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# Human neural stem cells improve sensorimotor deficits in the adult rat brain with experimental focal ischemia

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#### Abstract

Ischemic stroke is caused by the interruption of cerebral blood flow that leads to brain damage with long-term sensorimotor deficits. Stem cell transplantation may recover functional deficit by replacing damaged brain. In this study, we attempted to test whether the human neural stem cells (NSCs) can improve the outcome in the rat brain with intravenous injection and also determine the migration, differentiation and the long-term viabilities of human NSCs in the rat brain. Focal cerebral ischemia was induced by intraluminal thread occlusion of middle cerebral artery (MCA). One day after surgery, the rats were randomly divided into two groups: NSCs-ischemia ys. Ischemia-only. Human NSCs infected with retroviral vector encoding  $\beta$  galactosidase were intravenously injected in NSCs-ischemia group ( $5 \times 10^6$  cells) and the same amount of saline was injected in Ischemia-only group for control. The animals were evaluated for 4 weeks using turning in an alley (TIA) test, modified limb placing test (MLPT) and rotarod test. Transplanted cells were detected by X gal cytohistochemistry or  $\beta$  gal immunohistochemistry with double labeling of other cell markers. The NSCs-ischemia group showed better performance on TIA test at 2 weeks, and MLPT and rotarod test from 3 weeks after ischemia compared with the Ischemia-only group. Human NSCs were detected in the lesion side and labeled with marker for neurons or astrocytes. Postischemic hemispheric atrophy was noted but reduced in NSCs-ischemia group. X gal<sup>+</sup> cells were detected in the rat brain as long as 540 days after transplantation. Our data suggest intravenously transplanted human NSCs can migrate and differentiate in the rat brain with focal ischemia and improve functional recovery.

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# 1. Introduction

Ischemic stroke is caused by abrupt and near-total interruption of cerebral blood flow that leads to ischemic changes with long-term sensorimotor deficits (see Ref. [31]

for a review). Cell transplantation in the experimental ischemic models has been reported [31]. Bone marrow cells [5,20], human umbilical cord blood cells [6,41], rodent embryonic hippocampal cells [24], MHP36 cells (conditionally immortalized neuroepithelial cell line derived from embryonic mouse) [21,22,39] and hNT cells (human neuroteratocarcinoma-derived neurons) [17,30] could integrate in the ischemic brain and reduce the neurological deficits induced by experimental brain ischemia.

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Human neural stem cells (NSCs) can be transplanted in rodent and primate CNS development [11,25], creating chimeras with different species and migrating to the damaged area of brain. Transplantation of human NSCs was tried in rodent models with transient global ischemia [8], intracerebral hemorrhage [13] or the aging brain [28] by intracisternal or systemic injections. However, human NSCs transplantation in focal cerebral ischemia in the rat brain is unknown.

In this study, we attempted to test whether systemically injected human NSCs can improve the functional deficits caused by focal cerebral ischemia in rat, and to determine the distribution, migration and differentiation of human NSCs. Furthermore, long-term survival and effect of human NSCs on brain atrophy were evaluated.

### 2. Materials and methods

#### 2.1. Focal ischemia model

All experimental procedures were approved by the Care of Experimental Animals Committee of University Hospital and by IRB for the human cell use. One-hundred and seventy-seven Sprague-Dawley male rats weighing 220-240 g (Genomics) were divided into three experimental groups of the NSCs-ischemia (n = 104), the Ischemia-only (n=43) and NSCs-normal control (n=30)groups. Transient focal cerebral ischemia was induced using the intraluminal thread occlusion of middle cerebral artery (MCA) for 90 min in the first two groups, as described elsewhere [19]. During recovery period, rats were assessed for forelimb flexion and contralateral circling to confirm ischemia. Rectal temperature was maintained at  $37 \pm 0.5$  °C using a thermistor-controlled heat blanket. The animals were maintained in each separate cage at room temperature, 25 °C, with free access to food and water under a 12-h light-dark cycle. After 2 weeks, the amount of pellets was restricted to 30 g/day to control body weight, until 28 days for the efficient rotarod performance, as described elsewhere [13]. The diet restriction was done both in NSCs-ischemia and Ischemiaonly groups.

#### 2.2. Cell preparation and transplantation procedure

Primary dissociated cell cultures were prepared from embryonic human brains of 15 weeks gestation as described previously in detail [7,8,13,25]. To provide an unambiguous molecular tag for identifying the implanted cells, cell line was infected with a replication incompetent retroviral vector encoding  $\beta$  galactosidase (*Lac Z*) and puromycin resistant genes. The cerebrum cultures were retrovirally transduced with *v*-myc oncogene 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. Twenty-four hours after surgery, we performed behavioral tests and randomly selected rats (NSCs-ischemia, n=104) received 500 µl suspensions of HB1.F3 cells ( $5 \times 10^6$  cells) slowly for 5 min via tail vein. The same volume of saline was injected into Ischemia-only group (n=43). For control experiment, human NSCs suspension was injected to the normal rats (n=30) with the same amount.

#### 2.3. Behavioral testing

Rotarod test, modified limb placing test (MLPT) and turning in an alley (TIA) test was performed at 1 day before and after ischemia, and further weekly (n = 15 per)group), as previously described [13]. In brief, the rats, assigned to the behavior test, were pre-trained for 3 days in order to alleviate the learning effect in rotarod test [5]. In the rotarod test, the rats were placed on the accelerating rotarod cylinder (4-40 rpm), and the time the animals remained on the rotarod was measured three times [5,13]. Rotarod test data were presented as percentages of the maximal duration compared with the internal baseline control (before ischemia). MLPT consists of two limbplacing tasks that assess the sensorimotor integration of the forelimb and the hindlimb, by checking responses to tactile and proprioceptive stimulation [13,35]. First, the rat is suspended 10 cm over a table and the stretch of the forelimbs towards the table is observed and evaluated: normal stretch, 0 points; abnormal flexion, 1 point. Next, the rat is positioned along the edge of the table, and its forelimbs are placed freely out of the table suspended over the edge and allowed to move freely. Each forelimb (forelimb-second task, hindlimb-third task) is gently pulled down and retrieval and placement is checked. Finally, the rat is placed towards the table edge to check for lateral placement of the forelimb. The three tasks are scored in the following manner: normal performance, 0 points; performance with a delay (2 s) and/or incomplete, 1 point; no performance, 2 points; 7 points means maximal neurological deficit and 0 points means normal performance. TIA test was used as a measure of coordinated muscle control. The animal was placed facing the back wall of an alley (10 cm wide with walls 100 cm high). The amount of time (up to 1 min) required for the animal to turn around and face the open end of the alley was recorded repeatedly (10 times per rat). The TIA data were presented as percentages of the maximal duration compared with the internal baseline control (before ischemia). The body weights of all animals were checked weekly for 4 weeks.

#### 2.4. Histological examinations

At 1 (n=12), 7 (n=12), 14 (n=12), 21 (n=12), 28 (n=12), 56 (n=27), 180 (n=6) and 540 (n=7) days after transplantation, the rats with NSCs-ischemia group was

reanesthetized and perfused through the heart with 100 ml cold saline and 100 ml of 4% paraformaldehyde in 0.1 mol/ 1 phosphate-buffered saline. The rats in the Ischemia-only group [P7 (n=12), P28 (n=12), P56 (n=15)] and NSCsnormal control group [P1 (n=6), P7 (n=6), P14 (n=6), P28 (n=6), P56 (n=6)] were sacrificed with the same procedure. After 24 h of fixation in 4% paraformaldehyde, the brains were cryoprotected with 30% sucrose for 24 h and cut by cryostat (Leica CM 1900) into 30-µm sections. To compare the infarction distributions and hemispheric areas of the two groups, we measured the infarction volume (at 7 days after transplantation; P7; n = 12 per group) and hemispheric areas (P56; n = 12 per group) using Nissl staining. The infarcted and total hemispheric areas of each section were traced and measured using an image analysis system (Image-Pro Plus<sup>™</sup>, Media Cybernetics). The morphometric analyses involved computer-assisted delineation of the area of the striatum, cerebral cortex, ventricle and the whole hemisphere as described elsewhere [13,35].

Adjacent serial coronal sections were processed for  $\beta$  gal immunofluorescent staining with anti- $\beta$  gal antibody (1:200, Sigma) to identify the Lac  $Z^+$ -transplanted cells. NF (1:200; Sigma), NeuN (1:200; Chemicon), GFAP (1:1000; Sigma), endothelial barrier antigen (EBA, 1:200; Sternberger Monoclonals), vimentin (1:100; Sigma) and CNPase (1:300; Sigma) antibodies were used as cell-type-specific markers. Primary antibodies were incubated overnight at 4 °C using the free floating method [15]. FITC-conjugated anti-mouse IgM or Cy3-conjugated anti-mouse IgG antibodies (1:300, Jackson Immunoresearch) were used as secondary antibody and incubated for 1 h at room temperature. The colocalization of  $\beta$  gal with neuronal and glial markers was performed by laser scanning confocal microscopy using a Bio-Rad MRC 1024 (argon and krypton). For immunofluorescence double-labeled coronal sections, green (B gal and EBA) and red (NeuN, NF, CNPase, vimentin and GFAP) fluorochromes on the sections were excited by laser at 488 and 550 nm.

To determine graft survival semiquantitatively, transplanted cells in the same focal plane were counted bilaterally in six regions of interest (ROIs) situated within the periinfarct striatum and cortex and their corresponding, contralateral areas, and the measurement was made in a predefined field  $(0.3 \times 0.3 \text{ mm})$  [8,13,35].  $\beta$  gal<sup>+</sup> cells (n=12–15 with different sacrifice timing) and double-labeled cells (P7, P14, P28, P56; n=12, respectively) with GFAP, NeuN or CNPase for each three sequential sections (six ROIs per section) were counted.

X gal EM (n=4 per group, P28) was performed on semithin serial sections (1 µm) from tissues stained with 5bromo-3-indolyl-beta-D-galactopyranoside, post-fixed in 2% glutaraldehyde and 1% osmium tetroxide and embedded in Epon as described [18,27]. Toluidine blue staining was also performed on semithin sections.

We examined the systemic organs to detect any adverse reaction produced by the NSCs (n=6 per group), with

Fig. 1. Effects of human neural stem cell transplantation on the sensorimotor recovery of ischemic rats. (a, b, c) Time course of rotarod test (a), modified limb placing test (b) and turning in an alley test (c) results from human NSCs transplantation. All data are mean values  $\pm$  S.D. from 4 to 10 experiments. Significant differences from Ischemia-only group are marked with a single (p < 0.05) asterisk.



different sacrifice timing (from P1 to P540). Systemic organs such as the kidneys, lungs, spleens, livers and hearts of each NSCs transplanted animal were examined and four sections for each organ were stained with H&E, standard X gal histochemistry and anti-Ki67 antibody (1:200, Boeringer-Ingelheim; ABC kit, Vector Laboratories) [8,13].

### 2.5. Statistical analysis

All data in this study are presented as mean  $\pm$  standard deviations. Data were analyzed by repeated-measures analysis of variance and unpaired Student's *t* test, if they were normally distributed (Kolmogorov–Smirov test, *p*>0.05). Otherwise, we used the Mann–Whitney *U* test and specified the test used. Two-tailed probability value of < 0.05 was considered significant.

## 3. Results

# 3.1. Human neural stem cells improved sensorimotor deficits in rats with focal cerebral ischemia

To determine whether the transplanted human NSCs can improve sensorimotor deficit, the behavior tests were compared (n=15 for each group). TIA tests began to show improvement at P14 (p=0.01). At P21 and P28, NSCsischemia group showed better performance on the MLPT, TIA, and rotarod test (P21; MLPT, p<0.01; TIA, p<0.01; rotarod, p=0.03; P28; MLPT, p<0.01; TIA, p<0.001; rotarod: p=0.01; Fig. 1). The initial body weights were similar (p=0.87) and those after 4 weeks were not different (p=0.85). Physiological parameters were unchanged between experimental groups before, during or after focal



Fig. 2. Distribution of *Lac Z*<sup>+</sup> human neural stem cells after intravenous injection in the ischemic rat brain. (a, b) Migration of X gal positive NSCs in the ischemic striatum. (c, d) Linear migration of NSCs in the peri-ischemic cortex. (e, f, g)  $\beta$  gal<sup>+</sup> NSCs were found in the ischemic striatum (e, g) and cortex (f). (h, i) Schematic diagram of NSCs population. The early migration pattern was along the vascular endothelium, in the pial or parenchymal areas (h) at P7, and NSCs migrated toward the corpus callosum, then to the ischemic areas (i) at P14. The green asterisk indicates  $\beta$  gal<sup>+</sup> cells. This pattern of stem cell distribution is consistent through P56. Scale bar = 50 µm (a-g).

ischemia. The overall mortality in this experiment was 17% (NSCs-transplanted group) vs. 18% (Ischemia-only group), which was generally limited within the first week of the ischemic surgery.

# 3.2. Temporal and spatial distribution of transplanted human NSCs

One day after transplantation (P1), none of the intravenously injected cells were detected within the brain in normal control rats (n=6).  $\beta$  gal<sup>+</sup> cells were rarely found in the NSCs-normal control group until P56. In contrast, in NSCs-ischemia group, Lac  $Z^+$  cells were found within meningeal spaces, superficial cortex, corpus callosum (CC) and around post-capillary venules (Fig. 2) at day 1. Seven days after transplantation, Lac  $Z^+$  cells were detected in the lesion side, toward the infarcted striatum and the cortex along the CC. They were also found in or near the post-capillary venules around the damaged area. But in the contra-lesional side, only a few Lac  $Z^+$  cells were detected (Table 1; Student's t test, p < 0.01). The number of Lac  $Z^+$  cells in the lesion side increased between at P7 and at P14, both in the cortex and in the striatum (p < 0.01, Student's t test; see Table 1, Fig. 2). However, the increase of cell number did not occur on the contralateral side (Table 1). Two weeks following transplantation, Lac  $Z^+$  cells were mainly detected around the ischemic areas, but some Lac  $Z^+$  cells were found in the ischemic core (see Fig. 2, schematic diagram). At 180 days after transplantation, Lac  $Z^+$  cells were found along the whole neural axis, especially in the subventricular zone (SVZ) and bilateral cortices. The Lac  $Z^+$  cells were detected as long as 540 days observation period with increase number of cells (Table 1), especially on the contralateral side (p = 0.03, Student's t test).

The liver and heart contained no  $Lac Z^+$  cells. In the kidneys, lungs and spleens,  $Lac Z^+$  cells were found, even in the human NSCs-injected normal control rats. However, no evidence of tissue damages, such as leukocyte infiltration or tumor formation were detected by H/E staining or Ki67 immunohistochemical study during the 540 days

follow-up period. Also, in the brain, no tumor growth was found.

### 3.3. Differentiation of human neural stem cells

To determine the pattern of differentiation of hNSCs in the ischemic brain, antibody against GFAP, NeuN, CNPase or vimentin were colabeled with anti- $\beta$  gal antibody at P7, P14, P28 and P56. Most (49–53%) *Lac*  $Z^+$  cells were GFAP<sup>+</sup> and took the morphology of the astrocytes at P7 (Fig. 3). They were found along the infarct border.

Twenty percent of Lac  $Z^+$  cells were labeled with NeuN in the lesioned hemispheres at P56 (Fig. 4), and these NeuN<sup>+</sup>  $\beta$  gal<sup>+</sup> cells were found in the vicinity of the infarct and were intermingled with the GFAP<sup>+</sup>  $\beta$  gal<sup>+</sup> astrocytes. NeuN<sup>+</sup>  $\beta$  gal<sup>+</sup> cells began to show at P28, which were found primarily in the perilesional cortex and less frequently in the striatum. The density of NeuN<sup>+</sup>  $\beta$ gal<sup>+</sup> cells was increased in the clusters of GFAP<sup>+</sup>  $\beta$  gal<sup>+</sup> astrocytes. No double-labeled cell for the oligodendrocytic marker (CNPase) was observed during experimental period. Vimentin positivity was sustained from P1 until P28, whereas GFAP appeared as early as P7, and NeuN on P28 (Fig. 3). Most vimentin<sup>+</sup>  $\beta$  gal<sup>+</sup> cells were found in the pial areas with long processes toward the deep cortical areas and CC. Vimentin<sup>+</sup>  $\beta$  gal<sup>+</sup> cells were also found in the CC, olfactory bulb and peri-infarct areas.

Lac  $Z^+$  cells were also found on and around the cerebral microvessels. Electron microscopic examinations at P28 showed the rod-shaped electron-dense precipitates (Fig. 3). Vimentin<sup>+</sup> cells around vessels showed immunoreactivity to EBA and GFAP in the peri-ischemic areas, but not in the contralateral side of Ischemia-only group in all experimental periods.

# 3.4. Human neural stem cell transplantation reduced the hemispheric atrophy following focal ischemia

The infarct size at P7 was not different statistically between two groups (NSCs-ischemia group,  $142.8 \pm 11.9$ 

Table 1

Quantification	of Lac 2	Z <sup>+</sup> human	neural	stem	cells	(NSCs)
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Survival time (rat number)	Striatum		Cortex			
	Lesion	Contralesional	Lesion	Contralesional		
1  day  (n=12)	$75.4 \pm 20.4$	$26.1 \pm 10.4$	$37.1 \pm 10.7$	$30.1 \pm 9.6$		
7 days $(n = 12)$	$177.8 \pm 34.2$	$23.0 \pm 13.7$	$104.2 \pm 35.7$	$33.4 \pm 16.3$		
14 days $(n = 12)$	$1678.0 \pm 234.7$	$90.2 \pm 46.5$	$1327.8 \pm 140.8$	$55.2 \pm 21.1$		
21 days $(n = 12)$	$2118.2 \pm 173.2$	$84.4 \pm 25.5$	$1680.7 \pm 172.4$	$59.6 \pm 16.0$		
28 days $(n = 12)$	$2125.8 \pm 216.1$	$74.4 \pm 46.0$	$1735.8 \pm 104.1$	$61.0\pm20.3$		
56 days $(n = 12)$	$2218.1 \pm 117.9$	$91.8 \pm 12.4$	$1812.6 \pm 189.8$	$104.5 \pm 59.7$		
180 days $(n=12)$	$2112.8 \pm 514.3$	$672.1 \pm 89.7$	$1708.7 \pm 198.5$	$514.4 \pm 105.8$		
540 days $(n = 12)$	$2087.1 \pm 324.7$	$694.8 \pm 245.7$	$1824.6 \pm 289.1$	$797.4 \pm 265.6$		

Mean number of  $\beta$  gal<sup>+</sup> cells observed in NSCs-ischemia group. Data shown are mean values  $\pm$  S.D./mm<sup>2</sup> in the ischemic striatum and cortex and the contralateral, corresponding areas.



Fig. 3. Differentiation of human neural stem cells in the ischemic brain. (a, b, c) Vimentin<sup>+</sup>  $\beta$  gal<sup>+</sup> NSCs in the pial area had long processes directed toward the deep cortical areas, which seemed to form a migratory route for implanted NSCs. Vimentin<sup>+</sup>  $\beta$  gal<sup>+</sup> NSCs took the morphology of monopolar or bipolar cells. (d, e, f)  $\beta$  gal<sup>+</sup> NSCs at P28 were stained for NeuN in the peri-ischemic cortex. (g) Schematic diagram of NSCs migration. Note the early (black arrow) and late (red arrow) migratory routes. (h, i, j)  $\beta$  gal<sup>+</sup> NSCs at P14 were stained for GFAP in the peri-infarct striatum. (k, l, m) Vimentin<sup>+</sup> cells were found to be anchored to the EBA<sup>+</sup> endothelial cells in the lesion side. This finding was not seen in the contralesional side. (n, o) NSCs located across the blood–brain barrier and in close contact with endothelial cells of the blood–brain barrier were also visible. See the dense cytoplasmic deposit of X gal product (black arrows). Scale bar: (a–c, h–m) 200 µm, (d–f) 30 µm.

mm<sup>3</sup>; Ischemia-only group,  $138.3 \pm 21.4$  mm<sup>3</sup>; n = 12 for each group; p = 0.52, Student's *t* test). Hemispheric area analysis showed more significant atrophy (progressive

cavitations) of the lesioned hemisphere in the Ischemia-only group than NSCs-ischemia group at P56 (Fig. 4; n = 12 for each group; p < 0.01, Student's *t* test).

# *3.5.* Correlation between behavior data and Lac $Z^+$ cells in the ischemic brain

We analyzed further whether the degree of behavioral recovery could be correlated with survival of  $Lac Z^+$  cells in the brain. Pearson correlation analysis of these data showed that functional improvement in the rotarod test was correlated well with the number of  $Lac Z^+$  cells at P56



 $(r^2 = 0.895;$  Fig. 4). Other variables, such as different cell marker positivity (such as NeuN or GFAP), were not correlated with the behavioral data.

## 4. Discussion

In this study, we attempted to test whether systemically injected human NSCs can improve the functional deficits in the ischemic rat brain and to determine the distribution of human NSCs. We also evaluated the possibility of long-term survival of human NSCs in the rat brain and the protective effect from brain atrophy. Our results show that intravenously transplanted human NSCs improved functional deficits. They can migrate to the damaged areas and are detected as long as 540 days without tumor formation. Human NSCs can reduce progressive hemispheric atrophy in the rat brain.

The exact mechanism of functional improvement by cell transplantation in the ischemic brain is unknown. Increased neurogenesis, neuroprotection by neurotrophic and growth factors or new synaptic formation with reorganization has been suggested [2,13,15,16,23,26,31,42,43]. However, the mechanism by human NSCs transplantation in our ischemic rat brain is unknown. It takes 2-6 weeks for the development of newly generated NeuN<sup>+</sup> neurons by the transplanted cells in vivo [3,9,13,34,36,38,40]. Hippocampal rodent NSCs were also found to differentiate into functionally mature neurons with synapses formation (in vitro and in vivo) [34]. Neural progenitors can differentiate into neurons with functional glutamatergic and GABAergic synaptic transmission and exhibited action potentials 12 days after differentiation with synapse formation in 28–35 days [36]. These results support the new synaptic formation between 2 and 6 weeks from newly generated neurons by neural progenitors could mediate functional improvement. In our study, functional improvement began to occur in 2 weeks. It suggests that newly generated neurons from the transplanted NSCs might play role for the synaptic networks in the periischemic areas [38].

Fig. 4. Results of differentiation pattern, functional improvement and progressive hemispheric atrophy on neural stem cell transplantation. (a) Differentiation pattern of NSCs with different time periods.  $GFAP^+ \beta gal^+$ , NeuN<sup>+</sup>  $\beta$  gal<sup>+</sup> and  $\beta$  gal<sup>+</sup> only cells were counted in the adjacent sections (three sections, six ROIs per rats). In all sites, about 50% of the transplanted  $\beta$  gal^+ cells were positive to GFAP, and NeuN^+  $\beta$  gal^+ cells were found at P56 (about 20%). About 40–50% of the transplanted  $\beta$  gal<sup>+</sup> cells were not reactive to any markers that we used in this experiment. Data shown are mean values  $\pm$  S.D. (%). (b) The degree of atrophy at P56 was calculated as (area of contralesioned side - area of lesioned side)/(area of contralesioned side)  $\times$  100 (%). In the NSCs-ischemia group, progressive atrophy was less prominent than Ischemia-only group, in the striatum, cortex, as well as in whole hemisphere. Student's t test was used for statistical significance. Significant differences from Ischemia-only group are marked with double (p < 0.01) asterisks. (c) Correlation analysis of rotarod test results with  $\beta$ gal<sup>+</sup> cells in the brain at P56. Pearson correlation analysis was used for statistical significance.

However, the protective effect of human NSCs from progressive brain atrophy cannot be explained by the synapse formation from newly generated neurons alone. Transplantation of MHP36, a mouse neural progenitor cell line, in the focal ischemia model in rat showed lesser degree of cerebral atrophy compared with sham-grafted group [39]. The increased BDNF and NGF secretion were noted in the mesenchymal stem cell-transplantated group [20]. NSCs might exert a neuroprotective effect and prevent the ischemic brain from progressive atrophy through the induction of various, endogenous growth factors [14,20]. They might induce neural plasticity and enable the damaged brain to generate new connections. In our results, NSCs transplantation may serve constitutive and inducible mechanisms of plasticity and protect host brain from further destruction, such as progressive cavitation following cerebral ischemia [4,10]. Further study will be needed to test these possibilities.

In our study, the transplanted human NSCs rarely cause immune response in the host rat brain. No signs of immunologic rejection were found until 540 days after transplantation. The exact mechanisms of this immune tolerance were unknown. We did not use cyclosporin as an immunosuppressant. Since NSCs rarely express MHC classes I, II, CD45 and CD 11b in the transplanted host brain, they might be less immunogenic [22]. However, further investigation will be needed.

The mechanism how the systemically injected human NSCs migrate to the damaged areas is unknown [1,7,8,13]. In our study, NSCs were found in the pia and vascular endothelium at P1, in the CC at P7, then in the lesioned area at P14. NSCs were known to express various surface adhesion molecules, and have ability for vascular attachment, especially in the areas of blood-brain barrier (BBB) destruction [7]. In our experiment, possible pathways to the brain parenchyma might be via parenchymal endothelium in the lesion side or via pial vessels to the newly generated Vimentin<sup>+</sup> pial fibers. X gal<sup>+</sup> cells around the endothelium in the lesioned hemispheres may implicate the migration via parenchymal endothelium. The implanted human NSCs may migrate into corpus callosum and the lesion areas along the self-generated radial glial fibers, which need verification in further experiment [12].

The transplanted human NSCs in our experiment preferably became astrocytes. At P56, 50% of  $\beta$  gal<sup>+</sup> was GFAP<sup>+</sup>, whereas 20% of cells were NeuN<sup>+</sup> in the peri-infarct areas. Astrocytes are known to support the proliferation, survival and maturation of developing neurons that have already committed to neural lineages [33]. Astrocytes can induce and stabilize synapses and may be capable of integrating neuronal inputs and modulating synaptic activity [29,33,37]. The role of differentiated GFAP<sup>+</sup> cells from human NSCs need to be determined [29]. Some proportions (20–60%) of the human NSCs were not labeled with either neuronal or glial markers, which may implicate that NSCs can remain as quiescent progenitors or differentiate into other cell types [32]. Further experiment will be needed. There are several limitations in our study. Intravenous route were performed for transplant delivery but the comparison between intravenous vs. intraparenchymal was not done. Therefore, advantages or efficacy of intravenous route over intraparenchymal or other transplantation procedure were not evaluated. The mechanism of increase of X gal<sup>+</sup> cells from P7 to P14 was undetermined. It is unknown whether initially entered human NSCs proliferate or entered the brain thereafter. Changes in blood brain permeability, trophic factors or other possible factors need to be evaluated with BrdU staining, different time window with different cell dosages.

In summary, our data showed that intravenously transplanted human NSCs can reach the ischemic-injured adult rat brain and improve functional recovery with long-term safety. These results suggest the possibility of injecting NSCs systemically may have clinical benefit in focal ischemia.

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