Experimental Neurology 313 (2019) 1–9

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research paper

Repetitive transcranial magnetic stimulation promotes functional recovery and differentiation of human neural stem cells in rats after ischemic stroke

Jiao-Jiao Peng, Rong Sha, Ming-Xing Li, Lu-Ting Chen, Xiao-Hua Han, Feng Guo, Hong Chen*, Xiao-Lin Huang*

Department of Rehabilitation Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

ARTICLE INFO

Keywords:
Human neural stem cells
Ischemic stroke
Repetitive transcranial magnetic stimulation
Transplantation
Functional recovery
Brain-derived neurotrophic factor
Differentiation

ABSTRACT

Stem cells hold great promise as a regenerative therapy for ischemic stroke by improving functional outcomes in animal models. However, there are some limitations regarding the cell transplantation, including low rate of survival and differentiation. Repetitive transcranial magnetic stimulation (rTMS) has been widely used in clinical trials as post-stroke rehabilitation in ischemic stroke and has shown to alleviate functional deficits following stroke. The present study was designed to evaluate the therapeutic effects and mechanisms of combined human neural stem cells (hNSCs) with rTMS in a middle cerebral artery occlusion (MCAO) rat model. The results showed that human embryonic stem cells (hESCs) were successfully differentiated into forebrain hNSCs for transplantation and hNSCs transplantation combined with rTMS could accelerate the functional recovery after ischemic stroke in rats. Furthermore, this combination not only significantly enhanced neurogenesis and the protein levels of brain-derived neurotrophic factor (BDNF), but also rTMS promoted the neural differentiation of hNSCs. Our findings are presented for the first time to evaluate therapeutic benefits of combined hNSCs and rTMS for functional recovery after ischemic stroke, and indicated that the combination of hNSCs with rTMS might be a potential novel therapeutic target for the treatment of stroke.

1. Introduction

Stroke, one of the most common cerebrovascular disease, induces long-term neurological disability (Feigin et al., 2014). Early systemic thrombolysis is the current treatment of ischemic stroke (Grossman and Broderick, 2013). Following the acute phase, no effective and available treatments exist for survivors except physiotherapy. Nevertheless, the functional recovery is incomplete after receiving systematic physiotherapy (Knecht et al., 2011). Therefore, there is an urgent need to develop alternative therapies for ischemic stroke.

Stem cell transplantation is promising and attractive for neural repair following stroke. Several types of cells such as bone marrow cells (Yoo et al., 2015), pluripotent stem cells (Emborg et al., 2013), umbilical cord blood cells (Cui et al., 2012), and neural stem/precursor cells (Doeppner et al., 2015) have been used for transplantation as cellular-based therapy. We mainly focused on the efficacy of human neural stem cells (hNSCs). Following transplantation into the rodent stroke models, hNSCs have been demonstrated to recover neurological function (Abeysinghe et al., 2015; Haus et al., 2016; Yuan et al., 2013). Numerous studies have shown that transplanted hNSCs not only substitute lost cells and integrate into neural circuits (Weick et al., 2011), but also promote tissue repair by alleviating inflammation, secreting neurotrophic factors, promoting neurogenesis, and so on (Oki et al., 2012; Ryu et al., 2016; Watanabe et al., 2016). To apply stem cell therapy to clinic, the neural derivatives of human embryonic stem cells (hESCs) showed a great potential for treating ischemic injury. Moreover, hESCs are pluripotent cells and can be differentiated into a great number of neural cells (Zhang et al., 2001). Here, neural precursors differentiated from hESCs were used for transplantation.

Repetitive transcranial magnetic stimulation (rTMS) induces an electrical current in the nerve tissue and stimulates the human brain by producing an electromagnetic field. Both clinical and pre-clinical studies indicated that rTMS has therapeutic effects on a variety of nervous system disorders, such as trauma, stroke, depression, and Parkinson's disease (Rossi et al., 2009; Tang et al., 2017). Its therapeutic effects may be mediated by synaptic plasticity, increasing neurotrophic factors as well as enhancing neurogenesis (Chervyakov et al., 2015). Although the underlying mechanism has not been yet fully elucidated, rTMS has advanced to clinical trials as a noninvasive neuroprotectant in ischemic stroke (Du et al., 2016).

* Corresponding authors.
E-mail addresses: chenhong1129@hotmail.com (H. Chen), xiaolinh2006@126.com (X.-L. Huang).

https://doi.org/10.1016/j.expneurol.2018.12.002
Received 5 September 2018; Received in revised form 22 November 2018; Accepted 3 December 2018
Available online 04 December 2018
0014-4886/

NSCs also locate in subventricular zone (SVZ) and subgranular zone (SGZ) in adult mammals apart from the embryonic brain (Gage, 2000). Promotion of endogenous neurogenesis is one goal of stroke rehabilitation to support tissue repair and functional restoration after brain damage (Bellenchi et al., 2013). Our previous studies showed that treatment of stroke with rTMS promoted neurogenesis (Guo et al., 2014). Luo et al. reported that the underlying mechanism is likely through activating brain-derived neurotrophic factor (BDNF)/tropomyosin-related kinase B (TrkB) signaling pathway (Luo et al., 2017), both of which improved brain functional recovery. Interestingly, a previous study showed that human-derived neuron-like cells could respond to rTMS in vitro (Hellmann et al., 2012). However, there is no study to evaluate therapeutic effects of combined treatment of rTMS and hNSCs for stroke rehabilitation. We hypothesized that combination of hNSCs with rTMS would result in greater improvements in motor function in rats after ischemic stroke, and that combined effect was mediated by enhanced neurogenesis and activation of the BDNF-TrkB signaling pathway. Whether rTMS could affect hNSCs has still remained unclear in vivo, hence, we investigate the effects of rTMS on hNSCs in this study.

2. Materials and methods

2.1. hESCs culture and neural differentiation

The hESCs (H9, passages 25–55; WiCell Research Institute) were grown on a feeder layer of irradiated mouse embryonic fibroblasts (MEFs) and cultured in defined medium: DMEM/F12 (Gibco, 11330-032) with 20% knockout serum replacement (Gibco, 10828028), 1% Non-essential amino acids (NEAA; Gibco, 11140050), 1% GlutaMAX (Gibco, 35050061), 0.1 mM β-Mercaptoethanol (Sigma-Aldrich, M3148), and 20 ng/ml basic fibroblast growth factor (PeproTech, 100-18B). Cells were digested by dispase (Roche, LD4693) and passaged every 5 days. The cultures were free of mycoplasma throughout the experiment.

The neural differentiation was performed using the dual SMAD inhibition differentiation protocol, as previously described (Chambers et al., 2009). In brief, the hESCs medium (1 day after passing) was replaced with neural differentiation medium, including DMEM/F12: neurobasal medium (1:1; Gibco, 21103049), 1 × GlutaMAX, 1 × NEAA, 1 × N_2 (Gibco, 17502048) and 1 × B_{27} (Gibco, 12587010) in presence of 2 μM SB431542 (Stemgent, 040010), 2 μM DMH1 (Tocris, 4126) for 6 days. The medium was changed every other day and hESCs were induced into neuroepithelial cells. On the 6th day, the neuroepithelial cells were dissociated and split at 1:2 with the same medium. After 12 days, the neural rosettes were mechanically detached by a 1 ml pipette and expanded as free-floating neurospheres for 6 days. For in vitro analysis, the neurospheres were dissociated by Accutase (Innovative Cell Technologies, AT-104) and plated onto poly-ornithine/laminin-coated coverslips in the neurobasal medium supplemented with 1 × B_{27} and a set of neurotrophic factors including 10 ng/ml BDNF (PeproTech, 450-02), 10 ng/ml glial-derived neurotrophic factor (GDNF; PeproTech, 450-10), 10 ng/ml insulin-like growth factor 1 (IGF1; PeproTech, 100-11), and 1 μM cAMP (Sigma-Aldrich, D-0260).

2.2. Animals and middle cerebral artery occlusion (MCAO) model

Male Sprague-Dawley (SD) rats, weighing 230–270 g, (SPF grade; Center of Experimental Animals, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) were used in the study and maintained at 25 ± 2 °C under a 12 h light/dark cycle with food and water ad libitum. All procedures were approved by Animal Care and Use Committee of the Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. The model of cerebral ischemia was generated by transient right MCAO as previously described (Daadi et al., 2009). Besides, reperfusion was initiated by filament removal after 60 min. All rats suffered from the injury of MCAO, and then were allowed for recovery. Animals (n = 11–15 rats/per group) were randomly divided into four groups as follows: (i) vehicle group, ischemic rats were injected medium; (ii) hNSCs group, ischemic rats were injected hNSCs; (iii) rTMS group, ischemic rats received rTMS treatment following medium injection; (iv) hNSCs + rTMS group, ischemic rats were given hNSCs and rTMS treatment.

2.3. Cell transplantation

Differentiated forebrain hNSCs in suspension were enzymatically dissociated with Accutase and prepared at approximately 100,000 cells/μl in medium. Rats were anesthetized and placed in a stereotactic apparatus 4 days after MCAO. The skull was exposed and a small hole was drilled on the hemisphere. A total of 2.5 μl of cell suspension was transplanted into the striatum site (anterior-posterior (AP) = +1.0 mm, medial-lateral (ML) = +2.0 mm, dorsal-ventral (DV) = −5 mm), using a tipped and sterile glass pipette connected to a microsyringe. The needle was left for 5 min before removal. All rats were injected intraperitoneally with 10 mg/kg of Cyclosporine A (Sandimmune; Novartis Pharmaceuticals) one day before transplantation and daily thereafter.

2.4. rTMS

Animals received rTMS 24 h after recovering from transplantation surgery. rTMS was administrated using a magnetic stimulation device (YRD-CCI, Wuhan, China) with a 60 mm figure-of-eight coil. The coil was placed perpendicular to the cortex approximately 0.5 cm to the right of the bregma. The rTMS parameters were as follows: stimulating frequency = 10 Hz, stimulating pulse intensity = 26% of the maximum output of the stimulator, and train duration = 3 s. Ten successive trains of rTMS were applied (300 pulses per day) with a 50 s interval between trains. The rTMS parameter was shown to be effective and safe in our lab (Guo et al., 2014). During stimulation, the rats were restrained by hand. No abnormal symptoms were observed during the experiment.

2.5. Behavioral evaluation

The prehensile traction test and grip strength test were used to assess forepaw power of animals. In the prehensile test, rat's forepaws were hung on a horizontal rope (60 cm in length and 0.5 cm in diameter). Subsequently, the time to fall was recorded (Yoon et al., 2011). For the grip strength test, the rats were allowed to grasp a plastic grid with the left paretic forelimbs while being dragged backwards from the base of tail, and that maximal grip strength was recorded until they loosened their grip (Lee et al., 2017). The tests were repeated three times and the results were averaged.

The elevated body swing test was used to assess the behavior of asymmetric motor (Fujimoto et al., 2012). The ischemic rats were held above the table and the rats tended to turn toward the contralateral side. The number of left-side turns over 10 trials was recorded as well. The tests were repeated three times and the results were averaged.

2.6. Tissue processing

Animals were deeply anesthetized with 10% chloral hydrate. The brains were post-fixed in paraformaldehyde (PFA) overnight, and then consecutively transferred to 20% sucrose and 30% sucrose at 4 °C for dehydration. The specimens were embedded in optimum cutting temperature (OCT) compound and cut into 30 μm tissue sections with a cryostat. The free-floating sections were serially collected into 24-well plates for immunohistochemical analysis.

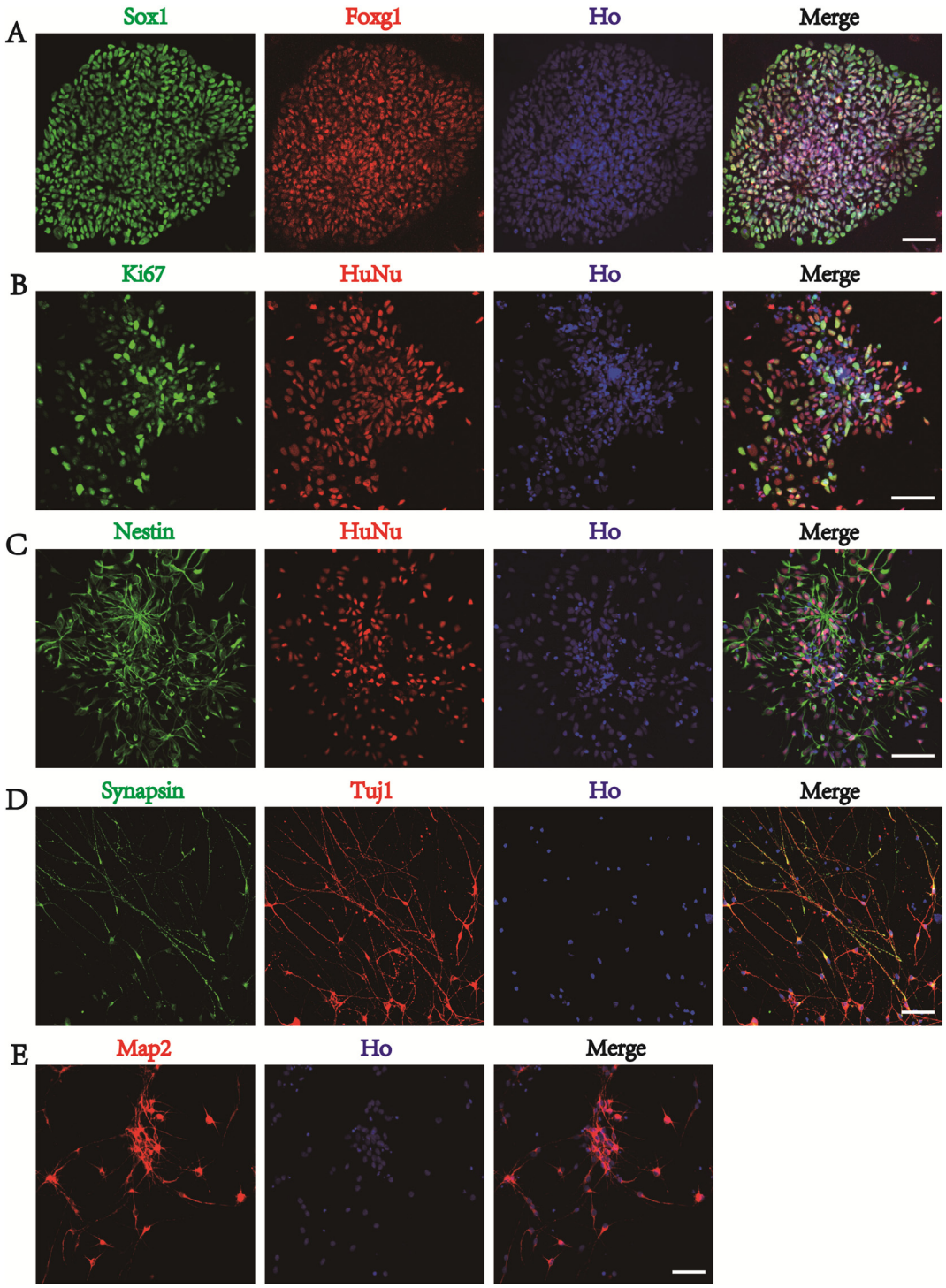

Fig. 1. Generation of hNSCs for transplantation and the phenotype of neural markers during the process of differentiation. (A) Representative images were immunoreactive for forebrain marker Foxg1 and neural stem cell marker Sox1 in the form of neural rosettes at day 10. (B–C) Differentiated hESCs expressed HuNu and demonstrated immunoreactivity for proliferative marker Ki67 (B) and neural stem cell marker Nestin (C) at day 21. (D–E) By 50 days in culture, neurons became mature and were positive for Synapsin (D) and Map2 (E). Scale bars: A, B, C, D, E = 100 μm. Ho: Hoechst.

2.7. Immunofluorescence staining

Immunofluorescence staining was performed on in vitro coverslip cultures as previously described (Yuan et al., 2015). In brief, cells were fixed with 4% PFA for 30 min, washed with phosphate buffered saline (PBS), and then blocked in 10% donkey serum (Jackson ImmunoResearch, 017-000-121) with 0.2% Triton X-100 for 1 h before being identified using primary antibodies at 4 °C overnight. Primary antibodies included rabbit anti-Foxg1 (Abcam, Ab18259, 1:1000), goat anti-Sox1 (R&D Systems, AF3369, 1:1000), mouse anti-human nuclei (HuNu; Millipore, MAB1281, 1:200), rabbit anti-Ki67 (Invitrogen, PA5-19462, 1:500), rabbit anti-Nestin (Millipore, ABD69, 1:1000), rabbit anti-Synapsin (Millipore, 574777, 1:1000), mouse anti-neuronal class III β-tubulin (Tuj1; Biolegend, 801201, 1:2000), mouse anti-microtubule-associated protein-2 (Map2; Sigma-Aldrich, M1406, 1:1000). The corresponding fluorescently conjugated secondary antibodies were

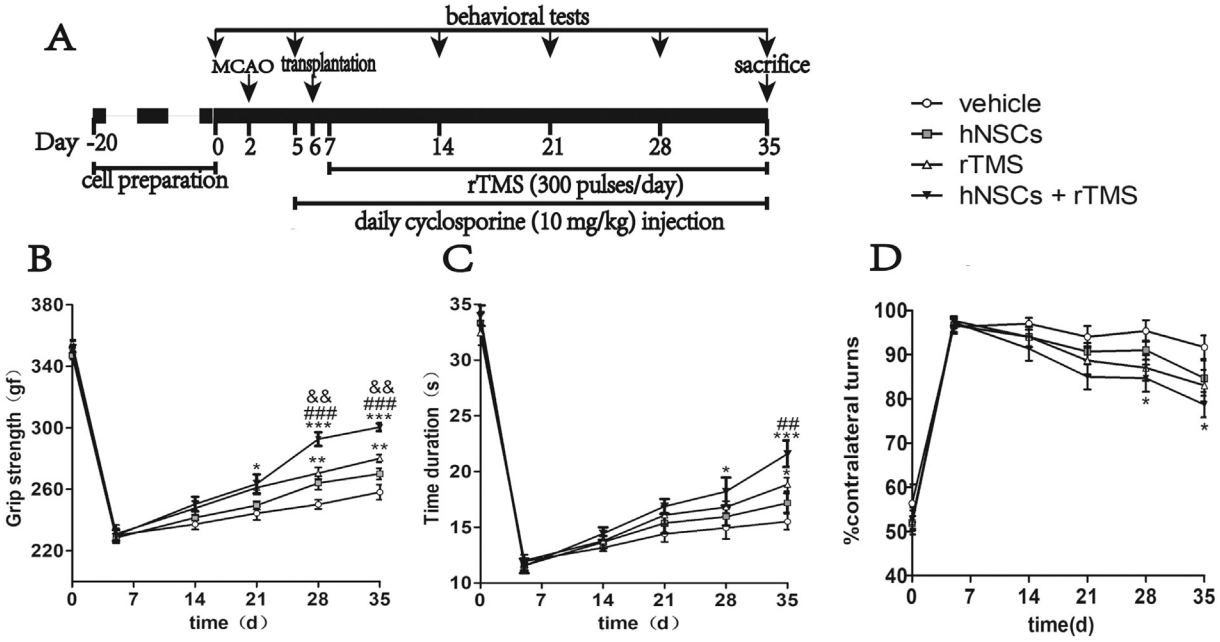

Fig. 2. Combining treatment rTMS with hNSCs improved functional outcomes in post-ischemic rats. (A) Experimental design: hNSCs transplantation was performed 4 days after stroke. Behavioral tests were assessed at the indicated times. Rats were intraperitoneally injected cyclosporine (10 mg/kg) and rTMS was administrated from day 7 until animals were sacrificed. (B–C) In the grip strength test (B) and prehensile traction test, behavioral performance was significantly improved in rTMS and hNSCs + rTMS groups. (D) In the elevated body swing test, only the hNSCs + rTMS group had significantly functional recovery compared with the vehicle group. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus vehicle group; $^{\#}p < .01$, $^{\#\#\#}p < .001$ versus hNSCs group; $^{\&\&}p < .01$ versus rTMS group. $N = 10$ rats/per group. Mean ± SEM. One-way ANOVA.

applied for 1 h and nuclei were stained with Hoechst 33342 (Invitrogen, H1399). For in vivo immunohistochemical analysis, free-floating tissue sections were processed as above described. Primary antibodies included rabbit anti-Tuj1 (Biolegend, 802,001, 1:1000), rabbit anti-Ki67 (Invitrogen, PA5-19462, 1:500), mouse anti-HuNu (Millipore, MAB1281, 1:200), mouse anti-Nestin (Abcam, ab6142, 1:500).

2.8. Western blot analysis

Under deep anesthesia, rats were sacrificed and brain tissue encompassing the transplant site was immediately collected. Total protein was isolated by homogenization and supernatants were collected after centrifuging at 12,000 rpm for 15 min. Next, a total of 40 μg protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were incubated at 4 °C overnight with primary antibodies: rabbit anti-BDNF (Abcam, ab108319, 1:1000, 15 kDa), rabbit anti-TrkB (Abcam, ab18987, 1:500, 92 kDa), rabbit phosphorylated-TrkB (p-TrkB; Millipore, ABN1381, 1:500, 140 kDa), followed by incubation with appropriate horseradish peroxidase conjugated secondary antibodies. Blots were detected using the ECL Western Blotting Substrate. The intensity of protein band was quantified using Gel-Pro Analyzer 4.0 software (Media Cybernetics, USA).

2.9. Microscopical analysis and cell quantification

Images were captured with an Olympus fluorescence microscope or an Olympus FV1000 confocal microscope. Three sections taken from similar positions in each rat.

were chosen. The number of positively $Nestin^+/Ki67^+$ double labeled cells in the SVZ were manually counted using ImageJ software in a blind manner. For analysis of differentiation of transplanted cells, all nuclei of grafted cells were identified based on HuNu immunostaining. The ratio of Ki67 and Tuj1 were determined by counting $Ki67^+/HuNu^+$ and $Tuj1^+/HuNu^+$ double labeled cells, and then dividing by the total number of HuNu cells. The images were taken from areas where HuNu-labeled cells were located. The ratio of Ki67 and Tuj1 was averaged for each subject. At least 1000 cells per animal were counted.

2.10. Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). For multiple comparisons, data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests between different groups. For comparison between two groups, the differences were compared by Student's unpaired *t*-test. GraphPad Prism 5 and SPSS 18.0 software were used for statistical analyses. *P*-value $< .05$ was statistically considered significant.

3. Results

3.1. Characterization of hNSCs in vitro

To obtain a high quantify of hNSCs for transplantation, the dual SMAD inhibition-based adherent culture was applied. Neural rosettes emerged evidently after 10 days of differentiation and the cells expressed the forebrain marker Foxg1 and neural stem cell marker Sox1 (Fig. 1A), showing forebrain identity. The differentiated hNSCs labeled with HuNu, a human specific antigen that detects transplanted cells, were positive for proliferative marker Ki67 and neural stem cell marker Nestin (Fig. 1B, C). Subsequently, these neurons expressed pre-synaptic marker Synapsin (Fig. 1D) and mature neural cell marker Map2 (Fig. 1E).

3.2. hNSCs transplantation combined with rTMS enhanced functional recovery

In the acute phase of stroke, the microenvironment is hostile for the survival of hNSCs and transplantation may not be practical in clinical settings at this time. Therefore, we transplanted hNSCs 4 days after

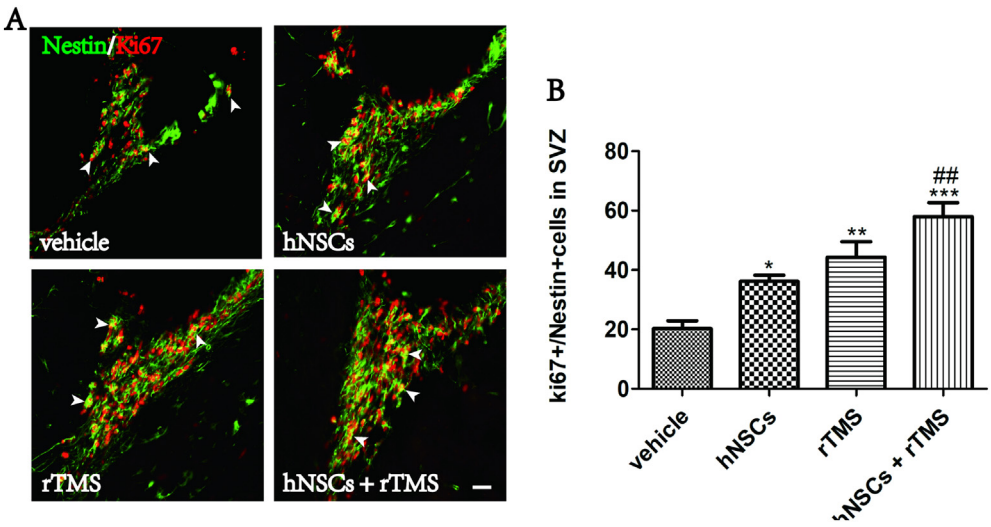

Fig. 3. Effects of hNSCs and rTMS treatment on neurogenesis in SVZ 33 days after MCAO. (A) Fluorescence photomicrographs of $Ki67^+/Nestin^+$ positive cells from vehicle, hNSCs, rTMS, hNSCs + rTMS group. White arrows indicate examples of colocalized cells. Scale bar = 50 μm. (B) Quantification of $Ki67^+/Nestin^+$ positive cells in each group. $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus vehicle group; $^{\#\#}p < .01$ versus hNSCs group. $N = 5$ rats/per group. Mean ± SEM. One-way ANOVA.

ischemic stroke, and then followed by treatment with rTMS (Fig. 2A). In the prehensile traction and grip strength tests, there were no significant differences among the four groups at 14th day. Thereafter, forepaw power was significantly improved in rTMS group compared with the ischemic rats receiving medium, and improvements were earlier observed with notably significant differences in hNSCs + rTMS group (Fig. 2B, C). The elevated body swing test revealed that only animals treated with hNSCs + rTMS showed significantly enhanced functional recovery compared with other three groups (Fig. 2D). These results suggest that hNSCs transplantation combined with rTMS rapidly promotes functional recovery in post-ischemic rats.

3.3. hNSCs transplantation combined with rTMS promoted neurogenesis in the SVZ

To investigate the neurogenesis of ipsilateral SVZ in response to combined treatment of hNSCs and rTMS, the proliferative marker Ki67 and neural stem cell marker Nestin were employed for double immunofluorescence (Fig. 3A). The $Nestin^+/Ki67^+$ double-positive cells were significantly higher in number in hNSCs and rTMS groups. We found that rats treated with rTMS and hNSCs exhibited a significant increase in double positive cells compared with rats treated with medium or hNSCs. There were no significant differences between rTMS and hNSCs + rTMS rats (Fig. 3B). These findings suggest that rTMS and hNSCs could synergistically enhance neurogenesis in the SVZ.

3.4. hNSCs transplantation combined with rTMS increased BDNF and p-TrkB expression

To examine whether the beneficial effects of rTMS and hNSCs treatment on functional recovery and neurogenesis were related to BDNF, we investigated the protein expression of BDNF, TrkB and p-TrkB. We found that the expression of BDNF and p-TrkB were significantly higher in the rTMS and hNSCs + rTMS groups than in the vehicle group. Compared with the hNSCs group, the expression of BDNF and p-TrkB in the hNSCs + rTMS group were significantly increased (Fig. 4A, B, C). The expression of total TrkB revealed that there were no significant differences among the four groups (Fig. 4A, D). The ratio of p-TrkB/TrkB was significantly increased in the rTMS and hNSCs + rTMS groups compared with the vehicle group. Particularly, significantly higher ratio was observed in the hNSCs + rTMS group (Fig. 4E). The results indicate that hNSCs transplantation combined with rTMS significantly activates BDNF/TrkB signaling pathway compared to the vehicle group or hNSCs group.

3.5. rTMS promoted the neural differentiation of hNSCs after transplantation in MCAO rats

To study whether rTMS had an influence on the proliferation and differentiation of hNSCs, we next examined the grafts in transplanted animals. Reflective of the proliferative ability of grafted cells, double immunostaining for Ki67 and HuNu were performed (Fig. 5A). A significantly higher proliferation rate for hNSCs animals was observed compared to hNSCs + rTMS group (Fig. 5C). Next, we found more neuronal cells, which were identified by the marker Tuj1, differentiated from grafted cells in the presence of rTMS (Fig. 5B) and the proportion of Tuj1/HuNu in the hNSCs + rTMS group was > 2 times higher than in the hNSCs group (Fig. 5D). The results demonstrate that rTMS stimulates neural production from grafted hNSCs in vivo.

4. Discussion

After stroke, multiple types of brain cells are lost and stem cell therapy is considered as a potential regenerative strategy for stroke (Boese et al., 2018). Basic studies and ongoing clinical studies have demonstrated that stem cell therapy is effective (Andres et al., 2011; Bang, 2016; Hess et al., 2017). Though some advancements have been made, there are still many difficulties to overcome in regenerative medicine, such as the production of cells for transplantation, low survival rate of grafted cells and limited differentiation of transplanted cells. This highlights the importance of seeking an effective treatment strategy when using exogenous stem cell treatment (Le Friec et al., 2017). rTMS may be an appropriate option to enhance stem cell therapy on the basis of effectiveness and safety of human treatment. In the present study, the grafted cells could be differentiated into mature neurons in vitro and survive 1 month after transplantation. We investigated the effects of combination therapy on neurological recovery in ischemic rats and examined the effects of rTMS as a post-stroke rehabilitation on the hNSCs after transplanted into the brain.

rTMS has been applied to stroke to alleviate neurological impairments (Dafotakis et al., 2008). Previous studies have shown that rTMS exerts a neurotrophic effect on the neurogenesis, promoting functional recovery (Luo et al., 2017). It has also been demonstrated that hNSCs have an neuroprotective effect on brain through secretion of trophic factors (Redmond et al., 2007; Ryu et al., 2004), among which BDNF is one of the most important neurotrophins (Lee et al., 2010). BDNF can bind to the receptor TrkB and then is induced autophosphorylation (Ohira and Hayashi, 2009). BDNF/TrkB signaling pathway plays an important role in endogenous ischemic-induced neurogenesis (Luo et al., 2017). Our behavioral results suggest that combined therapy can

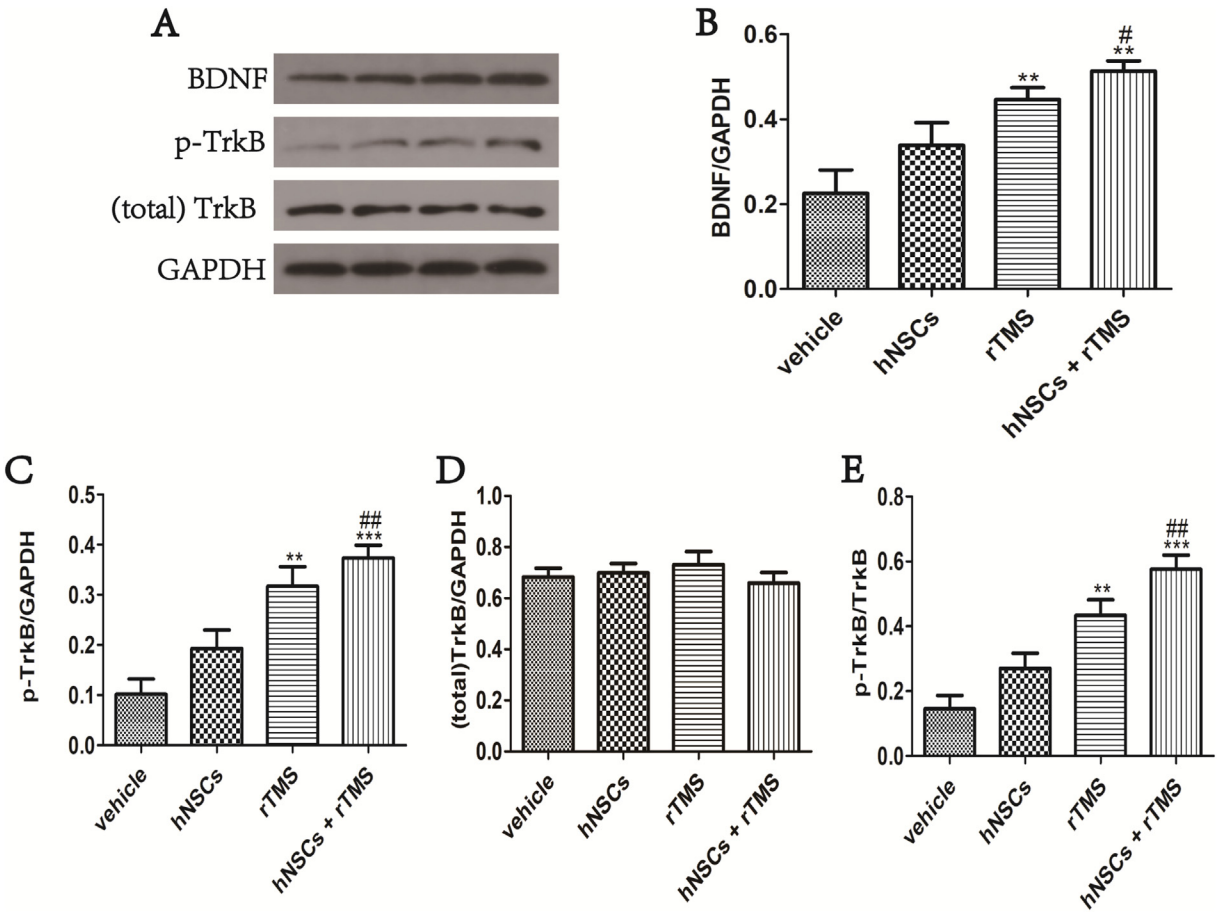

Fig. 4. Effects of hNSCs and rTMS treatment on the expression of BDNF, TrkB, p-TrkB were evaluated by western blot 33 days after MCAO. (A-D) Gel electrophoresis (A) and the densitometric analyses of BDNF (B), p-TrkB (C) and TrkB (D). (E) Combination treatment of hNSCs and rTMS increased p-TrkB/TrkB expression. $^{*}p < .01$, $^{**}p < .001$ versus vehicle group; $^{\#}p < .01$ versus hNSCs group. $N = 6$ rats/per group. Mean ± SEM. One-way ANOVA.

lead to better functional improvements than hNSCs or rTMS treatment alone. In addition, rTMS and hNSCs can synergistically enhance endogenous neurogenesis and increase BDNF levels that may directly contribute to functional recovery. There may be some interactive communications and signaling cross talk between rTMS and hNSCs for this synergistic enhancement.

In this study, rTMS treatment seems to be more effective than hNSCs transplantation. It may be due to the limited number of grafted cells that survive in ischemic rats. Although there is no report concerning detailed quantification of survival grafted cells after transplantation in post-ischemic animals (Hicks et al., 2009; Ishibashi et al., 2004), some studies have demonstrated that the survival of transplanted stem cells is poor owing to the hostile environment after stroke (Watanabe et al., 2016), which is consistent with our results as well. Because in our study, only a minority of surviving grafts was detected in some animals. A subtherapeutic dose of grafted cells may not improve functional outcomes (Daadi et al., 2013), while the survival of grafted cells is not proportional to functional recovery (Oki et al., 2012). Whether a higher dose of surviving grafts lead to a better outcome is still ambiguous. Many factors can affect the survival of transplanted cells, such as cerebral microenvironment, glia scar, and the donor cell type for transplantation (Kim et al., 2006; Molcanyi et al., 2007; Tom et al., 2017). As such it is difficult to draw a conclusion about the quantification of graft survival because of variability between different studies and it is not our aim of the present investigation. The results confirmed that rehabilitation was necessary for early functional improvements.

Our findings showed that rTMS decreases the proliferation of hNSCs about 1 month after transplantation in post-ischemic rats, which is contradictory to previous studies. Abbasnia et al. demonstrated that rTMS increased the proliferation and neural differentiation of NSCs (Abbasnia et al., 2015). Consistently, a recent research has also shown that high frequency rTMS reduced death of neural cells by increasing cell proliferation as well as reducing apoptosis in vitro (Baek et al., 2018). This discrepancy could be attributed to origin of the cells and the environment in which the cells are situated. In our research, the grafted hNSCs were differentiated from hESCs rather than animal origin. rTMS had been shown to activate cAMP-responsive element-binding protein (cAMP-CREB) pathway in human-derived neuronal cells by increasing the expression of BDNF (Hellmann et al., 2012; Ma et al., 2013). Furthermore, the beneficial effect of rTMS in brain was correlated with the enhancement of BDNF levels (Wang et al., 2011). It is known that BDNF plays an important role in the neuronal differentiation of stem cells (Leschik et al., 2013). Our results suggested that rTMS could promote the neural differentiation of hNSCs in ischemic rats, which may partially result from the up-regulation of BDNF levels. Interestingly, increased neuronal production from grafted hNSCs has been documented to promote functional recovery and the beneficial effects of neurological recovery could be abolished after ablating the role of neuronal production from hNSCs (Wang et al., 2016). The beneficial effects of rTMS through the neurotrophin BDNF to enhance neuronal differentiation implied that rTMS might be a potential intervention to treat stroke when applying stem cell therapy. To our knowledge, there were rare studies that assessed the effects of rTMS on the proliferation and differentiation of exogenous neural stem cells in vivo. Previous studies were mainly constructed in vitro (Baek et al., 2018; Grehl et al., 2015; Hellmann et al., 2012; Ma et al., 2013). Only one research showed that

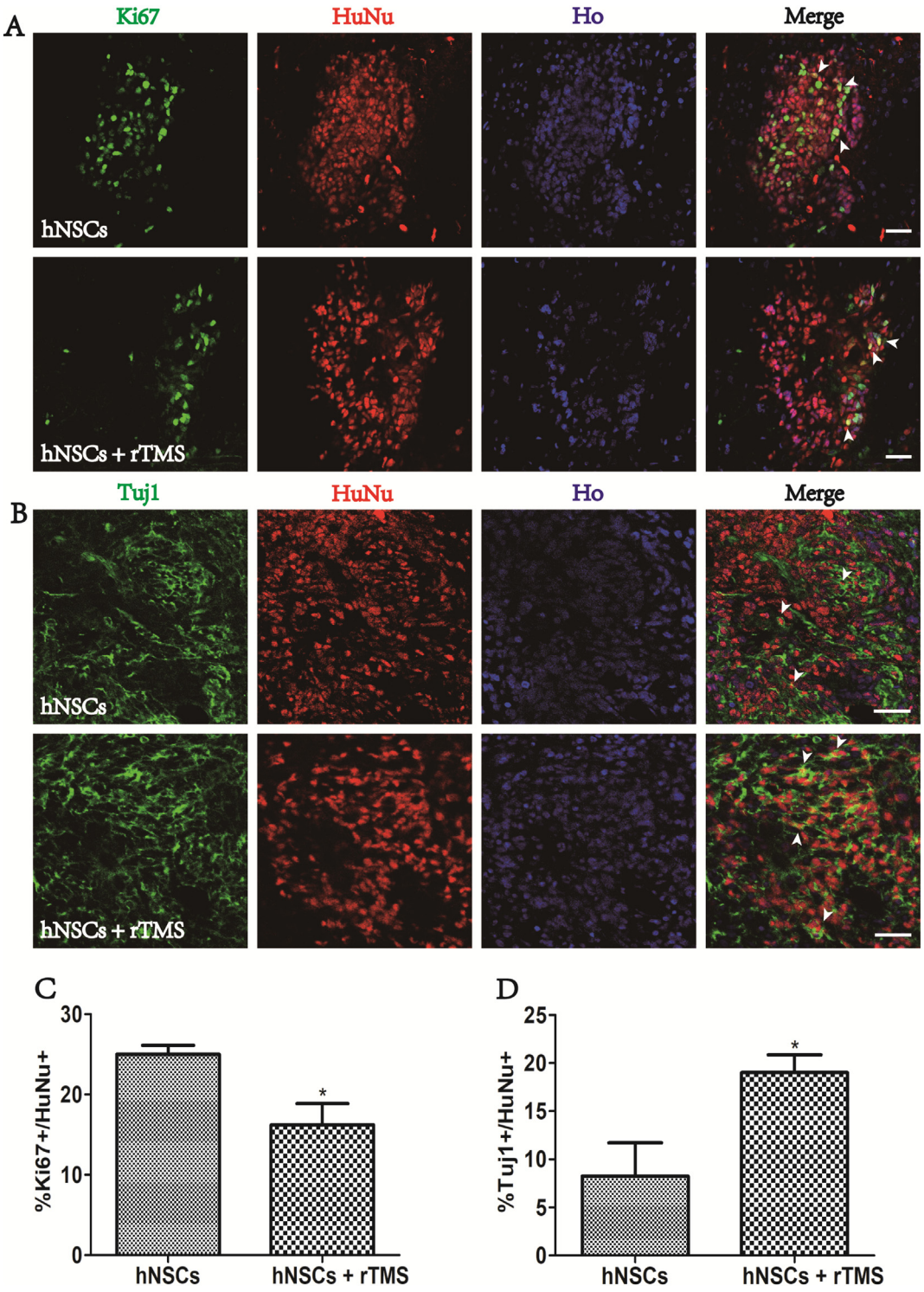

Fig. 5. rTMS promoted the differentiation of hNSCs after transplantation in MCAO rats 33 days after MCAO. (A) Confocal photomicrographs showing Ki67 and HuNu co-immunostaining in rats treated with hNSCs and hNSCs + rTMS. (B) Confocal photomicrographs showing Tuj1 and HuNu co-immunostaining in rats treated with hNSCs and hNSCs + rTMS. White arrows indicate examples of colocalized cells. Scale bars: A, B = 50 μm. Ho: Hoechst. (C-D) The percentage of $Ki67^+/HuNu^+$ (C), $Tuj1^+/HuNu^+$ (D) positive cells in hNSCs and hNSCs + rTMS groups. $^*p < .05$ versus hNSCs group. $N = 5$ rats/per group. Mean ± SEM. Student's unpaired *t*-test.

rTMS provided an unfavorable environment for human dental pulp stem cells in vivo, which was conducted in normal rats (Kremer et al., 2016). The complexity of stem cells and large scale of rTMS parameters may cause this situation. The ultimate purpose of cellular-based therapy is to reconstruct lost neural circuit and promote the functional repair of the injured brain. Interestingly, we observed that rTMS promotes the differentiation of hNSCs and decreases their proliferation. The higher rate of successful differentiation from rTMS-mediated grafts suggests that rTMS could be used as a new approach to promote exogenous hNSCs to differentiate into mature neural cells, ultimately integrating into existing neural circuits in the injured brain. To increase the therapeutic potential of cell transplantation therapy, rTMS may provide an effective way of optimizing neural stem cell transplantation.

In summary, we show for the first time that a combination of rTMS

and hNSCs treatment significantly improved functional recovery, promoted neurogenesis, and increased the expression of neurotrophin BDNF. We also find that rTMS promoted the neural differentiation of hNSCs, which may partially result from the up-regulation of BDNF and then contribute to functional recovery. This observation may indicate that the combination of hNSCs with rTMS might be an effective approach to the treatment of stroke. However, further studies and long-term observation will be required to determine whether rTMS-based clinical rehabilitation can enhance stem cell therapy, and clarify the specific mechanisms of action of rTMS/hNSCs treatment.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was supported by National Natural Science Foundation of China (Grant Nos. 81572238, 81702231, and 81774404).

Authors' contributions

H.C. and X.L.H. conceived and designed the project. J.J.P., R.S., and L.T.C. carried out the experiment. M.X.L., X.H.H., and F.G. analyzed the data. J.J.P. drafted the manuscript.

References

Abbasnia, K., Ghanbari, A., Abedian, M., Ghanbari, A., Sharififar, S., Azari, H., 2015. The effects of repetitive transcranial magnetic stimulation on proliferation and differentiation of neural stem cells. Anat Cell Biol 48, 104–113.

Abeysinghe, H.C., Bokhari, L., Quigley, A., Choolani, M., Chan, J., Dusting, G.J., Crook, J.M., Kobayashi, N.R., Roulston, C.L., 2015. Pre-differentiation of human neural stem cells into GABAergic neurons prior to transplant results in greater repopulation of the damaged brain and accelerates functional recovery after transient ischemic stroke. Stem Cell Res Ther 6, 186.

Andres, R.H., Horie, N., Slikker, W., Keren-Gill, H., Zhan, K., Sun, G., Manley, N.C., Pereira, M.P., Sheikh, L.A., McMillan, E.L., Schaar, B.T., Svendsen, C.N., Bliss, T.M., Steinberg, G.K., 2011. Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain. Brain 134, 1777–1789.

Baek, A., Kim, J.H., Pyo, S., Jung, J.H., Park, E.J., Kim, S.H., Cho, S.R., 2018. The differential effects of repetitive magnetic stimulation in an in vitro neuronal model of ischemia/reperfusion injury. Front Neurol 9, 50.

Bang, O.Y., 2016. Clinical trials of adult stem cell therapy in patients with ischemic stroke. J Clin Neurol 12, 14–20.

Bellenchi, G.C., Volpicelli, F., Piscopo, V., Perrone-Capano, C., di Porzio, U., 2013. Adult neural stem cells: an endogenous tool to repair brain injury? J Neurochem 124, 159–167.

Boese, A.C., Le, Q.E., Pham, D., Hamblin, M.H., Lee, J.P., 2018. Neural stem cell therapy for subacute and chronic ischemic stroke. Stem Cell Res Ther 9, 154.

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., Studer, L., 2009. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nature biotechnology 27, 275.

Chervyakov, A.V., Chernyavsky, A.Y., Sinitsyn, D.O., Piradov, M.A., 2015. Possible mechanisms underlying the therapeutic effects of transcranial magnetic stimulation. Front Hum Neurosci 9, 303.

Cui, X., Chopp, M., Shehadah, A., Zacharek, A., Kuzmin-Nichols, N., Sanberg, C.D., Dai, J., Zhang, C., Ueno, Y., Roberts, C., Chen, J., 2012. Therapeutic benefit of treatment of stroke with simvastatin and human umbilical cord blood cells: neurogenesis, synaptic plasticity, and axon growth. Cell Transplant 21, 845–856.

Daadi, M.M., Li, Z., Arac, A., Grueter, B.A., Sofilos, M., Malenka, R.C., Wu, J.C., Steinberg, G.K., 2009. Molecular and magnetic resonance imaging of human embryonic stem cell–derived neural stem cell grafts in ischemic rat brain. Molecular Therapy 17, 1282–1291.

Daadi, M.M., Hu, S., Klausner, J., Li, Z., Sofilos, M., Sun, G., Wu, J.C., Steinberg, G.K., 2013. Imaging neural stem cell graft-induced structural repair in stroke. Cell Transplant 22, 881–892.

Dafotakis, M., Grefkes, C., Eickhoff, S.B., Karbe, H., Fink, G.R., Nowak, D.A., 2008. Effects of rTMS on grip force control following subcortical stroke. Exp Neurol 211, 407–412.

Doeppner, T.R., Kaltwasser, B., Teli, M.K., Sanchez-Mendoza, E.H., Kilic, E., Bahr, M., Hermann, D.M., 2015. Post-stroke transplantation of adult subventricular zone derived neural progenitor cells–a comprehensive analysis of cell delivery routes and their underlying mechanisms. Exp Neurol 273, 45–56.

Du, J., Tian, L., Liu, W., Hu, J., Xu, G., Ma, M., Fan, X., Ye, R., Jiang, Y., Yin, Q., Zhu, W., Xiong, Y., Yang, F., Liu, X., 2016. Effects of repetitive transcranial magnetic stimulation on motor recovery and motor cortex excitability in patients with stroke: a randomized controlled trial. Eur J Neurol 23, 1666–1672.

Emborg, M.E., Liu, Y., Xi, J., Zhang, X., Yin, Y., Lu, J., Joers, V., Swanson, C., Holden, J.E., Zhang, S.C., 2013. Induced pluripotent stem cell-derived neural cells survive and mature in the nonhuman primate brain. Cell Rep 3, 646–650.

Feigin, V.L., Forouzanfar, M.H., Krishnamurthi, R., Mensah, G.A., Connor, M., Bennett, D.A., Moran, A.E., Sacco, R.L., Anderson, L., Truelsen, T., O'Donnell, M., Venketasubramanian, N., Barker-Collo, S., Lawes, C.M.M., Wang, W., Shinohara, Y., Witt, E., Ezzati, M., Naghavi, M., Murray, C., 2014. Global and regional burden of stroke during 1990–2010: findings from the Global Burden of Disease Study 2010. The Lancet 383.

Fujimoto, M., Hayashi, H., Takagi, Y., Hayase, M., Marumo, T., Gomi, M., Nishimura, M., Kataoka, H., Takahashi, J., Hashimoto, N., Nozaki, K., Miyamoto, S., 2012. Transplantation of telencephalic neural progenitors induced from embryonic stem cells into subacute phase of focal cerebral ischemia. Lab Invest 92, 522–531.

Gage, F.H., 2000. Mammalian neural stem cells. Science 287, 1433–1438.

Grehl, S., Viola, H.M., Fuller-Carter, P.I., Carter, K.W., Dunlop, S.A., Hool, L.C., Sherrard, R.M., Rodger, J., 2015. Cellular and molecular changes to cortical neurons following low intensity repetitive magnetic stimulation at different frequencies. Brain Stimul 8, 114–123.

Grossman, A.W., Broderick, J.P., 2013. Advances and challenges in treatment and prevention of ischemic stroke. Ann Neurol 74, 363–372.

Guo, F., Han, X., Zhang, J., Zhao, X., Lou, J., Chen, H., Huang, X., 2014. Repetitive transcranial magnetic stimulation promotes neural stem cell proliferation via the regulation of MiR-25 in a rat model of focal cerebral ischemia. PLoS ONE 9, e109267.

Haus, D.L., Lopez-Velazquez, L., Gold, E.M., Cunningham, K.M., Perez, H., Anderson, A.J., Cummings, B.J., 2016. Transplantation of human neural stem cells restores cognition in an immunodeficient rodent model of traumatic brain injury. Exp Neurol 281, 1–16.

Hellmann, J., Juttner, R., Roth, C., Bajbouj, M., Kirste, I., Heuser, I., Gertz, K., Endres, M., Kronenberg, G., 2012. Repetitive magnetic stimulation of human-derived neuron-like cells activates cAMP-CREB pathway. Eur Arch Psychiatry Clin Neurosci 262, 87–91.

Hess, D.C., Wechsler, L.R., Clark, W.M., Savitz, S.I., Ford, G.A., Chiu, D., Yavagal, D.R., Uchino, K., Liebeskind, D.S., Auchus, A.P., Sen, S., Sila, C.A., Vest, J.D., Mays, R.W., 2017. Safety and efficacy of multipotent adult progenitor cells in acute ischaemic stroke (MASTERS): a randomised, double-blind, placebo-controlled, phase 2 trial. The Lancet Neurology 16, 360–368.

Hicks, A.U., Lappalainen, R.S., Narkilahti, S., Suuronen, R., Corbett, D., Sivenius, J., Hovatta, O., Jolkkonen, J., 2009. Transplantation of human embryonic stem cell-derived neural precursor cells and enriched environment after cortical stroke in rats: cell survival and functional recovery. Eur J Neurosci 29, 562–574.

Ishibashi, S., Sakaguchi, M., Kuroiwa, T., Yamasaki, M., Kanemura, Y., Shizuko, I., Shimazaki, T., Onodera, M., Okano, H., Mizusawa, H., 2004. Human neural stem/progenitor cells, expanded in long-term neurosphere culture, promote functional recovery after focal ischemia in Mongolian gerbils. J Neurosci Res 78, 215–223.

Kim, D.E., Tsuji, K., Kim, Y.R., Mueller, F.J., Eom, H.S., Snyder, E.Y., Lo, E.H., Weissleder, R., Schellingerhout, D., 2006. Neural stem cell transplant survival in brains of mice: assessing the effect of immunity and ischemia by using real-time bioluminescent imaging. Radiology 241, 822–830.

Knecht, S., Hesse, S., Oster, P., 2011. Rehabilitation after stroke. Dtsch Arztebl Int 108, 600–606.

Kremer, K.L., Smith, A.E., Sandeman, L., Inglis, J.M., Ridding, M.C., Koblar, S.A., 2016. Transcranial magnetic stimulation of human adult stem cells in the mammalian brain. Front Neural Circuits 10, 17.

Le Friec, A., Salabert, A.S., Davoust, C., Demain, B., Vieu, C., Vaysse, L., Payoux, P., Loubinoux, I., 2017. Enhancing plasticity of the central nervous system: drugs, stem cell therapy, and neuro-implants. Neural Plast 2017, 2545736.

Lee, H.J., Lim, I.J., Lee, M.C., Kim, S.U., 2010. Human neural stem cells genetically modified to overexpress brain-derived neurotrophic factor promote functional recovery and neuroprotection in a mouse stroke model. J Neurosci Res 88, 3282–3294.

Lee, I.H., Huang, S.S., Chuang, C.Y., Liao, K.H., Chang, L.H., Chuang, C.C., Su, Y.S., Lin, H.J., Hsieh, J.Y., Su, S.H., Lee, O.K., Kuo, H.C., 2017. Delayed epidural transplantation of human induced pluripotent stem cell-derived neural progenitors enhances functional recovery after stroke. Sci Rep 7, 1943.

Leschik, J., Eckenstaler, R., Nieweg, K., Lichtenecker, P., Brigadski, T., Gottmann, K., Lessmann, V., Lutz, B., 2013. Embryonic stem cells stably expressing BDNF-GFP exhibit a BDNF-release-dependent enhancement of neuronal differentiation. J Cell Sci 126, 5062–5073.

Luo, J., Zheng, H., Zhang, L., Zhang, Q., Li, L., Pei, Z., Hu, X., 2017. High-frequency repetitive transcranial magnetic stimulation (rTMS) improves functional recovery by enhancing neurogenesis and activating BDNF/TrkB signaling in ischemic rats. Int J Mol Sci 18.

Ma, J., Zhang, Z., Su, Y., Kang, L., Geng, D., Wang, Y., Luan, F., Wang, M., Cui, H., 2013. Magnetic stimulation modulates structural synaptic plasticity and regulates BDNF-TrkB signal pathway in cultured hippocampal neurons. Neurochem Int 62, 84–91.

Molcanyi, M., Riess, P., Bentz, K., Maegele, M., Hescheler, J., Schafke, B., Trapp, T., Neugebauer, E., Klug, N., Schafer, U., 2007. Trauma-associated inflammatory response impairs embryonic stem cell survival and integration after implantation into injured rat brain. J Neurotrauma 24, 625–637.

Ohira, K., Hayashi, K., 2009. A new aspect of the TrkB signaling pathway in neural plasticity. Current Neuropharmacology 7, 276–285.

Oki, K., Tatarishvili, J., Wood, J., Koch, P., Wattananit, S., Mine, Y., Monni, E., Tornero, D., Ahlenius, H., Ladewig, J., Brustle, O., Lindvall, O., Kokaia, Z., 2012. Human-induced pluripotent stem cells form functional neurons and improve recovery after grafting in stroke-damaged brain. Stem Cells 30, 1120–1133.

Redmond, D.E., Bjugstad, K.B., Teng, Y.D., Ourednik, V., Ourednik, J., Wakeman, D.R., Parsons, X.H., Gonzalez, R., Blanchard, B.C., Kim, S.U., Gu, Z., Lipton, S.A., Markakis, E.A., Roth, R.H., Elsworth, J.D., Sladek, J.R., Sidman, R.L., Snyder, E.Y., 2007. Behavioral improvement in a primate Parkinson's model is associated with multiple

homeostatic effects of human neural stem cells. Proc Natl Acad Sci U S A 104 (12), 175–12,180.
Rossi, S., Hallett, M., Rossini, P.M., Pascual-Leone, A., Safety of, T.M.S.C.G, 2009. Safety, ethical considerations, and application guidelines for the use of transcranial magnetic stimulation in clinical practice and research. Clin Neurophysiol 120, 2008–2039.
Ryu, J.K., Kim, J., Cho, S.J., Hatori, K., Nagai, A., Choi, H.B., Lee, M.C., McLarnon, J.G., Kim, S.U., 2004. Proactive transplantation of human neural stem cells prevents degeneration of striatal neurons in a rat model of Huntington disease. Neurobiol Dis 16, 68–77.
Ryu, S., Lee, S.H., Kim, S.U., Yoon, B.W., 2016. Human neural stem cells promote proliferation of endogenous neural stem cells and enhance angiogenesis in ischemic rat brain. Neural Regen Res 11, 298–304.
Tang, A., Thickbroom, G., Rodger, J., 2017. Repetitive transcranial magnetic stimulation of the brain: mechanisms from animal and experimental models. Neuroscientist. 23, 82–94.
Tom, C.M., Younesi, S., Meer, E., Bresee, C., Godoy, M., Mattis, V.B., 2017. Survival of iPSC-derived grafts within the striatum of immunodeficient mice: Importance of developmental stage of both transplant and host recipient. Exp Neurol 297, 118–128.
Wang, H.Y., Crupi, D., Liu, J., Stucky, A., Cruciata, G., Di Rocco, A., Friedman, E., Quartarone, A., Ghilardi, M.F., 2011. Repetitive transcranial magnetic stimulation enhances BDNF-TrkB signaling in both brain and lymphocyte. J Neurosci 31, 11,044–11,054.
Wang, Y., Zhao, Z., Rege, S.V., Wang, M., Si, G., Zhou, Y., Wang, S., Griffin, J.H., Goldman, S.A., Zlokovic, B.V., 2016. 3K3A-activated protein C stimulates post-ischemic neuronal repair by human neural stem cells in mice. Nat Med 22, 1050–1055.
Watanabe, T., Nagai, A., Sheikh, A.M., Mitaki, S., Wakabayashi, K., Kim, S.U., Kobayashi, S., Yamaguchi, S., 2016. A human neural stem cell line provides neuroprotection and improves neurological performance by early intervention of neuroinflammatory system. Brain Res 1631, 194–203.
Weick, J.P., Liu, Y., Zhang, S.C., 2011. Human embryonic stem cell-derived neurons adopt and regulate the activity of an established neural network. Proc Natl Acad Sci U S A 108, 20,189–20,194.
Yoo, J., Seo, J.J., Eom, J.H., Hwang, D.Y., 2015. Enhanced recovery from chronic ischemic injury by bone marrow cells in a rat model of ischemic stroke. Cell Transplant 24, 167–182.
Yoon, K.J., Lee, Y.T., Han, T.R., 2011. Mechanism of functional recovery after repetitive transcranial magnetic stimulation (rTMS) in the subacute cerebral ischemic rat model: neural plasticity or anti-apoptosis? Exp Brain Res 214, 549–556.
Yuan, T., Liao, W., Feng, N.H., Lou, Y.L., Niu, X., Zhang, A.J., Wang, Y., Deng, Z.F., 2013. Human induced pluripotent stem cell-derived neural stem cells survive, migrate, differentiate, and improve neurologic function in a rat model of middle cerebral artery occlusion. Stem cell research & therapy 4, 73.
Yuan, F., Fang, K.H., Cao, S.Y., Qu, Z.Y., Li, Q., Krencik, R., Xu, M., Bhattacharyya, A., Su, Y.W., Zhu, D.Y., Liu, Y., 2015. Efficient generation of region-specific forebrain neurons from human pluripotent stem cells under highly defined condition. Sci Rep 5 (18), 550.
Zhang, S.C., Wernig, M., Duncan, I.D., Brüstle, O., Thomson, J.A., 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nature biotechnology 19, 1129.