Transplantation of Ngn2-expressing human neural stem/progenitor cells into hypoxic-ischemic injured neonatal mouse brain

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Transplantation of Ngn2-expressing human neural stem/progenitor cells into hypoxic-ischemic injured neonatal mouse brain

Directed by Professor Kook In Park

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Kyo Yeon Koo

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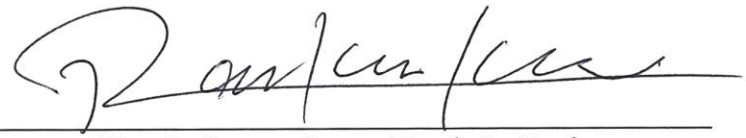
This certifies that the Doctoral

Dissertation of Kyo Yeon Koo is approved.



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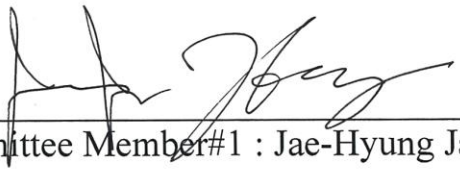

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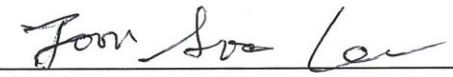

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ABSTRACT

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Neonatal hypoxic–ischemic (HI) brain injury represents a major cause of cerebral palsy, developmental delay, epilepsy, and even death. Stem cell transplantation offers new treatment hope, but significant questions remain regarding how grafted cells exert effects. HI brain spontaneously undergoes reinnervation and rewiring of surviving neurons after injury and this is widely known as the innate repair capacity of the brain. Here, we demonstrate that adenovirus-mediated overexpression of neurogenin 2 (Ngn2) of human neural

stem/progenitor cells (hNSPCs), which directly controls neurogenesis in the embryonic cerebral cortex, and transplantation into mice with HI brain injury, enhances functional recovery, host cell survival, and innate neuroplasticity after HI brain injury. Transplanted Ngn2-expressing hNSPCs differentiated into mostly neurons, and we suggest that these effects are mediated by the increase of the Ngn2-expressing hNSPCs-secreted factor neurotrophin 3 during hNSPCs neurogenesis. We also carefully propose the possibility that Ngn2-expressing hNSPCs may form synapses with other surviving neuron in peri-infarcted areas based on our observation that Ngn2-expressing hNSPCs expressed synaptophysin and growth cone. Our results are the first evidence that grafted hNSPCs with neural induction could have play a bifunctional role in that they replace lost cells and secrete neurotrophic factors to promote survival and connectivity.

Key Words: Hypoxia-ischemia, Brain, Neurogenin 2 protein, Mouse, Neural stem cells (NSCs), Transplantation, Differentiation

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I. INTRODUCTION

Hypoxic-ischemic (HI) brain injury in newborn infants is a major cause of mental retardation, sensory neural hearing loss, blindness, cerebral palsy, epilepsy, learning disabilities, and even death. Perinatal HI brain injury occurs in 2-4 per 1000 live term births, and approximately 60% of preterm HI infants sustain permanent brain damage even though therapeutic hypothermia has been shown to improve recovery[1]. The pathogenesis of the ischemic brain is

not described only by the pathway of hypoxic cell death, but also reflects continuous changes in genetic expression and levels of intrinsic oxidant radicals, growth factors, and inflammatory mediators such as cytokines, adhesion molecules, etc[2-4]. Hypoxic ischemic encephalopathy is a condition of cell death when the brain does not receive enough oxygen and blood flow. Cell death is caused by impairment of ATP-dependent Na^+-K^+ pump, generation of oxidative stress, excessive release of excitatory neurotransmitter with accumulation of polymorphonuclear leukocytes and activated microglia[2]. Furthermore, Arvin et al.[3] delineated change after ischemic event based upon molecular point such as transcription factors, heat-shock proteins, pro-inflammatory mediators, chemokines, adhesion molecules and growth factors. Li et al.[4] reported growth associated gene and protein expression in the region of axonal sprouting after stroke.

Regeneration of the central nervous system (CNS) was previously considered impossible, despite the observation that focal cerebral ischemia promotes neurogenesis of endogenous neural stem/progenitor cells (NSPCs) in the subventricular zone and subgranular zone of the dentate gyrus and induces their migration towards the ischemic boundary[5]. Recent studies suggest that transplantation of exogenous NSPCs may have beneficial effects on outcome following HI brain injury[6-8]; however, the underlying recovery mechanisms are unknown.

Stem cell therapies have been attempted with various stem/progenitor cell preparations, including mesenchymal stem cells, multipotent astrocytic stem cells, and human umbilical cord blood cells (hUCBCs)[9]. Among these, NSPCs show more restricted neural differentiation capabilities committed to specific subpopulation lineages. NSPCs are described as cells that are derived from specific spatiotemporal neural tissues or are generated from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)[10,11]. NSPCs have capacity for self-renewal and can give rise to neurons, astrocytes, and

oligodendrocytes[12,13]. hNSPCs can also be derived from developing human fetal brain between 8-20 weeks of gestation[14,15]. These multipotent NSPCs derived from the developing or adult brain can be cultured and grown in the presence of mitogens, such as epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF2) either as monolayers or as free floating spherical aggregates named the neurosphere[14-17]. When NSPCs are implanted into a diseased or injured nervous system, they show not only preferential extensive migration to and engraftment within areas of discrete abnormalities but the capability to replace diseased tissue in an appropriate manner (cell replacement effect)[18-21]. Apart from replacing lost cells, NSPCs based therapy can also provide a regenerative microenvironment for other cells residing of diseased brains (bystander effect)[21-25]. Early studies aimed to establish whether exogenous stem/progenitor cells could engraft and replace dying cells, and many showed that the donor cells survived and migrated toward the injury site. However, the effect derived from the net increase in cell numbers was generally negligible[9]. Notably, the transplanted NSPCs were usually maintained with little differentiation, and it is not clear that these treatments resulted in fully functional and integrated neurons or glia[8,26,27]. These findings suggest that the key mechanisms leading to therapeutic effects may be the release of neurotrophic and immunomodulatory/immunosuppressive factors[9,28]. In the case of neonatal HI brain injury, stem cell treatment at different time points during the evolution of injury may allow a range of potentially complementary mechanisms to be targeted. If stem cells are administered relatively soon after HI, then many cells may be saved by attenuation of the immune response and decreased release of excitotoxins, cytotoxins, and oxygen free radicals, as well as reduced infiltration of leukocytes[9].

One proposed mechanism to explain the repair effects of NSPCs was suggested by Andres et al.[26], who posited that hNSPCs may promote the

innate repair capacity during recovery after cerebral ischemia. Innate repair capacity plays a major role in the spontaneous recovery observed in patients and animals after stroke. It is thought to mediate the dramatic reorganization and rewiring of surviving circuits that enable the healthy brain to take over the function of the damaged area[29-31]. Functional imaging and stimulation studies in patients have shown a remapping of the brain after stroke that, at least in the first few weeks, indicates recruitment of both ipsi- and contralesional brain areas. Animal studies yielded similar results[32,33], suggesting that this innate repair capacity arises by local and long distant changes in axonal sprouting and dendritic arborization, also known as synaptic plasticity. Synapses in the brain are housed between dendritic spines, which contain receptors and associated proteins (the post-synaptic density), and axonal terminals which contain the synaptic vesicles for neurotransmitter release[34]. Synaptic plasticity describes the ability of the connection, or synapse, between two neurons to change in strength[35]; thus, plastic change results not only from the alteration of the number of synaptic vesicles or receptors located on a synapse but also from changes in axonal sprouting or dendritic arborization[36,37]. Several underlying mechanisms promote the formation and growth of synapses and spines; for example, involvement of the Rho GTPases regulatory proteins has been described[36]. Andres et al.[26] further hypothesized that the functional recovery seen with hNSPC therapy is correlated with changes in synaptic plasticity induced by secretion factors derived from the transplanted hNSPCs, such as neurotrophic factors like vascular endothelial growth factor (VEGF), basic fibroblast growth factor, other growth factors, and extracellular matrix molecules like the secreted protein acidic and rich in cysteine (SPARC) and thrombospondins. These factors are involved in signaling axonal and dendritic changes after stroke. They are poorly understood, but many of these molecules are known to promote neurite sprouting, either in culture or *in vivo*[26].

Ngn2 belongs to a family of basic helix-loop-helix (bHLH) transcription factors and was first isolated from neural crest cell by Sommer in 1996[38]. It is a proneural gene that is a critical regulator of neurogenesis through its effects on a neuron-specific signaling pathway associated with a Notch signaling during cortical development. In the developing brain, endogenous NSPCs give rise to neurons or glial cells that are later under control of delta/notch ligands (Notch signaling). Neuronal fate cell is determined by expression of bHLH genes, which include Mash1, Math, and Neurogenin[39,40]. Ngn2 induces expression of neuron-specific gene while inhibiting transcription factors that promote the maintenance of NSPC undifferentiated status[39]. Ngn2 also regulates the initial axon guidance of cortical pyramidal neurons projecting to the target[41].

Several lines of evidence show that overexpression of select proneural genes enhances neuronal differentiation *in vitro* and *in vivo*[42,43]. Fetal hNSPCs can be efficiently, rapidly, and safely expanded in vitro as well as rapidly differentiated toward mature neural lineages by overexpression of transcription factors such as Ngn1, Ngn2, Ngn3, and Mash1 in various ways. Ngn2 acts as a master regulator of neuronal differentiation that requires no additional external or internal factors and occurs even under pluripotency-promoting conditions[42]. Upon transplantation into adult rat brains, Mash1- or Ngn2- expressing NSPCs yielded large grafts enriched with neurons when compared to control LacZ-transduced NSPCs[43] and transplantation of Ngn2-expressing NSPCs into injured spinal cord improves recovery of hindlimb locomotor function and sensory responses by increasing myelin[27]. From the perspective of regenerative medicine, multipotent hNSPCs therefore offer a therapeutic advantage over pluripotent stem cells in that they are already invariantly "neurally committed" and lack tumorigenicity[42]. However, while grafts of hNSPCs into HI brain showed targeted homing and integration into ischemic areas with ~ 5% differentiation into neuronlike cells[44], we expected

to see greater differentiation of transplanted NSPCs into neurons, as this would be more ideal than cells remaining in undifferentiated status or differentiating into other cell types *in vivo*. We predicted that expression of Ngn2 would induce neurogenesis of grafted NSPCs, which then might replace lost cells and secrete transcription factors that might be physiologically relevant to CNS repair in the HI brain.

In vitro, NSPCs expressing Ngn2 also expressed higher levels of neurotrophic factors compared to control, which is important because the survival effect of Ngn2 is mediated via neurotrophin 3 (NT3) and its receptor[43]. The neurotrophins are a family of closely related proteins that were first identified as survival factors for sympathetic and sensory neurons in both the central and peripheral nervous systems. Neurotrophic factors promote the survival of neuronal cells and control their synaptic plasticity and differentiation through activation of the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (TrkA, TrkB and TrkC) and the p75 neurotrophin receptor (p75(NTR)). The neurotrophic factor hypothesis contends that developing neurons survive and connect with target neuron using only a limited supply of a neurotrophic factor. This hypothesis provides an explanation for how neurotrophic factor influences the size of the neuronal populations and the innervations among them in the neural network. Some aspects of neurotrophin action do not conform to the classic neurotrophic hypothesis; for example, neurotrophins act on at least some sensory neurons by an autocrine route. However, the neurotrophic hypothesis has been broadened by the demonstration that multiple neurotrophic factors regulate the survival of certain populations of neurons[45-51].

In this study, we transplanted hNSPCs transfected with an Ngn2-encoding adenoviral vector into ischemic neonate mouse brain to determine if we could successfully induce hNSPCs to differentiate into neurons. In addition, we use the mouse HI brain model to determine whether differentiated hNSPCs

transfected with neurogenin 2 also enhance their therapeutic effects via greater physiological secretion of factors or by increased functional integration.

II. MATERIALS AND METHODS

1. Human neural progenitor cell culture

Human fetal tissue from embryos therapeutically aborted at 13 weeks of gestation was obtained with full parental consent and the approval of the research ethics committee of Yonsei University College of Medicine, Seoul, Korea. The methods of acquisition conformed to NIH and Korean Government guidelines. The telencephalic region of CNS tissue was freshly dissected, dissociated in trypsin (0.1% for 30 minutes), and seeded into tissue culture-treated 100-mm plates (Corning) at a density of 200,000 cells/ml of serum-free growth medium, which consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (GIBCO) supplemented with penicillin/streptomycin (1% vol/vol; GIBCO) and N2 formulation (1% vol/vol; GIBCO). Mitogenic stimulation was achieved by adding 20 ng/ml fibroblast growth factor-2 (FGF-2; R&D) and 10 ng/ml leukemia inhibitory factor (Sigma). Heparin (8 μg/ml; Sigma) was added to stabilize FGF-2 activity. All cultures were maintained in a humidified incubator at 37℃ and 5% CO_2 in air, and half of the growth medium was replenished every 3-4 days. Passage of these cells was undertaken every 7-8 days by the dissociation of bulk neurospheres with 0.05% trypsin/EDTA (T/E; GIBCO).

2. Viral vector construction and preparation of Ngn2-expressing hNSPCs

We constructed recombinant adenoviral vectors bearing human Ngn2 and green fluorescent protein controlled by an IRES (Adeno-Ngn2-IRES-eGFP; Fig. 1). The adenoviral vectors were produced in conformity with the AdEasy™ Adenoviral Vector System (Stratagene) manual. The infectious recombinant virus was purified by CsCl-gradient centrifugation and titrated

on 293A cells by Tissue Culture Infecting Dose 50 (QBiogene). Adeno-Ngn2-IRES-eGFP-infected hNSPCs were seeded onto cell culture dishes with 15 multiplicity of infection (MOI). Ngn2 expression was checked by reverse transcriptase polymerase chain reaction (RT-PCR), and cell transplantation was conducted after 3 days.

Figure 1. Adenoviral vector construct containing a promoter. An E1-deleted adenoviral type 5 construct was used to express a bicistronic transcript of Ngn2 and human GFP.

3. **Induction of experimental focal HI brain injury and cell transplantation**

We used CD-1 mice to induce hypoxic-ischemia injury. The right common carotid artery of anesthetized mice of postnatal day 7 was ligated with a 6-0 surgical silk. The incision was closed, and the animals were kept warm (37-38℃) until awake and returned to their dams for 1.5-2 hr. The stabilized mice were placed in an acrylic chamber with a hypoxic atmosphere of 8% O_2 and 92% N_2 and a 39℃ heating pad for 1.5 hr. The animals recovered in room air and were returned to their dams. All mice received the same care and housing, and were evenly distributed between control and treatment groups. On day 7 after HI injury (postnatal day 14), the pups were anesthetized, and an incision was made through the dorsal midline of the head to inject Ngn2-expressing hNSPCs (Ad-Ngn2-hNSPCs or Ngn2 group; 12 μl cell suspension at 8×10^4 cells/μl), GFP-expressing hNSPCs (Ad-GFP-hNSPCs or GFP group), or vehicle into the infarct cavity of each mouse brain with a glass micropipette (diameter, 0.3 mm). Cyclosporine (10 mg/kg) was intraperitoneally

administered daily beginning a day before surgery and until sacrifice. The procedures were approved by the Animal Care and Use Committees of Yonsei University College of Medicine (Seoul, Korea).

4. In vitro differentiation studies of Ngn2-expressing hNSPCs

To identify Ngn2-expressing hNSPCs, we stained Ngn2-expressing hNSPCs (Ad-Ngn2-hNSPCs), GFP-expressing hNSPCs (Ad-GFP-hNSPCs), and compared the cell fates. hNSPCs were infected with an Ngn2-encoding adenoviral vector or GFP-encoding adenoviral vector at each 10 MOI for 18 hr. 4 days later they were trypsinized and plated on poly-L-lysine (10 μg/ml; Sigma)-coated eight-well chamber slides (Nunc) at $8x10^4$cells/well density and these two distinct hNSPCs were differentiated for 7 days. The cells were fixed with 4% paraformaldehyde (PFA) in 0.1 M PIPES buffer (Sigma) for 10 min, rinsed three times with phosphate-buffered saline (PBS), and treated as described below. The fixed cells were blocked with 3% bovine serum albumin (BSA) and 10% normal horse serum with 0.2% Triton X-100 and incubated with the following primary antibodies: human nestin (1:200; Millipore, Billerica, MA, USA); GFAP (1:1000; DAKO, Glostrup, Denmark); β-tubulin III (Tuj1, 1:500; Covance, Princeton, NJ, USA); O4 (1:30; Millipore); Olig2 (1:500; Millipore). Following rinsing in PBS, the cultures were incubated with species-specific secondary antibodies conjugated with fluorescein (Vector, 1:180) or Texas Red (Vector, 1:180), and DAPI (4', 6'-diamidino-2-phenylindole, Vector) was used as the nuclear stain. After immunofluorescence staining, the percentage of immunoreactive cells was evaluated using a fluorescence microscope (Olympus Optical Co.). For each group, we counted cells in 10 fields that included 100–500 cells. The total number of cells was evaluated by counting DAPI-positive nuclei and marker-positive cells.

5. Immunohistochemistry *in vivo*

The animals were sacrificed and transcardially perfused with cold PBS followed by cold 4% PFA in 0.1M PIPES solution. The brain was carefully extracted, fixed for overnight in 4% PFA in 0.1 M PIPES solution at 4℃, and subsequently transferred to 30% sucrose solution at 4℃ until embedding. Optimal cutting temperature compound was used to embed the brains, and 16-μm sections were cut on a freezing cryostat and stored at -20℃. For fluorescence immunohistochemistry, sections were first blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 3% BSA in PBS with 0.1-0.3% Triton X-100. BrdU antibody requires a pre-treatment step of 30 minutes in 2 N HCl at 37℃. Primary antibodies were incubated overnight at 4℃. Appropriate secondary antibodies were applied for 70 minutes at 37℃ followed by three PBS washes before they were mounted. Primary antibodies included anti-BrdU fluorescein (1:20; Roche Applied Science), human nuclei (hNuc, 1:100; Millipore), and SC121 (1:500; StemCell INC) for tracing hNSPCs and anti-human nestin (1:200; Millipore, Billerica, MA, USA), Tuj1 (1:500; Covance, Princeton, NJ, USA), GFAP (1:1000; DAKO, Glostrup, Denmark), Olig2 (1:500; Millipore) for identifying hNSPCs differentiation patterns. Mitogen-activated protein 2 (MAP2) (1:50; Cell Signaling), human NT3 (hNT, 1:50; Santa Cruz Biotechnology), human synaptophysin (hSYP,1:25; Sigma), and GAP43 (1:200; Abcam) were also used used. Secondary antibodies were conjugated to fluorescein, or Texas Red (Jackson ImmunoResearch Laboratories, Inc). Immunolabeled cells were observed with a fluorescence microscope (Olympus), and a Zeiss LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany) was used to acquire *Z*-stacks to assess merged images.

To investigate the grafted hNSPCs differentiation profile, 100 or more human nuclear antigen- or SC121-positive cells in the peri-infarct area were

scored for each marker: nestin as a marker of immature NSCs, GFAP as a marker of astrocytes, Tuj1 as a marker of immature and mature neurons, and Olig2 as markers of oligodendrocyte progenitors at 4 weeks post-transplantation.

Pixel intensity of human NT3 staining was analyzed using ImageJ in six brain slices encompassing the peri-infarct area (*n*=3 per group at 3 days post-transplantation).

6. Behavior testing

Neurological, cylinder, and grip strength tests were performed at 3, 5, and 7 weeks post-transplantation in the following four groups of mice with moderate infact volume (35~60%): Ad-Ngn2-hNSPCs (*n*=18), Ad-GFP-hNSPCs (*n*=13), vehicle (*n*=15), and intact (*n*=16). The neurological functions of all animals were evaluated using the following five reflexes. Each exam was scored as '0' if the response was normal and '1' if the animal showed an abnormal reflex during the following: (i) When the mouse was suspended by the tail above the ground (abnormal postures include rotating the body [torso twisting], and limb flexing [forelimb flexion]); (ii) When the mouse was placed on its side, it immediately turned over to rest in the normal position with all four feet on the ground (right reflection); (iii) When the dorsum of the paw contacted the edge of the table, the mouse immediately placed their paw on the surface (placing reaction); and (iv) the mouse was placed on a board, and if the board was suddenly bounced, the mouse spread its toes (toe spreading). In the cylinder test, all mice were located in the acrylic cylinder (diameter 10 cm and height 20 cm) and recorded for 5 minutes. The recorded video was used to evaluate how many times the mice placed their forelimbs on the cylinder walls and the left:right forelimb ratio was calculated. In the grip strength test, the grip strength meter was positioned horizontally, and

mice were allowed to grasp the triangular pull bar with their left paw only and pull backward in the horizontal plane. The test was repeated five consecutive times within a single session, and the highest value was recorded. The force applied to the bar at the moment the grasp was released was recorded as the peak tension (kg/g/N).

7. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The cryopreserved brain sections were fixed with 4% PFA in PBS for 1 hr at 4℃ and washed with PBS. The slides were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate buffer for 10 minutes at 4℃. After three washes, the sections were treated with TUNEL reaction mixture (Roche Applied Science) and incubated for 1 hr at 37℃. TUNEL-positive cells (six sections per animal, *n*=3 per group at 6-weeks post-transplantation) were microscopically analyzed in each scanned field (400 μm^2).

8. Infarct volume measurement

We estimated lesion size as a percentage of the whole brain by using the following formula: [(area of contralateral hemisphere) - (area of remaining ipsilateral hemisphere)/(area of contralateral hemisphere) × 100]. The area of both hemispheres was measured in eight serial coronal sections per brain (200 μm apart) stained with Hematoxylin Gill's formula (Vector, Burlingame, CA, USA) and Eosin-Y (Sigma, St. Louis, MO, USA) using Virtual microscope (Olympus BX51, Tokyo, JAPAN) and Image J (Broken symmetry software, NIH). The area of the infarct was averaged over all eight sections per animal. We analyzed *n*=10-12 per group at 11 weeks post-transplantation.

9. Bielschowsky's silver stain and neurite analysis

Brain sections were washed in distilled water, and the slides were placed in pre-warmed 20% silver nitrate solution for 15 minutes and washed in distilled water. Slides were placed back in the ammonium silver solution in a 37℃ oven for 10 minutes followed by 1 minute in 0.1% ammonium hydroxide solution. Lastly, slides were placed in 5% sodium thiosulfate solution for 1 minute and dehydrated through 95% ethyl alcohol, absolute alcohol, and xylene for mounting with resinous medium. Seven sections per selected lesion per animal were analyzed. The total length of neurite in each scanned field (1000 μm^2 for silver stain) was determined using the measuring tool on the NeuroJ software (Neurite Tracer Manual).

10. Axonal tracing studies

At 9 weeks after cell transplantation (postnatal day 11 weeks), biotinylated dextran amine (BDA; 0.5 μl 10% BDA in PBS, 10,000 d; Invitrogen) was stereotactically injected with a Hamilton infusion pump into the forelimb sensorimotor cortex of ischemic hemisphere (0.5 mm anterior, 2.25 mm lateral, 0.55 mm depth relative to bregma) in each group (n=5 per group). Animals were killed 2 weeks after BDA injection. The brain and spine were removed and perfused with 4% PFA in PBS. Six coronal brain sections from each animal were taken to analyze BDA staining. These sections were taken starting at bregma +1.2 mm, and every 540 μm. For corticospinal tract tracing, three axial sections were taken at 20-μm intervals from the spinal cord at the level of C5. BDA was detected using streptavidin-conjugated antibody (Streptoavidin-DTAF, 1:250; Jackson) and confocal images were systematically acquired with the same anatomical landmarks in all animals, including the ipsi-/contralesional cortex, ipsi-/contralateral corpus callosum,

internal capsule, ipsilateral striatum, thalamus, and dorsal funiculus at the level of C5. BDA-positive fibers were calculated using ImageJ software by determining the number of pixels above a set intensity threshold in each scanned field (400 μm^2 for BDA). In the spinal cord, all BDA-labeled axons were calculated in a region of interest measuring 0.2 mm^2. The accuracy of the ImageJ counts was confirmed by manual counting. The total number of BDA^+ cells at the injection site was determined and used to normalize the total number of BDA-labeled axon terminals for each experimental case.

11. RT-PCR and Real-time PCR Quantification

Total RNA was extracted from hNSPCs that were infected without (named hNSPCs) or with an Ngn2-encoding or GFP-encoding adenoviral vector with conditioned medium after the 3-day period following plating under differentiation conditions *in vitro* (*n*=3 per group). *In vivo*, RNA was extracted from the ipsilateral brain tissue of mice at 1 week post-transplantation in each group (*n*=6-8 per group) using TRI Reagent (Molecular Research Center, Inc, Cincinnati, OH, USA). First, we synthesized cDNA for RT-PCR. RNAs were quantified spectrophotometrically and 5 μl isolated RNA was reverse-transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in a 20-μl reaction volume. Primers were used to detect Ngn2 and NeuroD expression (Table 1). Human GAPDH mRNA was used as an internal standard. The RT-PCR products were separated on a 1.5% agarose gel that was stained with ethidium bromide.

Real-time PCR experiments were conducted with an ABI Prism 7700 (Applied Biosystems) and were detected with a SYBR premix Ex Taq kit (TAKARA). Human gene expression was determined using human-specific Taqman Gene Expression Assays (Applied Biosystems, Carlsbad, CA) according to the manufacturer's protocol (Table 1). Prior to result

quantification, optimization procedures were performed by running PCR to identify the melting temperatures of the primer dimers and the specific product. Serial dilutions of the GAPDH plasmid DNA (102–109 molecules) were used to generate standard curves. PCR conditions were 10 s at 94℃ followed by 50 cycles of 5 s at 94℃, 30 s at 58℃, and 30 s at 72℃. For analysis of the result, we calculated the Ct value (the fractional cycle number at which the fluorescence generated by the reporter dye exceeds a fixed level above baseline). The selected genes' signals were normalized against the relative quantity of GAPDH, and the relative amount of each cDNA was calculated by determining the $\triangle C_T$ value: $\triangle C_T = C_{T,\ target} - C_{T,\ GAPDH}$. The fold difference in the target gene relative to the endogenous GAPDH control gene was determined by the $2^{-\triangle(\triangle CT)}$ method: $\triangle(\triangle C_T) = C_{T,\ sample} - \triangle C_{T,\ control}$.

Table 1. Primer sequences used in semi-quantitative RT-PCR and/or Real-Time PCR.

Gene	Forward primer	Reverse primer
Mash1	GGCTCAACTTCAGCGGCTTT	CGGCCATGGAGTTCAAGTCG
NeuroD	TCCCTGTACACCCCTACTCC	CAGTGTCGCTGCAGGATAGT
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
Ngn2	CGCATCAAGAAGACCCGTAG	GTGAGTGCCCAGATGAGTTGTG
THBS1	CGGCCTCCCCTATGCTATCA	GGTAACTGAGTTCTGACAGTGAC
NT3	GTGGGGGAGACTTTGAATGA	TGAGGGAATTGAGCGAGTCT
SPARC	AGCACCCCATTGACGGGTA	GGTCACAGGTCTCGAAAAAGC
BDNF	TAACGGCGGCAGACAAAAAGA	TGCACTTGGTCTCGTAGAAGTAT
VEGFA	CGCAGCTACTGCCATCCAAT	GTGAGGTTTGATCCGCATAATCT
Slit1	GGCTGCGACTGAACCGAAA	GGATGGCGTTCTCACTCAAGT
THBS2	GACACGCTGGATCTCACCTAC	GAAGCTGTCTATGAGGTCGCA
TNFα	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC
GAPDH	CCATGAGAAGTATGACAACAGCC	GGGTGCTAAGCAGTTGGTG

12. Statistical analysis

Statistical significance was set as $P<0.05$ with two-tailed testing. All

statistical analyses were performed using SAS version 9.2 (SAS institute, Cary, NC) and SPSS version 19 (SPSS, Chicago, IL). Correlational analyses were performed with Pearson's and Spearman's correlations. For parametric analyses, differences among multiple means were calculated by one-way analysis of variance (ANOVA) and scheffe post hoc tests. For non-parametric analyses, differences were evaluated by Kruskal-Wallis tests.

III. RESULTS

1. Ngn2-expressing hNSPCs show increased differentiation into early neurons *in vitro*/*in vivo*

Real time PCR and RT-PCR analysis of cultured cells confirmed that the hNSPCs infected with adeno-Ngn2-IRES-eGFP generate Ngn2 and its downstream molecules under differentiation conditions. Expression level of Ngn 2 (54.7±4.8) was significantly higher in Ad-Ngn2-hNSPCs than other treated hNSPCs on Real time analysis. Ad-Ngn2-GFP-hNSPCs also express much greater Mash1 and NeuroD than Ad-GFP-hNSPCs on RT-PCR analysis. Mash1 and NeuroD are downstream molecules that are directly transduced through the transcriptional activator Ngn2 [12]. These results show that Ngn2-expressing hNSPCs underwent neurogenesis through a neuron-specific signaling pathway (Fig. 3A).

Under *in vitro* differentiation conditions, the two distinct hNSPCs groups showed different differentiation potential. The Ngn2-expressing hNSPCs had higher expression levels of Tuj1 (85.8±4.0 %) and O4 (11.7±8.1 %) compared to GFP-expressing hNSPCs and lower expression levels of nestin (37.8±4.6 %) and GFAP (15.7±2.5%). The GFP-expressing hNSPCs relatively exhibited lower expression of Tuj1 (30.9±8.5 %) and O4 (1.6±0.8 %), and higher level of GFAP (36.7±12.2 %) and nestin expression (85.3±3.4 %; Fig. 3B and C).

In vivo, the brains of these mice showed that large populations of grafted Ngn2 and EGF-expressing hNSPCs widely integrated in the penumbra (Fig. 4A). We also observed grafted donor cells expressed Tuj1, Olig2, nestin or GFAP in the penumbra region 6 weeks after transplantation in Ngn2 group and 4 weeks after transplantation in GFP group. These results demonstrate that transplanting Ngn2-expressing hNSPCs into the brains of HI-injured mice

survive and engraft in the peri-infarct area (Fig. 4B).

The differentiation profile 4 weeks following transplantation indicated that co-localization with the immature neuronal marker Tuj1 of grafted Ngn2-expressing hNSPCs (77±1.6%) was more prominent compared to the GFP-expressing hNSPCs (9±1.9%; Fig. 4A). The majority of Ad-Ngn2-hNSPCs were also positive for nestin (65±1.1%) and GFAP (60±1.2%) and a small proportion of cells expressed the Oligodendroglial marker Olig2 (10±1.6%). In the GFP group, a few hNSPCs were positive for Olig2 (7±2.8%), and a large proportion of cells expressed GFAP (77±1.9%) and nestin (82±1.3%). These findings imply that most Ngn2-expressing hNSPCs preferentially differentiated into early neurons, consistent with control group that transplanted hNSPCs remained in an immature state.

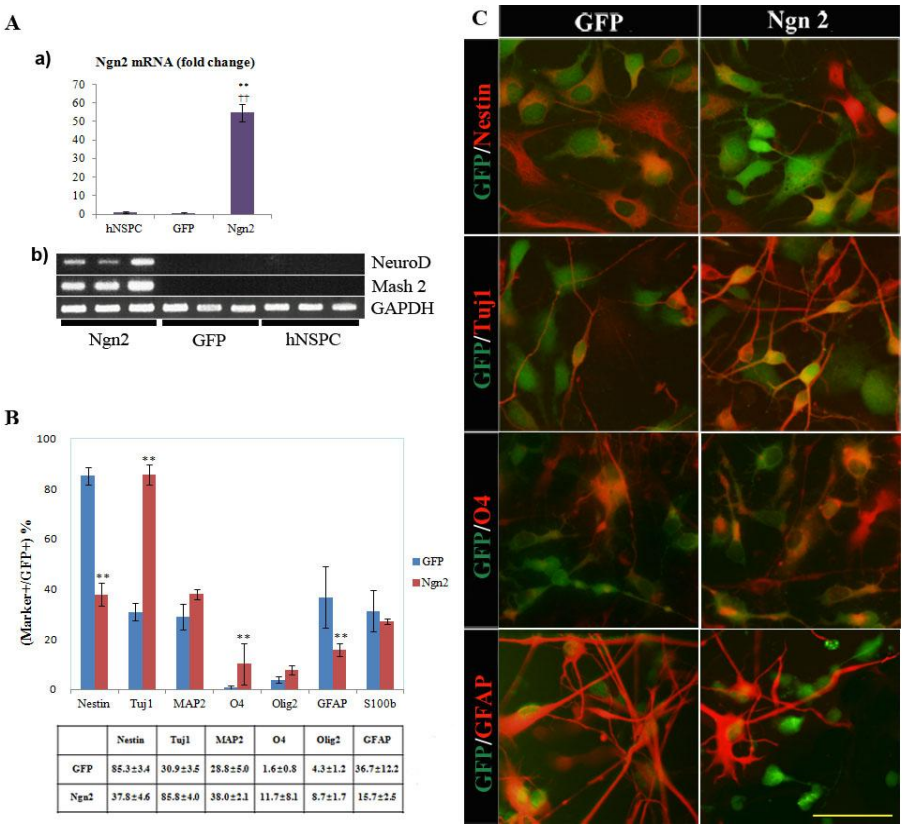


Figure 2. *In vitro* differentiation of hNSPCs.

(A) Relative expression of Ngn2, Mash1 and NeuroD mRNA in quantified by real time PCR using the $2^{-\triangle(\triangle CT)}$ method for relative quantification (a) and RT-PCR respectively (b). Data are presented as mean ± standard error of the mean (SEM). ***P*<0.01 compared with vehicle group; ††*P*<0.01 compared with GFP group.

(B) The percentage of each marker-positive/GFP-positive cells in each group was calculated and compared. Data are presented as mean ± SEM. ***P*<0.01 compared with GFP group (two sample t-test).

(C) Seeded cells were cultured for 7 days in PLL-coated 8-well chamber slides without mitogen, and subjected to immunofluorescence staining for

human-specific nestin (hNestin), Tuj1 (class III β-tubulin), O4, and GFAP (×400). Scale bar = 100 μm.

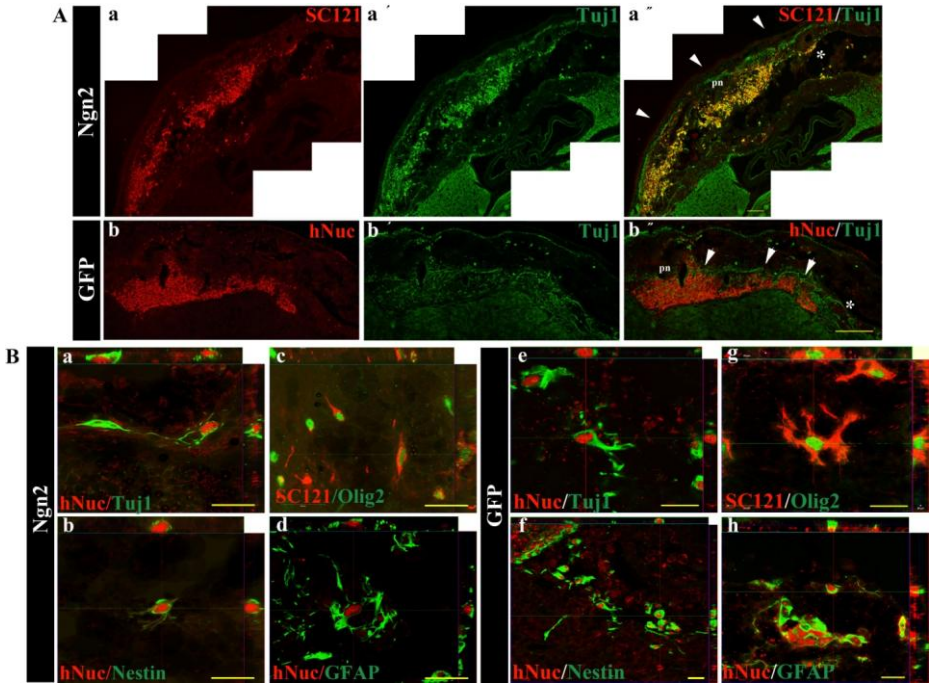


Figure 3. Integration, engraftment and differentiation of transplanted hNSPCs *in vivo*.

(A) Immunofluorescence staining reveals hNSPCs integration (arrow) into the penumbra at 4 weeks post-transplantation and most Ngn2-expressing hNSPCs preferentially differentiated into immature neurons. The majority of SC121-positive hNSPCs in Ngn2 group are co-labeled with antibodies against Tuj1 (a-a") whereas the majority of hNuc-positive hNSPCs in GFP group were not labeled with Tuj1 antibody (b-b"). (a) SC121-positive cells (red) and (a') Tuj1-positive cells (green) and (a") merged image (×200). (b) hNuc-positive cells (red) and (b') Tuj1-positive cells (green) and (b") merged image (×200). Scale bar = 100 μm, pn=penumbra; *=infact cavity

(B) Differentiation statuses of grafted hNSPCs were confirmed by confocal microscopy (*Z*-stacks) with immunohistochemistry using each marker. Ngn2-expressing hNSPCs expressed **(a)** Tuj1, **(b)** Nestin, **(c)** Olig2, and **(d)** GFAP in the penumbra region 6 weeks after transplantation. GFP-expressing

hNSPCs expressed **(e)** Tuj1, **(f)** Nestin, **(g)** Olig2, and **(h)** GFAP in the penumbra region 4 weeks after transplantation. Scale bars = 20 μm.

2. **Ngn2-expressing hNSPCs-transplanted mice exhibited functional recovery**

We evaluated neurological, sensory (or asymmetry of limb), and motor changes using a neurological exam and cylinder and grip strength tests. Compared to the vehicle group, the Ad-Ngn2-hNSPCs group showed significantly improved neurological and motor functions at 3, 5, and 7 weeks post-transplantation and improved sensory function at 7 weeks post-transplantation. Our results suggest that there were significant motor effects of Ad-Ngn2-hNSPCs transplantation, but sensory function improvement was more delayed. Compared to the Ad-GFP-hNSPCs group, the Ad-Ngn2-hNSPCs group showed significant improvements on the grip strength test at 3 weeks after transplantation. The Ad-GFP-hNSPCs group showed significant neurological improvement at 3 and 5 weeks post-transplantation and improved motor function at 5 weeks post-transplantation compared to the vehicle group. Except for these time points, there were no significant differences between the Ad-GFP-hNSPCs and vehicle groups; however, the Ad-GFP-hNSPCs group showed a tendency of increased functionality compared to the vehicle group in all of the behavioral tests, and functional recovery peaked 5 weeks after transplantation (Fig. 4).

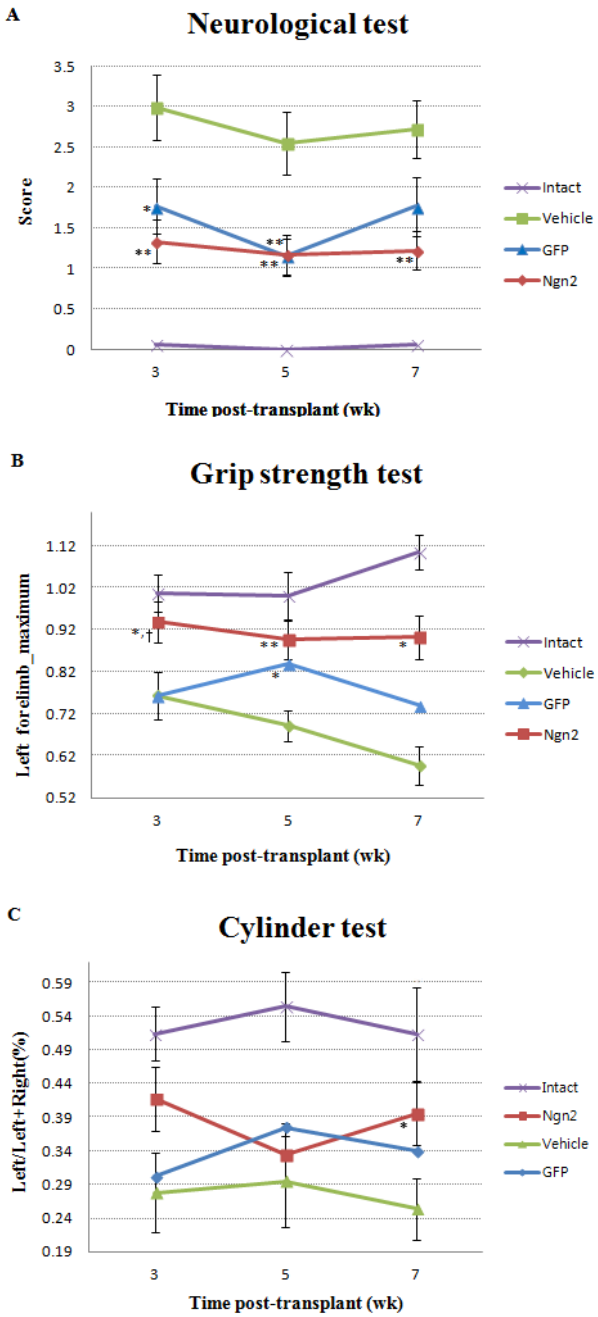


Figure 4. Behavioral tests

At 3, 5, and 7 weeks after transplantation, the Ad-Ngn2-hNSPCs group

showed a significant difference in **(A)** neurological by Kruskal-Wallis test, and **(B)** grip strength, **(C)** cylinder tests by one way ANOVA compared to the other groups. All data are presented as mean±SEM. **P*<0.05, ***P*<0.01 compared with vehicle group; †*P*<0.05, ††*P*<0.01 compared with GFP group. Ad-Ngn2-hNSPCs (*n*=18), Ad-GFP-hNSPCs (*n*=13), vehicle group (*n*=15), intact (*n*=16).

3. Ngn2-expressing human NSPCs increase host cell survival in HI brain

To investigate whether Ad-Ngn2-hNSPCs grafting prevented apoptosis in HI brain, we performed TUNEL staining to label apoptotic cells in the penumbra 6 weeks after transplantation. A significant decrease of TUNEL-positive cells was found in the Ad-Ngn2-hNSPCs group compared to H-H buffer-injected mice. Ad-GFP-hNSPCs-injected mice showed a tendency of fewer TUNEL-positive cells compared to H-H buffer-injected mice, but the difference was not significant (Fig.5A and B). Infarction volume was not significantly different among groups at 11 weeks post-transplant (52.8±10.5%, 54.6±9.6%, and 56.2±11.7%, respectively; Fig.5C). These data suggest that Ad-Ngn2-hNSPCs can protect host cells against the ischemic environment in the cellular level, but do not effectively reducing of infaction volume.

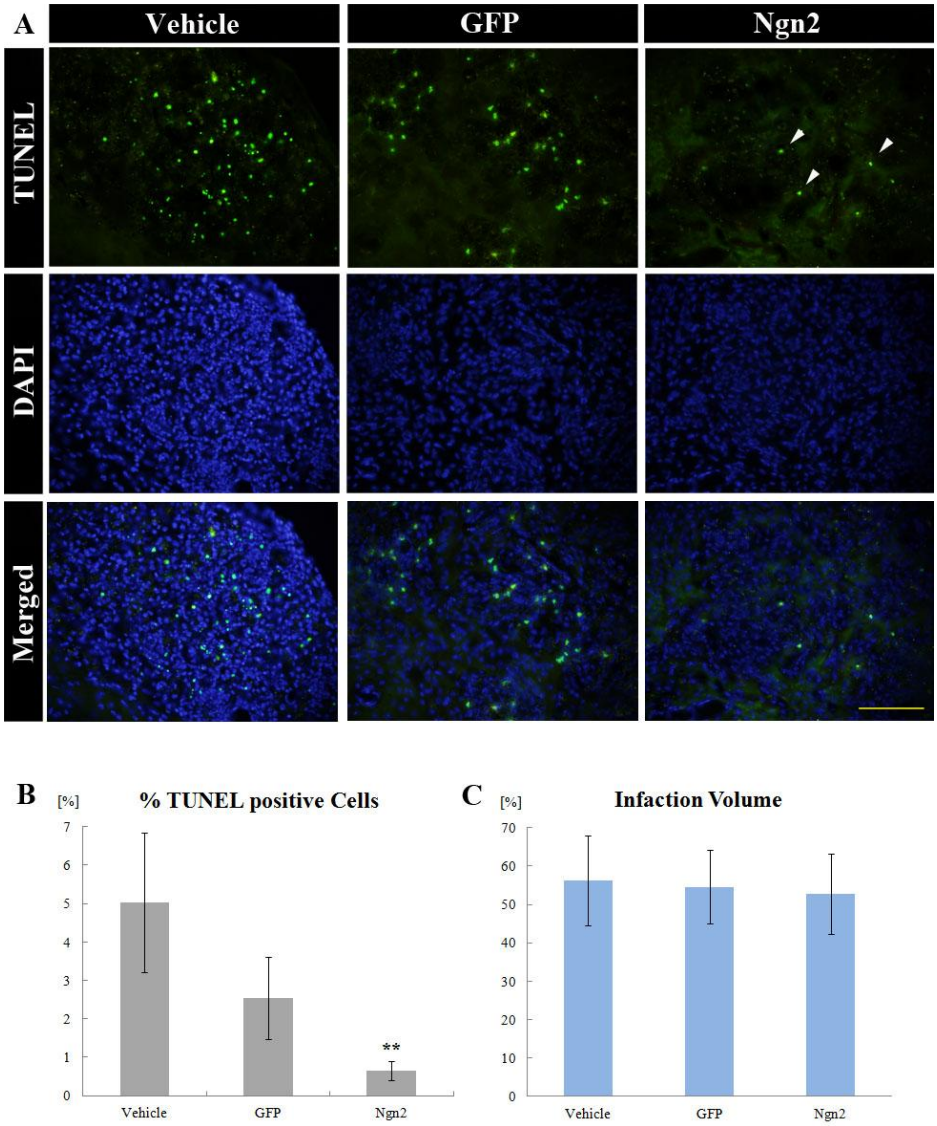


Figure 5. TUNEL assay and infarction volume analysis.

(A) TUNEL assay in Ad-Ngn2-hNSPCs, Ad-GFP-hNSPCs, and vehicle groups. TUNEL-positive cells are stained in green (arrow), and all nuclei are stained blue with DAPI. Scale bar = 100 μm.

(B) Ngn2-expressing hNSPCs-treated mice exhibited significantly decreased TUNEL-positive cell density in the penumbra at 6 weeks post-transplantation

(*n*=3 per group). Bars represent mean ± SEM. ***P*<0.01 compared with vehicle group (Kruskal-Wallis analysis of variance, Dunn).

(C) Infarction volume was not significantly different among groups at 11 weeks post-transplant (*n*=10-12 per group). Bars represent mean ± SEM (one way ANOVA).

4. **Ngn2-expressing human NSPCs enhance structural plasticity**

Silver-stained neurites from layer V cortical pyramidal neurons were analyzed in the ipsilateral penumbra and ipsi-/contralateral sensorimotor cortex at 9 weeks post-transplantation. Ad-Ngn2-hNSPCs treatment enhanced total neurite length in the ipsilateral penumbra and sensorimotor cortex compared with Ad-GFP-hNSPCs and vehicle controls (Fig. 6) but did not induce neuritic changes in the contralateral sensorimotor cortex. Ad-Ngn2-hNSPCs-treated mice also showed relatively well-aligned neurite arrays, whereas other groups showed non-oriented alignment in cortical layer V of ischemic mice. Changes in neurites were not significant between Ad-GFP-hNSPCs and vehicle treatment. MAP2 staining, which is visible in dendrites of pyramidal cells, showed that Ad-Ngn2-hNSPCs promote dendrite outgrowth similar to the result of silver staining (Fig. 7).

The anterograde axonal tracer BDA was injected into the ipsilateral forelimb sensorimotor cortex to visualize corticostriatal, corticothalamic, and corticospinal tract projections. BDA was analyzed at 11 weeks post-transplantation to assess the chronic phase of HI brain injury. Compared to the Ad-GFP-hNSPCs or vehicle groups, mice in Ad-Ngn2-hNSPCs group showed increased BDA-labeled fiber density in the ipsilesional cortex, corpus callosum, internal capsule, striatum, thalamus, and the contralateral dorsal funiculus of the spinal cord at the C5 level, which suggests enhanced axonal sprouting of the corticospinal tract. The Ad-GFP-hNSPCs group showed increased BDA-labeled fiber density compared to the vehicle group in the corpus callosum, internal capsule and thalamus, and there were no significant differences in the contralesional cortex. It suggests that proximity to the grafted hNSPCs is important to maintain enhanced axonal sprouting, and it may denote local effects of transplanted hNSPCs. Compared with vehicle-treated mice, Ad-GFP-hNSPCs-grafted mice exhibited significant differences

in the ipsilesional corpus callosum, internal capsule, thalamus, and spinal cord (Fig. 8). Moreover, grip strength test positively correlated with the BDA signal in the injured corticospinal tract (Spearman's correlation coefficient p=0.864;P<0.01) and silver stain (Spearman's correlation coefficient p=0.819; P<0.01). These data suggest that hNSPCs-induced changes of synaptic plasticity are important for functional recovery.

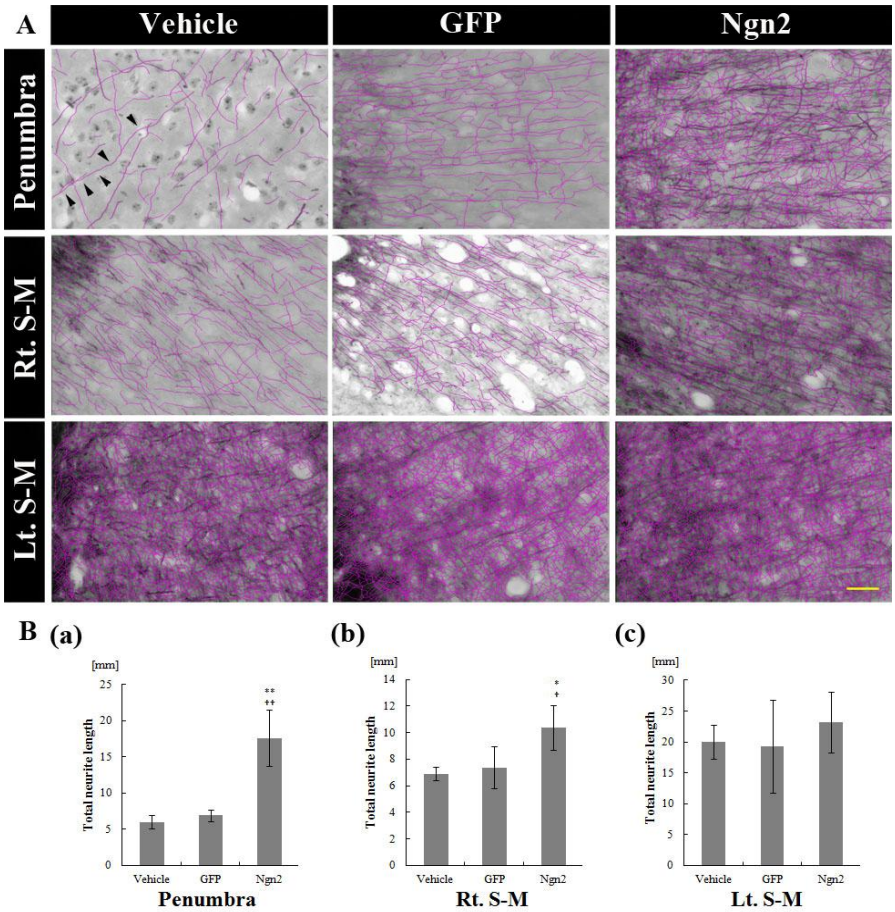


Figure 6. Ngn2-expressing hNSPCs enhance neurite outgrowth.

(A) Representative image of a Bielschowsky's silver stain of cortical neurons. Violet line (arrow) is generated using the NeuroJ software and it painted over the neurite. ×1000; Scale bar = 10 μm.

(B) Ngn2-expressing hNSPCs significantly enhance neuritic length in the ipsilateral (a) penumbra and (b) sensorimotor cortex compared with Ad-GFP-hNSPCs or vehicle-treated mice at 9 weeks post-transplantation. These effects were not observed in the (c) contralateral sensorimotor cortex (n=4-5 per group). All data are presented as mean±SEM. $^{**}P<0.01$ compared with vehicle group; $^{\dagger\dagger}P<0.01$ compared with GFP group by Kruskal-Wallis test. S-M= sensorimotor cortex.

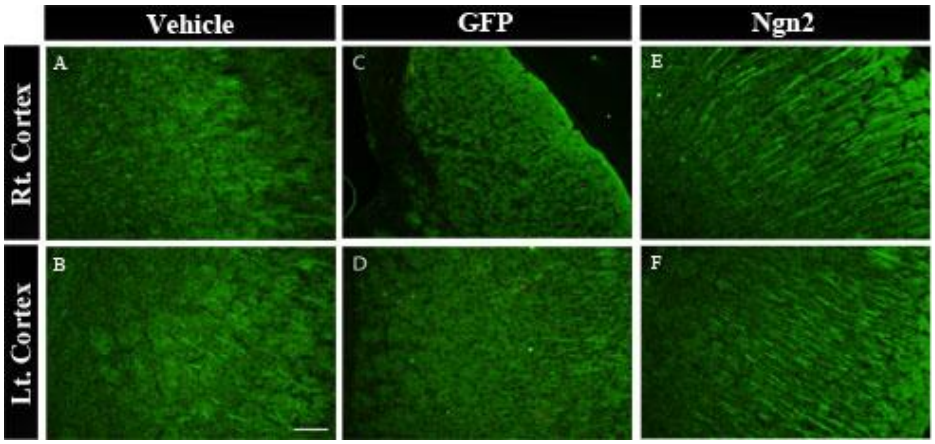
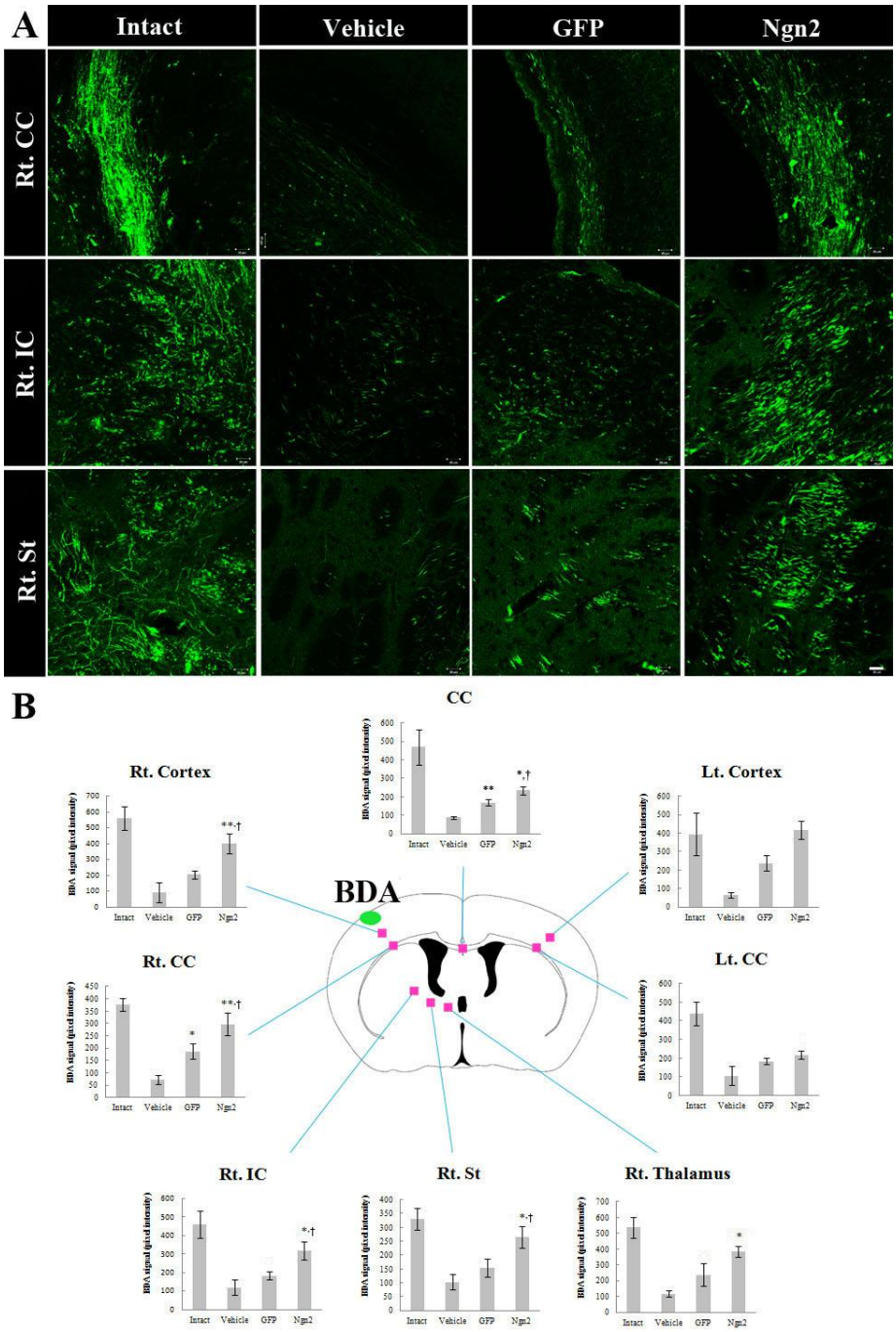


Figure 7. Representative coronal sections stained with anti-MAP2 FITC. These show dendrite length in cortical pyramidal neurons in the ipsilateral cortex (A, C, and E) and contralateral cortex (B, D, and F) among the three groups 9 weeks after cell transplantation. Vehicle (E and F), Ad-GFP-hNSPCs- (C and D) and Ad-Ngn2-hNSPCs-treated mice (A and B). ×200; scale bar = 100 μm.


A
Intact
Vehicle
GFP
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Rt. CC
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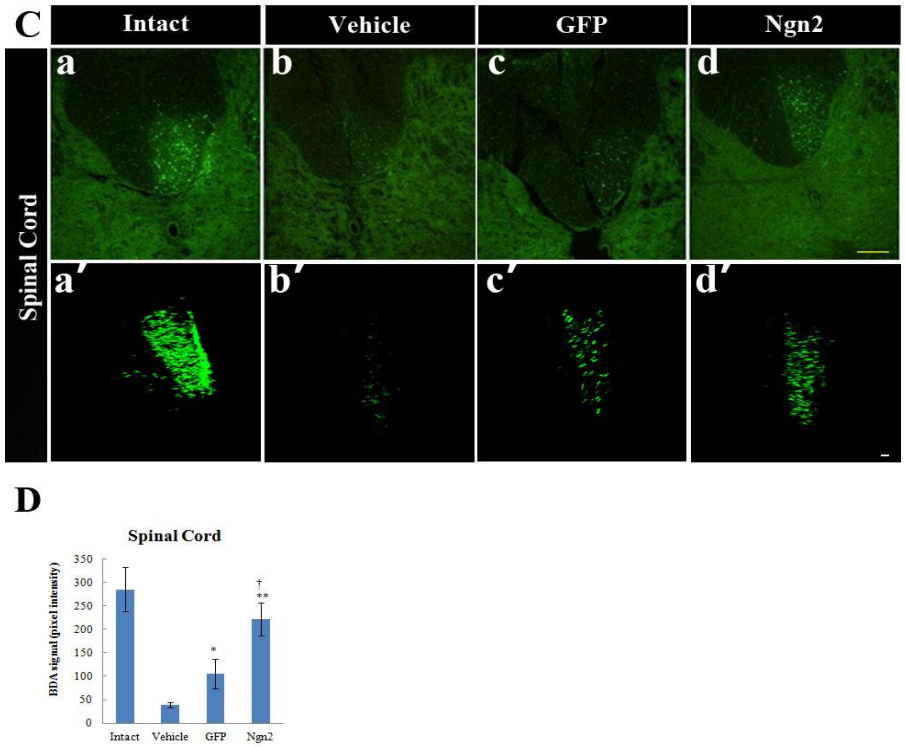

Figure 8. Ngn2-expressing hNSPCs enhance axonal sprouting in the HI brain. The anterograde axonal tracer BDA was injected into the forelimb sensorimotor cortex of the ipsilateral cerebral hemisphere to HI brain injury at 9 weeks post-transplantation, and the animals were sacrificed 2 weeks later. (A) Representative confocal images of BDA staining in the ipsilateral corpus callosum, internal capsule, and striatum in all three groups at 11 weeks post-transplantation. Scale bar = 20 μm.

(B) Compared to the Ad-GFP-hNSPCs or vehicle groups, mice in Ad-Ngn2-hNSPCs group showed increased BDA-labeled fiber density in the ipsilesional cortex, corpus callosum, internal capsule, striatum, and thalamus. Ad-GFP-hNSPCs-grafted mice also showed significant differences compared with vehicle-treated mice in the ipsilesional corpus callosum, internal capsule, and thalamus (n=5 per group). All data are presented as mean±SEM. *P<0.05,

**P<0.01 compared with vehicle group; $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01 compared with GFP group (Kruskal-Wallis test). Green oval = BDA injection site. CC = corpus callosum; IC = internal capsule; St = striatum.

(C) Representative microscopic (a-d) and confocal (a'-d') images of BDA staining in the contralateral dorsal funiculus of the spinal cord at C5 level, which suggests enhanced axonal sprouting of the corticospinal tract. ×200, Scale bars: (a) = 100 μm, (c) = 20 μm.

(D) Increased BDA-labeled fiber density in the spinal cord was observed in both hNSPCs groups, but the Ad-Ngn2-hNSPCs group exhibited greater BDA-labeled fiber density compared to the Ad-GFP-hNSPCs group (n=5 per group). Bar represent mean ± SEM. *P<0.05, **P<0.01 compared with vehicle group; $^{\dagger\dagger}P$<0.01 compared with GFP group (Kruskal-Wallis test).

5. **Identification of Ngn2-expressing hNSPCs-secreted factors *in vivo/vitro***

We compared mRNA levels among all three groups after 3 days of differentiation *in vitro* (*n*=3 per group). Target mRNAs known to be expressed in our hNSPCs based on our previous analysis were selected. We found that NT-3 (28.6±1.2) and thrombospondin-1 (TSP1, 4.22±1.1) were predominantly secreted by Ad-Ngn2-hNSPCs compared with naïve hNSPCs. The expression levels of thrombospondin 2 (TSP2) and SPARC were significantly lower in Ad-Ngn2-hNSPCs than naïve hNSPCs, which coincides with results of another study that NSPCs differentiation led to a marked decrease in trophic factor expression (Fig. 9A).

To determine whether these factors were secreted by hNSPCs in the HI brain, we conducted quantitative PCR analysis of the ipsilesional hemisphere at 1 week post-transplantation using primers specifically amplified for the human versions of NT3 and TSP1 (*n*=6-8 per group). In the Ad-Ngn2-hNSPCs-transplanted brain, NT3 (6.3±1.6) was more highly expressed than in the Ad-GFP-hNSPCs-transplanted brain. TSP1 expression was not significantly different between the two groups, *in vivo* (Fig. 9B).

To evaluate NT3 expression levels *in vivo*, immunohistochemical staining for NT3 was conducted within the penumbra of all three groups at 3 days post-transplantation (*n*=3 per group). We performed double immunolabeling for NT3 and human stem cell marker (SC121 or anti-BrdU) and found that Both Ad-Ngn2-hNSPCs and Ad-GFP-hNSPCs expressed NT3 in the their cytoplasm (Fig. 10A and B). We also observed that NT3 density was increased in the penumbra of Ad-Ngn2-hNSPCs-transplanted mice compared to the other groups (Fig. 10C) and it means that grafted Ngn2-expressing hNSPCs more secreted NT3 than GFP-expressing hNSPCs in HI brain . NT3 density also positively correlated with TUNEL staining results (Spearman's correlation coefficient *p*=0.800; *P*=0.01) and silver staining (Spearman's

correlation coefficient p=0.742; P<0.01). These data suggest that NT3 may have potential roles in neuroprotection and synaptic plasticity in the HI brain.

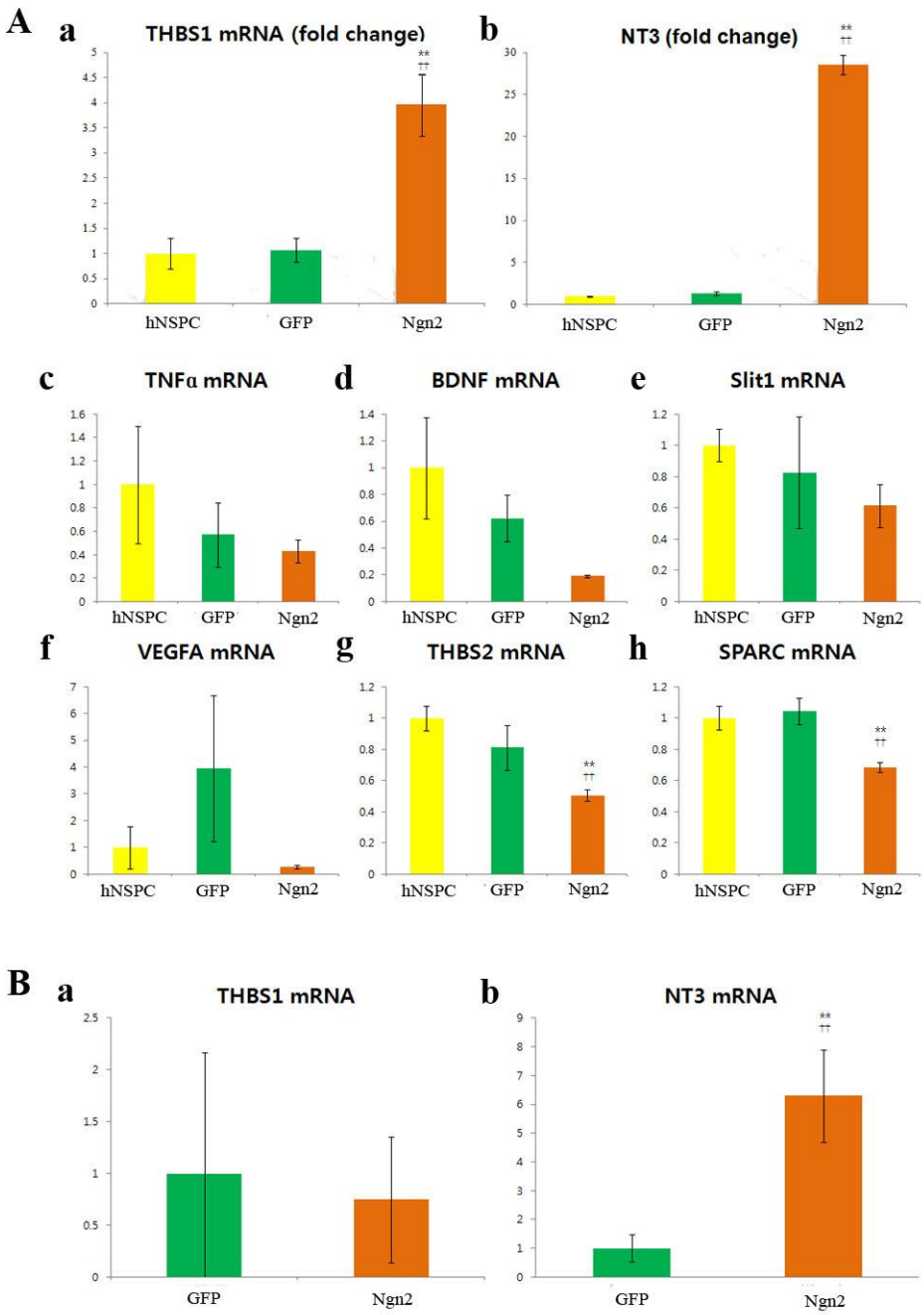

Figure 9. Trophic expression of hNSPCs *in vivo/vitro*.

(A) Real time analysis shows that Ad-Ngn2-hNSPCs secrete 4-times more

TSP1 (a) and 28-times more NT3 (b) than Ad-GFP-hNSPCs, whereas other factors are more secreted by Ad-GFP-hNSPCs than Ad-Ngn2 hNSPCs (c-h) *in vitro* (*n*=3 per group).

(B) Expression of control hNSPCs and GFP- and Ngn2-expressing hNSPCs *in vivo*. In Ad-Ngn2-hNSPCs-transplanted brain, NT3 was expressed 6 times more than in Ad-GFP-hNSPCs-transplanted brain (b) (*n*=6-8 per group). All data are presented as mean±SEM. ***P*<0.01 compared with control hNSPCs group; $^{\dagger\dagger}P$<0.01 compared with GFP group.

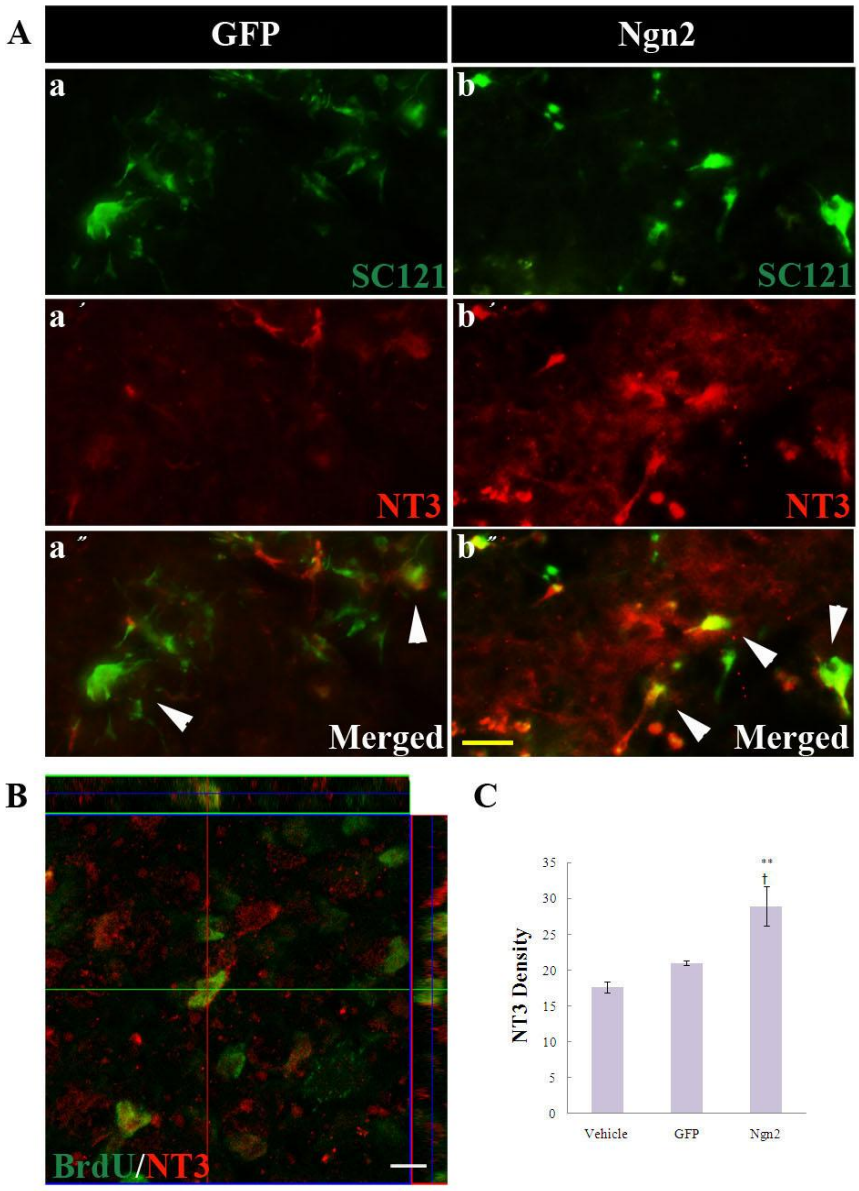

Figure 10. NT3 is secreted by Ngn2- and GFP-expressing hNSPCs.

In vivo microscopic (A) and confocal images (B) confirmed co-expression of NT3 (red) and SC121 (green) in grafted hNSPCs in the penumbra of HI 3 days after Ad-Ngn2-hNSPC (A,C) or Ad-eGFP-hNSPC (B) transplantation.

Double labeling, seen as yellow/orange fluorescence is indicated by arrows. ×400, Scale bars; A = 20 μm, B = 10 μm.

(C) Quantification of NT3 immunofluorescence density demonstrates a significant increase in the Ad-Ngn2-hNSPC-grafted group compared to vehicle and Ad-eGFP-hNSPC-grafted groups (n=3 per group). Bar represent mean ± SEM. $^{**}P<0.01$ compared with control hNSPCs group; $^{\dagger\dagger}P<0.01$ compared with GFP group (Kruskal-Wallis test).

6. **Ngn2-expressing hNSPCs expressed synaptophysin and axonal growth cone protein.**

We performed synaptophysin immunohistochemistry using confocal microscopy. At 6 weeks after transplantation, the engrafting Ad-Ngn2-hNSPCs were co-immunolabeled with anti-SC121 and anti-hSYP antibodies, which label the presynaptic terminals of functional synapses (Fig. 11A). These results suggest that transplanted Ad-Ngn2-hNSPCs may synapse with other neurons.

We also observed co-labeling of SC121 and GAP 43 on engrafting Ad-Ngn2-hNSPCs 6 weeks after transplantation (Fig.11B). It suggests that transplanted Ad-Ngn2-hNSPCs may have a growing axon seeking a synaptic target, although GAP-43 is not purely a marker of axonal growth cone protein and is also expressed on non-neuronal cells, such as astrocytes and oligodendrocytes. These findings were not observed in transplanted Ad-GFP-hNSPCs.

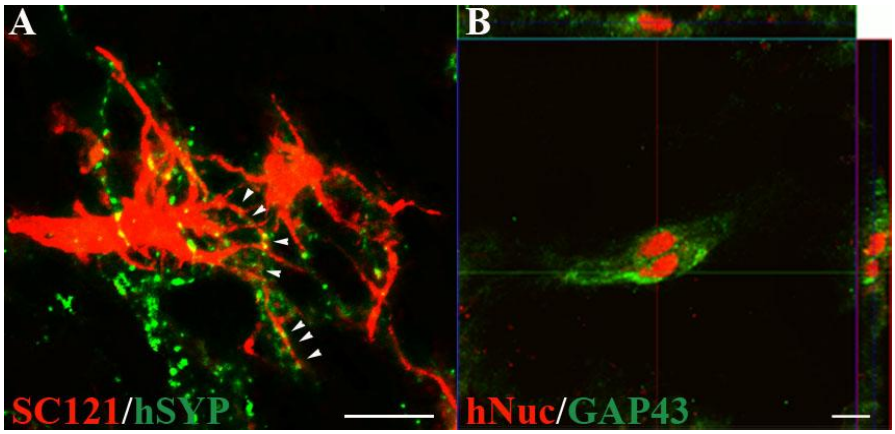

Figure 11. Ngn2-expressing hNSPCs expressed synaptophysin and axonal growth cone protein.

(A) Confocal image of engrafted Ad-Ngn2-hNSPCs 6 weeks after transplantation co-labeled with anti-SC121 and anti-hSYP antibodies (arrow). It suggests that transplanted Ad-Ngn2-hNSPCs may synapse with other neurons.

(B) Anti-SC121 and anti-GAP 43 antibodies merged on engrafted Ad-Ngn2-hNSPCs 6 weeks after transplantation. It suggests that transplanted Ad-Ngn2-hNSPCs may develop growth cones to guide them to their synaptic targets. Scale bars; A, B = 10 μm.

IV. **DISCUSSION**

We showed that hNSPCs infected with an Ngn2-encoding adenoviral vector differentiated into neurons *in vitro/in vivo*, and transplantation of these cells into the infarct cavity 1 week after ischemic brain injury significantly improved functional recovery and enhanced dendritic and axonal plasticity in the penumbra and ipsilesional sensorimotor cortex compared to the control group. We suggest that these effects may be related to increased NT-3 secretion from hNSPCs. Grafted donor cells also expressed synaptophysin *in vivo*, and this suggests the possibility that Ngn2-expressing hNSPCs could synapse with endogenous mouse neurons.

Following exposure to HI in the perinatal period, the brain suffer three phases of deterioration: the latent phase, which is associated with immediate cell death; the secondary phase, which is related to secondary cell death due to mitochondrial failure and cytotoxic edema; and the chronic phase, which is correlated with additional loss of neurons and glial cells due to chronic loss of trophic factors and synaptic input from neighboring cells[9]. Because of the complex etiology of HI, stem cell-based therapies have been tried at multiple time points with different expectations of the effects. We transplanted hNSPCs into the ischemic cavity 1 week (chronic phase) after HI and did not observe an effect on the extent of brain damage, but host cellular survival was improved. These findings are in line with the suggestion that early injection of hNSPCs is associated with immune suppression and reduction of infarction volume. Alternatively, we were also able to capture the effect of hNSPCs related to trophic factors and synaptic contact with neighboring cells.

We showed that grafted hNSPCs in the brains of HI-injured mice migrated to the penumbra boundary and integrated, as mentioned in a previous report. In this study, >70% of Ngn2-expressing hNSPCs stained with Tuj1 and nestin *in vivo*, which implies that they were differentiating into early neurons,

compared to >70% of GFP-expressing hNSPCs expressing the astrocyte marker GFAP and nestin. This is in obvious contrast with other previous studies in which transplanted hNSPCs remained in an immature state or differentiated into astrocyte both *in vitro* and *in vivo*[26,27,52,53]. In the GFP group, functional recovery peaked at 5 weeks post-transplantation and declined after that point. Andres et al.[26] produced similar results and noted that grafted hNSPCs exhibited robust survival and migration towards the lesion at 5 weeks post-transplantation. It is supposed that cell survival and differentiation affect functional recovery. We carefully suggest that Ngn2-expression induced hNSPCs to integrate earlier with differentiation into early neuron and survive longer than GFP-expressing hNSPCs; therefore, it hastened the time when functional recovery appeared and lasted longer in the Ngn2 group. We confirmed that Ngn2-expressing hNSPCs survived at 6-weeks post-transplantation and functional effect of Ngn2-expressing hNSPCs maintained at 7-weeks post-transplantation. We think that reason for the delay of sensory function recovery maybe is the slow pace of axonal growth and re-innervation of peripheral sensory neurons into the sensorimotor cortex.

TUNEL staining demonstrated that more neurons survived, and silver staining and BDA showed increased synaptic plasticity. Importantly, greater structural plasticity correlated with hNSPCs-enhanced functional recovery, especially the grip strength test, which measures forelimb muscle power. It could be that grip strength test is closely related with the increased BDA signal, which would imply axonal spouting in the CST[54]. Andres et al.[26] showed that hNSPCs enhanced BDA signal in corticocortical, corticostriatal, and corticothalamic pathways after stroke, and this therapeutic effect of hNSPCs was confirmed in our study. Additionally, we found that mice in the Ad-Ngn2-hNSPCs group showed enhanced neurite length in the peri-infarct area and increased BDA-labeled fiber density in the ipsilateral hemisphere and the spinal cord compared to the Ad-GFP-hNSPCs group. These

phenomena were observed late, 11 weeks after HI injury. BDA, an anterograde tracer, is specifically taken up by neuronal cell bodies located at the injection site (forelimb sensorimotor cortex) and anterogradely fill up the cell bodies in the CNS through the axonal transport system. Increased BDA axonal labeling reflects increased numbers of neurons in the corticospinal tract and greater axonal transport and dendritic arborization of neuron that uptake BDA. In our study, spinal BDA signal density negatively correlated with TUNEL-positive cells (Spearman's correlation coefficient p=-0.733; P=0.025) and positively correlated with silver staining in the ipsilateral forelimb sensorimotor cortex (Pearson's correlation coefficient p=0.588; P=0.035). Our BDA data also suggests that transplantation of Ngn2-expressing human NSPCs enhanced host cell survival and promoted neurite arborization during recovery from cerebral ischemia.

Changes in structural plasticity are observed more dominantly in the ipsilesional area that was treated with an injection of hNSPCs, which indirectly suggests that hNSPCs have local effects by secreting factors. We identified grafted hNSPCs-secreted factors, NT3 and TSP1, and the Ad-Ngn2-hNSPCs group had more over 30- fold and 3-fold levels of NT3 and TSP1 mRNA, respectively, compared to Ad-GFP-hNSPCs *in vitro* and more 5-fold NT3, *in vivo*. NT3 immunoreactivity correlated with neurite length (silver staining) and cell survival (TUNEL staining). These results suggest that transplanted hNSPCs secreted factors that may protect against CNS damage. Recently, many reports have suggested that NSPCs constitutively secrete neurotrophic factors and exert positive effects on structural plasticity[8]. There is some evidence that Ngn2-expressing hNSPCs induce NT3 expression. The mechanism has not been fully elucidated, but we observed that NeuroD, downstream of Ngn2, activates tropomyosin-related kinase B transcription[43]. However, in our study, neural induction of hNSPCs led to a marked decrease in the expression of trophic factors except NT3 and TSP1 compared to Ad-

GFP-hNSPCs *in vitro*. It is possible that more extensive stem cell differentiation could downregulate the expression of other growth factors, and genetic overexpression of one factor had a reciprocal effect on the expression of another factor as mentioned by Lu et al[55]. Nevertheless, the effects of NT3 expression were considered more important than other factors in the aspect of enhancement of neural plasticity after HI injury.

The known functions of NT3 and TSP1 correspond with the phenotype of Ad-Ngn2-hNSPCs-transplanted mice described here, e.g., improvement of cell survival, differentiation, and synaptic plasticity. It strongly implies that transplanted Ad-Ngn2-hNSPCs elicit their effects through direct secretion of relevant factors. NT3 has been shown to have three functions: cell survival, differentiation, and synaptic plasticity, which are mediated via nuclear factor-kappa B through p75 neurotrophin receptor (p75NTR) and MAP kinases pathway and phospholipase C-gamma 1 signaling through Trk receptors[56]. TSPs are a family of large oligomeric multidomain glycoproteins that participate in a variety of biological functions as part of the extracellular matrix. TSPs mediate cell–cell and cell–matrix interactions, and it was recently shown that TSPs promote the formation of new synapses in the CNS. Signaling that occurs downstream of TSP is currently unknown; however, it seems to be related to rearrangement of the actin cytoskeleton by the Rho GTPases pathway, which plays a pivotal role in neurite outgrowth and synaptic plasticity, including dendritic spine remodeling[57]. The Rho GTPases signaling pathway has a cyclic mode of action between extension and retraction that allows axonal growth cones, which are specialized structures at the tips of growing axons seeking their synaptic target, to advance[58]. In our study, transplanted Ad-Ngn2-hNSPCs expressed the axonal growth cone protein GAP-43, which indirectly proves TSP1's action *in vivo*, although GAP-43 labels structures other than regenerating axons.

We previously reported that when NSPCs transduced with a retrovirus

encoding NT-3 were transplanted into brains of HI mice, the 20% of donor cells differentiated into neurons in infarct region[59], and Lu et al.[55] demonstrated that NSPCs genetically modified to produce NT-3 significantly expanded NSPCs effects on host axons. However, we believe that NT3 secretion of Ngn2-expressing hNSPCs is more physiological because it may be transported into host neurons through synapses or via autocrine or paracrine signaling. If grafted hNSPCs secrete very large amounts of NT3, it would rather be toxic in HI brain. At 6 weeks after transplantation, engrafted Ad-Ngn2-hNSPCs expressed the presynaptic marker synaptophysin, suggesting the possibility that Ad-Ngn2-hNSPCs may form synapses with endogenous mice neurons. If hNSPCs secrete NT-3 into synapses between Ad-Ngn2-hNSPCs and mice neurons, it could influence survival and differentiation of the postsynaptic neuron. This is known as the neurotrophic factor hypothesis, which posits that postsynaptic neuron may uptake NT3 via endocytosis through Trk-p75NTR receptors and anterogradely transport it to the axonal terminal with newly synthesized NT3 to be transmitted to other neuron. NT3 is continuously recycled within neural circuits without degradation and can enhance the survival of postsynaptic neuron and afferents by providing trophic support. It also can induce morphological changes in postsynaptic neurons in aspects of differentiation and synaptic plasticity, including upregulation of neurotransmitter, synaptic molecules, and vesicle density[60].

Understanding what happens to implanted hNSPCs in the post-ischemic brain will be important for the successful clinical translation of cell transplantation strategies[8]. Our data imply that Ad-Ngn2-hNSPCs induced changes in survival, neurite length, and axonal sprouting would be useful predictors of cell efficacy. This phenomenon may be mediated through factors secreted by Ad-Ngn2-hNSPCs that exert obvious bystander effects. However, if Ad-Ngn2-hNSPCs form synapses with mouse neuron in the penumbra, it

means that grafted hNSPCs could differentiate into neurons and continuously transfer secreted NT3 into host neuron via synaptic transfer. In our study, it remains to be studied whether Ad-Ngn2-hNSPCs have an immunomodulatory effect and whether fully synapse with electrical firing between Ad-Ngn2-hNSPCs and host neuron exerts.

V. CONCLUSION

Ngn2 induce neurogenesis of hNSPCs via Notch/Delta signaling. We demonstrated that Ngn2-expressing hNSPCs exhibit greater differentiation into early neurons *in vitro* and *in vivo*. Ngn2-expressing hNSPCs enhance survival, neurite outgrowth, and axonal sprouting of host neurons after HI brain injury. Furthermore, we identified increases in Ngn2-expressing hNSPCs-secreted factors, NT3 *in vitro* and *in vivo*. We also showed that Ngn2-expressing hNSPCs expressed synaptophysin and axonal growth cone protein. Based on these lines of evidence, we carefully hypothesize that Ngn2-expressing hNSPCs may synapse with endogenous mouse cerebral neurons, and multistep transfer of NT3 through these synapses could induce plastic changes among neural networks.

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ABSTRACT(IN KOREAN)

신생아 저산소성-허혈성 뇌손상 동물모델에서 Neurogenin 2 발현하는 인간 신경줄기세포 유래 분화 신경세포의 뇌 이식

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구 교 연

신생아 저산소성-허혈성 뇌손상(neonatal hypoxic-ischemic brain injury)은 주산기 가사 (perinatal asphyxia)에 의해서 신생아에서 발병하는 대표적 중증 신경계질환으로 만삭아 1000명 출생아 중 2명에서 발병하고, 약 60%에서 신경발달 장애소견을 보인다. 현재까지의 연구에 의하면 손상된 중추신경계의 재생은 거의 불가능하거나 극히 제한적이기 때문에 대부분의 치료는 합병증을 예방하거나 비정상적인 운동 패턴과 경직을 감소시키는데 중점을 두어왔다. 신경줄기세포는 자가갱신(self-renew)하고, 신경원세포 및 신경교세포로 분화할 수 있는 분화의 다능성(multipotency)을 보이는 미성숙 신경세포이다. 시험관 내에서 증식되어 생체 내 이식이 가능한 신경줄기세포는 숙주 신경계에 이주, 생착, 통합되어 치료적으로 유용한 물질을 분비하고,세포구조학적 및 기능적으로도 적절한 신경세포로 분화 가능하기에 난치성

신경계 질환의 치료에 있어 잠재력을 가지고 있다.

Neurogenin2(Ngn2)는 신경능선세포(neural crest cell)로부터 분리된 basic helix-loop-helix 전사인자로서 성체 및 태아 신경계에서 신경줄기세포를 신경원세포로 분화시키는 핵심적인 인자이다. 본 연구에서는 Ngn2을 이용하여 인간 신경줄기세포를 신경원세포로 분화를 유도한 다음, 신생아 저산소성 허혈성 뇌손상 동물모델에 이식하여 세포치료 가능성을 연구하고자 하였다. 결과 Ngn2 발현하는 인간 신경줄기세포 유래 분화 신경세포는 뇌손상 부위에 이식된 후 뇌손상 부위와 그 주변 부위로 이주하면서 생착함을 보였고, 주로 초기 신경원세포로 분화함이 관찰되었다. Ngn2 발현하는 인간 신경줄기세포 유래 분화 신경세포들이 이식된 실험군은 대조군에 비해 신경행동학적 검사에서 전반적인 신경학적 기능이 유의하게 증가하였고, cylinder test 및 grip strength test상 손상된 쪽의 앞발의 운동기능이 유의하게 개선됨을 보였다. 또한 대조동물에 비해 실험동물의 뇌 세포 사멸이 유의하게 감소하였고,숙주의 신경세포 돌기 길이는 길어지고, 축삭재생과 곁발아가 증가하여 시냅스 가소성이 증가되었다. 공여세포는 인간 신경줄기 세포에 비해 생체 내와 생체 외에서 모두 Neurotrophin 3(NT3)의 분비가 유의하게 증가되었으며, 이는 숙주의 뇌세포사멸의 감소와 시냅스 가소성 증가와 밀접한 연관성을 보였다. 즉 공여세포가 분비한 NT3의 신경 보호 및 신경 영양 작용으로 인하여 저산소성 허혈성 손상을 받은 뇌의 신경 재생 능력이 향상되어 숙주동물에서 신경행동학적 기능이 개선됨을 보였다. 또한 생체내에서 Ngn2을 발현하는 인간 신경줄기세포 유래 분화 신경세포는 시냅스에 존재하는

당단백질인 synaptophysin을 발현하고, 정확한 시냅스 형성을 이끄는, 성장 중인 신경돌기의 첨단에 형성되는 성장원뿔(growth cone)을 발현하고 있어, 투여한 Ngn2 발현 인간 신경줄기세포 유래 분화 신경세포가 숙주의 신경세포와 시냅스를 형성하여 기능적인 생착을 하고 손상된 신경원세포를 대체할 수 있는 가능성을 제시하였다.

따라서 본 연구는 현재까지 특별한 치료법이 없는 신생아 저산소성 허혈성 뇌손상에서 Ngn2 발현 인간 신경줄기세포 유래 분화 신경세포의 뇌 이식이 향후 유용한 세포치료법으로 사용될 수 있음을 제시하였다.

핵심 되는 말 : 신생아 저산소성 허혈성 뇌손상, 인간 신경줄기세포, 분화 신경세포, Neurogenin 2, 세포이식