

Original Paper

# Neurogenin 2 Converts Mesenchymal Stem Cells into a Neural Precursor Fate and Improves Functional Recovery after Experimental Stroke

Feng Cheng<sup>a,b,c</sup> Xiao Cheng Lu<sup>a,c</sup> Huai Yong Hao<sup>a</sup> Xue Liang Dai<sup>a</sup> Teng Da Qian<sup>a</sup>  
Bao Sheng Huang<sup>a</sup> Lin Jun Tang<sup>a</sup> Wan Yu<sup>a</sup> Li Xin Li<sup>a</sup>

<sup>a</sup>Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, <sup>b</sup>Department of Neurosurgery, The First People's Hospital of Kunshan Affiliated with Jiangsu University, Suzhou, China; <sup>c</sup>The first two authors contributed equally to this work

## Key Words

Mesenchymal stem cells • Neurogenin 2 • Cell transplantation • Stroke

## Abstract

**Background:** Neurogenin2 (Ngn2) is a proneural gene that directs neuronal differentiation of progenitor cells during development. Here, we investigated whether Ngn2 can reprogram MSCs to adopt a neural precursor fate and enhance the therapeutic effects of MSCs after experimental stroke. **Methods:** *In vitro*, MSCs were transfected with lenti-GFP or lenti-Ngn2. Following neuronal induction, cells were identified by immunocytochemistry, Western blot and electrophysiological analyses. In a stroke model induced by transient right middle cerebral artery occlusion (MCAO), PBS, GFP-MSCs or Ngn2-MSCs were injected 1 day after MCAO. Behavioral tests, neurological and immunohistochemical assessments were performed. **Results:** *In vitro*, Ngn2-MSCs expressed neural stem cells markers (Pax6 and nestin) and lost the potential to differentiate into mesodermal cell types. Following neural induction, Ngn2-MSCs expressed higher levels of neuron-specific proteins MAP2, Tuj1 and NeuN, and also expressed voltage-gated Na<sup>+</sup> channel, which was absent in GFP-MSCs. *In vivo*, after transplantation, Ngn2-MSCs significantly reduced apoptotic cells, decreased infarct volume, and increased the expression of VEGF and BDNF. Finally, Ngn2-MSCs treated animals showed the highest functional recovery among the three groups. **Conclusions:** Ngn2 was sufficient to convert MSCs into a neural precursor fate and transplantation of Ngn2-MSCs was advantageous for the treatment of stroke rats.

Copyright © 2014 S. Karger AG, Basel

Li-Xin Li

Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University,  
300 Guangzhou Road, Nanjing, Jiangsu, 210029 (China)  
Tel. +86-25-83718836, Fax +86-25-84715666, E-Mail lilixin2@hotmail.com

## Introduction

Stroke, the number one cause of disability and a major cause of death, is characterized by progressive neurologic deficits [1]. Currently, there are no effective therapeutic approaches available besides thrombolytic treatment, which has a narrow time window and limited availability [2]. Preclinical studies suggested that cell transplantation may be one potential strategy for the treatment of stroke [3-6]. A surprising variety of cells have been demonstrated to reverse neuronal deficits associated with stroke, including mesenchymal stem cells (MSCs), neural stem cells (NSCs), embryonic stem cells, and induced pluripotent cells.

Transplantation of MSCs several hours to days after induction of stroke can reduce infarct size and improve functional outcome in rodent stroke models [7-9]. However, the proportion of neurons differentiated from MSCs was limited. NSCs represent an excellent cell therapy choice and have been widely employed in pre-clinical stroke studies with encouraging results [10, 11]. Recent meta-analysis of preclinical studies employing intravenous cell delivery indicated that NSCs yielded the greatest behavioral recovery when compared to MSCs or other cell types [12]. However, human neural tissue is generally obtained from which restricts their supply, while fetal NSCs derived from aborted human fetuses is associated with ethical controversies. Therefore, how to obtain NSCs without ethical problems remains a great challenge.

Recently, several studies have shown that defined sets of transcription factors can reprogram somatic cells into induced NSCs, which express multiple NSC-specific markers (Pax6, Nestin, sox2, and Olig2), and are capable of self-renewal and differentiating into electrophysiologically functional neurons [13, 14]. In the present study, we investigated whether Ngn2, a proneural gene that directs neuronal differentiation of progenitor cells, could reprogram MSCs into NSCs. In addition, we also studied whether transplantation of Ngn2-MSCs could reduce infarct volume and improve functional recovery effectively in a rat stroke model *in vivo*.

## Materials and Methods

### *Animals*

The laboratory animals used in all experimental procedures were provided by the Department of Animal Experiments, Nanjing Medical University. Animal studies were approved by the Animal Experimental Committee of the Nanjing Medical University and the procedures complied with relevant laws.

### *Isolation and Culture of MSCs*

Primary rat MSCs were isolated from male Sprague-Dawley rats (60–80 g). After sacrifice of rats, the tibias, femurs, and humeri were dissected and the bone marrow was flushed out with 0.01M phosphate buffered saline (PBS) using a needle and syringe. After centrifugation, the pellet was suspended in cell culture medium Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin, plated on a 25-cm<sup>2</sup> plastic flask at a density of  $1 \times 10^6$  cells/cm<sup>2</sup> and incubated in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. After 24 hours, non-adherent cells were removed and fresh complete medium was added and replaced every 3 days thereafter. When the cultures reached 90% confluence, MSCs were passaged. The cells were analyzed by flow cytometry for CD45, CD105, CD44 and CD34 [15].

### *Lentivirus Transduction and Neuronal Differentiation*

Lentivirus transfection of MSCs was performed as previous reports [16, 17]. The vector of lenti-Ngn2 contains a neomycin resistance gene for establishing a stable cell line and a coral GFP gene for tracking transfection efficiency, driven by the CMV promoter and SV40 promoter, respectively. The vector uses the Rous sarcoma virus (RSV) enhancer/promoter joined with HIV 5'LTR (PRSV/5'LTR) and HIV 3'LTR for viral transcription and packaging. For infections,  $5 \times 10^5$  MSCs were exposed to  $7 \times 10^7$  virus particles for 24 hours.

To induce neuronal differentiation, cells were grown on poly-D-lysine/collagen-coated culture dishes or coverslips in L-DMEM/2% FBS containing 10 ng/ml epidermal growth factor and 20 ng/ml basic fibroblast growth factor for 5 days, and then in L-DMEM/2% FBS medium containing 20 ng/ml brain-derived neurotrophic factor (BDNF) for 2 weeks.

#### *Immunocytochemistry*

MSCs, GFP-MSCs and Ngn2-MSCs were fixed and immunostained with rabbit anti-microtubule-associated protein 2 (MAP2) (1:100; Sigma-Aldrich), rabbit-anti beta III Tubulin (Tuj1) (1:200; Chemicon) followed by either goat anti-rabbit Alexa-647 (1:500; Abcam) or goat anti-rabbit Alexa-555 (1:500, Abcam). Nuclei were counterstained with DAPI (Sigma). Labeling was imaged using a confocal laser-scanning microscope (Olympus LSM-GB200, Tokyo, Japan) and epifluorescent images were acquired using a Zeiss Axiophot microscope.

#### *Western Blot Analysis*

Samples containing equivalent amounts of protein (20 ug) were separated by SDS-PAGE using 10% acrylamide gradients. Following electrophoresis, the separated proteins were transferred onto transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were incubated with a blocking solution [5% non-fat dried milk dissolved in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h, and then incubated overnight at 4 °C with the following antibodies: rabbit anti-neurogenin2 (1:1000, Abcam), rabbit anti-VEGF (1:1000, Abcam), rabbit anti-BDNF (1:500, Abcam), rabbit anti-nestin (1:500, Abcam), rabbit anti-pax-6 (1:500, Abcam), rabbit anti-MAP2 (1:500, Chemicon), rabbit anti-Tuj1 (1:500, Chemicon), or mouse anti-NeuN (1:200, Chemicon). The internal control was  $\beta$ -actin antibody (1:1000; Sigma-Aldrich, St Louis, MO, USA). After several washes with TBST buffer, the membranes were incubated for 1 hour at room temperature with horseradish peroxidase-linked secondary antibody. The membranes were then processed with enhanced chemiluminescence western blot detection reagents (Pierce Biotechnology Inc., Rockford, IL, USA). The films were scanned and densitometry was performed using the 'Quantity One' image software (Bio-Rad Laboratories Inc., Hercules, CA, USA). The relative level of protein was quantified from the scanned films.

#### *Electrophysiological Analyses*

The bath solution was 140 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM glucose, and 10 mM HEPES, and the pipette solution was 140 mM KCl, 5 mM NaCl, 1mM CaCl<sub>2</sub>, 10 mM HEPES, 5 mM EGTA, and 2 mM Mg-ATP, pH7.3. Electric currents were amplified using either an Axopatch-1C patch-clamp amplifier or an Axopatch 200B patch-clamp amplifier (Axon Instruments/Molecular Devices Corp., Foster City, CA). The signals were amplified with and digitized by an analog-to-digital interface (Gould 1425 [Gould Instruments, Hainault, U.K.] or Digidata 1320 [Axon Instruments]) and analyzed using the Clampfit version 6.0.3 software package or pClamp8.2 (Axon Instruments).

#### *Experimental Groups*

Adult (250–270 g) male SD rats (n=69) were divided into the following groups: a PBS group (n =23), a GFP-MSCs group (n =23) and an Ngn2-MSCs group (n=23). Six animals per group were sacrificed 14 days after MCAO to measure the infarct volume by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining. On 14 days following MCAO, 6 rats per group were sacrificed for assess neural differentiation and paracrine factors. Six animals per group were used in behavioral tests and were sacrificed at 4 weeks. To count terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cells in the cortex of the ischemic hemisphere, 5 rats were sacrificed at day 10.

#### *Middle Cerebral Artery Occlusion Model*

Anesthesia of adult male Sprague-Dawley rats weighing 250–270 g was induced with 10% chloral hydrate (0.35 ml/100 g, i.p.). Rectal temperature was maintained at 37 °C throughout the surgical procedure, using an electronic temperature controller linked to a heating pad. Transient middle cerebral artery occlusion (MCAO) was induced as previous [18] with a slight modification. Briefly, the right common carotid artery, external carotid artery, and internal carotid artery were exposed through a ventral midline

incision. A 4-0 monofilament nylon suture with a rounded tip was introduced into the CCA lumen and gently advanced into the ICA until it blocked the bifurcating origin of the MCA. At 1.5 hours after occlusion, animals were reanesthetized and reperfused by withdrawing the suture until the tip cleared the lumen of the CCA.

The degree of functional deficit at 60 min post occlusion was scored using a scoring system based on previous report [19]. Briefly, (0) no observable deficit; (1) forelimb flexion; (2) as for 1, decreased resistance to lateral push without circling (3) same behavior as grade 2, with circling (4) no movement or unconscious. Rats with scores of 2–3 were included and others (5 rats) were excluded from the study. Additionally, 3 rats were excluded from this study because of hemorrhage or death during or soon after surgical procedure. Finally, 69 of 77 rats were included in the present study.

#### *Cell transplantation*

Prior to transplantation, the GFP-MSCs and Ngn2-MSCs were incubated with 5 mg/mL bis-benzimide (Hoechst 33258; Sigma) for 12 hours to label the nuclei fluorescently. One day after MCAO, the animals were anesthetized as described above and received PBS, GFP-MSCs, or Ngn2-MSCs through a stereotactic apparatus (Kopf Instruments, Tujunga);  $5.0 \times 10^5$  MSCs in 10  $\mu$ l of PBS were injected into the striatum (0.5 mm posterior; 3.5 mm from the bregma right to midline; 4.5 mm deep from the dura) based on the atlas given by Paxinos et al [20], covering the ischemic perifocal territories. The needle was retained in the brain for an additional 10 minutes to avoid donor reflux. An equal volume of PBS was used as a control.

#### *Percentage Hemisphere Lesion Volume (%HLV)*

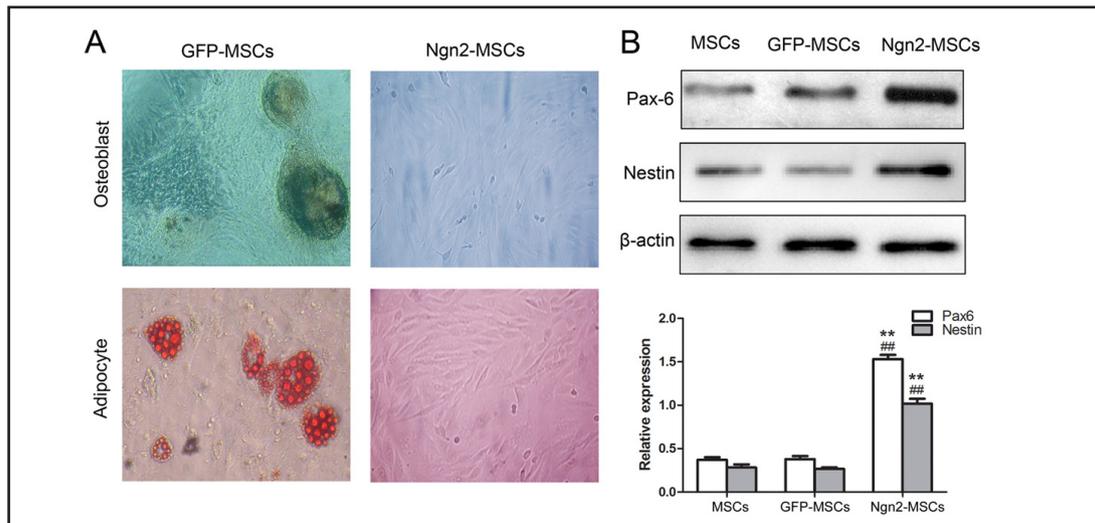
At 14 days after MCAO, the rats were sacrificed to quantify ischemic damage. Brains were dissected and cut into five coronal slices of 2-mm thickness, incubated in a 2% solution of TTC at 37 °C for 15 min and immersion-fixed in 4% paraformaldehyde. TTC-stained sections were photographed and the digital images were analyzed using image analysis software (Image-Pro Plus 5.1). The lesion volume was calculated by multiplying the area by the thickness of slices. Relative infarct volume was normalized as previously described [19], using the equation  $HLV(\%) = [LT - (RT - RI)] / LT \times 100$ , where LT and RT represent the areas of the left and right hemispheres, respectively, in square millimeters. RI is the infarcted area in square millimeters. Percentage Hemisphere Lesion Volume (%HLV) is expressed as percentages of the right hemispheric volume.

#### *Detection of the numbers of apoptotic cells*

The apoptotic cells were detected using the TUNEL method. Briefly, free-floating, 20- $\mu$ m brain sections were digested with proteinase K (Roche Molecular Biochemicals) and then covered with TUNEL reaction mixture followed by converter-POD/wash buffer. Then, sections were reacted with diaminobenzidine (DAB, Chemicon ES005-10ML). TUNEL-positive cells in the cortex of the ipsilateral hemisphere were counted in five random high-power fields ( $\times 400$ ) per slides. For each brain, a total 4 slides were selected.

#### *Immunohistochemistry*

Two weeks after transplantation, the expression of neuronal markers (MAP2 and neuron-specific enolase (NSE) in Ngn2-MSCs and GFP-MSCs was assessed by immunohistochemistry. Standard immunohistochemical procedures were performed as previously described [21, 22]. Briefly, rats were anesthetized with chloral hydrate and perfused transcardially with ice-cold 0.9% saline followed by 4% PFA. Brains were removed and fixed in 4% paraformaldehyde at room temperature overnight, transferred to 30% sucrose for 1–3 days at 4 °C, and then embedded in optimal cutting temperature compound (OCT). The brain tissues surrounding the injection needle were cut at a thickness of 20  $\mu$ m by cryostat in the coronal plane, and 6 sections were randomly selected. The sections were incubated in 0.5% Triton X-100 for 10 min to facilitate antibody penetration. After blocking in 5% normal serum for 60 min, brain sections were incubated overnight at 4°C with the following antibodies: rabbit anti-MAP2 (1:100; Chemicon), and rabbit anti-NSE (1:100). Then, the sections were incubated with Alexa Fluor 647-conjugated anti-IgG secondary antibodies (1:200, Abcam) for 2 hours at room temperature and nuclei were counterstained with Hoechst 33258 (Sigma). Confocal images were taken by a confocal laser-scanning microscope (Olympus LSM-GB200, Tokyo, Japan) and epifluorescent images were acquired using a Zeiss Axiophot microscope. The stained cells in six random fields ( $\times 200$ ) per each section were counted.



**Fig. 1.** A. GFP-MSCs differentiated into adipocytes (oil red+), osteoblasts (silver nitrate+); Ngn2-MSCs showed very low levels of oil red and silver nitrate activity. (X100) B. Western blot analysis of neural stem markers reveals a significant increase in the levels of nestin and pax6 of Ngn2-MSCs. \*\*= $p < 0.01$  vs MSCs, ##= $p < 0.01$  vs GFP-MSCs.

#### Behavioral Tests

Animals were pre-trained for 1 week. Behavioral tests were performed in all animals 2 days after MCAO and then at 0, 7, 10, 14, and 28 days after MCAO. Modified Neurological Severity Scale (mNSS) scores were used to evaluate the neurological function of each animal according to the method used by Chen et al and Li et al [23, 24]. The NSS is a composite of motor, sensory, reflex, and balance tests. For adhesive removal tests, square dots of adhesive-backed paper (100 mm<sup>2</sup>) were used as bilateral tactile stimuli occupying the distal-radial region on the wrist of each forelimb. Animals were given three trials with a cutoff time of 250 s. The data are presented as the mean time to remove the left dot

#### Statistical Analysis

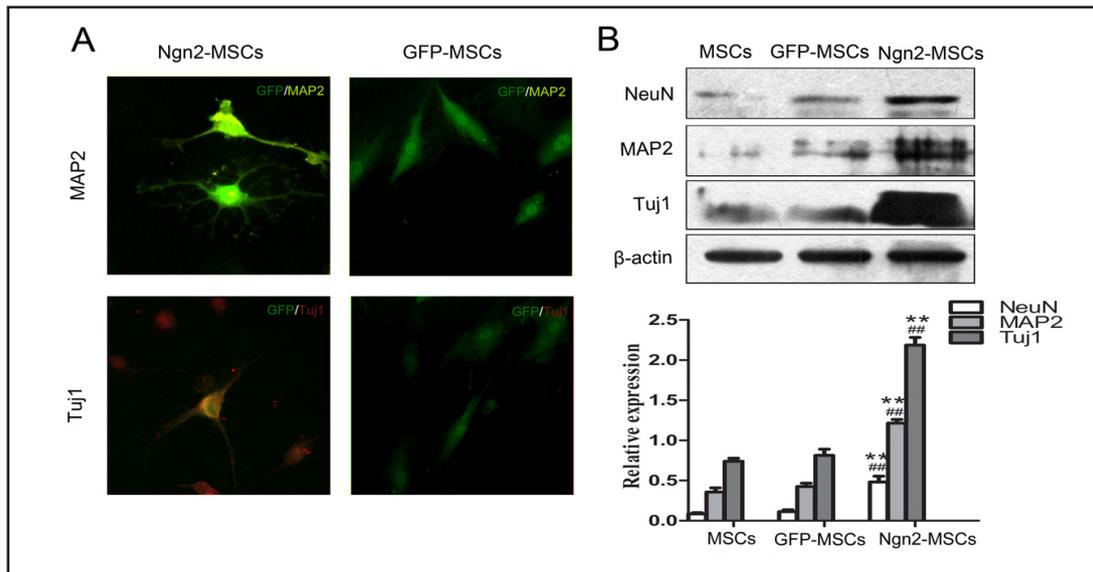
Sigma Stat Statistical Software (SPSS Inc) was used for all statistical analyses. All data represent at least three independent experiments and one-way analysis of variance (ANOVA) was used. Neurological scores were analyzed using repeated measures analysis of variance with independent variables of treatment groups and days of testing. Results are expressed as means  $\pm$  standard deviations (SD). Statistical significance was set at  $P < 0.05$ .

## Results

#### Reprogramming of MSCs into NSCs by Ngn2

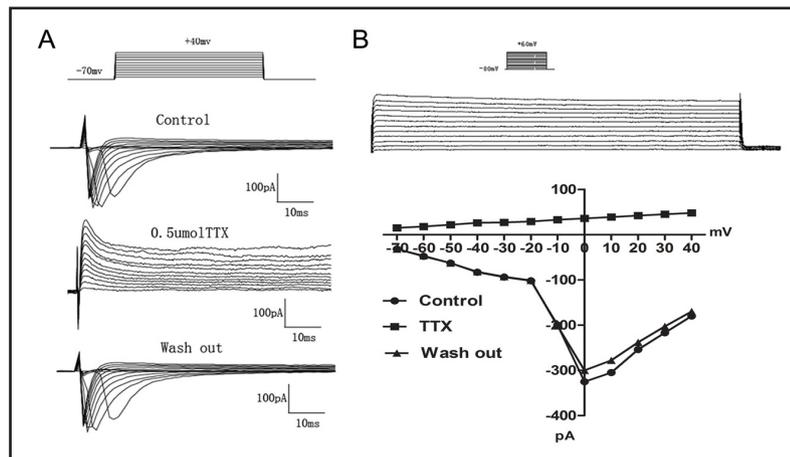
MSCs were isolated from male SD rats and identified by flow cytometry. These cells showed high expression of CD44 and CD105, and were negative for CD34 and CD45 (data not shown). MSCs were infected with Ngn2-expressing lentivirus with an efficiency of approximately 85%. After gene transduction, western blotting demonstrated a significantly increased in the level of Ngn2 protein compared with that seen in GFP-MSCs and parental MSCs (data not shown).

Five days after transduction of Ngn2, western blot analysis revealed a significantly increased in the levels of neural stem markers: nestin and pax6, when compared with those in GFP-MSCs and parental MSCs (Fig. 1B). Moreover, Ngn2-MSCs could proliferate in growth medium without apparent morphological changes, but lost the potential to differentiate into mesodermal cell types, such as adipocytes and osteoblasts (Fig. 1A).



**Fig. 2.** Neuronal differentiation of Ngn2-MSCs, GFP-MSCs, and MSCs. A. Immunocytochemistry revealed expression of MAP2 and Tuj1 in GFP-MSCs and Ngn2-MSCs groups B. Western blot analysis showing the expression of NeuN, MAP2, and Tuj1 in Ngn2-MSCs following neuronal induction. \*\*= $p < 0.01$  vs MSCs, ###= $p < 0.01$  vs GFP-MSCs.

**Fig. 3.** Patch-clamp recordings of Ngn2-MSCs derived cells following 2 weeks of neuronal induction. A. In Ngn2-MSCs, Voltage-gated Na<sup>+</sup> currents were activated and blocked reversibly by 0.5  $\mu$ M TTX. B. No inward current was detected in GFP-MSCs. Peak current-voltage relationship was plotted against the voltages.



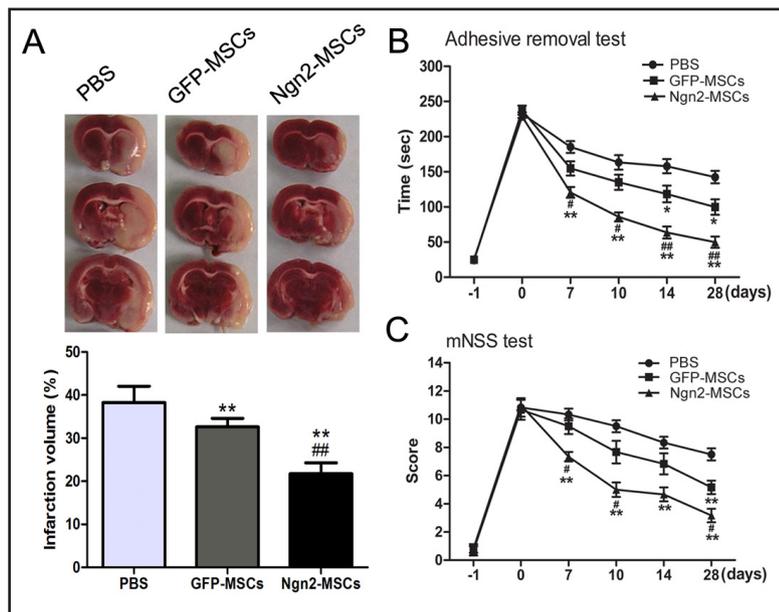
#### Neural differentiation of Ngn2-MSCs derived NSCs in vitro

Immunocytochemistry for MAP2, NeuN and Tuj1 was performed to identify the differentiated cells derived from Ngn2-MSCs. By day 10 following induction, Ngn2-MSCs expressed MAP2 and Tuj1, markers of mature neurons (Fig. 2A), however, which were absent in Ngn2-MSCs. Additionally, western blotting showed that the levels of NeuN, Tuj1 and MAP2 in Ngn2-MSCs derived cells were significantly higher than those derived from parental MSCs and GFP-MSCs (Fig. 2B). Finally, patch-clamp recordings of MSC-derived cells performed after 2 weeks of neuronal induction, indicating that Ngn2-MSCs expressed voltage-gated Na<sup>+</sup> channel, which could be blocked reversibly by 0.5  $\mu$ M TTX. No inward current was detected in GFP-MSCs derived neurons (Fig. 3).

#### Ngn2-MSCs decreased Infarct Volume

The percentage hemisphere lesion volume (%HLV) was evaluated by TTC staining on day 14 following MCAO. In the PBS group, the HLV spontaneously decreased to  $38.28\% \pm 3.78\%$  at day 14, and GFP-MSCs moderately reduced HLV to  $32.62\% \pm 1.97\%$  at 14 days.

**Fig. 4.** Infarction volume, adhesive removal test and mNSS test after transplantation. A. Infarction volume detected by TTC stain and data are presented as mean relative infarct volume  $\pm$  SD. The adhesive removal test (B) and mNSS test (C) were collected from six animals per group and are presented as means  $\pm$  SD, \*= $p < 0.05$ , \*\*= $p < 0.01$  vs PBS group; #= $p < 0.05$ , ##= $p < 0.01$  vs GFP-MSCs group.



Transplantation of Ngn2-MSCs decreased HLV to a lowest degree among the tested animals on 14 days after MCAO (21.72%  $\pm$  2.57%). (Fig. 4A)

#### *Ngn2-MSCs improved functional recovery*

One day after MCAO, Ngn2-MSCs, GFP-MSCs and PBS were injected into the striatum. Analysis of motor functions was performed using the mNSS test and adhesive removal test. As a result, animals received PBS injection spontaneously recovered to a limited degree over 14 days and reached a plateau thereafter. Animals received GFP-MSCs transplantation exhibited higher recoveries than PBS injected animals and the scores continued to decrease thereafter. In addition, the Ngn2-MSCs treated animals showed the highest recovery among the three groups. At 28 days, there are significant differences between Ngn2-MSCs treated and GFP-MSCs treated groups in adhesive removal test (49.92  $\pm$  19.55 vs. 99.97  $\pm$  27.02) (Fig. 4B) and mNSS test (3.17  $\pm$  1.15 vs. 5.16  $\pm$  1.17) (Fig. 4C).

#### *Neural differentiation of Ngn2-MSCs after transplantation*

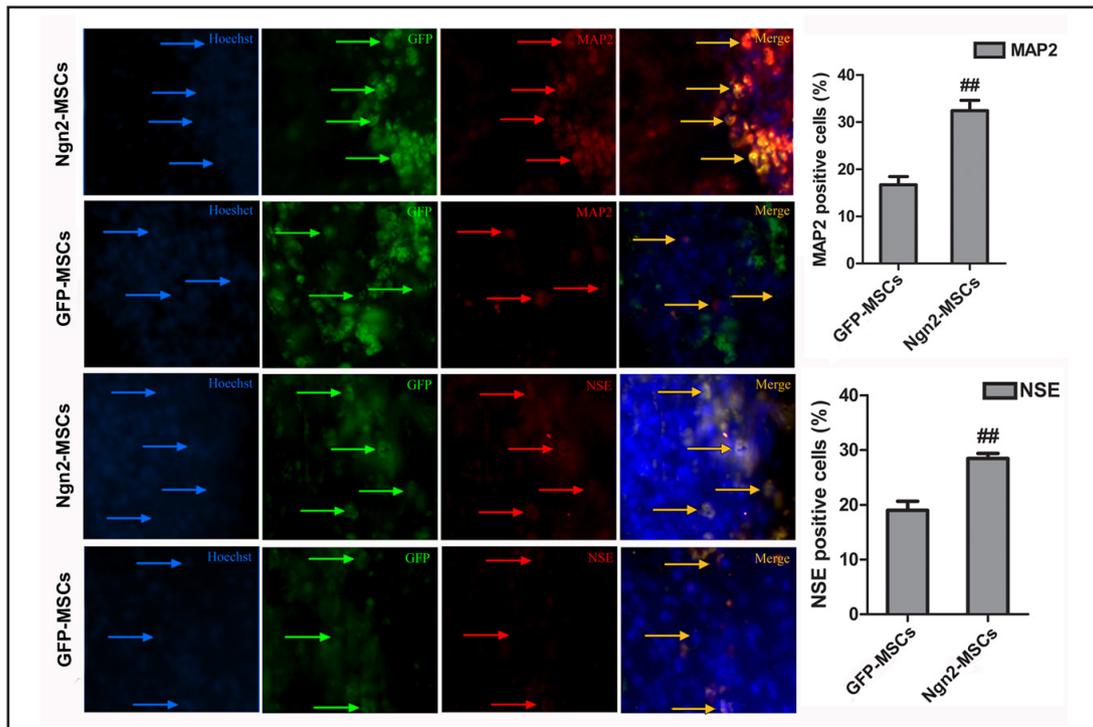
To determine whether the Ngn2-MSCs could transdifferentiate into neuronal cells *in vivo*, immunostaining for MAP2 and NSE was performed. The results showed that 32.40%  $\pm$  4.90% and 28.46%  $\pm$  2.05% of the transplanted Ngn2-MSCs were positive for MAP2 and NSE, respectively. However, only 16.70%  $\pm$  3.85% and 18.98%  $\pm$  3.76% of the transplanted GFP-MSCs expressed MAP2 and NSE, respectively. (Fig. 5)

#### *Ngn2-MSCs increased paracrine factors expression*

We measured VEGF and BDNF levels on 14 days after transplantation by western blot. When compared with PBS control group, expression levels of VEGF and BDNF were significantly higher in both GFP-MSCs and Ngn2-MSCs groups. In addition, in Ngn2-MSCs group, these paracrine factors significantly increased than those in GFP-MSCs and PBS control groups (Fig. 6A).

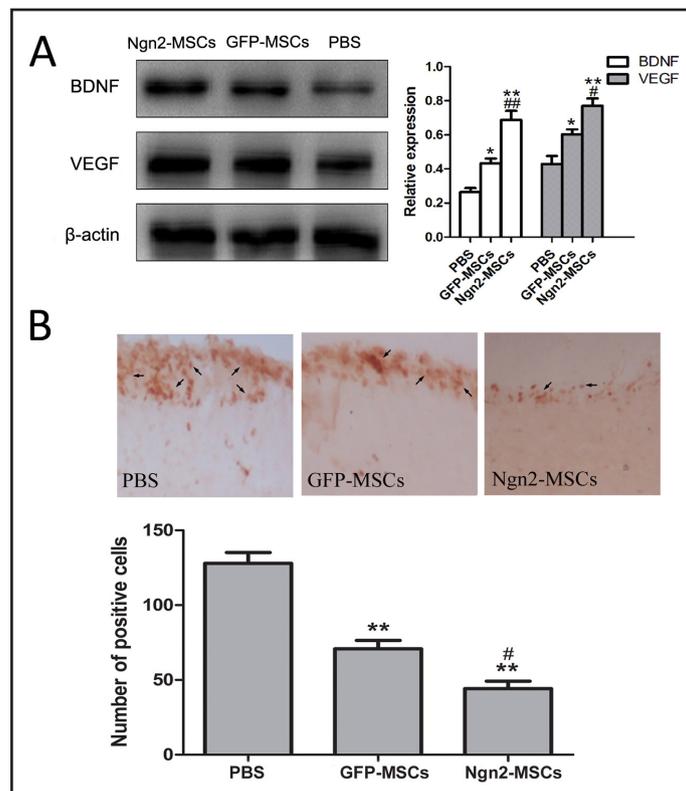
#### *Ngn2-MSCs reduced cell apoptosis in vivo*

TUNEL assays were carried out to detect the effect of transplantation on delayed cell death in the penumbra regions such as ischemic cortex. In the PBS control group, a massive amount of apoptosis were observed in the cortex 10 days after ischemic injury. However, compared with PBS-treated group, TUNEL positive cells in the GFP-MSC and Ngn2-MSCs



**Fig. 5.** *In vivo* differentiation of engrafted cells in the ischemic brain. A. Expression of Map2 in both Ngn2-NSCs and GFP-MSCs. B. Expression of NSE in both Ngn2-NSCs and GFP-MSCs  $**=p < 0.01$  vs GFP-NSCs group.

**Fig. 6.** Paracrine factors and apoptotic cells of the ipsilateral cortex after transplantation. A. Expression of BDNF and VEGF in ischemia brain in PBS, GFP-MSCs and Ngn2-MSCs groups. B. Apoptotic cells of the ipsilateral cortex detected by TUNEL in the PBS, GFP-MSC, Ngn2-MSCs groups. (X100)  $**=p < 0.01$  vs PBS group;  $\#=p < 0.05$  vs GFP-MSC group.



groups decreased 44.70% and 65.48%, respectively. Moreover, the difference between the GFP-MSC and Ngn2-MSCs groups was significant ( $P < 0.05$ ) (Fig. 6B).

## Discussion

In the current study, we showed that transfection with Ngn2 is sufficient to convert the mesodermal cell fate of MSCs into a neural stem one, which expressed neural stem cells markers and were capable of generating electrophysiologically functional neurons *in vitro*. *In vivo*, transplantation of these Ngn2-MSCs decreased apoptotic cells, reduced the infarct volume, increased expression of VEGF and BDNF, and further improved the neurological functional recovery more effectively in a rat model of stroke than MSCs.

Mesenchymal stem cells (MSCs) can be found in various locations in the body, such as adipose tissue, umbilical cord, and adenoid tissue [25-27]. Accumulated studies demonstrated neuroprotective effect of MSCs in rats after MCAO [28]. However, after injected into injured brain, MSCs often remained in an undifferentiated stem state and did not interact with host cells [29]. Ngn2 is a member of the basic helix-loop-helix (bHLH) transcription factor family, committing multipotent progenitors to a neuronal fate and inhibiting astrocytic differentiation [30]. Berninger et al. [31] showed that Ngn2 possessed the ability to reprogram astroglial cells from the early postnatal cerebral cortex to a truly neuronal fate. Combining with other factors, Ngn2 combined with other factors can reprogram somatic cells to induced neural stem cells (iNSCs) [32]. In this study, after Ngn2 transduction, as neural stem cells, MSCs expressed neural stem markers such as Nestin and Pax6, which were also detected in NSCs previously described [13, 14]. Coincidentally, Ngn2-MSCs continuously proliferated in growth medium but lost the potential to differentiate into adipocytes and osteocytes under conditions in which naive MSCs easily differentiated into these mesodermal cell types [33].

We then detected whether Ngn2-MSCs could differentiate into functional neurons. Following neural induction, compared with GFP-MSCs, immunocytochemistry showed that a much more proportion of Ngn2-MSCs were positive for Tuj1 and MAP2, which was also confirmed by western blot. Moreover, Ngn2-MSCs derived neurons could acquire voltage-gated Na<sup>+</sup> channels, which are critical elements for initiation and propagation of action potential in neurons [34], but those were absent in MSCs [35]. These results indicated that Ngn2-transfected might reprogram MSCs into a neural stem fate.

Previous studies have demonstrated that MSCs improved the neurological function after stroke in rats [36]. After transplantation, animals received GFP-MSCs injected exhibited higher motor recovery than PBS injected animals, which was accessed by adhesive removal tests and mNSS tests. In the PBS group, the time-dependent functional recovery may be due to compensatory neural plasticity or brain remodeling. Importantly, the Ngn2-MSCs treated animals showed the highest motor recovery among the three groups, which may be owing to that neural differentiation in the early period and paracrine effect of Ngn2-MSCs. The moderate functional recovery with GFP-MSCs was also reported in other studies [35, 37]. Additionally, the alteration of infarct volume was coincident with the result of motor improvement. Transplantation with GFP-MSCs did reduce lesion volume a rat stroke model as previously described [38], and Ngn2-MSCs group showed a significantly greater reduction in infarct volume than did the GFP-MSC engrafted group after 14 days of MCAO.

After transplantation, there are only 2%-20% transplanted MSCs typically differentiate into neuronal cells [23, 39]. In this study, we found that approximately 15% of transplanted GFP-MSCs adopted a MAP2<sup>+</sup> and NSE<sup>+</sup> neural cell fate, while others remained undifferentiated. Conversely, Ngn2-MSCs expressed neuronal markers such as MAP2 and NSE at a level that was about 2-fold higher than that in GFP-MSCs. These differentiated cells may replace the infarcted tissues. However, a moderate to severe MCAO would cause more than  $2 \times 10^7$  cells die, approximately 75% of which are neurons [40]. Thus, it is difficult to attribute all functional recovery to the small number of cells replaced.

Many studies have shown that vascular and neuronal growth factors, such as VEGF and BDNF, are shared between angiogenesis and neurogenesis during functional recovery from brain injury [28, 41]. The present study indicated that transfection with Ngn2 increased paracrine factors in the MCAO brain. The expression levels of VEGF and BDNF in the injured cerebral tissues were significantly evaluated in Ngn2-MSCs group than in both GFP-MSCs

and PBS control groups. Previous study indicated transplantation of BDNF-modified MSCs, which produced a high level of BDNF, could reduce apoptotic cells, decrease infarct volume and improve functional recovery in stroke rats [42]. In addition, Miki et al also indicated that VEGF-modified MSCs could significantly reduce infarct volume and improve neurological recovery in stroke [43]. Taken together, these results demonstrated that paracrine effect of Ngn2-MSCs might also contribute to the functional recovery.

The main target of acute stroke therapy is saving the penumbra, where the functions of neurons are impaired but potentially viable. We evaluated the effect of transplanted cells on apoptosis in the area of ischemic cortex by TUNEL staining. Consistent with the remarkable recovery of motor functions, the number of apoptotic cells of the GFP-MSC-engrafted group was about 1.3 times that in the Ngn2-NSCs-engrafted group. This rescue of the penumbra regions from ischemic injury may be the result of paracrine effect and transdifferentiation as above mentioned. Moreover, several other events may rescue the penumbra regions, including immune suppression and promotion of neurogenesis [44].

### Conclusions

In conclusion, the present study suggested that transfection with Ngn2 convert the mesodermal cell fate of MSCs into a neural stem one, and significantly improved functional recovery and reduced ischemic damage in a rat model of MCAO. These results highlight a possible advantage of this gene-modified cell therapy for treating central nervous system diseases.

### Conflict of Interests

The authors declare that they have no competing interests.

### Acknowledgements

The authors gratefully acknowledge professor Lee Sang-Hun for generous donation of neurogenin 2. The authors further want to thank Department of Cardiology, The First Affiliated Hospital of Nanjing Medical University. This study was supported by grants from the National Natural Science Foundation of China (No. 81171147), "Xingwei Project" Key Personal Medical Research Foundation of Health Department of Jiangsu Province (No. RC201156), "Six Categories of Key Person" Research Foundation of Jiangsu Province (No. 069), Nanjing Medical Science and Technique Development Foundation (No. QKK10194), Program Sponsored for Scientific Innovation Research of College Graduate in Jiangsu Province (No. CXZZ12\_0583), and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (No. JX10231801).

### References

- 1 Modan B, Wagener DK: Some epidemiological aspects of stroke: Mortality/morbidity trends, age, sex, race, socioeconomic status. *Stroke* 1992;23:1230-1236.
- 2 Kwiatkowski TG, Libman RB, Frankel M, Tilley BC, Morgenstern LB, Lu M, Broderick JP, Lewandowski CA, Marler JR, Levine SR, Brott T: Effects of tissue plasminogen activator for acute ischemic stroke at one year. National institute of neurological disorders and stroke recombinant tissue plasminogen activator stroke study group. *N Engl J Med* 1999;340:1781-1787.
- 3 Hassani Z, O'Reilly J, Pearse Y, Stroemer P, Tang E, Sinden J, Price J, Thuret S: Human neural progenitor cell engraftment increases neurogenesis and microglial recruitment in the brain of rats with stroke. *PLoS One* 2012;7:e50444.

- 4 Tornero D, Wattananit S, Gronning Madsen M, Koch P, Wood J, Tatarishvili J, Mine Y, Ge R, Monni E, Devaraju K, Hevner RF, Brustle O, Lindvall O, Kokaia Z: Human induced pluripotent stem cell-derived cortical neurons integrate in stroke-injured cortex and improve functional recovery. *Brain* 2013;136:3561-3577.
- 5 Ishikawa H, Tajiri N, Shinozuka K, Vasconcellos J, Kaneko Y, Lee HJ, Mimura O, Dezawa M, Kim SU, Borlongan CV: Vasculogenesis in experimental stroke after human cerebral endothelial cell transplantation. *Stroke* 2013;44:3473-3481.
- 6 Xin H, Li Y, Cui Y, Yang JJ, Zhang ZG, Chopp M: Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. *J Cereb Blood Flow Metab* 2013;33:1711-1715.
- 7 Lin YC, Ko TL, Shih YH, Lin MY, Fu TW, Hsiao HS, Hsu JY, Fu YS: Human umbilical mesenchymal stem cells promote recovery after ischemic stroke. *Stroke* 2011;42:2045-2053.
- 8 Tsai LK, Wang Z, Munasinghe J, Leng Y, Leeds P, Chuang DM: Mesenchymal stem cells primed with valproate and lithium robustly migrate to infarcted regions and facilitate recovery in a stroke model. *Stroke* 2011;42:2932-2939.
- 9 Mora-Lee S, Sirerol-Piquer MS, Gutierrez-Perez M, Gomez-Pinedo U, Roobrouck VD, Lopez T, Casado-Nieto M, Abizanda G, Rabena MT, Verfaillie C, Prosper F, Garcia-Verdugo JM: Therapeutic effects of hmapc and hmsc transplantation after stroke in mice. *PLoS One* 2012;7:e43683.
- 10 Chang DJ, Lee N, Choi C, Jeon I, Oh SH, Shin DA, Hwang TS, Lee HJ, Kim SU, Moon H, Hong KS, Kang KS, Song J: Therapeutic effect of bdnf-overexpressing human neural stem cells (hb1.F3.Bdnf) in a rodent model of middle cerebral artery occlusion. *Cell Transplant* 2013;22:1441-1452.
- 11 Hicks AU, Lappalainen RS, Narkilahti S, Suuronen R, Corbett D, Sivenius J, Hovatta O, Jolkkonen J: 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* 2009;29:562-574.
- 12 Janowski M, Walczak P, Date I: Intravenous route of cell delivery for treatment of neurological disorders: A meta-analysis of preclinical results. *Stem Cells Dev* 2010;19:5-16.
- 13 Han DW, Tapia N, Hermann A, Hemmer K, Hoing S, Arauzo-Bravo MJ, Zaehres H, Wu G, Frank S, Moritz S, Greber B, Yang JH, Lee HT, Schwamborn JC, Storch A, Scholer HR: Direct reprogramming of fibroblasts into neural stem cells by defined factors. *Cell Stem Cell* 2012;10:465-472.
- 14 Ring KL, Tong LM, Balestra ME, Javier R, Andrews-Zwilling Y, Li G, Walker D, Zhang WR, Kreitzer AC, Huang Y: Direct reprogramming of mouse and human fibroblasts into multipotent neural stem cells with a single factor. *Cell Stem Cell* 2012;11:100-109.
- 15 Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E: Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 2006;8:315-317.
- 16 Cho YH, Cha MJ, Song BW, Kim IK, Song H, Chang W, Lim S, Ham O, Lee SY, Choi E, Kwon HM, Hwang KC: Enhancement of msc adhesion and therapeutic efficiency in ischemic heart using lentivirus delivery with periostin. *Biomaterials* 2012;33:1376-1385.
- 17 Fierro FA, Kalomoiris S, Sondergaard CS, Nolte JA: Effects on proliferation and differentiation of multipotent bone marrow stromal cells engineered to express growth factors for combined cell and gene therapy. *Stem Cells* 2011;29:1727-1737.
- 18 Longa EZ, Weinstein PR, Carlson S, Cummins R: Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* 1989;20:84-91.
- 19 Neumann-Haefelin T, Kastrup A, de Crespigny A, Yenari MA, Ringer T, Sun GH, Moseley ME: Serial mri after transient focal cerebral ischemia in rats: Dynamics of tissue injury, blood-brain barrier damage, and edema formation. *Stroke* 2000;31:1965-1972.
- 20 Paxinos G, Watson C, Pennisi M, Topple A: Bregma, lambda and the interaural midpoint in stereotaxic surgery with rats of different sex, strain and weight. *J Neurosci Methods* 1985;13:139-143.
- 21 Hattori H, Suzuki S, Okazaki Y, Suzuki N, Kuwana M: Intracranial transplantation of monocyte-derived multipotential cells enhances recovery after ischemic stroke in rats. *J Neurosci Res* 2012;90:479-488.
- 22 Rana I, Stebbing M, Kompa A, Kelly DJ, Krum H, Badoer E: Microglia activation in the hypothalamic pvn following myocardial infarction. *Brain Res* 2010;1326:96-104.
- 23 Chen J, Li Y, Wang L, Zhang Z, Lu D, Lu M, Chopp M: Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke* 2001;32:1005-1011.

- 24 Li Y, Chen J, Chen XG, Wang L, Gautam SC, Xu YX, Katakowski M, Zhang LJ, Lu M, Janakiraman N, Chopp M: Human marrow stromal cell therapy for stroke in rat: Neurotrophins and functional recovery. *Neurology* 2002;59:514-523.
- 25 Schaffler A, Buchler C: Concise review: Adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies. *Stem Cells* 2007;25:818-827.
- 26 Xu J, Liao W, Gu D, Liang L, Liu M, Du W, Liu P, Zhang L, Lu S, Dong C, Zhou B, Han Z: Neural ganglioside gd2 identifies a subpopulation of mesenchymal stem cells in umbilical cord. *Cell Physiol Biochem* 2009;23:415-424.
- 27 Lee YS, Lee JE, Park HY, Lim YS, Lee JC, Wang SG, Lee BJ: Isolation of mesenchymal stromal cells (mscs) from human adenoid tissue. *Cell Physiol Biochem* 2013;31:513-524.
- 28 Liu X, Ye R, Yan T, Yu SP, Wei L, Xu G, Fan X, Jiang Y, Stetler RA, Chen J: Cell based therapies for ischemic stroke: From basic science to bedside. *Prog Neurobiol* DOI: 10.1016/j.pneurobio.2013.11.007.
- 29 Coyne TM, Marcus AJ, Woodbury D, Black IB: Marrow stromal cells transplanted to the adult brain are rejected by an inflammatory response and transfer donor labels to host neurons and glia. *Stem Cells* 2006;24:2483-2492.
- 30 Ross SE, Greenberg ME, Stiles CD: Basic helix-loop-helix factors in cortical development. *Neuron* 2003;39:13-25.
- 31 Berninger B, Costa MR, Koch U, Schroeder T, Sutor B, Grothe B, Gotz M: Functional properties of neurons derived from in vitro reprogrammed postnatal astroglia. *J Neurosci* 2007;27:8654-8664.
- 32 Sheng C, Zheng Q, Wu J, Xu Z, Wang L, Li W, Zhang H, Zhao XY, Liu L, Wang Z, Guo C, Wu HJ, Liu Z, He S, Wang XJ, Chen Z, Zhou Q: Direct reprogramming of sertoli cells into multipotent neural stem cells by defined factors. *Cell Res* 2012;22:208-218.
- 33 Chan J, O'Donoghue K, Gavina M, Torrente Y, Kennea N, Mehmet H, Stewart H, Watt DJ, Morgan JE, Fisk NM: Galectin-1 induces skeletal muscle differentiation in human fetal mesenchymal stem cells and increases muscle regeneration. *Stem Cells* 2006;24:1879-1891.
- 34 Westenbroek RE, Merrick DK, Catterall WA: Differential subcellular localization of the ri and rii na+ channel subtypes in central neurons. *Neuron* 1989;3:695-704.
- 35 Kim SS, Yoo SW, Park TS, Ahn SC, Jeong HS, Kim JW, Chang DY, Cho KG, Kim SU, Huh Y, Lee JE, Lee SY, Lee YD, Suh-Kim H: Neural induction with neurogenin1 increases the therapeutic effects of mesenchymal stem cells in the ischemic brain. *Stem Cells* 2008;26:2217-2228.
- 36 Wakabayashi K, Nagai A, Sheikh AM, Shiota Y, Narantuya D, Watanabe T, Masuda J, Kobayashi S, Kim SU, Yamaguchi S: Transplantation of human mesenchymal stem cells promotes functional improvement and increased expression of neurotrophic factors in a rat focal cerebral ischemia model. *J Neurosci Res* 2010;88:1017-1025.
- 37 Li Y, Chen J, Wang L, Lu M, Chopp M: Treatment of stroke in rat with intracarotid administration of marrow stromal cells. *Neurology* 2001;56:1666-1672.
- 38 Onda T, Honmou O, Harada K, Houkin K, Hamada H, Kocsis JD: Therapeutic benefits by human mesenchymal stem cells (hmscs) and ang-1 gene-modified hmscs after cerebral ischemia. *J Cereb Blood Flow Metab* 2008;28:329-340.
- 39 Chen J, Li Y, Katakowski M, Chen X, Wang L, Lu D, Lu M, Gautam SC, Chopp M: Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat. *J Neurosci Res* 2003;73:778-786.
- 40 Williams RW, Herrup K: The control of neuron number. *Annu Rev Neurosci* 1988;11:423-453.
- 41 Nakano-Doi A, Nakagomi T, Fujikawa M, Nakagomi N, Kubo S, Lu S, Yoshikawa H, Soma T, Taguchi A, Matsuyama T: Bone marrow mononuclear cells promote proliferation of endogenous neural stem cells through vascular niches after cerebral infarction. *Stem Cells* 2010;28:1292-1302.
- 42 Kurozumi K, Nakamura K, Tamiya T, Kawano Y, Kobune M, Hirai S, Uchida H, Sasaki K, Ito Y, Kato K, Honmou O, Houkin K, Date I, Hamada H: Bdnf gene-modified mesenchymal stem cells promote functional recovery and reduce infarct size in the rat middle cerebral artery occlusion model. *Mol Ther* 2004;9:189-197.
- 43 Miki Y, Nonoguchi N, Ikeda N, Coffin RS, Kuroiwa T, Miyatake S: Vascular endothelial growth factor gene-transferred bone marrow stromal cells engineered with a herpes simplex virus type 1 vector can improve neurological deficits and reduce infarction volume in rat brain ischemia. *Neurosurgery* 2007;61:586-594.
- 44 Honmou O, Onodera R, Sasaki M, Waxman SG, Kocsis JD: Mesenchymal stem cells: Therapeutic outlook for stroke. *Trends Mol Med* 2012;18:292-297.