ABSTRACT

Effects of intraventricular human neural stem cell transplantation on proliferation of endogenous neural stem cells and angiogenesis in focal cerebral ischemic rat model

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Stem cells can differentiate into several types of mature cells, including neurons and glial cells. Stem cell transplantation, a promising therapy, may be able to facilitate functional recovery in animal models. Cerebral ischemia often results in neuronal loss, leading to neurological deficits in stroke patients. To improve functional recovery after stroke, stem cell transplantation, enhancement of endogenous neurogenesis, and angiogenesis may have potential therapeutic applications. In this study, we investigated the route of human neural stem cell (hNSC) transplantation that conferred the greatest therapeutic benefit on functional recovery and infarct volume following focal cerebral ischemia. We also investigated endogenous stem cell proliferation and angiogenesis in the ischemic

rat brain after hNSC transplantation.

In order to compare the potential therapeutic benefits of cell transplantation after focal cerebral ischemia, we administered hNSCs by intrastriatal, intraventricular, and intravenous routes 24 h after middle cerebral artery occlusion (MCAo) in rats. Functional recovery was evaluated using the modified Neurological Severity Score. Fourteen days after transplantation, we evaluated infarct volume, survival, migration, and the phenotypic features of the grafted cells. The transplanted groups demonstrated better performance on both measures compared to the non-transplanted control group. Functional recovery was similar in the intravenous and intrastriatal transplantation groups; however, the intraventricular transplantation group had the most dramatic recovery of all of the transplanted groups ($P < 0.01$). Furthermore, the cerebral infarct size was reduced only in the intraventricular delivery group ($p < 0.05$). The hNSCs were preferentially localized to the ipsilateral ischemic hemisphere, as indicated by immunohistochemistry with a marker for neurons or astrocytes. Based on these findings, intraventricular delivery is more effective than the other delivery routes, producing functional improvement and reduced infarct volume.

In addition, we examined endogenous stem cell proliferation and angiogenesis in the ischemic rat brain after intraventricular hNSC transplantation. Focal cerebral ischemia was induced by MCAo in rats. One day after MCAo, hNSCs were transplanted to the lateral ventricle of the ischemic hemisphere. Daily intraperitoneal injections of bromodeoxyuridine (BrdU) were started on the seventh day after the hNSC transplant. On day 14 after transplantation, we histologically

evaluated the proliferation of endogenous neural stem cells and angiogenesis. Numerous transplanted hNSCs had survived and were preferentially localized to the ipsilateral ischemic hemisphere. Furthermore, BrdU-labeled endogenous neural stem cells were observed in the subventricular zone (SVZ) and dentate gyrus (DG) of the hippocampus, where they differentiated into cells immunoreactive for the neural markers doublecortin, neuronal nuclear antigen NeuN, and astrocyte marker GFAP in the hNSC-treated animals, but not controls. The number of BrdU+/ von Willebrand factor (vWF)+ proliferating endothelial cells was higher in the ischemic boundary zone of hNSCs-treated rats relative to controls.

Finally, these results suggest that hNSC transplantation may be an effective treatment for stroke. Intraventricular delivery may be more effective at producing functional benefits compared to other delivery routes. Furthermore, our study demonstrated that hNSC transplantation following focal cerebral ischemia in the rat promoted angiogenesis and the proliferation of endogenous neural stem cells to differentiate into mature neural-like cells. This study provides valuable insight into the effects of hNSC transplantation on focal cerebral ischemia, which could be applied to the development of an effective therapy for stroke.

Keywords: Focal Cerebral Ischemia; Human Neural Stem Cells; Transplantation; Differentiation; Infarct Size; Behavioral Analysis; Endogenous Neurogenesis; Angiogenesis; Rats

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

hNSCs: human Neural Stem cells

MCAo: middle cerebral artery occlusion

PBS: phosphate buffered saline

FBS: fetal bovine serum

FGF: fibroblast growth factor

CTL: control

TPL: transplantation

H-E : hematoxylin and eosin

GFAP: glial fibrillary acidic protein

FITC: fluorescein isothiocyanate

OB: olfactory bulb

Ctx: cortex

LV: lateral ventricle

Hp: hippocampus

BrdU: bromodeoxyuridine

hNu: human nuclei

NeuN: neuronal nuclei

vWF: von Willebrand factor

SVZ: subventricular zone

DG: dentate gyrus

LS: lateral striatum

IV: intravenous

RT-PCR: reverse transcriptase-polymerase chain reaction

GAPDH: glyceraldehydes 3-phosphate dehydrogenase

INTRODUCTION

Stroke is characterized by rapid loss of brain function caused by a lack of blood supply to the brain and is a major cause of death or permanent disability. Ischemic stroke, the most common type of cerebrovascular injury, accounts for over 80% of all strokes. Ischemia in the brain causes local and/or global neuronal death from oxygen deprivation, leading to permanent loss or impairment of body function (Donnan et al., 2008). Although thrombolytic approaches are available for acute phase stroke, this immediate treatment is often impossible in clinical settings. Despite enormous pharmacological and medical advances, effective treatments for stroke after the acute phase are still needed.

Stem cells are undifferentiated cells that have the capacity to self-renew and differentiate into a range of tissues. Stroke therapy has distinct features compared with those for other neurological diseases, like Parkinson's disease or multiple sclerosis, since stroke is not a progressive condition, involves a focal loss of tissue of all cell types, and is typically associated with a degree of endogenous recovery. Stem cell therapy is therefore not restricted to a paradigm of tissue replacement or a specific neuronal cell type (the focus in Parkinson's disease, for example), but potentially affects inflammation, immunomodulation, and the stimulation of endogenous recovery. Cell therapies probably act on multiple mechanisms following ischemic stroke, depending upon the timing and mode of administration; however, unlike neuroprotectant drugs, cell therapies have the advantage that they may be able to respond dynamically to an environment that varies both temporally and spatially after ischemia, rather than targeting a single pathway or mechanism of action. Interaction

with the host environment appears to dictate the phenotypic properties of stem cell grafts. Stem cells come from various sources, and although they share some common properties, they also differ in many respects and behave differently in terms of their rate of differentiation, trophic factor secretion, and the stimulation of endogenous processes under pathological conditions (Kalladka and Muir, 2014).

Neural stem cells (NSCs) are the primordial, multipotent, self-renewing cells that give rise to differentiated progeny within all neuronal and glial cell fates. NSCs continue to produce new neurons throughout the lifetime in the subventricular zone (SVZ) and dentate gyrus (DG) of the hippocampus in the adult mammalian brain (Gage, 2000) and this process of neurogenesis is proliferated under pathological conditions (Jin et al., 2001, Jin et al., 2004). Interestingly, experimental stroke not only induces the proliferation of neuronal precursor cells in these discrete regions, but also stimulates migration into the injured regions of the ischemic brain, including the striatum and cerebral cortex (Arvidsson et al., 2002, Jin et al., 2003b), suggesting that the brain may engage in self-repair under these conditions. However, the capacity for self-repair appears to be limited; in one study, about 80% of migrating newborn neurons died within six weeks and only about 0.2% of the damaged cells were ultimately replaced via neurogenesis (Nadareishvili and Hallenbeck, 2003). This limitation might be overcome through the pharmacological manipulation of endogenous NSCs in vivo to enhance their propensity for survival, migration, differentiation, and functionality (Abe, 2000).

Cell-based therapy for stroke subsumes at least two possible approaches: enhancement of endogenous cytogenesis and transplantation of exogenous cells, both of which have had success in animal models. A variety of drugs and growth factors promote

endogenous neurogenesis and improve histological and functional outcomes following experimental stroke (Kernie and Parent, 2010), and a range of transplanted cell types are likewise effective (Locatelli et al., 2009). However, little is understood about the possible interaction between transplanted and endogenous neural precursor cells following stroke.

There are several reasons to hypothesize that crosstalk between exogenous and native neuroproliferative cells might be important. First, injury stimulates endogenous neurogenesis (Gould and Tanapat, 1997) and transplantation by certain (e.g., intracerebral or intraventricular) routes proliferated necessity induces at least some degree of injury. Second, transplantation may elicit an inflammatory response that can modify endogenous neurogenesis (Whitney et al., 2009). Third, transplanted cells release growth and other soluble factors that may contribute to their beneficial effects after stroke (Li and Chopp, 2009) and are also known to promote neurogenesis (Bath and Lee, 2010).

Previous studies support the notion that transplanted neural cells can modify endogenous neurogenesis in normal rodents. Intrahippocampal transplantation of glial-restricted progenitors from the embryonic rat spinal cord or neural stem cells from the mouse neural tube (Hattiangady et al., 2007) enhanced neurogenesis, as evidenced by increased doublecortin (Dcx) staining in the dentate gyrus (DG) of middle-aged (12-month old) rats. In a study of aged (22-month-old) rats, neurogenesis in the DG detected by bromodeoxyuridine (BrdU) and Dcx staining increased following the intraventricular administration of conditionally immortalized human fetal brain (CTX0E03) cells (Park et al., 2010).

Another study has described the effect of neural cell transplantation on neurogenesis following cerebral ischemia. CTX0E03 cells were transplanted into the putamen four weeks after middle cerebral artery occlusion (MCAo) in the rat (Stroemer et al., 2009). In contrast to most other reports (Jin et al., 2001, Zhang et al., 2001, Arvidsson et

al., 2002, Parent et al., 2002), these investigators found that MCAo decreased cell proliferation in the subventricular zone (SVZ) as measured by Ki67 immunostaining. Transplantation partially restored the number of Ki67-positive cells, from approximately 25% to 50% of the levels in non-ischemic animals at 3 months post-transplant. However, the cell type affected was not examined and transplantation had no effect on infarct volume in that study.

Enhancement of endogenous neurogenesis and angiogenesis may have potential applications for functional recovery following stroke. In this study, we investigate the route of human neural stem cell transplantation that produces the greatest therapeutic benefit in functional recovery and infarct volume following focal cerebral ischemia in the rat. We also investigated endogenous stem cell proliferation and angiogenesis in the ischemic brain after hNSCs transplantation.

PART Ⅰ

Evaluation of functional recovery and infarct volume after intrastriatal, intraventricular, and intravenous human neural stem cells transplantation in focal cerebral ischemic rat model

Neural stem cells (NSCs) are the primordial, multipotent, self-renewing cells that give rise to differentiated progeny of all glial and neuronal cell fates within all neuronal and glial. NSCs for transplantation can be obtained from embryonic stem cells, induced pluripotent stem cells, bone marrow, adipose-derived mesenchymal stem cells, embryonic NSCs, and fetal and adult nervous systems (Garzon-Muvdi and Quinones-Hinojosa, 2009). The differentiation of NSCs is restricted to neurons, astrocytes, or oligodendrocytes, and can be influenced by intrinsic signals (Massirer et al., 2011) such as neuron-restrictive silencing factors and extrinsic signals such as experimental hypoxia and epidermal growth factors.

These characteristics make NSCs promising candidates for the replacement of dead or damaged neurons from neurodegenerative disorders including stroke. Human fetal NSCs are less tumorigenic than embryonic stem cells; in a clinical trial using human fetal NSCs to treat Batten disease, no tumors were detected in the five patients two years after transplantation (Lindvall and Kokaia, 2011). Further, NSCs express few MHC molecules, which obviates immunorejection (Hori et al., 2003).

Mounting evidence has confirmed that the transplantation of NSCs derived from various origins via different routes reduced the infarct area and promoted neurological and functional recovery (Ishibashi et al., 2004, Jiang et al., 2006, Mochizuki et al., 2008) in animal models with ischemic stroke, although individual contradictory results also exist. For example, intracerebral injection of NSCs derived from human iPSCs did not reduce the infarct volume nor improve functional recovery in a rat ischemic stroke model, although NSCs could survive and differentiate into neurons (Jensen et al., 2013). Following striatum implantation, proximity to the lesion influences survival while very few cells reach the brain following intravenous

administration as they are filtered by the pulmonary vascular bed and sequestered in the spleen (Pendharkar et al., 2010). The time, dose, route, and type of transplanted cell might account for these differential results.

The cell delivery route chosen for the therapy may influence the migration and final destination of the transplanted cells. In previous experimental studies, various cell delivery routes, such as intravenously (Chen et al., 2001a, Chu et al., 2004), intra-arterially (Shen et al., 2006, Walczak et al., 2008), intracisternally (Zhang et al., 2004), intraperitoneally (Gao et al., 2001), intraventricularly (Ohta et al., 2004), intracerebrally (Ishibashi et al., 2004, Chen et al., 2008), or directly into the lesioned site (Amsalem et al., 2007), have been investigated for their therapeutic benefit and underlying mechanisms.

However, it remains unclear how the route of cell administration affects functional recovery and infarct volume, which may influence its therapeutic efficacy. In this study, we investigated the effects of lateral intrastriatal (LS), lateral intraventricular (LV), and intravenous (IV) injection of hNSCs on functional recovery and cerebral infarction in rats with middle cerebral artery occlusion.

MATERIALS AND METHODS

1. Animal groups

Sprague-Dawley male rats (Samtaco, Korea, weighing 270–300 g) were housed under 12 h light/12 h dark cycle with unlimited access to food and water. Animal housing, care, and experimental procedures were in accordance with the National Institutes of Health Guide of Experimental Animals and the Committee of the Clinical Research Institute at Seoul National University Hospital, Korea. An effort was made to minimize the pain and suffering of the animals. Experiments were performed as outlined in Figure 1.1. Thity two rats were subjected to MCAo in the left hemisphere, and implanted with human NSCs into the left lateral ventricle, into the ischemic region of the left striatum, and tail at 24 hours after MCAo and were sacrificed 14 days. The control group (n = 8) was no injected hNSCs.

2. Human NSC preparation

Immortalized human neural stem cells (F3) were established and prepared as described previously (Kim et al., 2009). Briefly, primary dissociated cell cultures were prepared from embryonic human brains of 15 weeks gestation as described previously in detail. To provide an unambiguous molecular tag for identifying the implanted cells, cell line was infected with a replication incompetent retroviral vector encoding galactosidase (Lac Z) and puromycin resistant genes. The cerebrum cultures were retrovirally transduced with v-myconcogene and subsequently cloned. One of these clones was named HB1.F3 and was further studied, which could be subcultured and passaged weekly over a period of 6 months.

3. Focal ischemia model

Transient focal cerebral ischemia was induced using the endovascular internal carotid artery (ICA) suture method of (Longa et al., 1989) with minor modifications. In brief, after the intraperitoneal injection of 1% ketamine (30mg/kg) and xylazine hydrochloride (4 mg/kg), the left common carotid artery was exposed at its bifurcation by a midline cervical incision. The branches from the external carotid artery (ECA) were then coagulated and the pterygopalatine artery was ligated with a 4-0 silk suture. The ECA was then transected and a 3-0 nylon monofilament suture, its tip rounded by heating, was inserted into the ECA stump. To occlude the origins of the MCA and proximal anterior cerebral artery, the suture was advanced into the ICA 15 mm beyond the ICA–pterygopalatine artery bifurcation. The suture was then secured in place with a ligature and the wound closed. After 2 h of occlusion, the monofilament was removed. No case of seizure occurred during the experiments at any time following the MCA occlusions. Rectal temperature was maintained at 37 ± 0.5℃ using a thermistor-controlled heating blanket. Free access to food and water was allowed after recovery from anesthesia. Cannulation of a femoral artery allowed mean arterial blood pressure (MABP), arterial blood gases, pH, and blood glucose to be monitored. MABPs were determined during occlusion and during the first 30 min of reperfusion. Arterial blood samples obtained using a femoral catheter were analyzed at baseline and after ischemia. Rats were kept in air-ventilated cages at 24 ± 0.5 ℃ for the duration of the experiment.

4. Human NSC transplantation

Immortalized human neural stem cells (F3) were transplanted 24 h after induction of

focal ischemia. Rats (n= 3–9/group) were reanesthetized and placed in stereotaxic frames with a rat head holder. Burr holes were drilled with a dental drill, which was irrigated continuously with saline at room temperature to prevent overheating of the underlying cortex. hNSCs (1.2×10^5 cells/μl in PBS) were injected with a Hamilton syringe either (1) into the left lateral ventricle (−0.8 mm anterior to the bregma, 1.3 mm lateral to the midline, and 3.7 mm beneath the dura), in a volume of 5 μl over 5 min, or (2) into the ischemic region of the left striatum (0.7 mm anterior to the bregma, 3.2 mm lateral to the midline, and 5.5 mm beneath the dura), in a volume of 3 μl over 5 min; in either case, the needle was left in place afterwards for an additional 5 min. After injections were completed, bone wounds were closed with bone wax, anesthesia was discontinued, and animals were returned to their cages. Another group of rats received 3×10^6 cells by tail vein injection in a volume 200 μl. Rats were perfused with 0.9% saline and 4% paraformaldehyde in PBS (pH 7.5) at 14 days after transplantation.

5. Behavioral test

5.1. modified neurologic severity scores (mNSS)

Table 1 shows a set of the mNSS. One of the most common neurological scales used in animal studies of stroke is the modified neurologic severity scores (mNSS), which includes the assessment of motor (muscle status and abnormal movement), sensory (visual, tactile, and proprioceptive), and reflex (pinna, corneal, and startle) functions (Chen et al., 2001b). The testing was performed before MCAo and at days 1, 7, and 14 after MCAo.

5.2. Rotarod test

In the rotarod motor test (Chen et al., 2001b), the rats were placed on the rotarod cylinder, and the time the animals remained on the rotarod was measured. The speed was slowly increased from 4 to 40 rpm within 5 minutes. A trial ended if the animal fell off the rungs or gripped the device and spun around for 2 consecutive revolutions without attempting to walk on the rungs. The animals were trained 3 days before MCAo. The mean duration (in seconds) on the device was recorded with 3 rotarod measurements 1 day before surgery. Motor test data are presented as percentage of mean duration (3 trials) on the rotarod compared with the internal baseline control (before surgery).

6. Measurement of infarct volume

Infarct volume was assessed 15 days post ischemia. After cardiac perfusion-fixation with 4% paraformaldehyde in 0.1 M PBS, the brains were removed and cut into 40-μm-thick coronal sections by using a cryostat microtome. In total, six brain sections from each brain were mounted onto glass slides, and processed for hematoxylin and eosin (H-E) staining; infarct volumes were measured using an image analysis program (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA). The indirect lesion area, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, was calculated. Relative infarct volume was presented as a volume percentage of the indirect lesion compared with the contralateral hemisphere (Swanson et al., 1990)

7. Immunofluorescence histochemistry

After transcardial perfusion with ice-cold 4% paraformaldehyde (PFA), brains were removed and post fixed in PFA overnight following cryoprotection in 30% sucrose, and then cut in 40 μm thick coronal sections on powdered dry ice using a freezing microtome (Leica, Germany). For immunofluorescent immunohistochemical staining, the sections were incubated in blocking buffer. The following primary antibodies were used: mouse monoclonal anti-human nuclei antibody to label human cells (1:100, Chemicon International, Temecula, CA, USA), mouse monoclonal anti-neuronal nuclei antigen (NeuN) to label mature neurons (1:100; Chemicon International), and rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) to label mature astrocytes (1:1000; Chemicon International). The sections were incubated with the primary antibodies at 4°C for 16 h. After washing, the sections were incubated for 2 h at room temperature with the following fluorophore-conjugated secondary antibodies diluted at 1:200: Alexa 488-conjugated goat anti-mouse (Molecular Probes, Eugene, OR, USA), or Alexa 568-conjugated goat anti-rabbit (Molecular Probes).

8. Statistics

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) with significance of difference between the groups assessed using a one-way ANOVA, followed by Tukey post hocmeans comparison test, or Student's t test. The data are presented as the mean ± standard deviation (SD); $P < 0.05$ was considered statistically significant.

RESULTS

Intraventricular administration of hNSCs after transient MCAo significantly increases functional recovery compared to LS or IV administration.

We evaluated the effects of hNSC transplantation on functional recovery after MCAo in rats, using the mNSS and rotarod tests. The hNSC transplants, regardless of administration route, performed better than the nontransplanted group. The degree of functional recovery was similar by intravenous or intrastriatal transplantation (Figure 1.2A, $P < 0.05$), whereas there was significantly improved functional recovery in the intraventricular transplantation group (Figure 1.2A, $P < 0.01$). However, there were no significant differences on the rotarod test between the hNSC-transplanted groups and the non-transplanted group (Figure 1.2B; $P > 0.05$). These results indicate that neural stem cells enhance recovery after focal cerebral ischemia.

Intraventricular administration of hNSCs following transient MCAo reduces infarct volume

We assessed which hNSC transplantation routes influenced infarct volume following MCAo using H-E staining. Cerebral infarct size was reduced only in the intraventricular delivery group ($175.0\% \pm 18.34\%$, $P < 0.05$) when compared to the non-transplanted group ($232.5\% \pm 14.17\%$, $P < 0.05$) (Figure 1.3). However, there were no significant differences between the intravenous and intrastriatal transplantation groups compared to the non-transplanted group (Figure 1.3).

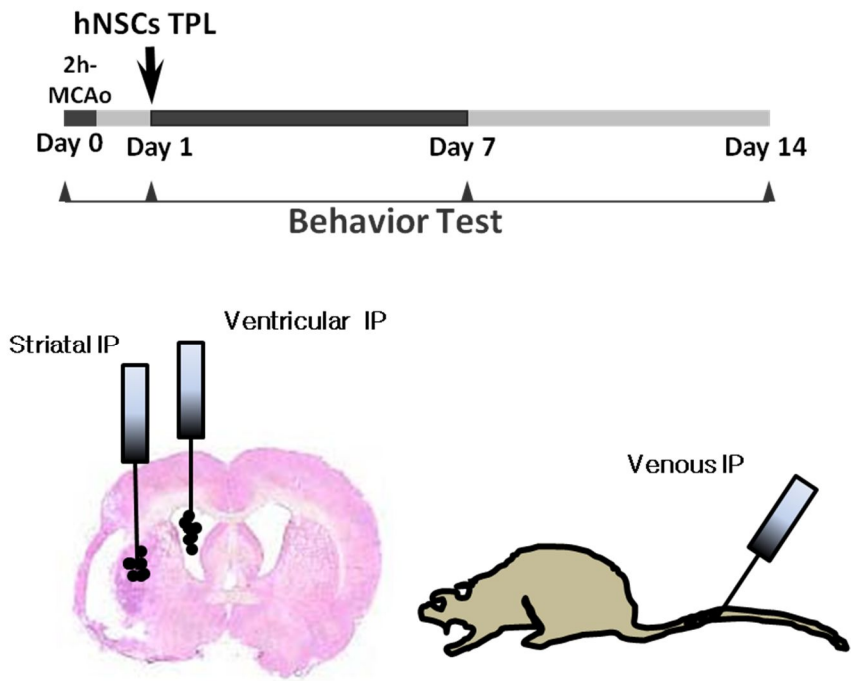

Figure 1.1 Experimental design of the present study. Ischemic stroke was induced by occlusion of the middle cerebral artery (MCA) and hNSCs were transplanted into the ipsilateral striatum (LS), lateral ventricle (LV), or tail vein (IV) of rats on the following day. Behavioral tests were performed days 1, 7, and 14 after MCAo.

Table 1. Modified neurologic severity scores (mNSS)

Neurological Severity Scores (NSS)	Points
Motor tests	
Raising rat by the tail (total 3)	3
Flexion of forelimb	1
Flexion of hindlimb	1
Head moved >10 ° to vertical axis within 30 s	1
Placing rat on the floor (normal=0; maximum=3)	3
Normal walk	0
Inability to walk straight	1
Circling toward the paretic side	2
Fall down to the paretic side	3
Sensory tests (total 2)	2
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation, pushing the paw against the table edge to stimulate limb muscles)	1
Beam balance tests (normal=0; maximum=6)	6
Balances with steady posture	0
Grasps side of beam	1
Hugs the beam and one limb falls down the beam	2
Hugs the beam and two limb fall down the beam, or spins on beam (>60s)	3
Attempts to balance on the beam but falls off (>40 s)	4
Attempts to balance on the beam but falls off (>20 s)	5
Falls off: No attempt to balance of hang on the beam (<20 s)	6
Reflexes absent and abnormal movements (total 3)	4
Pinna reflex (head shake when touching the auditory meatus)	1
Corneal reflex (eye blink when lightly touching the cornea with cotton)	1
Startle reflex (motor response to a brief noise from snapping a clipboard paper)	1
Seizures, myoclonus, myodystony	1
Limb placement test	10
Visual Forward	1
Visual Lateral –3times	3
Proprioperception (forepaw) –3times	3
Proprioperception (hindpaw) –3times	3

One point is awarded for the inability to perform the tasks or for the lack of a tested reflex.

Chen et al., 2001

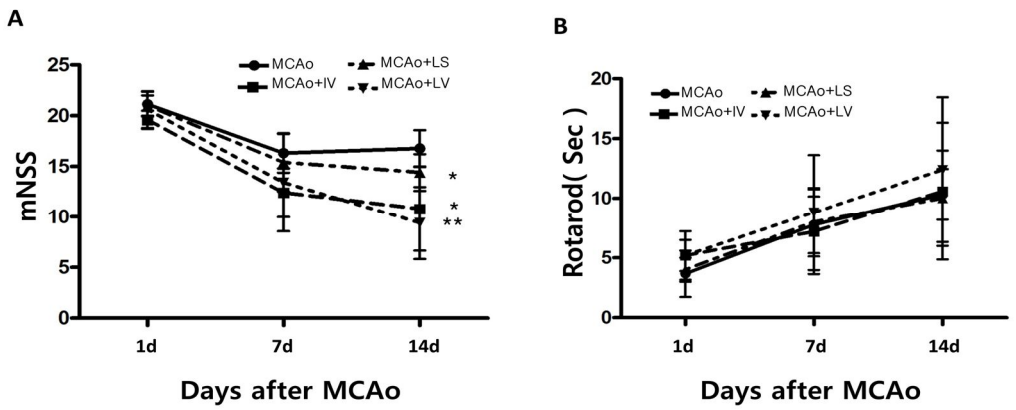

Figure 1.2 Intraventricular administration of hNSCs after transient MCAo significantly increases functional recovery compared to LS or IV administration. Behavioral tests were performed using the modified Neurological Severity Score (mNSS) and rotarod test at days 1, 7, and 14 after hNSC transplantation. (A) Functional recovery was similar between the intravenous and intrastriatal transplantation groups, whereas there was significantly improved functional recovery in the intraventricular transplantation group. (B) There were no significant differences on the rotarod test between the hNSC transplanted groups and the non-transplanted group (B) The data are expressed as the Mean ± SD (n = 8); Data were analyzed using ANOVA. * $P < 0.05$, ** $P < 0.01$ vs. control group.

Transplanted hNSCs exhibit directional migration in ischemic lesions following intrastriatal, intraventricular, and intravenous administration

The hNSCs migrated in ischemic brains following ST, LV, or IV administration. The amount of transplanted cells was presented in the host brain. We observed that hNSCs were preferentially localized to the ipsilateral ischemic hemisphere (Figure 1.4).

Transplanted hNSCs exhibit differentiation in the ischemic lesion site following intrastriatal, intraventricular, or intravenous administration

To determine the differentiation potential of transplanted hNSCs in the ischemic brain, we conducted double labeling with antibodies specific to human nuclei (hNu) and GFAP, or NeuN. We found that hNu-positive cells were located in the infarct border zone and were co-localized with the marker for mature neurons, NeuN, and a small percentage expressed the astrocyte marker, GFAP (Figure 1.5). However, transplanted hNSCs were not observed in the contralateral hemisphere.

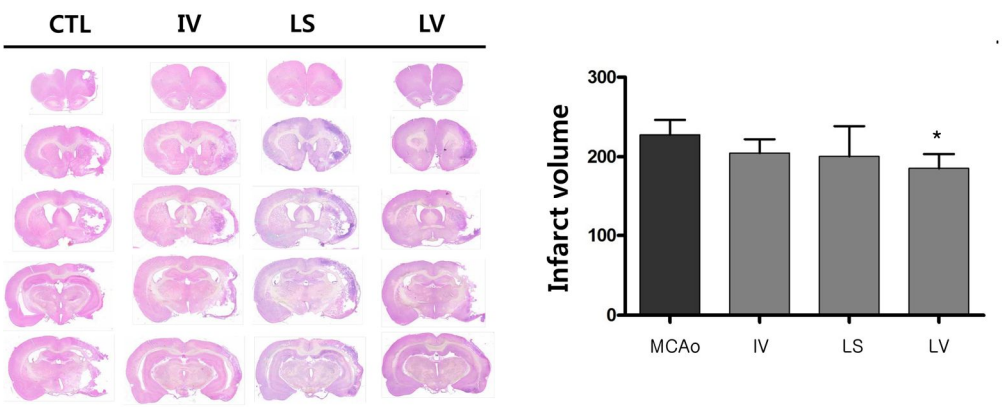

Figure 1.3 Intraventricular hNSC transplants resulted in reduced infarct volume at day 14 after MCAo. (A) Images show H-E staining of serial coronal brain sections on day 14 after MCAo in the non-transplanted and hNSC-transplanted rats. (B) Bar graph represents the quantification of the infarct volume. The data are expressed as the mean ± SD (n = 8); Data were analyzed using ANOVA. *P < 0.05 vs. control group.

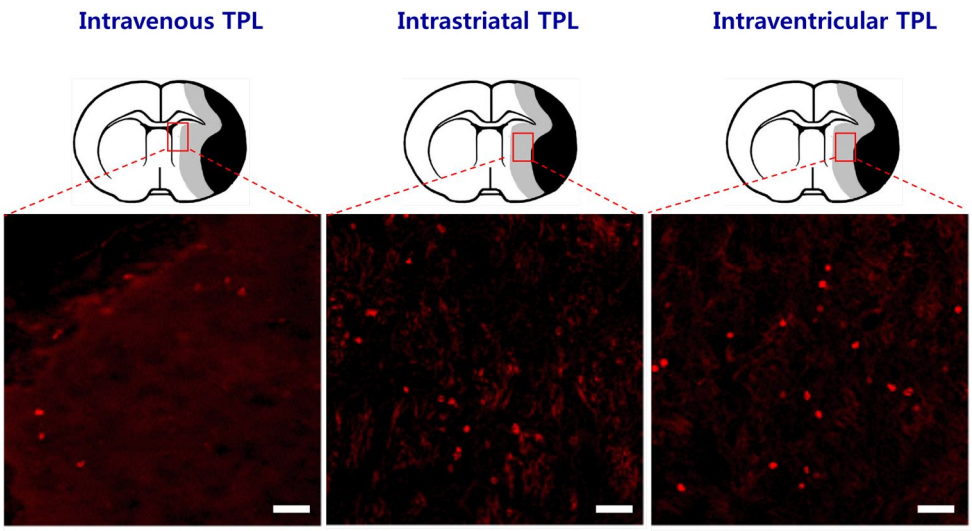

Figure 1.4 Transplanted hNSCs exhibit directional migration in ischemic lesions following intrastriatal, intraventricular, or intravenous administration. Survival and migration patterns of hNSCs transplanted into the ipsilateral subventricular zone of the rat brain subjected to ischemic stroke. hNSC (anti-human-specific nuclei, red) are localized to the striaum, mainly in the ischemic region. Scale bar is 100 μm.

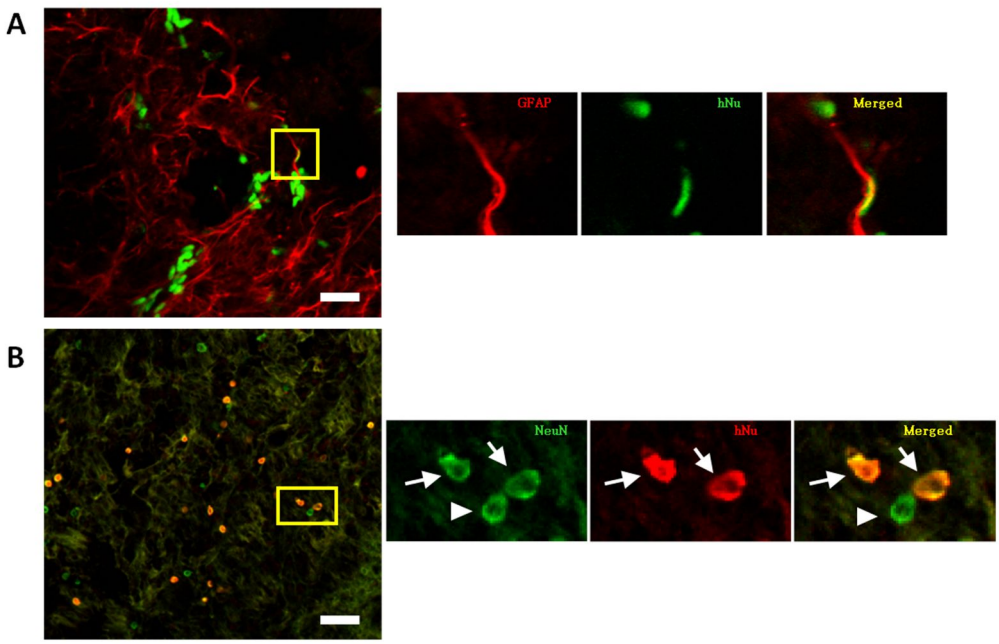

Figure 1.5 Transplanted hNSCs exhibit differentiation in the ischemic lesion following intrastriatal, intraventricular, or intravenous administration. Differentiation pattern of hNSCs transplanted into the ipsilateral subventricular zone of the rat brain subjected to ischemic stroke. Immunohistochemical staining for human-specific nuclei (hNu) to identify transplanted hNSCs in the ischemic penumbra (A, B), hNu + GFAP (A), and hNu + NeuN (B) in the ischemic striatum 14 days after stroke. Arrows, double-labeled cells; arrowhead, single-labeled cells. Scale bar, 100 μm

DISCUSSION

In the present study, we demonstrated that intralateral ventricular administration of hNSCs after transient MCAo significantly increased functional recovery and reduced infarct volume compared to LS or IV administration. Transplanted hNSCs can migrate to the ischemic brain areas via all three delivery routes and preferentially localize to the hemisphere ipsilateral to the ischemic lesion. This is in agreement with the previous finding that transplanted cells are attracted by and interact with tissue regions undergoing degeneration and reorganization (Modo et al., 2002).

As previously shown, locations in the brain selected for cell injection after stroke are associated with different functional benefits (Modo et al., 2002, Mochizuki et al., 2008), This suggests that different transplantation sites may have unique utility and the type and extent of functional recovery is affected by the localization of grafted cells within the brain (Modo et al., 2002, Roitberg, 2004). In addition, functional outcome after cell-based therapy is dose-dependent and a certain number of grafted cells is necessary to significantly attenuate the functional deficits induced by ischemic injury (Chen et al., 2001a).

The precise mechanism by which the transplanted cells are guided to the site of damaged tissue remains unknown, but the brain expressed chemotactic signals that attract the cells and direct their migration to damaged areas in response to ischemic injury (Chopp and Li, 2002, Magnitsky et al., 2005, Shyu et al., 2006, Bacigaluppi et al., 2008). At the molecular level, matrix metalloproteinases (Lee et al., 2006, Wang et al., 2006) and the SDF-1/CXCR4 system (Wang et al., 2008) are involved in directing cell migration. Our observations show that whichever route of

cell administration is chosen, hNSCs have the capacity to migrate to the lesioned area. This supports the hypothesis that the chemoattractive gradient in the brain lesion influences selective and specific homing of transplanted cells to the brain area with ischemic injury. Therefore, the status of the ischemic brain may play a critical pathobiological role in mediating the cell grafting process.

The exact mechanism underlying the functional improvement observed in the ischemic brain after NSC-based therapy remains unclear. Cell replacement was initially considered the main mechanism responsible for the beneficial effects of NSC implantation because a number of animal studies have demonstrated that transplanted NSCs differentiate into neuronal and/or glial phenotypes (Lee et al., 2009, Song et al., 2009). However, enhanced functional recovery could be observed even in the absence of neuronal differentiation (Hao et al., 2014), suggesting that cell replacement may not directly contribute to the neuronal functional improvement caused by NSC transplantation. Therefore, our focus in this study was not on the replacement of ischemia-damaged tissue with NSCs but rather on functional evaluation and infarct volume.

The functional mechanisms and therapeutic effects of stem cells in stroke have been investigated using various delivery routes including intravenous, intrastriatal, and intraventricular transplantation (Jin et al., 2005). Although intravenous delivery is the easiest route of stem cell administration, it yields very few donor cells in the ischemic brain region (Jin et al., 2005), prompting the investigation of more effective ways to achieve NSC transplantation. A previous study has shown that the intraventricular implantation of NSCs to be less invasive and yield more cells, resulting in increased cell engraftment compared to intrastriatal administration (Jin et al., 2005). The mechanism

underlying the migration of intraventricularly transplanted hNSCs to the ischemic lesion remains unclear. Several studies have reported that NSCs have specific receptors activated by chemokines released in the injury site that govern cell migration towards the lesion (Kelly et al., 2004). However, the specific mechanisms involved in NSC chemotaxis after transplantation have yet to be investigated.

We did not conduct a systematic or quantitative comparison of the efficiency of cell transplantation by different routes, because different numbers of cells were used (3.6×10^5 cells into the striatum, 6×10^5 cells into the ventricle, and 3×10^6 cells intravenously) and it is difficult to know what endpoint (cell lineage and state of differentiation) to consider. Even when more cells were infused through the tail vein, IV administration did not result in a more improved functional recovery in the ischemic brain than with the other administration routes, possibly due to the long migration distance or loss of cells trapped in the filtering organs (Kraitchman et al, 2005; Hauger et al, 2006; Parr et al, 2007). Therefore, delivery route is the determining factor for functional recovery, not the number of transplanted cells, at least in the range employed in this study.

Cell-induced therapeutic benefits following stroke are evoked using various cellular delivery routes (Chopp and Li, 2002, Parr et al., 2007, Bacigaluppi et al., 2008). IV injection, a minimally invasive and clinically relevant technique, offers the most comfortable strategy for both subjects and operators. Some more invasive administration routes, such as intracerebral injection, are widely used in experimental studies and may be applied in future clinical practice (Li et al., 2001, Zhang et al., 2004, Shen et al., 2006, Walczak et al., 2008). We, therefore, assessed functional recovery and infarct volume through these three delivery mechanisms.

In summary, LV hNSC administration after transient MCAo significantly increases functional recovery and infarct volume compared to LS or IV administration. Our findings demonstrate that delivery routes influence functional recovery and infarct volume.

PART Ⅱ

Effects of intraventricular human neural stem cell transplantation on the proliferation of endogenous neural stem cells and angiogenesis in focal cerebral ischemic rat model

Stroke is an acute cerebrovascular disorder caused by an diminished blood supply to the brain due to ischemia or hemorrhage, resulting in extensive loss of neurons and their connections in the damaged brain regions (Jin et al., 2005). The current therapy for patients with stroke is restricted to fast vessel recanalization and pharmacological approaches based on thrombolytic drugs (1995, Wardlaw et al., 2014). Despite considerable research efforts, few treatment options for stroke are presently available and there is an urgent need for approaches that are more clinically efficacious.

Numerous observations in the past decade have altered Cajal's "harsh decree" that the adult brain has a fixed number of neurons (Hallbergson et al., 2003). There is now a consensus that neural stem cells (NSCs) capable of neurogenesis are found in the adult brain within the subventricular zone lining the lateral ventricles and in the subgranular zone of the dentate gyrus (DG) (Gage, 2000). Neurogenesis by NSCs and survival of newly differentiated cells can contribute to self-repair after neuron loss (Arvidsson et al., 2002). The process can be stimulated in response to CNS injury (Aharoni et al., 2005, Emery et al., 2005) and by signaling from astroglia (Song et al., 2002). However, neurogenesis by endogenous NSCs cannot fully compensate for the neural loss observed in CNS disorders and normal aging. These observations have stimulated a search for agents that will increase neurogenesis or enhance neuroprotection. Many factors have been recently evaluated for their role in neurogenesis: nerve growth factor (NGF) (Fiore et al., 2002), brain-derived neurotrophic factor (Scharfman et al., 2005), neurotrophin-4/5 (NT-4/5) (Scarisbrick et al., 2000), neurotrophin-3 (NT-3) (Collazo et al., 1992, Barnabe-Heider and Miller, 2003), ciliary neurotrophic factor (CNTF) (Emsley and Hagg, 2003), VEGF (Schanzer et al., 2004), fibroblast growth factor 2 (FGF-2), (Palmer et al., 1995, Jin et al., 2003a), erythropoietin(EPO) (Shingo et al., 2001), and the polycomb family transcriptional repressor

BMI-1 (Molofsky et al., 2003, Molofsky et al., 2005).

Stem cell-mediated regeneration has emerged as a promising therapeutic strategy to promote regeneration after stroke (Lindvall et al., 2004). Several studies have demonstrated that transplanted neural stem cells (NSCs) restore neurological function and secretion of neurotrophic factors. Furthermore, NSCs were able to differentiate into mature neurons and promote endogenous neurogenesis and angiogenesis in a cerebral ischemia model (Chu et al., 2004, Zhang et al., 2011, Mine et al., 2013). The role that neuroplasticity, neurogenesis, and angiogenesis play in mediating recovery following stroke has been recently reviewed (Ergul et al., 2012, Hermann and Chopp, 2012). However, only a few studies have investigated the relationship between the transplantation of exogenous NSCs and endogenous neurogenesis and angiogenesis after stroke (Jin et al., 2011, Zhang et al., 2011). Previous studies have shown that grafting human stem/progenitor cells or neural stem cells into the dentate gyrus (DG) or ventricles of rodents promotes neurogenesis of endogenous NSCs (Munoz et al., 2005, Park et al., 2010). In this study, we examined whether hNSCs directly transplanted into the neuroproliferative subventricular zone (SVZ) would promote endogenous neurogenesis and angiogenesis in the brain of ischemic rats as intraventricularly injected NSCs can be transplanted into the ischemic brain without cell loss (Jin et al., 2005)

MATERIALS AND METHODS

1. Focal ischemia model

Animal housing, care, and experimental procedures were in accordance with the National Institutes of Health Guide of Experimental Animals and the Committee of the Clinical Research Institute at Seoul National University Hospital, Korea. An effort was made to minimize the pain and suffering of the animals. Transient focal cerebral ischemia was induced in Sprague-Dawley male rats (weight, 270–300 g) by intraluminal thread occlusion of the middle cerebral artery (MCA) for 2 h followed by reperfusion (Song et al., 2009). During brain ischemia, rectal temperature was maintained at 37 ± 0.5°C using a thermistor-controlled heated blanket

2. Human NSC preparation and transplantation

Immortalized human neural stem cells (F3) were established and prepared as described previously, (Kim et al., 2008) and transplanted 24 h after MCA occlusion (MCAo). MCAo rats (n = 7) were reanesthetized and placed in a stereotaxic frame (David Kopf instruments, Tujunga, CA, USA) with a rat head holder, and burr holes were drilled with a dental drill. HNSCs (1.2×10^5 cells/μl in PBS) were injected with a Hamilton syringe into the left lateral ventricle (-0.8 mm anterior to the bregma, 1.3 mm lateral to the midline, and 3.7 mm beneath the dura) in a volume of 5 μl over 5 min, and the needle was left for an additional 5 min after injection (Jin et al., 2005). The control group (n = 7) was injected with vehicle. Subsequently, bone wounds were sealed with bone wax, and the animals were returned to their cages. The rats were perfused with 4% paraformaldehyde in PBS 14 days after hNSC transplantation.

3. BrdU administration

To detect cell mitosis, which is an indirect marker for cell proliferation, bromodeoxyuridine (BrdU) incorporation was used to label DNA-replicating cells. All rats (n = 14) received intraperitoneal injections of 50 mg/kg BrdU (Sigma, St. Louis, MO, USA) in saline daily for 7 days beginning 24 h after hNSC transplantation, and BrdU labeling in the brain was analyzed by immunohistochemistry.

4. Quantitative reverse transcriptase - real time polymerase chain reaction (qPCR)

The brains were removed, and the hippocampi were quickly frozen in liquid nitrogen and homogenized using a Polytron homogenizer. Total RNA was isolated using the RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA, USA) and treated with DNase. Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), based on the manufacturer's protocol. Primer sequences were designed using a software program (Applied Biosystems) and synthesized commercially (Bioneer, Korea). Primers for proliferating cell nuclear antigen (*PCNA*) were as follows: forward, 5′-GAGCAACTTGGAATCCCAGAACAGG-3′; reverse, 5′-CCAAGCTCCCCACTCGCAGAAAACT-3′. GAPDH was used as an internal control to normalize the expression levels of the target genes; the primers were as follows: forward, 5′-AATGCATCCTGCACCACCAA-3′; and reverse, 5′-GTAGCCATATTCATTGTCATA-3′. Quantitative PCR was performed with SYBR Green Master Mix (Applied Biosystems) using the ABI 7700 system (Applied Biosystems). Amplification was carried out under the following conditions: 20-s

denaturation at 94℃ followed by 1-min annealing and extension at 62℃ for 40 cycles. PCR products were then electrophoresised on 1% agarose gel and stained with ethidium bromide.

5. Immunohistochemistry

The sections were placed into 2 N HCl at 37℃ for 10 min and then 0.1 M boric acid at room temperature for 3 min. After blocking in normal serum, sections were treated with mouse monoclonal anti-BrdU (Calbiochem,La Jolla, CA) diluted at 1:100 in phosphate buffered saline (PBS) at 4℃ overnight. Following sequential incubation with biotinylated secondary anti-mouse IgG (dilution 1:200; Vector Laboratories, Inc., Burlingame, CA), the sections were treated with an ABC kit (Vector Laboratories). DAB was then used as a sensitive chromogen for light microscopy.

For immunofluorescence staining with a BrdU-specific antibody, the sections were first incubated in 1.5 M HCl for 30 min at 37°C and then in blocking solution. For other immunofluorescence staining, the sections were incubated in blocking solution and then the following primary antibodies were used: mouse and sheep monoclonal anti-BrdU (1:200; Biodesign, San Diego, CA, USA), goat polyclonal anti-doublecortin (Dcx) to label migrating neuroblasts (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-human nuclei antibody to label human cells (1:100, Chemicon International, Temecula, CA, USA), mouse monoclonal anti-neuronal nuclei antigen (NeuN) to label mature neurons (1:100; Chemicon International), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) to label mature astrocytes (1:1000; Chemicon International), and rabbit monoclonal anti-von Willebrand factor (vWF) to label endothelial cells (1:200;

Abcam, Cambridge, UK). The sections were incubated with the primary antibodies at 4°C for 16 h. After washing, the sections were incubated for 2 h at room temperature (22±3℃) with the following secondary antibodies diluted at 1:200: Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA), Alexa 568-conjugated donkey anti-sheep IgG (Molecular Probes), FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA), or rhodamine-conjugated donkey anti-goat IgG (Jackson ImmunoResearch). BrdU-positive cells and cells double-labeled with BrdU and NeuN, Dcx, GFAP, or vWF were counted in the lesioned hemisphere by using a laser scanning confocal microscope (Carl Zeiss, Weimar, Germany).

7. Data quantification and analysis

All analyses were accomplished using objective counting methods on the two regions: the SVZ and the SGZ of dentate gyrus in the hippocampus. BrdU-positive cells were quantified in the SVZ of individual animal in two coronal sections between 5.5mm and 7.0mm apart from the front of the brain. Two sections were selected in anterior and posterior portion of the SVZ region. BrdU-positive cells and double labeling with NeuN, Dcx, or GFAP were counted in the entire SVZ area at lesioned side using fluorescence microscope imaging system, respectively. Cell counting in the SGZ of hippocampus was performed in the entire SGZ area of dentate gyrus using two sections between 10.0mm and 10.8mm from front of the brain. For confocal microscopy, a laser-scanning confocal microscope (Carl Zeiss) was used.

8. Statistics

Two independent samples *t*-test was used to compare mRNA expression, and cell counts between the groups. Prism 5 for Windows (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analyses. The data are presented as the mean ± standard deviation (SD); $P < 0.05$ was considered statistically significant.

RESULTS

Transplanted hNSCs exhibit directional migration in the ischemic lesion

We observed that hNSCs intraventricularly transplanted within the focal cerebral ischemic region migrated toward the ischemic striatum, mostly located in the infarct border zone (Figure 2.2).

Transplanted hNSCs promoted endogenous cell proliferation in MCAo rats

To explore in detail the effect of transplanted hNSCs on the endogenous NSCs, we analyzed the expression of *PCNA* in the hippocampus. At day 14 after transplantation, the level of *PCNA* mRNA in hNSC-transplanted rats was significantly higher than that in the control PBS-injected rats (Figure 2.3, $P < 0.05$), suggesting an increase in cell proliferation. To further evaluate the influence of hNSC grafting on cell proliferation, we assessed the proliferation of newly generated cells in the SVZ and hippocampus. BrdU was administered daily for 7 days after hNSC transplantation and the proportion of BrdU-positive cells was measured at day 14 after transplantation. There were significantly more BrdU-positive cells in the ischemic hemisphere (the SVZ and DG) of hNSC-treated rats than in PBS-injected rats (Figure 2.4; $P < 0.05$), whereas human-specific nuclei (hNu) were not detected, indicating that hNSCs did not migrate to these brain regions (Figure 2.5).

Transplanted hNSCs promote the proliferation of endogenous NSCs in the SVZ and DG of the hippocampus of ischemic brain

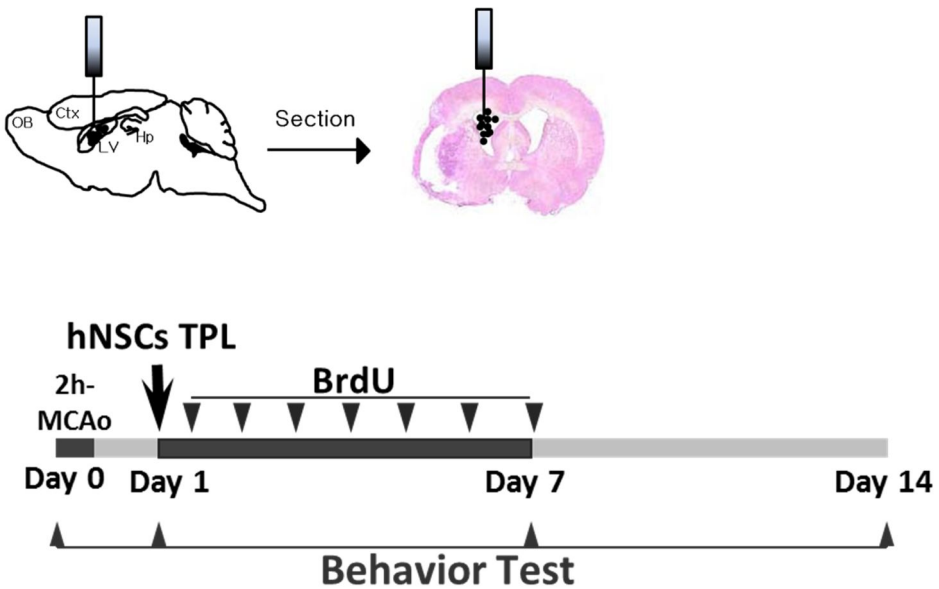

Figure 2.1 Experimental design of the present study. Ischemic stroke was induced by occlusion of the middle cerebral artery (MCA), and hNSCs were transplanted into the ipsilateral ventricle of the brain at 1 day post-stroke. The following day, daily i.p. injections of BrdU were started and continued for 7 days. Behavioral testing was performed at the indicated intervals.

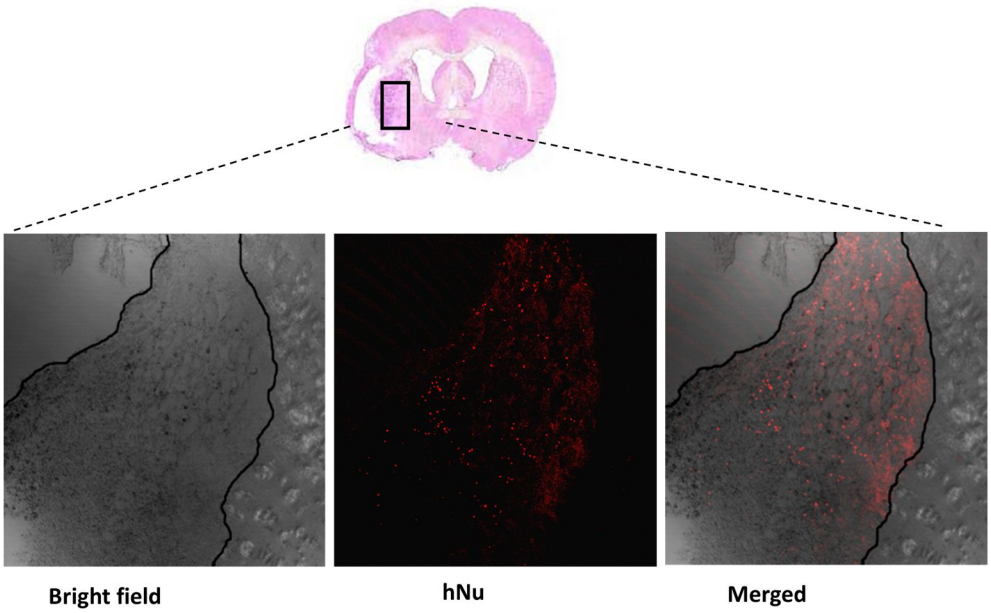

Figure 2.2 Transplanted human neural stem cells exhibit directional migration in the ischemic lesion. Survival and migration pattern of hNSC transplanted into the ipsilateral subventricular zone of the rat brain subjected to ischemic stroke. Immunohistochemical staining for human-specific nuclei (hNu, red) to identify transplanted hNSCs in the ischemic penumbra in the ischemic striatum 14 days after stroke , Magnification, 50×

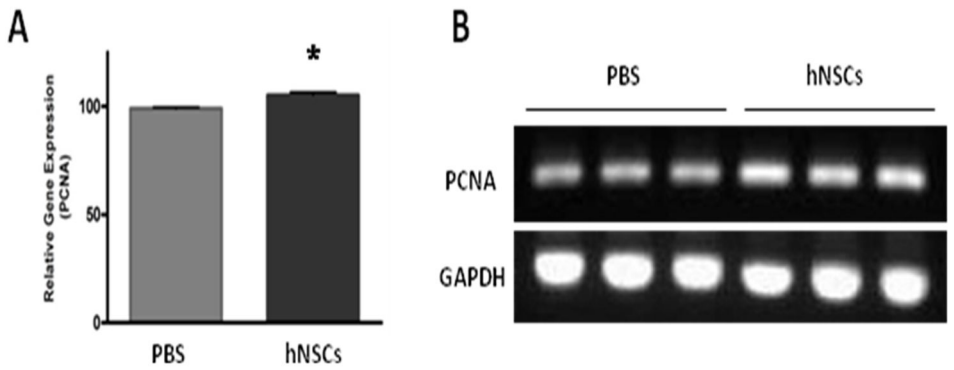

Figure 2.3 Transplanted human neural stem cells promote mRNA expression of proliferation cell nuclear antigen (*PCNA*) in the DG of the hippocampus of ischemic rats. (A) Quantification of mRNA expression of PCNA in the hippocampus. (B) Gel visualization of the PCR products. GAPDH was used as an internal control .The data are presented as the mean ± SD of triplicate independent experiments. (n = 3); Data were analyzed using t-test. * $P < 0.05$, vs. PBS injection

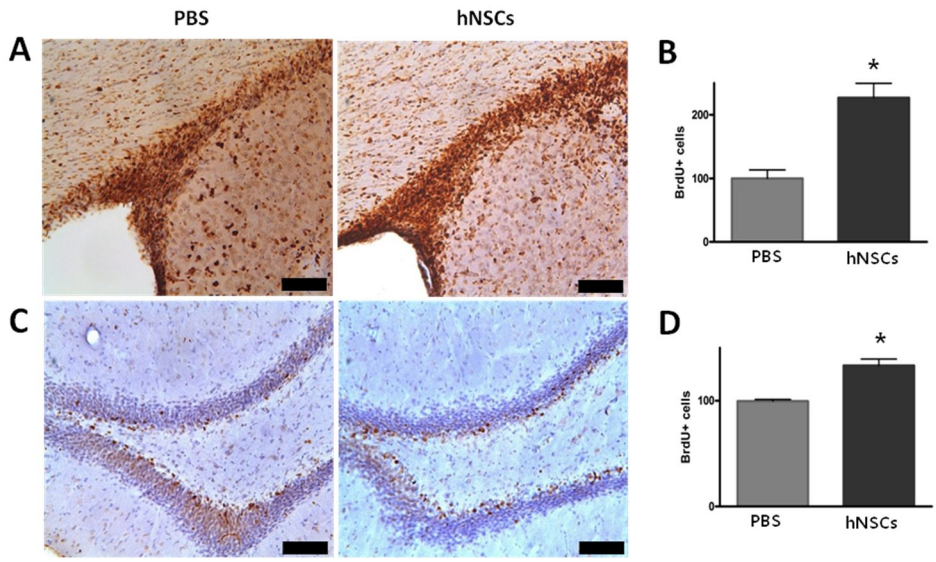

Figure 2.4 Transplanted human neural stem cells promote proliferation of endogenous stem cells in the SVZ and DG of the hippocampus of ischemic rats. Images show immunostaining for BrdU + in the SVZ and DG of the hippocampus of of hNSC-treated (hNSCs) and control (PBS) rats at day 14 after MCAo (A). Bar graphs show quantification of BrdU+ cells (B). The data are expressed as the mean ± SD (n = 7); Data were analyzed using t-test. * P < 0.05, hNSC transplantation vs. PBS injection. Scale bar, 100 μm.

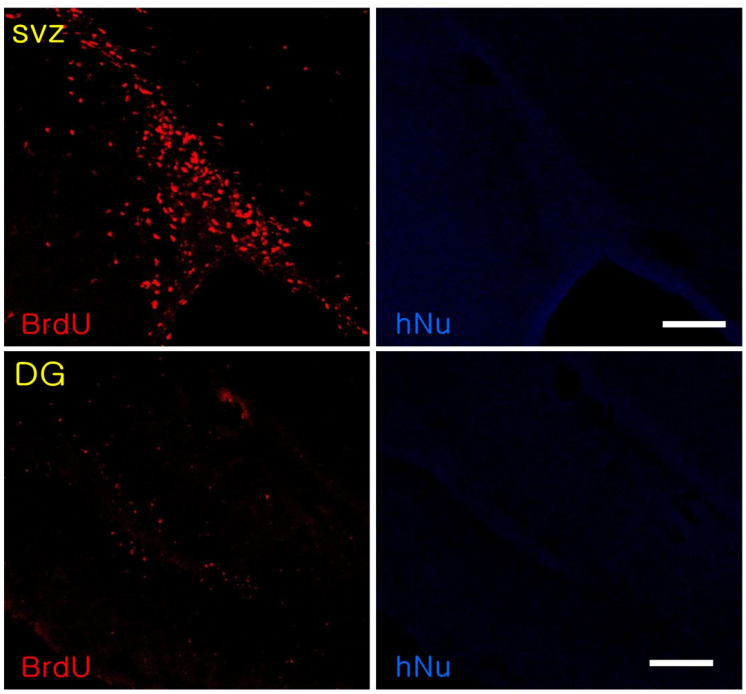

Figure 2.5 Transplanted human neural stem cells did not exhibit in the SVZ and DG of hippocampus of ischemic rats. Human neural stem cells (hNSCs) (anti- human-specific nuclei, blue) localized to the striatum, mainly ischemic region, whereas were not localized in the SVZ and DG of hippocampus of ischemic rats, Scale bar, 100 μm.

The results demonstrate that transplanted hNSCs stimulated the proliferation of endogenous rat stem cells within the SVZ and DG of the hippocampus. To analyze the differentiation potential of endogenous NSCs in rats after hNSC transplantation, we performed double labeling with the antibodies against BrdU and against Dcx, GFAP, or NeuN. BrdU-positive cells expressed Dcx, a marker for migrating neuronal cells. The total number of BrdU+/Dcx+ (Figure 2.6A, B; $P < 0.05$, 2.7A, B; $P < 0.05$) and BrdU+/GFAP+ (Figure 2.6E, F; $P < 0.05$, 2.7E, F; $P < 0.05$) cells was significantly higher in the SVZ and DG of hNSC-treated rats than in PBS-injected rats, whereas the proportion of BrdU+/NeuN+ cells was slightly higher in the hNSC-treated rats than in the PBS-treated rats, although this difference was statistically insignificant (Figure 2.6C, D; $P > 0.05$, 2.7C, D; $P > 0.05$). These results demonstrate that hNSC transplantation promoted the expansion and differentiation of endogenous rat NSCs within the SVZ and DG of the hippocampus.

Transplanted hNSCs promote focal angiogenesis in ischemic rats

To determine the effect of hNSC transplantation on the proliferation of endothelial cells in ischemic rats, we performed double labeling with BrdU and vWF antibodies. In MCAo rats treated with hNSCs, the number of BrdU+/vWF+ cells in the ischemic penumbra was higher than that in the PBS-injected rats at day 14 post MCAo (Figure 2.8, $P < 0.05$), indicating that hNSC transplantation enhanced angiogenesis after focal ischemic stroke.

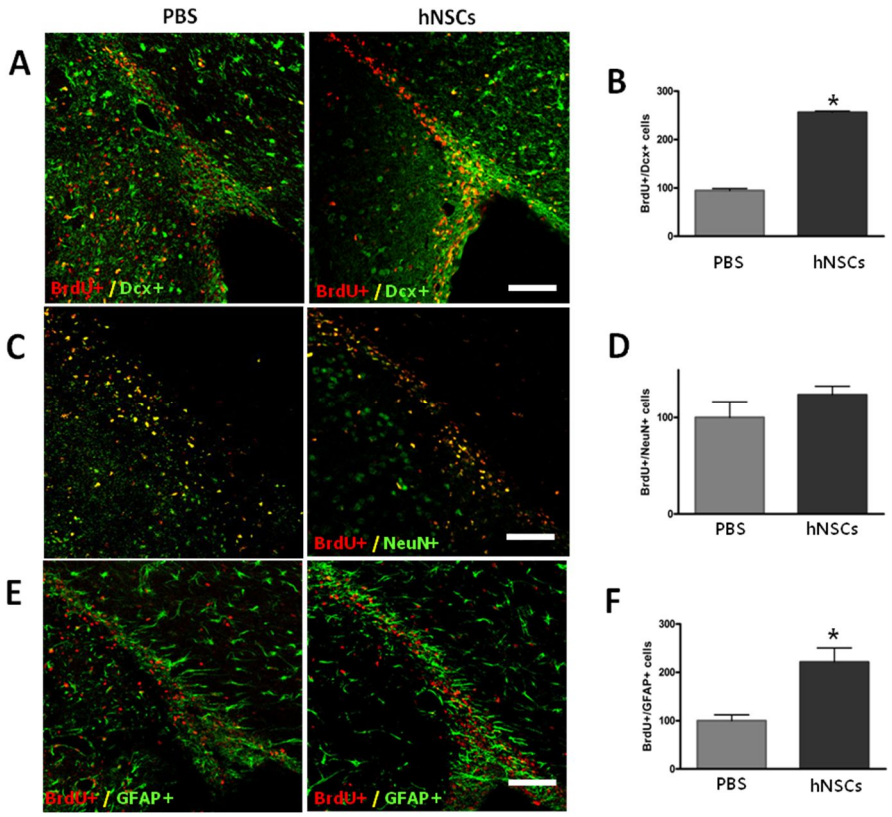

Figure 2.6 Transplanted human neural stem cells promote proliferation of endogenous NSCs in the SVZ of ischemic rats. Transplanted hNSCs enhanced proliferation and differentiation of endogenous neural stem cells in the subventricular zone of ischemic rats. Images show immunostaining for BrdU (red) /Dcx (green) (A), BrdU (red) /NeuN (green) (C), and BrdU (red) /GFAP (green) (E) double-positive cells in the SVZ of hNSC-treated (hNSCs) and control (PBS) rats at day 14 after MCAo. Bar graphs show quantification of BrdU+/Dcx+ (B), BrdU+/NeuN+ (D), and BrdU+/GFAP+ (F) cells. The data are expressed as the mean ± SD (n = 7); Data were analyzed using t-test. * $P < 0.05$, hNSC transplantation vs. PBS injection. Scale bar, 100 μm.

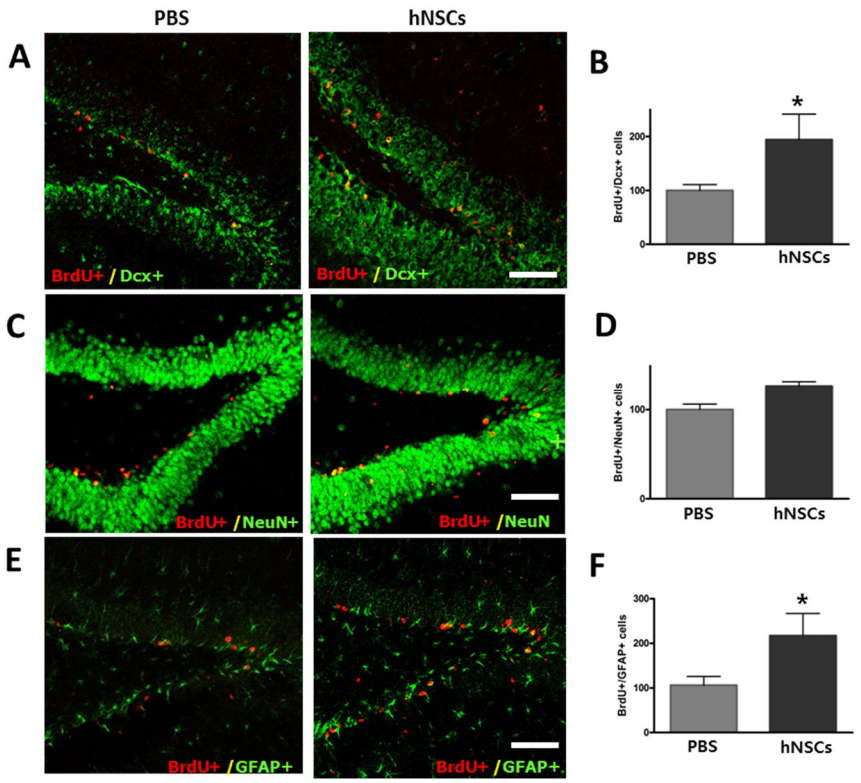

Figure 2.7 Transplanted human neural stem cells promote the proliferation of endogenous NSCs in the DG of the hippocampus of ischemic rats. hNSCs enhanced the proliferation and differentiation of endogenous neural stem cells in the dentate gyrus (DG) of ischemic rats. Images show immunostaining for BrdU (red) /Dcx (green) (A), BrdU (red) /NeuN (green) (C), and BrdU (red) /GFAP (green) (E) double-positive cells in the DG of hNSC-treated (hNSCs) and control (PBS) rats at day 14 after MCAo. Bar graphs show quantification of BrdU+/Dcx+ (B), BrdU+/NeuN+ (D), and BrdU+/GFAP+ (F) cells. The data are expressed as the mean ± SD (n = 7); Data were analyzed using t-test. * $P < 0.05$, hNSC transplantation vs. PBS injection. Scale bar, 100 μm.

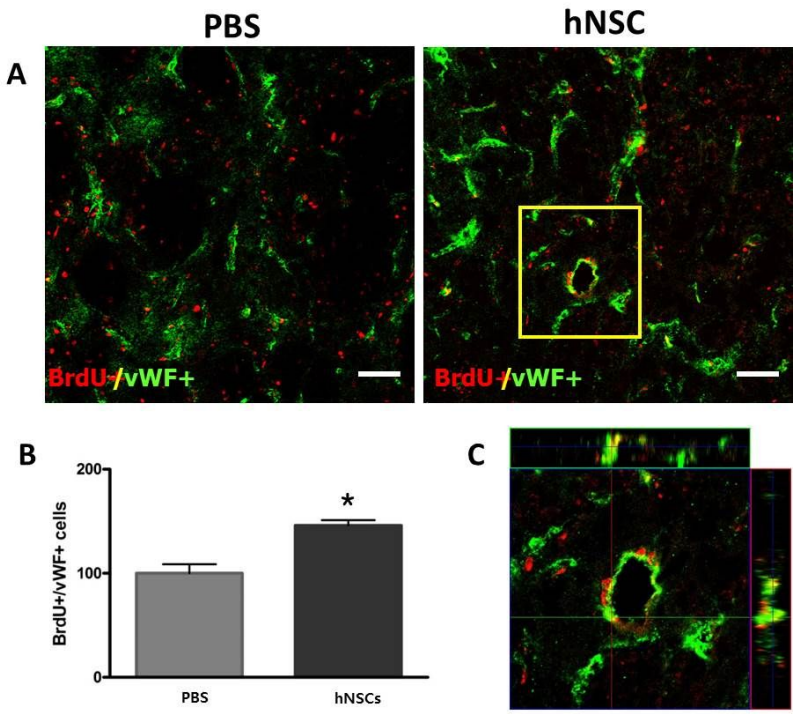

Figure 2.8 Transplanted human neural stem cells promote focal angiogenesis in ischemic rats. (A) Images show immunohistochemistry staining for von Willebrand factor (vWF, green) and BrdU (red) in the ischemic penumbra. (B) Bar graph shows quantification of BrdU+/vWF+ cells in hNSC-treated (hNSCs) and control (PBS) rats. (C) Orthogonal reconstructions of double-labeled cells are presented as viewed in the x-z (top) and y-z (right) planes. The data are expressed as the mean ± SD (n = 7); Data were analyzed using t-test. * $P < 0.05$, hNSC transplantation vs. PBS injection. Scale bar, 100 μm.

DISCUSSION

In the present study, we demonstrated that transplanted hNSCs survive and migrate toward ischemic damaged areas, resulting in the proliferation of endogenous neural stem cells and angiogenesis in the ischemic brain.

Intraventricular implantation of NSCs has been shown to be less invasive and deliver more cells, resulting in increased cell integration compared to intrastriatal administration (Jin et al., 2005). Our data suggest that direct transplantation of hNSCs into the neuroproliferative SVZ and DG regions of the hippocampus is potentially a more effective delivery method that also promotes endogenous neurogenesis. The mechanism underlying the migration of intraventricularly-transplanted human NSCs to the ischemic lesion remains unclear. Several studies have reported that NSCs sensitive to chemokines released in the injured site govern cell migration towards the lesion (Kelly et al., 2004). However, the specific mechanisms involved in NSC chemotaxis after transplantation have yet to be investigated.

We demonstrated that transplanted hNSCs stimulated the proliferation and differentiation of endogenous neural stem cells within the SVZ and DG of the hippocampus in rats in the acute phase following ischemic stroke (14 days). A previous study has shown that human neural precursor cell grafts increased the number of Dcx-positive cells in the SVZ after MCAo in both young adult and aged rats (Zhang et al., 2011). It has been demonstrated that NSC transplantation increased the number of BrdU-positive cells in the SVZ 14 days after focal cerebral ischemia (Zhang et al., 2011), and that intrastriatal implantation of hNSCs promoted several stages of neurogenesis in the striatum of rats after MCAo (Mine et al., 2013). Our results were consistent with these

previous reports.

The enhancement of endogenous neurogenesis contributes to the improvement of neuronal function after ischemia, whereas its ablation has negative effects on functional recovery (Jin et al., 2011, Wang et al., 2012). Our results, together with our previous findings, suggest that the stimulation of neurogenesis by NSC transplantation is associated with functional improvement after ischemic stroke. Understanding the molecular mechanisms involved in the proliferation, migration, and differentiation of neural stem cells may provide insights into how to best implement neural stem cells for cell therapies directed at stroke and other neurological disorders.

Angiogenesis is coupled to neurogenesis and plays a critical role in neuronal repair (Shen et al., 2004). We observed that the number of BrdU+/vWF+ cells in the ischemic region increased following hNSC transplantation, suggesting that hNSCs could promote angiogenesis in the acute phase following ischemic injury. Our results are consistent with those of a previous study that elucidated that NSC transplantation increased the proportion of BrdU+/vWF+ cells in the cortical peri-infarct region 14 days after focal cerebral ischemia; however, the effect was insignificant at later time points (Zhang et al., 2011). Further study is required to investigate the specific mechanisms underlying hNSC-induced stimulation of endothelial cells after stroke. The finding that intracerebral transplantation of NSCs can induce angiogenesis is important because angiogenesis is implicated in the formation of neurovascular units and functional recovery after ischemic stroke and thus may significantly contribute to more favorable clinical outcomes for stroke patients (Zhang et al., 2011).

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CONCLUSION

In the present study, we demonstrated the beneficial effects of hNSC therapy on functional recovery and infarct volume after ischemic brain injury. Lateral ventricular administration of hNSCs after transient MCAo significantly increased functional recovery and reduced infarct volumes relative to LS or IV administration. Transplanted hNSCs survived and migrated toward the damaged areas in the ischemic region. To examine the effects of hNSC transplantation on endogenous neurogenesis and angiogenesis in the ischemic rat brain, we transplanted hNSCs and PBS in the lateral ventricle after MCAo. Finally, we found that hNSCs promoted stem cell proliferation and angiogenesis in rats.

Stem cells in circulation can migrate to the injured brain area, probably in response to chemoattractant stimuli through rolling on and adhering to endothelium and endothelial transmigration. VCAM-1 and the integrins α2, α6, and β1 may be involved in the communication between stem cells and endothelium. NSCs are then guided into target brain area via chemokines such as SDF-1, MCP-1, Ang-1, and Slit (Pluchino et al., 2003, Imitola et al., 2004, Pluchino et al., 2005, Mueller et al., 2006, Ohab et al., 2006, Sawamoto et al., 2006, Gaudier et al., 2007). The CCL2/CCR2 interaction has recently been demonstrated to be critical for transendothelial recruitment of intravascularly delivered NSCs in response to ischemic injury (Andres et al., 2011a).

A recent study revealed that the advantageous effect of intracerebral NSC transplantation after stroke is dependent on high numbers of intracerebrally grafted cells; however, systemic NSC delivery initiates sustained neuroprotection despite low

intracerebral numbers of grafted cells via different mechanisms, like stabilization of the BBB and reduction of ROS during early reperfusion (Doeppner et al., 2012). Therefore, compared to intracerebral transplantation, intravascular delivery of NSCs achieves an improved distribution in the injured areas and does not require invasive surgery.

The mechanisms underlying the improved functional recovery in this ischemic stroke animal model following NSC therapy remain unclear. Cell replacement was initially determined to be the main mechanism of the advantageous effects of transplanted NSCs. In most animal studies, NSCs were found to differentiate into neuronal and/or glial phenotypes (Riess et al., 2002, Chu et al., 2004, Kim et al., 2004, Guzman et al., 2008a, Zhu et al., 2011). Synaptogenesis and functional electrophysiological integration of exogenous NSC into the neuronal circuitry of the host brain has also been demonstrated (Englund et al., 2002). However, improved functional recovery could be observed prior to neuronal differentiation. This suggests that cell replacement may not be a prerequisite for the efficacy of transplanted stem cells in neuronal recovery.

Ten surviving NSCs in the lesion area were too few to replace the neurons following MCAo. Hence, it was supposed that neuroprotective cytokines secreted by exogenous NSCs, host stem cells, and/or other cells, such as VEGF, BDNF, NGF, and neurotrophins, might play pivotal roles in functional recovery after ischemic stroke directly or indirectly via angiogenesis, immunomodulation, and/or endogenous neurogenesis (Nan et al., 2005, Schaller et al., 2005, Harms et al., 2010). Increased dendritic plasticity and axonal re-writing in stroke have been linked with the paracrine effects of NSCs, possibly via VEGF andthrombospondins 1 and 2 (Andres et al.,

2011b).

Injection of NSCs into the cortical infarct cavity in the rat has been found to stimulate neurogenesis in the SVZ ipsilateral to stroke, as demonstrated by increased numbers of cells expressing the early neuronal lineage marker Dcx 60 days post-transplant (Jin et al., 2011). Transplantation of human embryonic NSCs into the cortical peri-infarct in rat 24 h after ischemia increased the number of BrdU+ cells in the SVZ (Zhang et al., 2011). Although the experiment did not directly address whether enhanced neurogenesis contributes to neurological function improvement, ablation of endogenous neurogenesis in transgenic mice expressing the Herpes simplex virus thymidine kinase under the control of the Dcx promoter increases infarct size and exacerbates post-ischemic sensorimotor behavioral deficits (Jin et al., 2010).

Angiogenesis is important for the formation of new brain microvessels and functional recovery after ischemic stroke. Enhanced angiogenesis has been associated with functional recovery after NSC transplantation following stroke (Jiang et al., 2005, Guzman et al., 2008b, Pluchino and Martino, 2008, Zhang et al., 2011). For example, angiogenesis, as indicated by BrdU and vWF staining in cortical peri-infarct regions, has been enhanced by grafted human embryonic NSCs in a rat model of stroke at 7 and 14 days post-injury (Zhang et al., 2011).

In conclusion, LV hNSC administration after transient MCAo significantly increases functional recovery and infarct volume compared to LS or IV administration. Our findings demonstrate that delivery routes influence functional recovery and infarct volume. We have shown that NSC transplantation promotes the proliferation of endogenous neural stem cells and enhances angiogenesis, suggesting that the

transplantation of NSCs provides a microenvironment that activates endogenous restorative mechanisms in the ischemic brain. Therefore, the results of the present study suggest that a NSC-based therapy may be an effective treatment for stroke patients. Future studies are required to identify the exact mechanisms underlying the therapeutic effects of transplanted hNSCs after ischemic stroke and to evaluate its effects on functional outcomes.

The current study was limited in its focus on one time point of ischemia; however, we have demonstrated for the first time that intraventricular transplantation of hNSCs promotes neurogenesis in the acute phase of stroke in rats. Further work is needed to determine whether NSCs are therapeutic in later phases of cerebral ischemia and to test whether NSC therapy alters the expression of neurotrophins or angiogenic proteins following cerebral ischemia.

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국문초록

줄기세포는 스스로의 복제능력(self-renewal capacity)을 유지하면서 다양한 종류의 세포로 분화할 수 있는 능력(pluripotency)을 지닌 미성숙 세포를 뜻한다.

치료법으로써 줄기세포이식은 동물 모델에서 기능적 회복을 향상시킬 수 있는 가능성을 제시한다. 뇌허혈은 신경세포의 손상을 초래하는 질병으로 뇌졸중 환자에서 신경학적인 장애를 유발시킨다. 뇌 손상후 기능적 회복을 향상시키기 위하여 뇌허혈 동물모델에서 내인성 신경줄기세포를 증진시키고 혈관내피생성을 촉진시키고자 하는 연구들이 보고되어 왔다. 본 연구에서는 흰쥐 국소뇌허혈 동물모델에서 기능적 회복과 뇌경색 부피감소에 있어서 가장 효율적인 인간신경줄기세포 이식 방법을 찾아보고자 하였고, 더불어 인간신경줄기세포의 뇌실 내 이식이 내인성 줄기세포의 증식과 혈관내피생성에 미치는 영향에 대해 연구하고자 하였다.

국소뇌허혈 모델은 내경동맥 혈관내 폐색법으로 제작하였고, 국소뇌허혈 유발 후 24 시간 후에 세 가지의 이식방법으로 분류하여 인간신경줄기세포를 이식하였다. 이식방법으로는 뇌선조체 내 이식, 뇌실 내 이식, 꼬리의 정맥주사방법으로 분류하였다. 줄기세포 이식 후 7 일, 14 일에 행동학적 분석을 위하여 mNSS, rotarod test 를 이용하였으며, 14 일에 뇌 경색 부피를 측정하였다. 이식된 인간신경줄기세포의 생존과 분화과정을 확인하기 위해 인간유래세포만을 염색하는 항 인간핵 항체를 이용하여 면역형광염색을 시행하였다. 이식된 인간신경줄기세포는 세

그룹에서 동일하게 뇌 손상 부위에서 펴져있는 것을 발견하였으며, 성체신경세포 및 성체희소돌기아교세포로의 분화가 확인되었다. 세 그룹에서 동일하게 기능적 호전을 보였으나($p < 0.05$), 그 중에서도 뇌실 내로 이식한 그룹에서 가장 높은 기능적 호전을 보였고($p < 0.01$), 뇌 경색 부피는 뇌실 내로 이식한 그룹에서만 감소되는 것으로 나타났다($p < 0.05$).

다음, 뇌졸중 유발 동물모델에서 인간신경줄기세포 이식이 내인성 줄기세포의 증식과 혈관내피생성에 미치는 영향을 조사하기 위하여, 국소 뇌허혈 유발 후 24 시간 후에 인간신경줄기세포를 뇌실 내에 이식하였다. 뇌에서 증식하는 세포를 확인하기 위해서 BrdU (50mg/kg) 를 매일 복강주사 하였고, 세포이식 후 14 일에 내인성 줄기세포의 증식과 혈관내피생성을 확인하기 위하여 이중면역형광염색을 시행하였다. 이식된 인간신경줄기 세포는 뇌 손상 부위에 퍼져있는 것을 발견하였으며, 뇌실 하 부위와 해마의 치아이랑내 과립세포층에서 인간신경줄기세포를 이식한 그룹이 대조군에 비해 내인성 줄기세포의 증식과 분화가 증가 되는 것 을 확인 하였고, 선조체의 뇌 손상부위에서는 신생혈관생성을 촉진 시키는 것을 확인하였다.

본 연구에서는 뇌허혈 동물모델에서 인간신경줄기세포의 이식이 손상된 기능의 회복을 촉진시키는 것을 확인하였다. 또한 이식방법에 있어서 뇌실 내 이식방법이 기능적 호전도와 뇌 경색 부피를 비교했을 때 뇌선조체 내 이식과 정맥 내 이식방법보다 더 효과적인 방법인 것으로 사료된다. 또한, 인간신경줄기세포 치료의 효과에 있어서 내인성 줄기세포의 증식과 신경세포로 분화를 증가시키고 신생혈관생성을 촉진시키는 효과가 있음을 제시하고 있다.

주요어: 뇌허혈, 인간신경줄기세포, 이식방법, 기능적 회복, 뇌 경색 부피, 뇌실 내, 내인성 줄기세포, 신생혈관생성, 뇌실 하 부위, 해마의 치아이랑

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