

Anwen Shao and Jianmin Zhang have equally contributed to this work as cocorresponding authors.

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