Behavioral improvement after transplantation of neural precursors derived from embryonic stem cells into globally ischemic brain of adolescent rats

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Behavioral improvement after transplantation of neural precursors derived from embryonic stem cells into globally ischemic brain of adolescent rats

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Research on stem cells is advancing knowledge about how an organism develops from a single cell and how healthy cells replace damaged cells in adult organisms. This promising area of science is also leading neurologists to investigate the possibility of cell-based therapies to repair damaged brain. Especially, embryonic stem cells can proliferate extensively in vitro while maintaining an undifferentiated state and differentiate into most cell types under certain conditions. Large numbers of embryonic stem cells can be relatively easily grown in culture compared to other sorts of stem cells and efficiently differentiated into neural precursors.

It is an honor for me to be able to experience this up-to-date research, evaluating the possibility of mouse embryonic stem cells for repairing damaged brain in adolescent rat model. I wish to thank all of the superivsors, Prof. Dong Wook Kim, Prof. Baik Lin Eun, Prof. Heung Dong Kim, Prof. KooK In Park, Prof. Jin-Sung Lee and the assistants, Dae Sung Kim, Hyung Chul Choi, Ji Young Kim, and Min Kyoung Kang. Especially, I also owe a special debt of gratitude to my guiders, Prof. Dong Wook Kim and Prof. Baik Lin Eun.

I hope this trifling paper can give a help to advance of cell-based therapy for pediatric patients with damaged brain.

I dedicate this paper to my parents, Dr. Young Seup Kang, Yeun Ja Lee and Prof. Chong Youl Kim, Young Sook Hong as well as Eun Soo Kim, who honors me as my wife, and to our beloved daughter, Da Hyen.

<TABLE OF CONTENTS>

LIST OF FIGURES

serotonergic neuronal marker, TPH and gabaergic neuronal marker, GAD and in addition, oligodendrocyte precursor marker, Olig2 and astrocyte precursor marker, GFAP were upregulated, consistent with the results of immunocytochemical stainings.

Figure 5. Efficient integration, migration and generation of neural cells from grafted embryonic stem cells at 8 weeks after transplantation. A. mouse embryonic stem cells with enhanced yellow fluorescent protein in injection site (a), migration around hippocampus (b), migration into the ipsilateral thalamus (c), B. dispersion around hippocampus and adjacent cortex (d), NeuN staining showed neuronal cells originated from transplanted embryonic stem cells (e), C. dispersion around hippocampus (f), GFAP positive astrocytes (g), merged imaging showed differentiated cells into astrocytes from transplanted embryonic stem cells (h), D. dispersion of embryonic stem cells around hippocampus (f), O4 positive oligodendrocytes (g), merged imaging showed differentiated cells into oligodendrocytes from transplanted embryonic stem cells (h), scale bar of a, b, c, 200 μm. scale bar of d, e, 100 μm. scale bar of f-k, 50 μm.

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Figure 7. Morris water-maze method at 8 weeks after transplantation. Transplant and sham groups improve significantly in the time to reach the escape platform more than vehicle group ($F=8.797$, $p=0.004$, repeated measures ANOVA, Tukey-Kramer methods).

Figure 8. Spatial probe trial, transplant and sham groups have a significantly better cognitive function than vehicle group ($F=9.137$, $p=0.001$, one-way ANOVA, Tukey-Kramer methods).

ABSTRACT

Behavioral improvement after transplantation of neural precursors derived from embryonic stem cells into globally ischemic brain of adolescent rats

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(Directed by Professor Jin-Sung Lee)

Recent research into cell replacement therapy of the central nervous system has raised hopes that researchers can find ways to actually repair central nervous system damage. The purpose of the present study was to determine whether transplantated neurally-predifferentiated mouse embryonic stem (ES) cells can integrate and differentiate into mature neurons and glial cells in damaged brains and improve functional deficits caused by globally cerebral ischemic injury in adolescent rats. From predifferentiated precursors, we were able to generate neural lineages, including neurons and glial cells. Specifically, mouse ES cells that display enhanced expression of yellow fluorescent protein were co-cultured in N2 supplemented media with a stromal line of PA6 cells that had stromal derived inducing activity. We confirmed that the ES cells became neurons and glia by immunostaining, RT-PCR and electrophysiologic analysis. The cells that had been co-cultured were transplanted into bilateral hippocampal C3 regions of post-globally ischemic brains of adolescent rats at 14 days after 20-minute occlusion of both carotid arteries combined with cauterization of bilateral vertebral arteries. After 8 weeks following transplantation, efficient integration, migration and generation of neural cells including neurons, astrocytes, and oligodendrocytes from grafted ES cells could be observed. ES cells-transplanted animals exhibited enhanced functional recovery on neurological and behavioral tests, compared to vehicle control-treated animals. Therefore, transplantation of neurally-predifferentiated mouse ES cells shows promise for improving recovery after global ischemia in adolescent rats.

Key words: embryonic stem cells, neural precursors, globally ischemic brain, adolescent rats

I. INTRODUCTION

Stroke is caused by abrupt and near-total interruption of cerebral blood flow leading to long-term sensorimotor and cognitive deficits. The only specific therapies currently available for stroke are intervention to prevent inappropriate coagulation, surgical procedures to repair vascular abnormalities, and thrombolytic therapy. Recently, cell transplantation has been reported as a potential therapeutic strategy for aiding recovery after stroke, both in experimental models and also in the human brain. Bone marrow-derived cells[1,2], human umbilical cord blood cells[3], rodent embryonic hippocampal cells[4], MHP36 cells (a conditionally immortalized neuroepithelial cell line derived from an embryonic mouse)[5], human neuroteratocarcinoma-derived neurons and embryonic stem (ES) cells[6] have been successfully transplanted into the human brain.

ES cells, derived from the inner cell mass of preimplantation embryos[7,8], can proliferate extensively *in vitro* in an undifferentiated state and then differentiate into most cell types under certain conditions[9]. Large numbers of ES cells can be relatively easily grown in culture compared to other sorts of stem cells[9]. Implantation of mouse ES cells into rat striatum can cause teratomas[10] but tumorogenesis has been minimized by utilizing *in vitro* predifferentiated neural lineage cells and engineered ES cells with regulatable suicide genes[11]. In addition, recent evidence of patient-specific ES cells derived from human somatic cell nuclear transfer blastocysts makes ES cells a good candidate source for cell replacement therapy[12].

The purpose of the present study was to determine whether transplanted neurally-predifferentiated mouse ES cells can integrate and differentiate into mature neurons and glial cells in damaged brains and improve functional deficits caused by global cerebral ischemic injury in adolescent rats.

II. MATERIALS AND METHODS

1. Globally ischemic rat model

Studies were performed using 3 week-old male Sprague-Dawley rats weighing 52-67 g (Charles River Diagnostics, Seoul, Korea). Rats were maintained under controlled environmental conditions with an ambient temperature of 22°C, relative humidity of 65% and equal 12-hour light/dark cycles. Animals were grouped into control (n=10) and ischemia groups (n=20). Ischemia groups were further divided into a vehicle only cohort (n=10) and transplant cohort (n=10).

Ischemia was induced using the four-vessel occlusion method. In brief, the rats were anesthetized by ketamine (50 mg/kg i.p.). Both vertebral arteries were electrocauterized, and reversible clasps were placed loosely around the common carotid arteries. The wounds were closed and the animals allowed to recover for 24 hours. The following day, the animals were subjected to 20 minutes of global ischemia by tightening the ligatures around the carotid arteries. Body temperature was monitored and kept at 37°C during surgical procedures by using a heating pad. Sham-operated animals had both vertebral arteries cauterized and both common carotid arteries exposed but not occluded. Animals had free access to food and water after recovery from anesthesia. All rats were kept in air-ventilated cages with room temperature maintained at 22 ± 0.6 ℃. All experimental procedures involving animals were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Fig. 1).

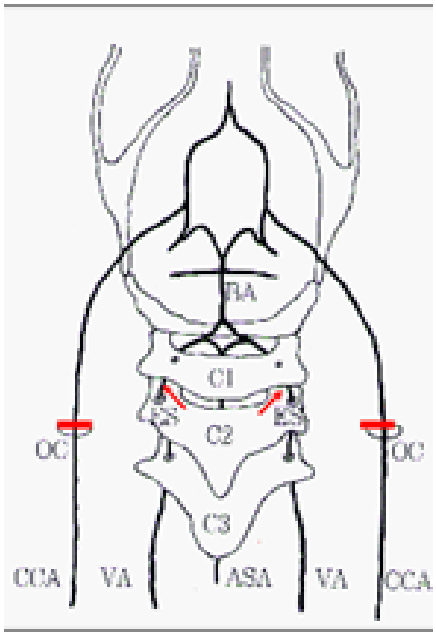

Fig. 1. Four-vessel occlusion method. Both vertebral arteries were electrocauterized, and reversible clasps were placed loosely around the common carotid arteries. The wounds were closed and the animals allowed to recover for 24 hours. The following day, the animals were subjected to 20 minutes of global ischemia by tightening the ligatures around the carotid arteries. VA, vertebral artery; CCA, common carotid artery; ASA, anterior spinal artery; BA, basilar artery; Arrow, ES, electrical stimulation; Bar, OC, occlusion.

Brain ischemia was morphometrically examined by Nissle stain of the hippocampus, the most vulnerable area to ischemia, contrasting before and at 72 hours after 4-vessel occlusion (Fig.2).

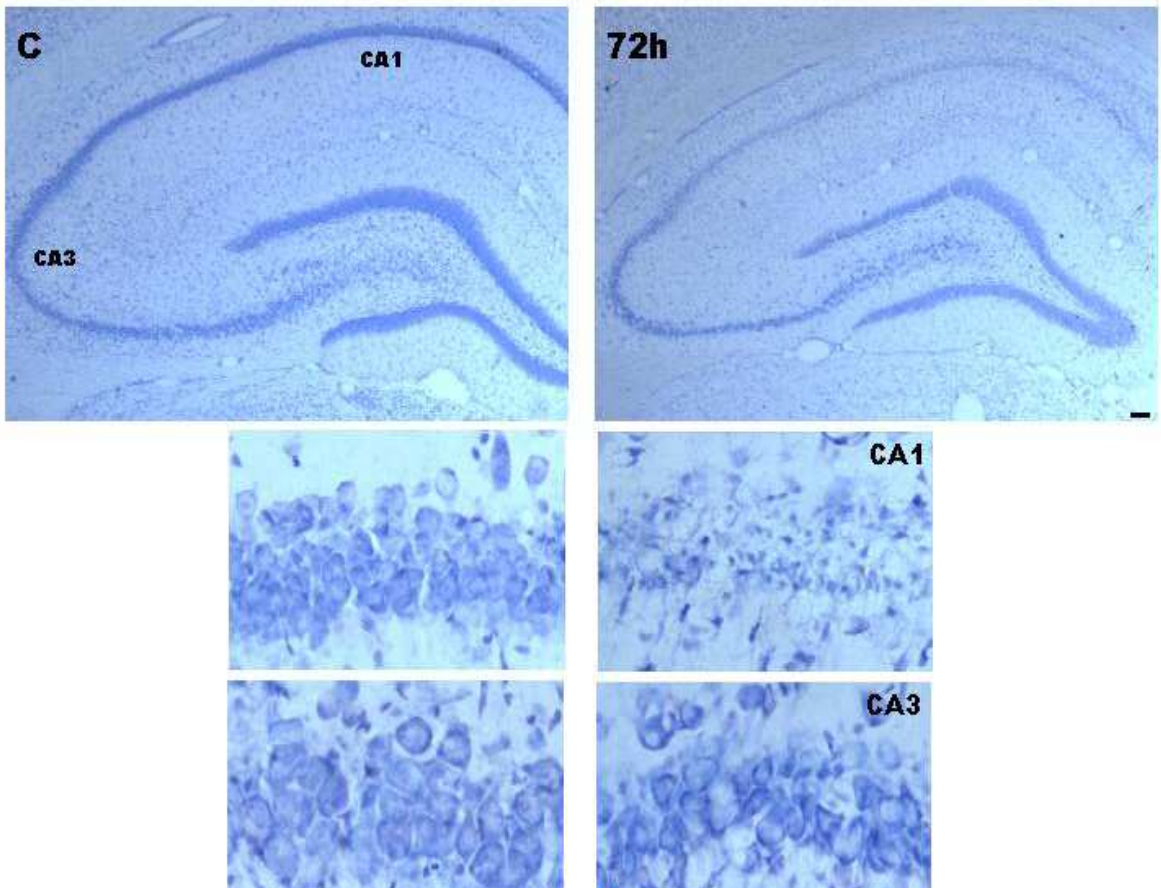

Fig. 2. Morphometrically examined brain ischemia by Nissle stain of hippocampus, comparing between before ischemia (C, control) and at 72 hours after 4-vessel occlusion.

2. Cell preparation and transplantation procedure

PA6 stromal feeder cells producing stromal cell-derived inducing activity (SDIA) were

purchased from Riken (Tsukuba, Japan) and maintained in PA6 cell culture medium [α-modified minimal essential medium (α-MEM) (Gibco, Rockville, MD, USA) supplemented with 0.5% penicillin-streptomycin (Gibco), and 10% fetal bovine serum (Hyclone, Logan, Utah, USA)]. The mouse blastocyst-derived ES cell line expressing enhanced yellow fluorescent protein (EYFP), was obtained from McLean Hospital/Harvard Medical School, Belmont, MA, USA. Undifferentiated ES cells were cultured on gelatin-coated dishes in Dulbecco's modified minimal essential medium (DMEM) supplemented with 2 mM glutamine, 0.001% β-mercaptoethanol, 0.1 mM nonessential amino acids, 10% donor horse serum, 0.5% penicillin-streptomycin (Gibco), and 1000 U/mL human recombinant leukemia inhibitory factor. To differentiate ES cells *in vitro*, PA6 cells at a density of 2.8 $X10^4$ cells/cm^2 were plated onto gelatin-coated culture dishes to form a uniform feeder monolayer one day before the addition of undifferentiated ES cells. ES cells were added at a density of 1,000 cells/cm^2. ES differentiation medium I [GMEM medium (Gibco) supplemented with 15% knockout serum replacement (Gibco), 0.001% β-mercaptoethanol (Gibco), 0.1 mM nonessential amino acids (Gibco), 0.1 mM sodium pyruvate (Sigma), and 0.5% penicillin-streptomycin (Gibco)] was used for 8 days and then replaced with ES differentiation medium II [GMEM medium (Gibco) supplemented with N2 supplement (Gibco) instead of 15% knockout serum replacement (Gibco)] for one additional day. The culture medium was changed on day 3 after co-culture and every other day thereafter. ES cells colonies that had differentiated into neural precursors were isolated from PA6 stromal feeder cells at differentiation day 9, one day after addition of medium II, by incubation with 2.5 ml of papain solution (Papain Dissociation Kit, Worthington, NJ, USA) for 5 minutes at 37℃. The dissociated colonies were transferred to a 15 ml tube and incubated for an additional 5 minutes at 37℃ followed by gentle trituration using a 1 ml tip and a 5 ml pipette. The cells were suspended at a density of $5X10^4$ cells/μl in phosphate buffered solution (PBS).

Cells were transplanted at 2 weeks after induction of global ischemia. All rats received cyclosporine (10 mg/kg, i.p.) 24 hours before transplantation and daily thereafter, antibiotics (amoxicillin sodium, 100 mg/kg, i.p.) before surgery and for 3 days after surgery, and nutritional supplements throughout the experimental period after surgery. The transplant group (n = 10) was reanesthetized with ketamine (50 mg/kg, i.p.) and placed in stereotaxic frames with a rat head holder. Each animal received a total injection of 8 μl of cells (5 X 10^4 cells/μl) at 2 bilateral hippocampal C3 areas. Burr holes were drilled at 2 injection sites, 4 mm posterior to the bregma, 2 mm lateral to the midline, and 4.5 mm beneath the dura) with a dental drill, which was irrigated continuously with saline at room temperature to prevent overheating of the underlying cortex. The solution was injected using a glass pipette with a tip diameter of ~80 μm mated to a 10 μl Hamilton syringe and a Kopf microstereotaxic injection system (Hamilton Company, Remo, Nevada, USA). Cells were injected at 1 μl/min while withdrawing the pipette

in 100 μm increments every 10 s. A 1 minute waiting period allowed the ES cells to settle before needle removal. After injections were completed, scalp wounds were closed and animals were returned to their cages. The vehicle cohort (n=10) received 8 μl of PA6 cells using the same method.

3. Evaluation of behavioral recovery

After transplantation, 2 behavioral tests were conducted on the rats in all 3 cohorts. Open field activity level was tested using a black plexiglas box (60×60×25 ㎝) at 2, 4, 6, and 8 weeks after transplantation. Animals were exposed to the box for 10 minutes. Locomotive behaviors were monitored and analysed by an auto-tracking system (SmarTrack), in which locomotion was calculated as a pixel unit. The Morris water maze test for investigating behavioral and cognitive functions was carried out at 8 weeks after transplantation according to the standard method. The Morris water maze apparatus was a large stainless steel tank (1.55 m diameter /60 cm depth) containing water (27±1℃) to a depth of 40 cm made opaque by the addition of skimmed milk. In the task, rats learn to locate a submerged circular platform (10 cm diameter) in a fixed location for 4 days (5 trials per day). Various extra-maze cues (posters, door, and computer. etc) were held constant. One day before training, rats were given a pre-training trial for 2 minutes in the absence of platform to acclimate them to the training situation. On the day of training, rats learned to escape from water by finding the invisible platform in the center a quadrant of the tank. In each trial, the animal was placed into the water facing the wall at one of three designated start points. Animals that failed to find the platform within 60 seconds were guided by an experimenter, placed onto the platform, and kept there for 30 seconds. Escape latency (time to find the platform) was measured as a learning score in each trial by an auto-tracking system (SmarTrack). Twenty-four hours after the fourth session, a probe test was performed to see the retention of spatial memory without the platform.

4. Tissue processing

At 8 weeks after transplantation, all transplanted animals were deeply anesthetized with pentobarbital sodium (100 mg/kg) and perfused transcardially with 100 ml of 0.9% saline, followed by a freshly prepared fixative solution containing 4% paraformaldehyde in 0.1M PBS (4°C, pH 7.0). The brains were then removed, postfixed for 8 hours in the same solution, equilibrated in sucrose (30% in PBS), sectioned at 40 μm on a freezing microtome, and collected in PBS.

5. Immunocytochemical and immunohistochemical assessments

For immunocytochemical staining, samples were first fixed with 4% paraformaldehyde for 30 min. After 24 h incubation in blocking solution (5% donkey serum albumin, Sigma), samples were incubated with primary antibodies for 1 hour at room temperature and then secondary antibodies for 1 hour at room temperature. Fluorescent images were obtained with an Olympus microscope (Olympus Corp., Tokyo, Japan), C5060 Olympus digital camera (Olympus Corp., Tokyo, Japan), and Adobe Photoshop 6.0 software (Adobe Systems Inc., San Jose, CA, USA). The following primary antibodies (Ab) were used for immunostaining: a panneural NCAM (neural cell adhesion molecule) Ab (1:100), a polyclonal βIII tubulin Ab (1:100), a polyclonal O4 Ab (1:200), and a polyclonal GFAP (glial fibrillary acidic protein) Ab (1:1000) (all from Pel-Freez, Rogers, AK, USA). Fluorescent-labeled secondary antibodies used to localize primary antibodies included Alexa Fluor® 488 donkey anti-mouse IgG, Alexa Fluor® 488 donkey anti-rabbit IgG, Alexa Fluor® 594 donkey anti-mouse IgG, Alexa Fluor® 594 donkey anti-rabbit IgG (1:500, Molecular Probes, Eugene, OR, USA), and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-sheep IgG (1:500, Chemicon).

EYFP was useful for identifying transplanted mouse ES cells in the rat brain. For immunohistochemical staining, brain sections were labeled with specific markers (antibodies) to identify cell types. These included antibodies (all from Pel-Freez, Rogers, AK, USA) directed against the neuron-specific nuclear antigen NeuN (1:200) and the oligodendrocyte developmental marker O4 (1:100). GFAP (1:800) staining was used to identify astrocytes. Secondary antibodies included Alexa Fluor® 488 donkey anti-mouse IgG, Alexa Fluor® 488 donkey anti-rabbit IgG, Alexa Fluor® 594 donkey anti-mouse IgG, Alexa Fluor® 594 donkey anti-rabbit IgG (1:500, Molecular Probes, Eugene, OR, USA), and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-sheep IgG (1:500, Chemicon). Primary antibodies were applied overnight at room temperature. Sections were washed 3-5 minutes in PBS and then incubated with a cross adsorbed secondary antibody for 45 min at room temperature. Sections were washed 3-5 minutes in PBS before coverslips were applied with Vectashield for immunofluorescence under a fluorescence microscope (Olympus Corp., Tokyo, Japan). For immunofluorescence double-labeled coronal sections, FITC and Cy3 fluorochromes on the sections were excited at 488 and 647 nm. Emissions were acquired sequentially with two separate photomultiplier tubes at 522 and 680 nm, respectively. Confocal microscopy immunofluorescence measurements were made using a Zeiss LSM5 PASCAL laser scanning confocal microscope equipped with a plan-Apochromat 63 X/1.4 oil DIC objective (Carl Zeiss, Germany). Alexa and CY3 signals were detected by excitation with the 488 nm and 543 nm lines, respectively. Images were scanned at scales of 0.02 Am (X), 0.02 Am (Y), and 0.4 Am

(Z), with the pinhole size of 106 Am (channel 1) and 158 Am (channel 2). Fluorescence data were collected using the LP560 filter (channel 1) and BP 505–550 (channel 2) and PASCAL software (Carl Zeiss, Germany). Off-line analysis was done using Adobe Photoshop (Adobe Systems, Mountain View, CA).

6. Semi-quantitative RT-PCR analyses

At pre- and 5, 10, 15, and 20 days after differentiation, total RNA from ES cell-derived neural cells was prepared using TriReagent (Sigma) followed by treatment with DNase I (Ambion, Austin, TX). cDNA was obtained using 5 μg of RNA with the SuperScript ™first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). The resulting cDNA was used as a template for the PCR reactions. We selected primer sets for Oct4, Pax6, MAP2 (microtubule-associated protein), Olig2, GFAP, TH (tyrosine hydroxylase), 5-HT (hydroxytryptamine), and GAD (glutamic acid decarboxylase). To analyze relative expression of different mRNAs, the amount of cDNA was normalized based on the signal from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Total RNA was extracted from cultured cells using easy-BLUE™ (Intron Biotechnology, South Korea) and the cDNA synthesis was carried out using AMV reverse transcriptase (RT) and oligo-dT as a primer, according to the manufacture's instructions (Power cDNA Synthesis Kit, Intron Biotechnology, South Korea). PCR amplification was performed using a standard procedure with Taq Polymerase (i-MAX™ DNA Polymerase, Intron Biotechnology, South Korea). To analyze relative expression of different mRNAs, the amount of cDNA was normalized based on the signal from glyceraldehyde-3-phosphate dehydrogenase(GAPDH) mRNA.. The number of cycles varied from 25 to 35 cycles depending on the particular mRNA abundance with denaturation at 94°C for 30 seconds, annealing temperatures at 55°C to 65°C for 30 seconds according to the primers, and elongation at 72°C for 30 seconds. Primer sequences (forward and reverse 5'-3') and the length of the amplified products were as follows:

Oct4 5’-CGTTCTCTTTGGAAAGGTGTTC-3’
5’-ACACTCGGACCACGTCTTTC-3’
Pax6: 5’-GGCAACCTACGCAAGATGGC-3’
5’-TGAGGGCTGTGTCTGTTCGG-3’
MAP2 5’-AGCCGCAACGCCAATGGATT-3’
5’-TTTGTTCCGAGGCTGGCGAT-3’
Olig2 5’-GGCGGTGGCTTCAAGTCATC-3’
5’-TAGTTTCGCGCCAGCAGCAG-3’

GFAP 5’-GCGCTCAATGCTGGCTTCAA-3’
5’-ACGCAGCCAGGTTGTTCTCT-3’
TH 5’-TTGGCTGACCGCACATTTG-3’
5’-ACGAGAGGCATAGTTCCTGAGC-3’
TPH 5’-CTACACTCCAGAGCCAGACAC-3’
5’-GACATCAAGGTCATACCGCAAC-3’
GAD 5’-GGGTTTGAGGCACACATTGATAAG-3’
5’-GCGGAAGAAGTTGACCTTGTCC-3’
G3PDH 5’-ACGACCCCTTCATTGACCTCAACT-3’
5’-ATATTTCTCGTGGTTCACACCCAT-3’

7. Electrophysiological experiment

Differentiated ES cells were examined using the whole-cell recording configuration of the conventional ‘dialysed’ whole-cell patch-clamp technique. Differentiated cells were obtained by seeding ES cells onto 12-mm round coverslips and culturing for 14 days. Patch electrodes were fabricated from a borosilicate glass capillary (Sutter Instrument Co., San Rafael, CA) by using a vertical micropipette puller (Narishige, Tokyo, Japan). The patch electrodes were fire-polished on a microforge (Narishige) and had resistances of 1 to 3 MΩ when filled with the internal solution described below. The cell membrane capacitance and series resistance were compensated (typically > 80 %) electronically using a patch-clamp amplifier (Axopatch-200A; Axon Instruments, Foster City, CA). Current protocol generation and data acquisition were performed using pClamp 8.0 software on an IBM computer equipped with an analog-to-digital converter (Digidata 1322A; Axon Instruments). Voltage traces were filtered at 2 KHz by using the four-pole Bessel filter in the clamp amplifier and stored on the computer hard drive for later analysis. All experiments were performed at room temperature (21-24 ºC). For recording of membrane potential in current clamp mode, the patch pipette solution contained (in mM): KCl 134, MgCl2 1.2, MgATP 1, Na2GTP 0.1, EGTA 10, glucose 14 and HEPES 10.5 (pH adjusted to 7.2 with KOH). The bath solution contained (in mM); NaCl 126, KCl 5, CaCl2 2, MgCl2 1.2, glucose 14 and HEPES 10.5 (pH adjusted to 7.4 with NaOH).

8. Statistical analysis

Data were analyzed with Student's t-test. In addition, one-way analysis of variance (ANOVA) and repeated measures ANOVA was used to assess the statistical significance of differences, followed by Tukey-Kramer tests for multiple comparisons of means. A p value of less than 0.05 was considered significant.

III. RESULTS

1. Differentiation into neural lineaged cells from mouse ES cells

In vitro, undifferentiated mouse ES cells induced on the stromal line PA6 differentiated into a variety of neural cell types. After co-culturing in ES cell differentiation medium I for 7 days, ES-derived rosettes were noted and at 9 days for transplantation, 95% of the ES cells colonies were nestin and NCAM positive cells. At 6 days after the addition of differentiation medium II supplemented with N2, immunocytochemistry showed βIII tubulin-positive cells in 30% of ES cells. Meanwhile, O4 or GFAP-positive cells, respectively 5%, and below 1% of ES cells were detected at 12 days after changing to medium II (Fig. 3).

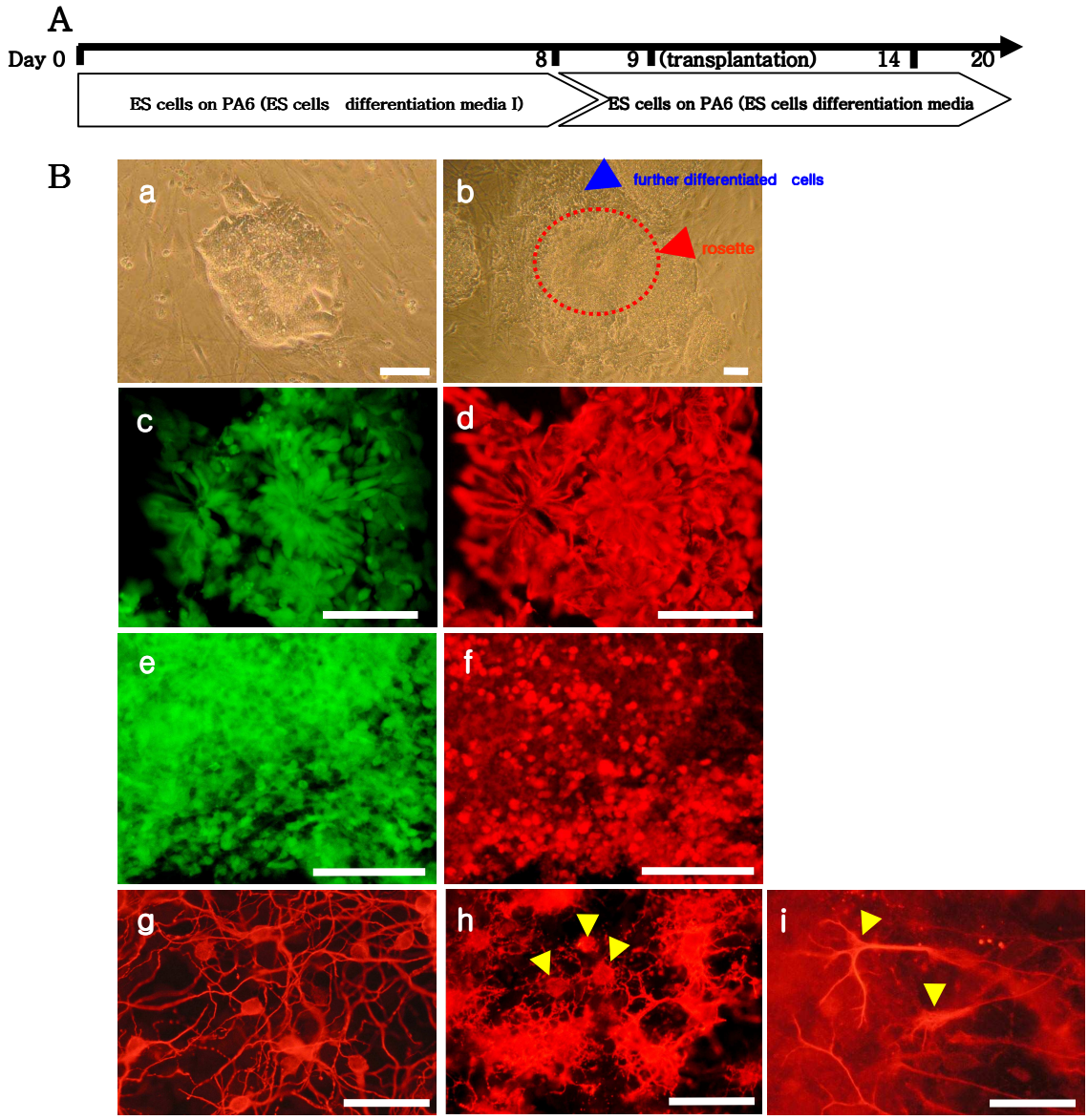

Fig. 3. A. Schematic procedures for *in vitro* differentiation into neural precusors from mouse embryonic stem cells on PA6 stromal feeder cell layer. B. After co-culturing in a embryonic stem cells differentiation medium I for 3 days, cell bodies were noted (a), at 7days, embryonic stem cells derived neural rosettes were noted (b), at 1 day after changing into medium II for transplantation, 95% of the embryonic stem cells colonies (c, e) were costained with nestin (d) and NCAM (f) positive cells, at 6 days after medium II, βIII tubulin-positive cells were noted in 30% of embryonic stem cells (g), meanwhile, O4 (h) or GFAP-positive cells (i), respectively 5%, and below 1% of embryonic stem cells were detected at 12 days after medium II. Scale bar of a-f, 100 μm. Scale bar of g-i, 50 μm.

The expression of several markers was further analyzed by RT-PCR throughout the differentiation procedure. Oct4, a marker of undifferentiated mouse ES cells, was not detected after differentiation. The disappearance of Oct4 expression was associated with increased expression of neural progenitor markers. Expression of Pax6, a neural precursor marker, was strongly upregulated in the transplantation stage and then maintained thereafter. RT-PCR demonstrated upregulation of other specific markers for neuronal precursor cells, including the dopaminergic neuronal marker MAP2, the serotoninergic neuronal marker TH, TPH, the GABAergic neuronal marker GAD, the oligodendrocyte precursor marker Olig2, and the astrocyte precursor marker GFAP, consistent with the results of immunocytochemical stainings (Fig. 4).

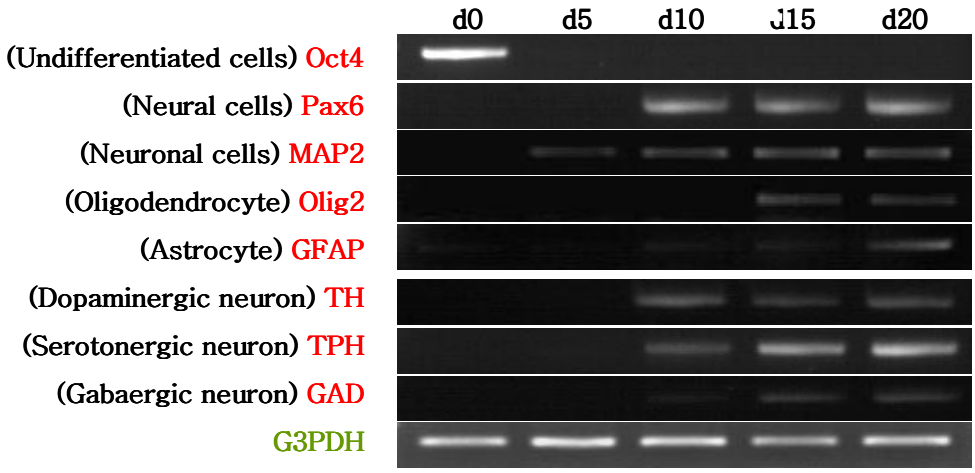

Fig. 4. Semi-quantitative RT-PCR Analyses. Oct4, a marker of undifferentiated mouse ES cells, was not detected after differentiation. The disapperance in expression of Oct4 was associated with increased expression of neural progenitor markers. Expression of Pax6, neural precursor marker was strongly upregulated in transplantation stage and then maintained thereafter. The

other specific markers for neuronal precursor cells, MAP2 including for dopaminergic neuronal marker, TH, serotoninergic neuronal marker, TPH and gabaergic neuronal marker, GAD and in addition, oligodendrocyte precursor marker, Olig2 and astrocyte precursor marker, GFAP were upregulated, consistent with the results of immunocytochemical stainings.

2. Efficient integration, migration and generation of neural cells from grafted ES cells

Cerebral ischemic lesions were consistently confirmed in both hippocampal CA1 and CA3 areas at 72 hours after four-vessel occlusion (Fig. 2). Immunohistochemistry conducted at 8 weeks after transplantation found surviving grafts in the ischemic brain. NeuN, O4, and GFAP-positive cells were found within the graft and migrated diffusely even into the contralateral hemisphere via the corpus callosum (Fig. 5). Teratoma formation was not found in any transplanted rat brains up to 12 weeks after transplantation.

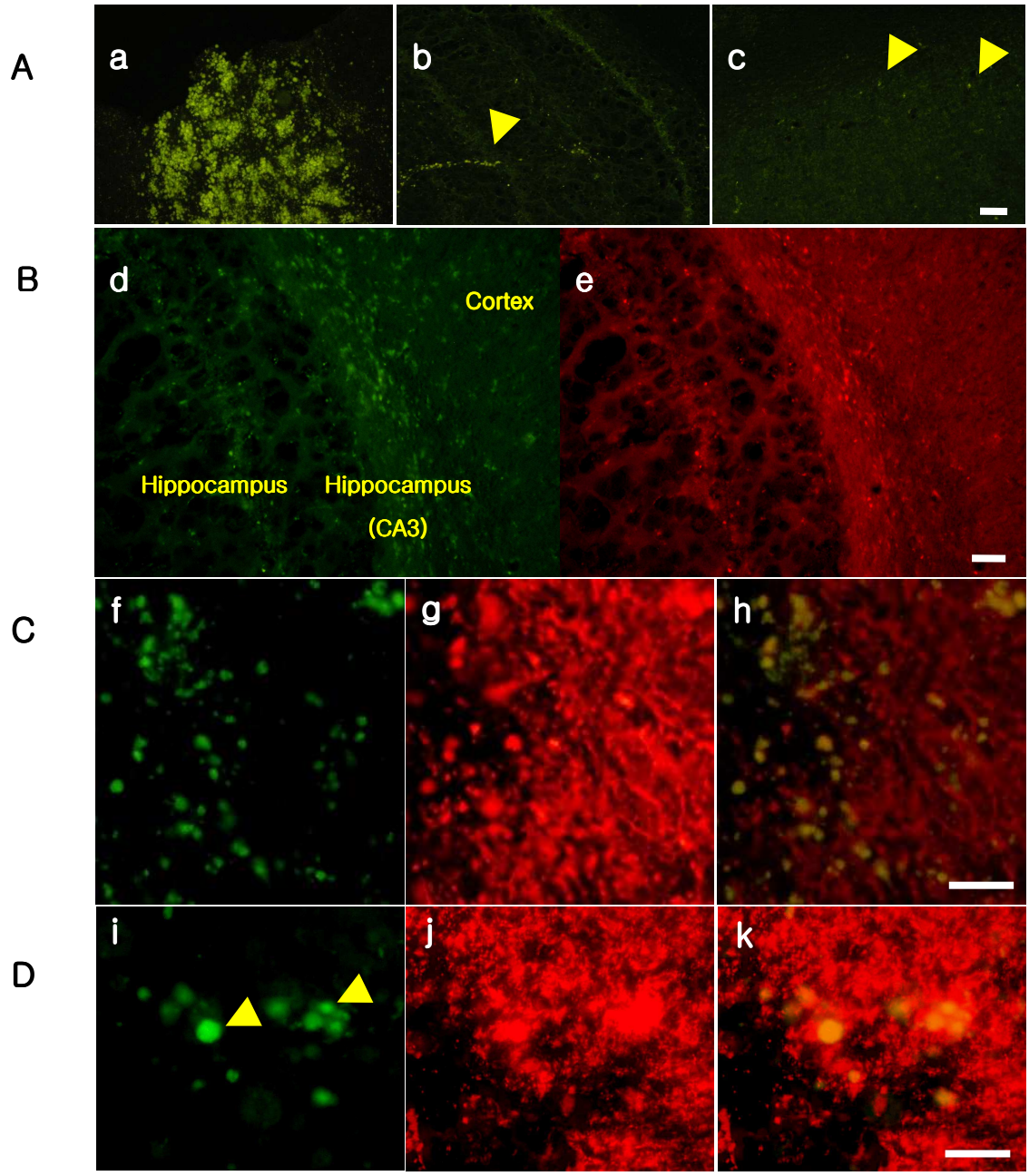

Fig. 5. Efficient integration, migration and generation of neural cells from grafted embryonic stem cells at 8 weeks after transplantation. A. mouse embryonic stem cells with enhanced yellow fluorescent protein in injection site (a), migration around hippocampus (b), migration into the ipsilateral thalamus (c), B. dispersion around hippocampus and adjacent cortex (d), NeuN staining showed neuronal cells originated from transplanted embryonic stem cells (e), C. dispersion of embryonic stem cells around hippocampus (f), GFAP positive astrocytes (g), merged imaging showed differentiated cells into astrocytes from transplanted embryonic stem cells (h), D. dispersion of embryonic stem cells around hippocampus (f), O4 positive oligodendrocytes (g), merged imaging showed differentiated cells into oligodendrocytes from

transplanted embryonic stem cells (h), scale bar of a, b, c, 200 μm. scale bar of d, e, 100 μm. scale bar of f-k, 50 μm.

3. Transplantation of neurally predifferentiated ES cells brings about behavior recovery in globally ischemic brains in adolescent rats

A. Open field activity test.

At the beginning of open field activity tests, mean body weight was 187.2 g in the sham group, 181.4 g in the vehicle group and 174.2 g in the transplant group. Rats in the transplant cohort performed better in the open field activity test from 4 to 8 weeks after the transplantation than did age-matched animals in the vehicle group but the difference was not statistically significant. While the activities of animals in all groups apparently were improved during the initial 6 weeks of testing, performance began to plateau from 6 to 8 weeks in the vehicle group. During the period from 6 to 8 weeks, however, activities of animals in the transplant and sham groups continuously improved and were better than those of the vehicle animals (Fig. 6).

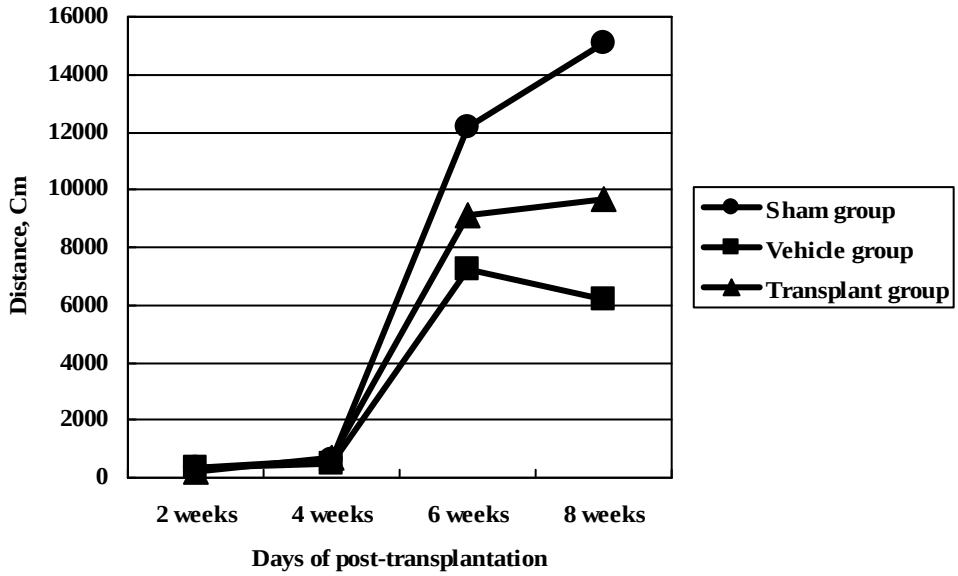

Fig. 6. Open field activities test. Transplant group performed better in the open field activities test from 4 to 8 weeks after the transplantation than did age-matched animals in the vehicle group but unfortunately no statistical significance was found between vehicle and transplant groups. However, statistically significant difference was found only between sham and vehicle groups at 4 weeks ($F=4.287$, $p=0.039$, repeated measures ANOVA, Tukey-Kramer methods).

and 8 weeks (F=4.462, p=0.036, repeated measures ANOVA, Tukey-Kramer methods) after the cell transplantation.

B. Morris water-maze learning.

During pretraining, it was observed that all animals could swim easily. Mean escape latency times differed significantly between the vehicle group and the transplant and sham groups (F=8.797, p=0.004, repeated measures ANOVA, Tukey-Kramer method). There was, however, not a significant difference between the transplant group and the sham group (Tukey-Kramer method) (Fig. 7).

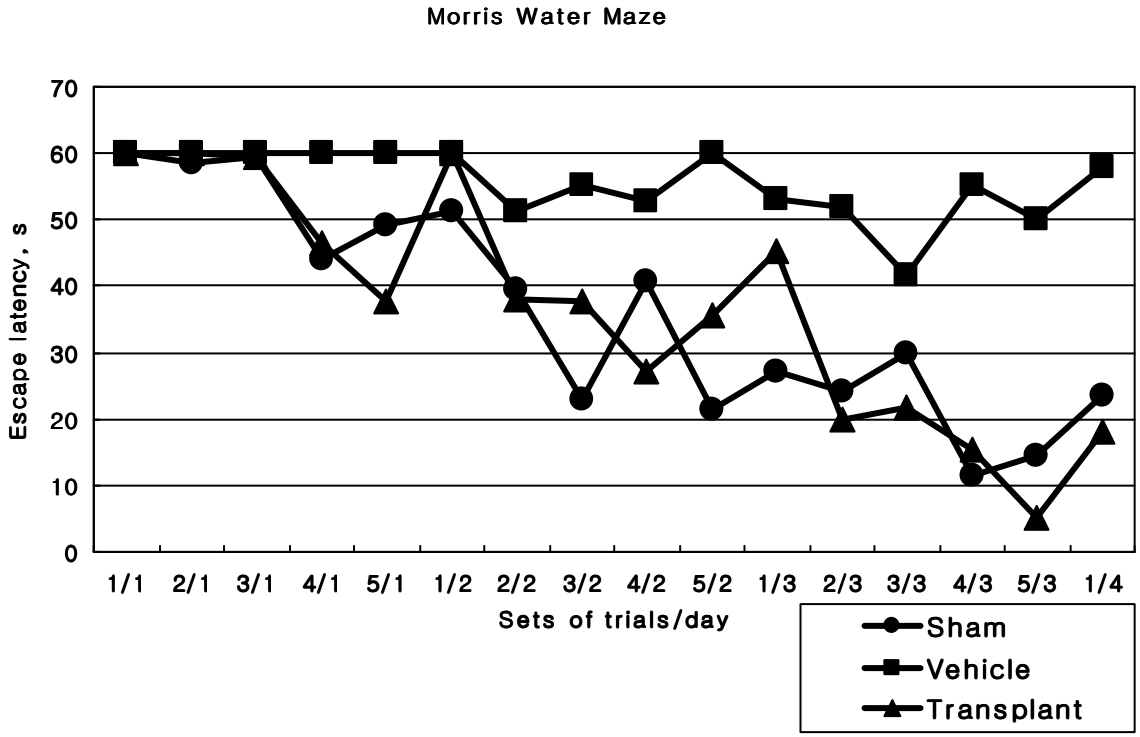

Fig. 7. Morris water-maze method at 8 weeks after transplantation. Transplant and sham groups improve significantly in the time to reach the escape platform more than vehicle group (F=8.797, p=0.004, repeated measures ANOVA, Tukey-Kramer methods).

In the ‘‘spatial probe’’ trial during the last set on the fifth day, the number of animals crossing the area in which the escape platform had been located differed significantly between the vehicle group and the transplant and sham groups (F=9.137, p=0.001, one-way ANOVA, Tukey-Kramer method). Again, there was no significant difference between the transplant group and the sham group (Tukey-Kramer method) (Fig. 8).

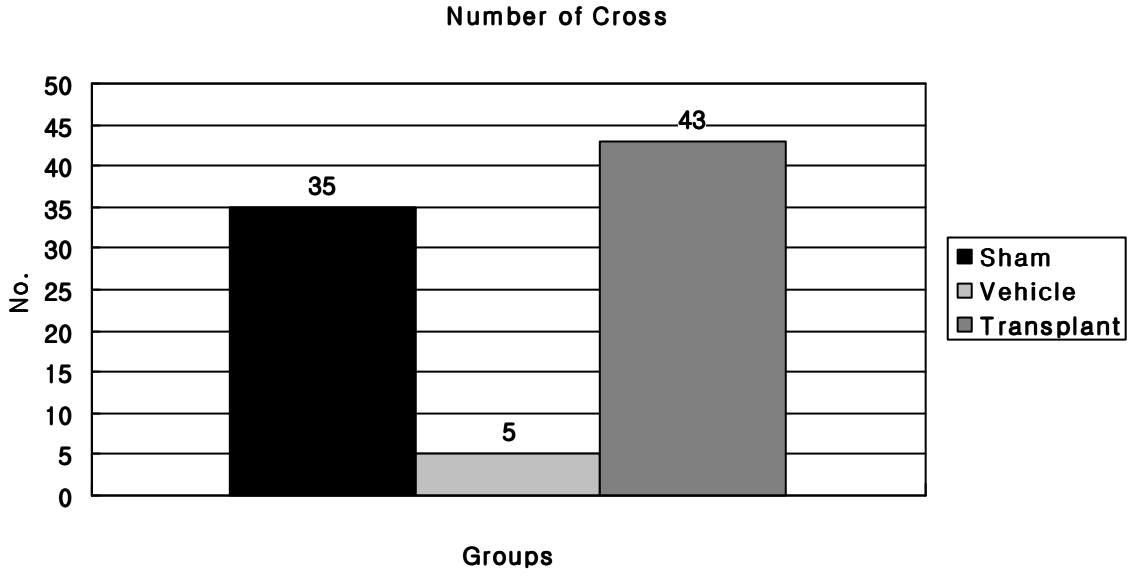

Fig. 8. Spatial probe trial, transplant and sham groups have a significantly better cognitive function than vehicle group (F=9.137, p=0.001, one-way ANOVA, Tukey-Kramer methods).

IV. DISCUSSION

Stroke causes serious damage to the nervous system, and can disrupt the interaction of neurons and glial cells. The reconstitution of the complex and widespread neuronal–glial interrelationships may require the involvement of a broad array of uncommitted neuronal subtypes. Moreover, the pathologic lesions found in pediatric ischemic brains are often globally distributed or dispersed within several foci throughout the brain[14]. Accordingly, the transplantation of a large number of uncommitted precursor cells is likely to be necessary to restore a globally ischemic brain, both anatomically and functionally.

Many recent trials of cell transplantation using neurospheres from fetal brain, umbilical cord blood, and bone marrow mesenchymal stem cells have been reported in experimental focal ischemic models, but these tissue sources share the disadvantage of being proliferative and phenotypically plastic[15]. Recent studies have used ES cells for the experimental treatment of heart[16] or brain ischemia[17] and spinal cord injury[18]. The distinguishing features of ES cells, including their capacity to be maintained indefinitely in an undifferentiated state, is an ability to develop into multilineage cells under certain conditions and the relative ease with which large cultures can be grown. These attributes make them a promising candidate for playing a major contribution to stem cell therapy[9]. In addition, recent evidence of patient-specific ES cells derived from human somatic cell nuclear transfer blastocysts makes ES cells a good source for cell replacement therapy[12].

In this study, we demonstrated that neurally-predifferentiated mouse ES cells transplanted into globally ischemic brains in adolescent rats survived and were differentiated into neurons and glial cells with functional recovery. The use of EYFP-expressing transgenic ES cells co-cultured on PA6 stromal feeder cells allowed visualization of the ES cells without fluorescent immunostaining. PA6 cells present a strong neuralizing activity, namely through SDIA on the cell surface. *In vitro*, neural rosettes were observed at 7 days of differentiation. In addition, 6 days after culture with ES differentiation medium II, a high yield 30% of βIII tubulin positive neuronal precursors was obtained. O4 and GFAP-positive cells were detected at differentiation 20 days but only 5% and below 1% differentiated to oligodendrocyte and astrocyte precursors. Such progressive differentiation of neural lineaged cells is similar to the differentiation seen when using MS-5 feeder cells [19]. In the graft experiment, many NeuN-, O4-, and GFAP-positive cells and neurons had integrated into the nearby host tissues and migrated diffusely even into the contralateral hemisphere via the corpus callosum. When neural stem cells were implanted into these regions of extensive ischemia, robust reciprocal interactions ensued spontaneously between the exogenous implant and the injured host brain. In addition, we should consider thant stem cell transplantation may also lead to clinically valuable improvemtns

through other mechanisms as well, including inflammation-induced stimulation of host plastic responses, neurotriphic factor secretion, release of missing transmitter [11]. The favorable behavioral outcomes in the transplanted group showed that transplantation of neural precursors, predifferentiated from ES cells, could improve sensorimotor integration and spatial cognition in the globally ischemic brain. A new neural network reconstructed between the graft and the host might result in the recovery of damaged higher cortical functions.

Implantation of ES cells can cause teratomas, and ES cells seem more prone to generate tumors when implanted into the same species from which they were derived [20]. However, the risk can be reduced if the cells are differentiated beforehand *in vitro.* For even greater safety, engineered ES cells with regulatable suicide genes can be used [11]. Our neurally predifferentiated mouse ES cells did not cause teratoma formation in rat brains following transplantation.

V. CONCLUSION

Neurally predifferentiated precursors generated from mouse ES cells with EYFP on a stromal line of PA6 with SDIA integrated, migrated and survived in extensively ischemic cerebral areas without teratoma formation, inducing functional recovery of ischemic adolescent rats. The present study provides hope that transplantation of expanded and partially differentiated stem cells may provide a method for repairing globally damaged brain structures and enhancing brain function recovery in adolescent rats.

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ABSTRACT(IN KOREAN)

광범위 뇌경색 유발 청소년 주령 흰쥐에서 배아 줄기 세포 기원 신경전구체 이식 후 행동 향상

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강훈철

최근 중추신경계 질환의 세포 치료에 대한 연구의 발전으로 인해 중추신경계 손상을 실질적으로 회복시킬 수 있는 가능성이 제시되고있다. 본 연구는 황색 형광을 발현하는 단백을 가진 쥐 배아줄기세포를 기질 유래 유발 요소를 배출하는 PA6 기질 세포주와 공동 배양하여, 신경세포와 아교세포를 포함한 신경전구세포로 분화시킨 후 광범위 뇌허혈 손상이 유발된 흰쥐의 양쪽 해마체 C3 영역에 이식 후, 해부학적 기능적 회복을 관찰하고자 하였다. 시험관내 배아줄기세포의 분화는 면역 형광, RT-PCR, 전기생리 검사를 통해 다양한 신경전구 세포로의 분화를 확인할 수 있었다. 이식 후 8 주간 생체내 배아 줄기세포의 신경세포 및 아교세포로의 분화 및 손상 조직 내 통합과 세포 이동을 관찰할 수 있었다. 인지 능력과 운동 능력을 평가하기 위해 시행한 Morris 수중 미로 검사에서 대조군에 비해 이식 쥐의 의미있는 능력 향상을 관찰할 수 있었고, 개방 영역 활동도 검사에서는 이식 쥐가 대조군에 비해 활동도가 증가하는 경향을 관찰하였다. 관찰 기간 2 개월 간 기형종 발생은 없었다. 신경전구세포로 분화된 배아줄기세포을 이식함으로서 종양의 형성없이 중주신경계 허혈성 손상의 회복을 관찰할 수 있었다.

핵심되는 말 : 신경전구세포, 배아줄기세포, 광범위 허혈성 뇌손상, 청소년 주령 흰쥐