

Figure 1. Characterization of hiPS-It-NES cells. (A): Expansion culture. hiPS-It-NES cells can be expanded continuously in the presence of both epidermal growth factor and fibroblast growth factor. hiPS-It-NES cells were uniformly immunopositive for Sox2 (green) and Nestin (red). Hoechst (blue) shows nuclear staining. (B): The pCII-EFp-luciferase-IRES2-GFP construct. (C): Flow cytometric analysis of GFP-positive cells in hiPS-It-NES cells. Uninfected cells (left) and cells infected with lentiviruses expressing luciferase and GFP (middle) were subjected to flow cytometric analysis. GFP-positive cells in the infected cell population were sorted on the basis of GFP fluorescence and reanalyzed for GFP expression (right). (D): After 4 weeks in differentiation conditions, infected hiPS-It-NES cells differentiated into Tuj1-positive neurons (red) and GFAP-positive astrocytes (blue). (E): Quantification of neural marker-positive cells after 4 weeks' differentiation. Lentiviral infection did not influence the differentiation tendency. Data are means \pm SD ($n = 3$). Scale bars = 100 μ m. Abbreviations: EFp, elongation factor promoter; GFP, green fluorescent protein; GFAP, glial fibrillary acidic protein; hiPS-It-NES, human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem; and IRES2, internal ribosomal entry site 2.

Sigma), rabbit anti- β -tubulin isotype III (Tuj1; 1:1,000, Covance, Madison, WI, <http://www.covance.com>), rabbit anti-GFP (1:1,000, Molecular Probes, Carlsbad, CA, <http://probes.invitrogen.com>), chick anti-GFP (1:500, Aves Labs, Tigard, OR, <http://www.aveslab.com>), rabbit anti-glial fibrillary acidic protein (GFAP, 1:2,000, DAKO, Carpinteria, CA, <http://www.dako.com>), mouse anti-human-specific GFAP (hGFAP, 1:1,000, STEM123, StemCells Science, Newark, CA, <http://www.stemcellscience.com>), mouse anti-MAP2ab (1:500, Sigma), chick anti-myelin basic protein (MBP) (1:200, Aves Labs), mouse anti-adenomatous polyposis coli CC-1 (APC, 1:200, Calbiochem-Merck, Darmstadt, Germany, <http://www.merckgroup.com>), mouse anti-synaptophysin (1:200, Chemicon), mouse anti-Bassoon (Bsn, 1:400, Stressgen-Enzo Life Sciences, Plymouth Meeting, PA, <http://www.enzo-life.com>),

www.StemCells.com

fesciences.com), mouse anti-human-specific synaptophysin (hSyn, 1:200, Chemicon), and mouse anti-NeuN (1:500, Millipore).

Immunocytochemistry

Immunocytochemistry experiments were performed as described previously [20]. Cells were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) in PBS for 10 minutes, and then washed with PBS and incubated in blocking solution (PBS containing 10% FBS and 0.1% Triton X-100). Subsequently, cells were incubated overnight at 4°C with the primary antibodies described above. After three washes in PBS, cells were incubated for 1 hour with the following secondary antibodies: FITC-conjugated donkey anti-chick/rabbit, Cy3-

conjugated donkey anti-chick/mouse/rabbit, Cy5-conjugated donkey anti-mouse/rabbit (1:500, Jackson ImmunoResearch, West Grove, PA, <http://www.jacksonimmuno.com>). After three washes with PBS, nuclei were stained with Hoechst (bisbenzimidazole H33258 fluorochrome trihydrochloride, Calbiochem-Merck). Samples were washed with PBS and mounted on glass slides with Immu-Mount (Thermo Scientific, Waltham, MA, <http://www.thermoscientific.com>). The cells were examined using a fluorescence microscope (Axiovert 200M, Zeiss, Jena, Germany, <http://www.zeiss.com>) equipped with the appropriate epifluorescence filters.

Immunohistochemistry

Animals were anesthetized and perfused with PBS followed by 4% PFA in 0.1 M PBS, pH 7.4. The spinal cords were dissected and postfixed overnight in the same fixative at 4°C. For cryosectioning, fixed tissues were cryoprotected in 10% sucrose in PBS overnight at 4°C, then in 20% sucrose in PBS overnight at 4°C, and embedded in optimal cutting temperature (OCT) compound (Tissue Tek, Sakura Finetek, Tokyo, Japan, <http://www.sakura-finetek.com>). Cryostat sections (20 μ m) were cut and affixed to matsunami adhesive slide (MAS)-coated glass slides (Matsunami Glass, Osaka, Japan, <http://www.matsunami-glass.co.jp>). The sections were permeabilized in PBS-T (PBS containing 0.1% Triton X-100) for 10 minutes and blocked with 10% FBS in PBS-T for 1 hour, and then incubated overnight at 4°C with the primary antibodies described above. After three washes with PBS, they were incubated in a mixture of the secondary antibodies described above for 1 hour. After a final rinse with PBS, nuclei were stained using Hoechst. Sections were mounted and examined under a fluorescence microscope (Axiovert 200M, Zeiss) and a scanning laser confocal imaging system (LSM 710, Zeiss).

In Vivo Imaging of Transplanted Cells

In vivo imaging experiments were performed as described previously [18]. A Xenogen-IVIS 100 cooled CCD optical macroscopic imaging system (SC BioScience, Tokyo, Japan, www.scbio.co.jp) was used for bioluminescence imaging. Mice were given an intraperitoneal injection of D-luciferin (150 mg/kg) and serial images were acquired from 20 minutes after administration until the maximum intensity was obtained with the field-of-view set at 10 cm. All images were analyzed using Igor (WaveMetrics, Portland, OR, <http://www.wavemetrics.com>) and Xenogen Living Image software, and optical signal intensity was expressed as photon flux in units of photons/s per cm²/steradian. To quantify the measured light, we defined regions of interest (ROIs) over the cell-implanted area and examined all values within the same ROI. The obtained photon count intensity was expressed as a percentage of the initial value.

Anterograde Labeling of the CST

Twelve weeks after injury, descending CST fibers were labeled with biotinylated dextran amine (BDA) (MW 10,000, 10% in saline, 2 μ l per cortex; Molecular Probes) [21, 22] by injection into the left and right motor cortices [23]. The skulls of anesthetized mice were tightly fixed to a stereotaxic apparatus (Narishige). Scalping and craniotomy over the motor cortex area were carried out using a micromotor system (Nakanishi). The injection site was 2.1 mm posterior to the bregma, 2 mm lateral to the bregma, and 0.7 mm in depth [23]. For pressure injections with a 20- μ m outer diameter glass capillary attached to a microsyringe (Narishige), we used 0.1- μ l steps per minute until the desired volume (1 μ l per injection site) was injected. Two weeks later, the animals were perfused and their spinal cords were fixed as described above. Sections (30 μ m) were cut and used for immunohistochemistry. To visualize the BDA, a tyramide signal amplification fluorescence system (Perkin Elmer, Waltham, MA, <http://www.perkinelmer.com>) was used.

Visualization of Multisynaptic Neural Pathways

To visualize selective and functional transsynaptic neural pathways, wheat germ agglutinin (WGA)-expressing recombinant adenoviruses were used [24, 25]. Twelve weeks after injury, 1 μ l of saline containing WGA-expressing virus at a titer of 1×10^{11} pfu/ml was injected into the bilateral motor cortices (0.5 μ l per injection site). The injection site was 2.1 mm posterior to the bregma, 2 mm lateral to the bregma, and 0.7 mm in depth. Two weeks later, animals were perfused and the spinal cords were fixed as described above. Sections (15 μ m) were cut and used for immunohistochemistry. Rabbit anti-WGA polyclonal antibody (3 μ g/ml, Sigma) was preabsorbed with 1% acetone powder of mouse brains in blocking solution overnight at 4°C.

Ablation of Transplant-Derived Cells

Cell ablation experiments were performed as described previously [26, 27]. Diphtheria toxin (DT) was purified from conditioned medium of the PW8 strain of *Corynebacterium diphtheriae* by diethylaminoethyl Sepharose column chromatography and diluted to an appropriate concentration with saline. Seven weeks after injury, DT solution (50 μ g/kg per day \times 2 days) was administered by intraperitoneal injection into seven hiPS-NES cell-transplanted mice.

Statistical Analysis

We performed statistical analysis with an unpaired two-tailed Student's *t* test for single comparisons. For BMS and BDA fiber analysis, we used repeated-measures analysis of variance (ANOVA) with Tukey-Kramer multiple comparison test at each point (Prism, GraphPad, LA Jolla, CA, <http://www.graphpad.com>). $p < .05$ was considered significant.

RESULTS

Characterization of hiPS-Lt-NES Cells In Vitro

We have previously shown that hiPS-NES cells, which are reliably derived from different hiPS cell lines, exhibit consistent characteristics such as continuous expandability, stable neuronal and glial differentiation, and the capacity to generate functional mature human neurons [11]. hiPS-Lt-NES cells can be expanded in the presence of FGF2 and EGF in monolayer culture, and express the NSC markers Sox2 and Nestin (Fig. 1A) and the radial glial marker BLBP (Supporting Information Fig. 1), but not the ES/iPS cell markers Oct3/4 or Nanog (not shown).

To visualize transplanted cells by both luminescence and fluorescence, hiPS-Lt-NES cells were infected with lentiviruses engineered to express luciferase and GFP (Fig. 1B). Almost all hiPS-Lt-NES cells were infected (Fig. 1C, middle), and we isolated only strongly GFP-expressing cells for use in subsequent in vitro and transplantation experiments (Fig. 1C, right).

We then examined whether lentiviral infection affected the differentiation potential of hiPS-Lt-NES cells. Uninfected and infected hiPS-Lt-NES cells were induced to differentiate by removal of growth factors and cultured in the presence of 1% FBS for 4 weeks. Both hiPS-Lt-NES cell population differentiated into large and small numbers of Tuj1-positive neurons and GFAP-positive astrocytes, respectively, as observed in our previous study [11] (Fig. 1D). Quantitative analysis revealed no significant differences in the proportions of differentiated cells between uninfected and infected hiPS-Lt-NES cells (Fig. 1E). Thus, we concluded that lentiviral infection did not influence the differentiation potential of hiPS-NES cells.

Transplantation of hiPS-Lt-NES Cells into the Injured Spinal Cord Improves Functional Recovery of Hind Limbs

In the present study, we used NOD-SCID mice, which are a suitable model for xenograft research because they are constitutively immunodeficient; their pathology and innate immune response after SCI are also similar to those of other mouse strains [4, 9, 28].

Previous studies have already shown that transplantation of human NSCs from fetal spinal cord or brain tissue improves locomotor functional recovery of SCI models [3, 4]. Nevertheless, only recently has the efficacy of hiPS-NSC transplantation into SCI begun to be investigated [9]. To determine whether our hiPS-Lt-NES cells have a therapeutic capability, we first sought to compare the effects of these cells with those of human fetal spinal cord-derived NSCs (hsp-NSCs). Seventy-kilodyne contusive SCI was applied at the ninth thoracic vertebral level of mouse spinal cords, and 1 week later we injected medium alone (SCI control), or medium containing hsp-NSCs or hiPS-Lt-NES cells, into the epicenter. We then monitored the animals' hind limb motor function using the BMS [19] for at least 8 weeks. All mice showed complete paralysis at 1-day after SCI (BMS scores were 0, Supporting Information Video 1). At 8 weeks after SCI, control mice could move their hind limbs but could not support their own weight (BMS scores were approximately 2, Supporting Information Video 2). In contrast, mice that received hsp-NSCs could touch the ground with their paws and/or support their body weight using their hind limbs (BMS scores were 3-4). The hiPS-Lt-NES cell-transplanted group also showed functional recovery (BMS scores were 3-4, Supporting Information Video 3) compared to the control group, and there was no significant difference in BMS scores between the hsp-NSC- and hiPS-Lt-NES cell-transplanted groups (Fig. 2A). These results indicate that hiPS-Lt-NES cells have a comparable therapeutic effect to hsp-NSCs on SCI.

Next, to evaluate the recovery of descending pathways from the motor cortex to the hind limb motor neurons, we monitored MEPs at 12 weeks after SCI. We stimulated motor cortices electrically and recorded MEP amplitudes from the hamstring muscles. The MEP amplitudes in the hiPS-Lt-NES cell-transplanted group were significantly higher than those in the SCI control group (Fig. 2B, 2C). Furthermore, the latency of MEP response in the hiPS-Lt-NES cell-transplanted group was significantly shorter than that in the SCI control group (Fig. 2D). These results suggest that transplantation of hiPS-Lt-NES cells into the injured spinal cord stimulates the functional recovery of hind limbs.

Transplanted hiPS-Lt-NES Cells Survive and Differentiate in the Injured Spinal Cord of NOD-SCID Mice

We have established hiPS-Lt-NES cells that express luciferase and GFP, enabling us to trace the survival of the transplanted cells with a bioluminescence imaging system which detects photon signals from living cells after administration of luciferin (a substrate of luciferase) to the mice [18]. The photon signals decreased gradually after transplantation (Fig. 3A) and approximately 20% of transplanted hiPS-Lt-NES cells survived in the injured spinal cord at 5 weeks after SCI (4 weeks after transplantation), whereafter the photon signals remained stable (Fig. 3B).

Using immunohistochemistry, we found that GFP-positive transplanted cells survived and migrated to both rostral and caudal areas around the lesion site in the injured spinal cord (Fig. 3C). High-magnification images showed that trans-

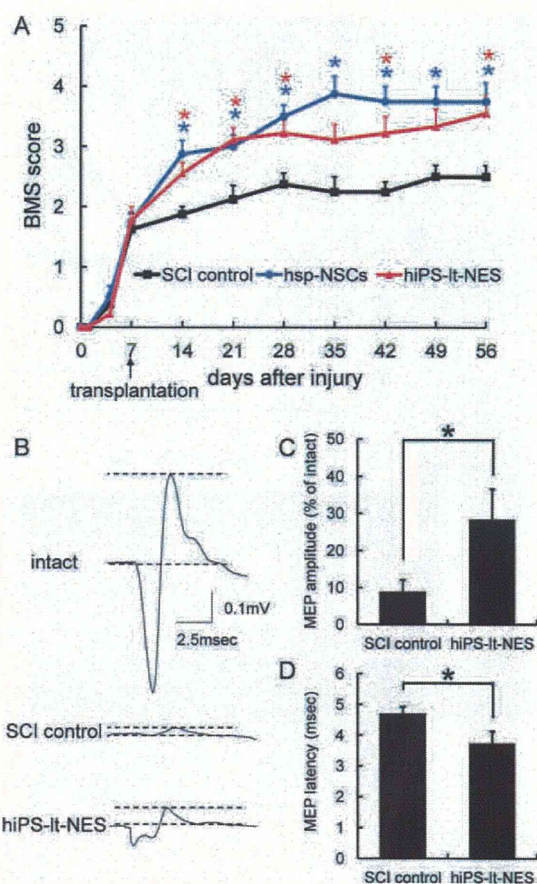


Figure 2. Transplantation of hiPS-Lt-NES cells into the injured spinal cord improves functional recovery of hind limbs. (A): Time course of functional recovery of hind limbs after SCI. Data are means \pm SEM (SCI control, $n = 8$; hsp-NSCs, $n = 8$; hiPS-Lt-NES cells, $n = 9$). Mean BMS values of hsp-NSC- and hiPS-Lt-NES cell-transplanted groups were compared with those of the SCI control group. *, $p < .05$. There was no significant difference between values in the hsp-NSC group and the hiPS-Lt-NES cell group. (B): Representative MEP waves of intact, SCI control, and hiPS-Lt-NES cell-treated mice at 12 weeks after injury. The motor cortices were stimulated and MEP amplitudes were recorded from hamstring muscles. Amplitudes from onset to peak of the negative deflection were measured. (C): Relative values of the mean MEP amplitudes. Values are expressed as percentages of those in intact mice. Mean relative MEP amplitude in the hiPS-Lt-NES cell group was significantly higher than that in the SCI control group. *, $p < .05$. Data are means \pm SD ($n = 3$). (D): Relative values of the mean MEP latency. Mean relative MEP latency in the hiPS-Lt-NES cell group was significantly shorter than that in the SCI control group. *, $p < .05$. Data are means \pm SD ($n = 3$). Abbreviations: BMS, Basso Mouse Scale; hiPS-Lt-NES, human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem; MEP, motor-evoked potential; hsp-NSC, human fetal spinal cord-derived neural stem cell; and SCI, spinal cord injury.

planted hiPS-Lt-NES cells extended processes into both gray and white matter (Fig. 3C-1, 3C-2).

We then examined the differentiation of the transplanted hiPS-Lt-NES cells in the injured spinal cord and found a similar differentiation tendency to that observed in vitro (Fig. 1D, 1E), with many GFP-positive transplant-derived cells having become Tuj1-positive neurons (75%) at 8 weeks after SCI (Fig. 3D-3F). Twenty percent of GFP-positive transplanted cells differentiated into GFAP-positive astrocytes, while their

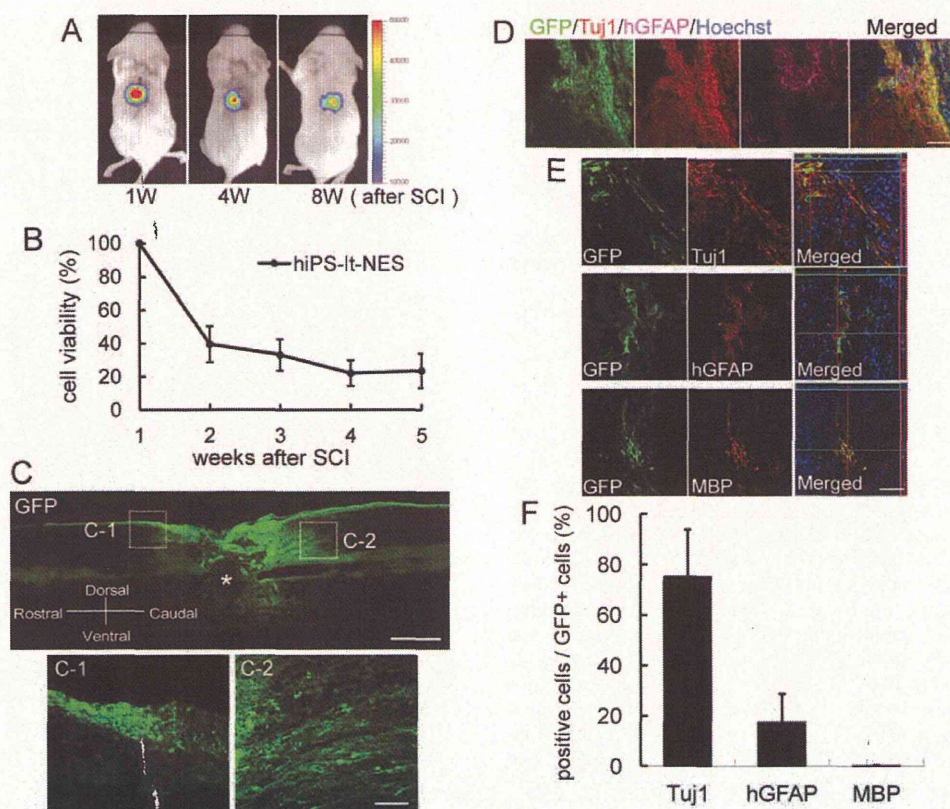


Figure 3. Transplanted human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem (hiPS-Lt-NES) cells survive and differentiate in the injured spinal cord of nonobese diabetic-severe combined immunodeficient mice. (A): Survival of transplanted cells was checked every week using a bioluminescence imaging system; left, 2 hours after transplantation; middle, 4 weeks after SCI; and right, 8 weeks after SCI. (B): Time course of transplanted hiPS-Lt-NES cell survival in SCI model mice. Optical signal intensity was measured using the bioluminescence imaging system. Quantification of the photon intensity revealed that approximately 20% of the transplanted cells survived 5 weeks after SCI; thereafter, the photon signals remained stable. Data are means \pm SEM ($n = 6$). (C): Sagittal sections of SCI model mice treated with hiPS-Lt-NES cells at 8 weeks after transplantation. Sections were stained with anti-GFP antibody (green). The epicenter of the SCI is indicated (*). Higher-magnification images of the white dotted boxes (C-1 and C-2) show GFP-positive transplant-derived cells extending their processes into gray and white matter. (D): Immunostaining images, 8 weeks after injury, of hiPS-Lt-NES cells grafted into spinal cord. Spinal cord sections were stained with anti-GFP (green), hGFAP (magenta) and Tuj1 (red) antibodies and with Hoechst (blue). (E): Confocal images, 8 weeks after injury, of hiPS-Lt-NES cells transplanted into spinal cord, revealing transplanted cells which were double-positive for GFP and markers of neural lineages. (F): Quantitative analyses of Tuj1-positive neurons, hGFAP-positive astrocytes, and MBP-positive oligodendrocytes as in E. Data are means \pm SD ($n = 3$). Scale bars = 500 μ m in C, 100 μ m in C-1, C-2, D, and E. Abbreviations: GFP, green fluorescent protein; hGFAP, anti-human-specific glial fibrillary acidic protein; MBP, and SCI, spinal cord injury.

differentiation into MBP- or APC-positive oligodendrocytes was less than 1% (Fig. 3E, 3F, Supporting Information Fig. 2). These data indicate that transplanted hiPS-Lt-NES cells differentiate preferentially into neurons in the injured spinal cord.

hiPS-Lt-NES Cell Transplantation Does Not Promote CST Axon Re-Extension After SCI

Since transplanted NSCs have been reported to play a supportive role in the re-extension of injured axons [29], we examined the effect of the transplanted hiPS-Lt-NES cells on CST first-order axonal regeneration. We injected BDA into the bilateral motor cortices and labeled CST first-order neuron axons by anterograde tracing [21, 22]. Because BDA is not transported from first- to second-order neurons across the synapse, only the axons of first-order neurons in the CST are visualized by this method.

In the region caudal to the injury site, >50% of the labeled fibers observed at 5 mm anterior to the lesion site were

detected in the intact mice, whereas almost no BDA-traced CST fibers could be seen in the SCI control or hiPS-Lt-NES cell-transplanted mice (Fig. 4A). Quantitative analysis revealed that there was no significant difference in the number of BDA-labeled fibers between the SCI control and hiPS-Lt-NES cell-treated groups at any position up to 5 mm on either side of the lesion site (Fig. 4B). Although we cannot exclude possibilities such as the re-extension of descending raphespinal axons, which are also important for the motor functional recovery of hind limbs [30, 31], these results suggest that the regeneration of CST axons, if it occurs, cannot be a major contributing factor in the recovery induced by hiPS-Lt-NES cell transplantation in our SCI model.

Local Neurons Reconstruct Disrupted CST Neuronal Circuits in a Relay Manner

Recent studies have revealed that local endogenous and transplant-derived neurons can form new neuronal circuits and make synaptic connections after SCI [32–34]. To determine

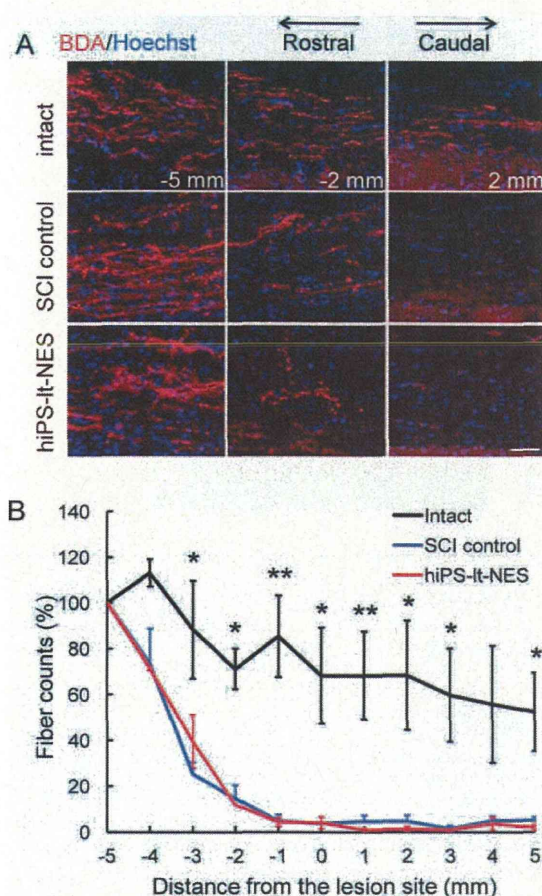


Figure 4. hiPS-lt-NES cell transplantation does not promote corticospinal tract (CST) axon re-extension after SCI. (A): Representative pictures of BDA-labeled CST fibers (red) at 5 and 2 mm rostral and 2 mm caudal to the lesion site at 12 weeks after SCI. Hoechst (blue) shows nuclear staining. Scale bar = 50 μ m. (B): Quantification of the labeled CST fibers in the spinal cords of untreated, SCI control, and hiPS-lt-NES cell-transplanted mice. The x-axis indicates specific locations along the rostro-caudal axis of the spinal cord, and the y-axis indicates the ratio of the mean number of BDA-labeled fibers at the indicated site to that at 5 mm rostral to the lesion site (thoracic vertebra [Th] 9). Intact mice were compared with SCI control mice. *, $p < .05$; **, $p < .01$. There was no significant difference in the number of BDA-labeled fibers between hiPS-lt-NES cell-treated (blue line) and SCI control groups (red line). Data are means \pm SEM ($n = 5$). Abbreviations: BDA, biotinylated dextran amine; hiPS-lt-NES, human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem; and SCI, spinal cord injury.

whether disrupted CSTs were reconstructed by forming a local neuronal relay, we injected WGA-expressing adenoviruses into the motor cortex of the hind limb area at 12 weeks after SCI. WGA, a plant lectin, has been widely used as a tracer of neuronal pathways [24, 25] because it is transported in axons and dendrites, and across synapses, to second- and even third-order neurons.

After injection, we could detect WGA-immunoreactive intracellular granule-like structures in MAP2ab-positive neurons (Fig. 5A). Furthermore, we found that hiPS-lt-NES cell-treated mice had more WGA/Map2ab double-positive cells than SCI control mice in the caudal region below the injured site (Fig. 5A, 5B). Taking these data and the results of the BDA experiment into consideration, it is suggested that WGA

was transferred to the caudal area through the lesion site via new synaptic connections, and that hiPS-lt-NES cell transplantation promoted the CST reconstruction without CST axonal re-extension. In support of this proposition, we could observe GFP-positive transplant-derived neurons adjacent to synaptophysin-positive patches (Fig. 5C-1 and 5C-1'). Furthermore, immunohistochemistry using species-specific antibodies for presynaptic markers revealed that transplant-derived neurons made synapses with endogenous neurons, suggesting that they reconstructed disrupted CST neuronal circuits in a relay fashion (Fig. 5C-2, 5C-2', 5C-3, and 5C-3').

hiPS-lt-NES Cell Transplantation Promotes the Survival of Endogenous Neurons

Massive endogenous cell death is observed in the injured spinal cord, probably due to environmental changes such as increased levels of inflammatory cytokines [35, 36]. Previous studies have suggested that neurotrophic factors secreted from transplanted cells could alleviate cell death and thereby contribute to improved locomotor function after SCI [37]. We therefore wanted to examine whether transplanted hiPS-lt-NES cells influence the survival of endogenous neurons. We performed immunohistochemistry and counted the number of NeuN-positive/GFP-negative endogenous neurons around the lesion site (Fig. 6A) and found that the number of endogenous neurons in the hiPS-lt-NES cell-treated mice was significantly higher than that in the SCI control (Fig. 6B). These findings indicate that transplantation of hiPS-lt-NES cells alters the environment of the injured spinal cord and promotes the survival of endogenous neurons.

Transplanted Cells Contribute Directly to Functional Recovery of Hind Limb Movement in SCI Model Mice

Ablation of specific cells is a useful method to evaluate their functional contribution in animal models. Mouse and rat cells are less sensitive to DT than human cells, which express human heparin-binding epidermal growth factor-like growth factor (hHB-EGF) as a DT receptor [26, 27]. We administered DT to hiPS-lt-NES cell-transplanted mice at 7 weeks after SCI. Using an in vivo imaging system, we were able to trace the survival of transplanted cells while evaluating hind limb function. Almost all the transplanted hiPS-lt-NES cells were specifically ablated following DT administration (Fig. 7A). After ablation of the transplanted cells, the BMS score of hiPS-lt-NES cell-treated mice dropped to a level similar to that of SCI control mice, although the reduction was not large enough to make the score differ significantly from that attained by hiPS-lt-NES cell-treated mice without DT administration (Fig. 7B). This may be because transplanted hiPS-lt-NES cells promoted the survival of endogenous neurons (Fig. 6) that participated in the reconstruction of disrupted neuronal circuitry. Nevertheless, these results suggest that transplanted hiPS-lt-NES cells play direct and important roles in the functional recovery of the injured spinal cord.

DISCUSSION

The advent of cell reprogramming and the establishment of iPSCs have provided new prospects for stem cell transplantation therapy [7, 8], and a large number of different types of iPSCs have already been generated by various methods [38–40]. Transplantation of NSCs into the injured spinal cord has been shown to be effective, and with recent advances in stem cell biology, particularly in human ES/iPSC cells, we anticipate

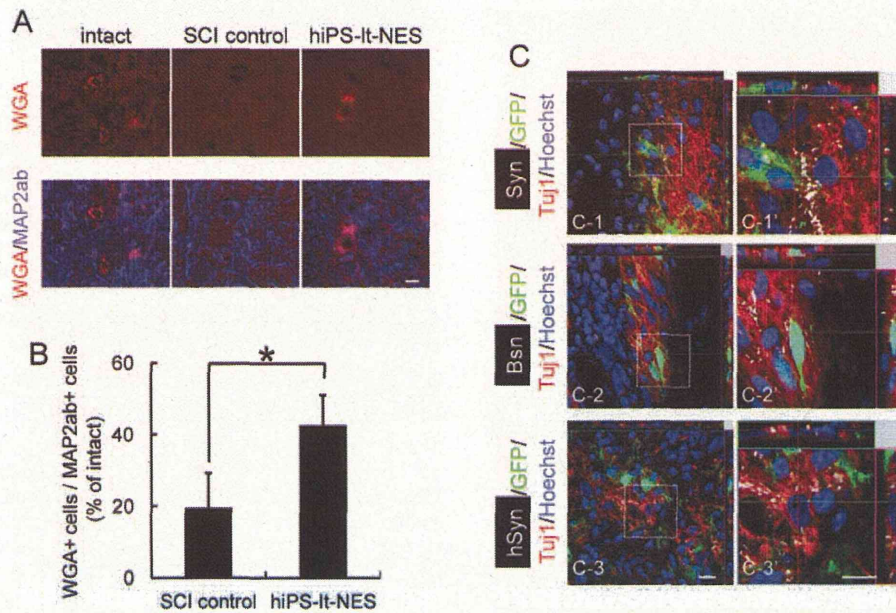


Figure 5. Local neurons reconstruct disrupted corticospinal tract neuronal circuits in a relay manner. (A): Representative pictures of WGA-labeled neuronal cell bodies located in the caudal area (Th11 to lumbar vertebra [L] 1) at 12 weeks after spinal cord injury (SCI; left, intact mice; middle, SCI control mice; and right, hiPS-lt-NES cell-treated mice). Spinal cord sections were stained with anti-WGA (red) and anti-MAP2ab (neuronal marker, blue) antibodies. WGA immunoreactivity was observed as intracellular granule-like structures. (B): WGA-positive cells/MAP2ab-positive neurons in the caudal area were quantified. Values are expressed as percentages of those in intact mice. The percentage of WGA-positive cells in hiPS-lt-NES cell-treated mice was significantly higher than that in SCI control mice. *, $p < .05$. Data are means \pm SD ($n = 3$). (C): Spinal cord sections were stained with anti-GFP and Tuji1 antibodies, Hoechst (blue), and Syn, Bsn, or hSyn antibodies. (C-1) Representative confocal image showing that GFP (green) and Tuji1 (red) double-positive transplant-derived neurons were adjacent to synaptophysin-positive patches (white). (C-1') High-magnification view of boxed area in (C-1). (C-2) Host and graft synapses were distinguished using a monoclonal antibody for the presynaptic marker Bsn that selectively recognizes rat and mouse, but not human, epitopes. Confocal image showing that GFP (green) and Tuji1 (red) double-positive transplant-derived neurons were in contact with Bsn-positive host synapses (white). (C-2') High-magnification view of boxed area in (C-2). (C-3) Confocal images showing that GFP (green)-negative and Tuji1 (red)-positive endogenous neurons were in contact with hSyn-positive synapses (white). (C-3') High-magnification view of boxed area in (C-3). Scale bars = 10 μ m. Abbreviations: Bsn, anti-Bassoon; GFP, green fluorescent protein; hiPS-lt-NES, human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem; hSyn, anti-human-specific synaptophysin; Syn, anti-synaptophysin; and WGA, wheat germ agglutinin.

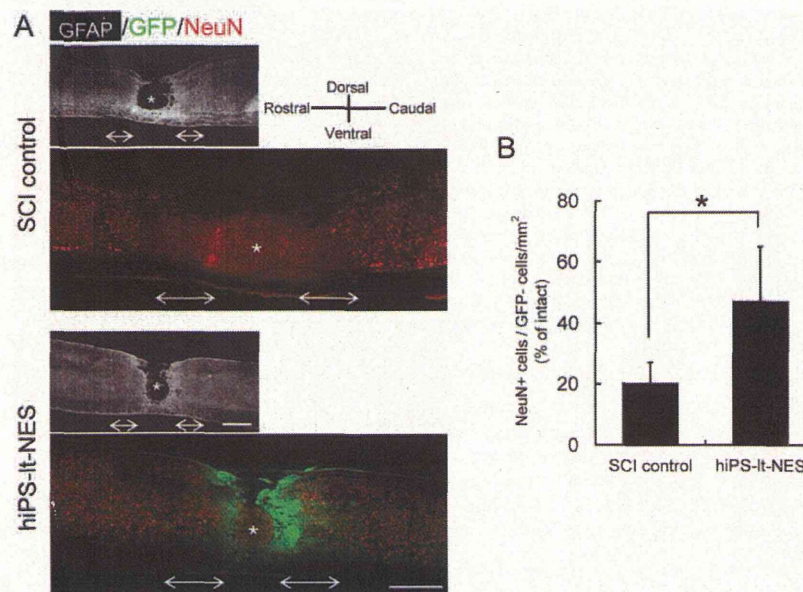


Figure 6. Transplanted hiPS-lt-NES cells promote the survival of endogenous neurons. (A): Representative sagittal sections of SCI control and hiPS-lt-NES cell-transplanted mice at 12 weeks after SCI. Sections were stained with anti-GFAP (white), anti-NeuN (mature neuronal marker, red) and anti-GFP (green) antibodies. Two 500- μ m regions, at the rostral and caudal edge of the lesioned site (double-headed arrows), were selected for the assessment. The epicenter of the SCI is indicated (*). Scale bars = 500 μ m. (B): Quantification of NeuN-positive/GFP-negative endogenous neurons. Values are expressed as percentages of those in intact mice. The number of NeuN-positive/GFP-negative endogenous neurons in hiPS-lt-NES-treated mice was significantly higher than that in SCI control mice. *, $p < .05$. Data are means \pm SD ($n = 3$). Abbreviations: GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; hiPS-lt-NES, human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem; and SCI, spinal cord injury.

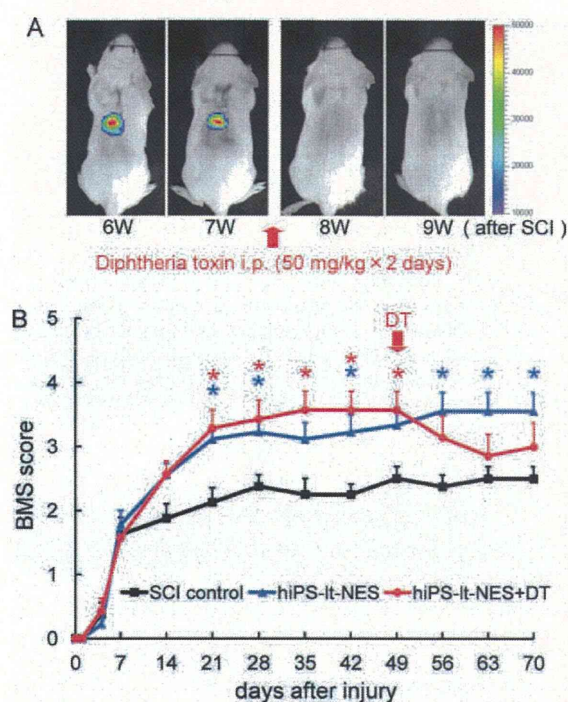


Figure 7. Transplanted cells contribute directly to the functional recovery of hind limb movement in SCI model mice. (A): Survival of transplanted cells was monitored using a bioluminescence imaging system. Seven weeks after SCI (6 weeks after transplantation), each mouse was administered DT. One week later, luciferase activity had completely disappeared in hiPS-lt-NES cell-transplanted mice. (B): Time course of functional recovery of hind limbs after SCI and DT administration. Data are means \pm SEM (SCI control, $n = 8$; hiPS-lt-NES, $n = 9$; and hiPS-lt-NES+DT, $n = 7$). Hind limb function of hiPS-lt-NES cell-transplanted mice deteriorated after DT administration (red line). BMS values of the hiPS-lt-NES and hiPS-lt-NES+DT groups were compared with those of the SCI control group. *, $p < .05$. Abbreviations: BMS, Basso Mouse Scale; DT, diphtheria toxin; hiPS-lt-NES, human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem; and SCI, spinal cord injury.

further improvements in the treatment of SCI [41]. However, transplantation studies of hiPS-NSCs, initially using mouse models of SCI [9], are in their infancy.

We have previously shown that human lt-NES cells, derived from different hESC and iPSC lines in two independent laboratories, exhibit consistent characteristics and retain their ability to proliferate and differentiate even after several passages and long-term in vitro growth [11]. In the present study, we investigated the differentiation potential of transplanted hiPS-lt-NES cells and found that the majority of differentiated hiPS-lt-NES cells in the injured spinal cord were neurons (Fig. 3D–3F), even though endogenous or transplanted NSCs in the injured spinal cord have been reported by others to differentiate preferentially into the glial lineage [42, 43]. Recent studies have indicated that neurons derived from transplanted human NSCs can integrate into the injured spinal cord and form synaptic connections with host neurons [33, 34]. We therefore took advantage of the tendency of our hiPS-lt-NES cells to differentiate into neurons and tried to apply these cells to reconstruction of the injured spinal cord.

Multiple therapeutic effects of NSC transplantation have been reported, and many different types of stem cells have been grafted into the injured spinal cord, yielding improved

functional recovery in animal models [44, 45]. However, the growth of axons through the lesion to reinnervate the side caudal to the injury has so far been very limited. Our experiments using anterograde labeling of CST fibers revealed that this was also the case after transplantation of hiPS-lt-NES cells (Fig. 4). We have previously shown that neurons derived from mouse NSCs could restore disrupted neuronal circuits [15]. Consistent with that observation, we demonstrated here that WGA-positive cell numbers in hiPS-lt-NES cell-treated mice were higher than those in SCI control mice in the area caudal to the SCI (Fig. 5A, 5B). Furthermore, immunohistochemistry using species-specific antibodies for presynaptic markers suggested that transplant-derived neurons made synapses with endogenous neurons (Fig. 5C-2, 5C-2', 5C-3, and 5C-3'). These results suggest that WGA was transferred to the caudal area through the lesion site via new synaptic connections, and that hiPS-lt-NES cell transplantation promoted CST reconstruction in a relay fashion. Other studies have provided evidence that local neurons can form intraspinal neural circuits in the lesion site and make synaptic connections with descending-tract collaterals after SCI [32, 46], and human NSCs transplanted into the injured rat or mouse spinal cord differentiated into neurons that formed axons and synapses, and also established contacts with host neurons [4, 34].

As well as their roles in cell replacement, NSCs reportedly exert other effects through the secretion of neurotrophic factors [37], which, by means such as suppressing myelin inhibitors, prevent neuronal cell death, enhance remyelination and regenerate axons [47, 48]. We found that transplantation of hiPS-lt-NES cells supported the survival of endogenous neurons (Fig. 6). Conversely, ablation of transplanted cells decreased previously attained functional recovery (Fig. 7), suggesting that transplanted cells contribute directly to functional recovery of hind limb movement in SCI model mice. In summary, we suggest that not only neurons derived from transplanted hiPS-lt-NES cells but also surviving endogenous neurons contribute to functional improvement by forming multiple synaptic connections which restore disrupted neuronal circuits.

Following injury, demyelinated and reconstructed axons must be remyelinated to ensure proper functional recovery. Generally, endogenous or transplanted NSCs which differentiate into oligodendrocytes have been reported to contribute to remyelination of axons and thereby to help in recovery after SCI. Transplanted oligodendrocyte progenitor cells (OPCs) derived from human ESCs have been shown to differentiate into oligodendrocytes and enhance remyelination in the moderate rat model of contusion spinal cord injury [5, 6]. A further study reported that in complete spinal cord transection rats, locomotor recovery after transplantation with both OPCs and human ESC-derived motor neuron progenitor cells was significantly greater than after treatment with either cell type alone [49]. Given that the application of stem cells and their progenitors for transplantation is currently in its infancy, and that SCI pathology differs between contusion and transection [50], these findings indicate that neural cells in an appropriate lineage should be transplanted according to the degree and/or type of SCI.

When ES and iPSC cells are used for transplantation treatment, a key consideration is the likelihood of tumor formation, since the transplanted cell population may include undifferentiated cells [51, 52]. Indeed, the sources and methods of induction of NSCs are critical for differentiation and tumor formation [33, 53]. Neurosphere cultures are heterogeneous and sensitive to variations in methodological procedures [12], whereas monolayer cell cultures give rise to more homogeneous population [13, 14]. In this study, we detected no tumor

formation in more than 40 hiPS-It-NES cell-treated mice up to 12 weeks after SCI. This is probably attributable to complete differentiation of hiPS cells into NES cells in our robust and stable monolayer cell cultures, since the NES cells were established by initially purifying neural rosette structures from differentiating cultures [11]. Furthermore, we transplanted hiPS-It-NES cells which had been expanded in the presence of growth factors and passaged more than 20 times. Before human iPS cell-based therapies can be implemented for clinical application, the cells' proliferation and tumor formation after transplantation must be strictly evaluated.

CONCLUSION

In this study, we have demonstrated that hiPS-It-NES cells survived and differentiated in the injured spinal cord of NOD-SCID mice, and promoted functional recovery of hind limbs. Moreover, we have shown that transplanted hiPS-It-NES cells support the reconstruction of CST pathways, promote endogenous neuron survival, and directly contribute to improved hind limb movement. To achieve a more efficient treatment for SCI, detailed investigations of hiPS-It-NES cells and SCI pathology will be necessary. Nevertheless, our study raises

the possibility that hiPS-based therapy can be applied in the near future to SCI patients who currently have few or no therapeutic options.

ACKNOWLEDGMENTS

We thank M. Saito and K. Kohno for providing diphtheria toxin, H. Miyoshi, H. J. Okano, and H. Okano for lentiviral vectors, Y. Yoshihara for WGA-expressing adenovirus, N. Uchida for anti-human specific antibodies, and S. Nori and M. Nakamura for species-specific antibodies. We also thank Y. Bessho, T. Matsui, Y. Nakahata, T. Matsuda, S. Komai, and M. Arai for valuable discussions and technical advice, T. Matta for statistical analysis, I. Smith for editing the manuscript, and M. Tano for secretarial assistance. It-NES[®] is a registered trademark of LIFE & BRAIN GmbH, Bonn, Germany.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

- Ditunno JF, Formal CS. Chronic spinal cord injury. *N Engl J Med* 1994;330:550–556.
- Ogawa Y, Sawamoto K, Miyata T et al. Transplantation of in vitro-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats. *J Neurosci Res* 2002;69:925–933.
- Iwanami A, Kaneko S, Nakamura M et al. Transplantation of human neural stem cells for spinal cord injury in primates. *J Neurosci Res* 2005;80:182–190.
- Cummings BJ, Uchida N, Tamaki SJ et al. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. *Proc Natl Acad Sci USA* 2005;102:14069–14074.
- Keirstead HS, Nistor G, Bernal G et al. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *J Neurosci* 2005;25:4694–4705.
- Sharp J, Frame J, Siegenthaler M et al. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants improve recovery after cervical spinal cord injury. *Stem Cells* 2010;28:152–163.
- Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006;126:663–676.
- Takahashi K, Tanabe K, Ohnuki M et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;131:861–872.
- Nori S, Okada Y, Yasuda A et al. Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. *Proc Natl Acad Sci USA* 2011;108:16825–16830.
- Koch P, Opitz T, Steinbeck JA et al. A rosette-type, self-renewing human ES cell-derived neural stem cell with potential for in vitro instruction and synaptic integration. *Proc Natl Acad Sci USA* 2009;106:3225–3230.
- Falk A, Koch P, Kesavan J et al. Capture of neuroepithelial-like stem cells from pluripotent stem cells provides a versatile system for in vitro production of human neurons. *PLoS One* 2012;7:e29597.
- Kim HT, Kim IS, Lee IS et al. Human neurospheres derived from the fetal central nervous system are regionally and temporally specified but are not committed. *Exp Neurol* 2006;199:222–235.
- Conti L, Pollard SM, Gorba T et al. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol* 2005;3:e283.
- Pollard SM, Conti L, Sun Y et al. Adherent neural stem (NS) cells from fetal and adult forebrain. *Cereb Cortex* 2006;16 Suppl 1:i112–i120.
- Abematsu M, Tsujimura K, Yamano M et al. Neurons derived from transplanted neural stem cells restore disrupted neuronal circuitry in a mouse model of spinal cord injury. *J Clin Invest* 2010;120:3255–3266.
- Sun Y, Pollard S, Conti L et al. Long-term tripotent differentiation capacity of human neural stem (NS) cells in adherent culture. *Mol Cell Neurosci* 2008;38:245–258.
- Miyoshi H, Blömer U, Takahashi M et al. Development of a self-inactivating lentivirus vector. *J Virol* 1998;72:8150–8157.
- Okada S, Ishii K, Yamane J et al. In vivo imaging of engrafted neural stem cells: Its application in evaluating the optimal timing of transplantation for spinal cord injury. *FASEB J* 2005;19:1839–1841.
- Basso DM, Fisher LC, Anderson AJ et al. Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. *J Neurotrauma* 2006;23:635–659.
- Setoguchi T, Nakashima K, Takizawa T et al. Treatment of spinal cord injury by transplantation of fetal neural precursor cells engineered to express BMP inhibitor. *Exp Neurol* 2004;189:33–44.
- Hata K, Fujitani M, Yasuda Y et al. RGMa inhibition promotes axonal growth and recovery after spinal cord injury. *J Cell Biol* 2006;173:47–58.
- Kaneko S, Iwanami A, Nakamura M et al. A selective Sema3A inhibitor enhances regenerative responses and functional recovery of the injured spinal cord. *Nat Med* 2006;12:1380–1389.
- Pronichev IV, Lenkov DN. Functional mapping of the motor cortex of the white mouse by a microstimulation method. *Neurosci Behav Physiol* 1998;28:80–85.
- Yoshihara Y, Mizuno T, Nakahira M et al. A genetic approach to visualization of multisynaptic neural pathways using plant lectin transgene. *Neuron* 1999;22:33–41.
- Kinoshita N, Mizuno T, Yoshihara Y. Adenovirus-mediated WGA gene delivery for transsynaptic labeling of mouse olfactory pathways. *Chem Senses* 2002;27:215–223.
- Furukawa N, Saito M, Hakoshima T et al. A diphtheria toxin receptor deficient in epidermal growth factor-like biological activity. *J Biochem* 2006;140:831–841.
- Saito M, Iwawaki T, Taya C et al. Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat Biotechnol* 2001;19:746–750.
- Luchetti S, Beck KD, Galvan MD et al. Comparison of immunopathology and locomotor recovery in C57BL/6, BUB/BnJ, and NOD-SCID mice after contusion spinal cord injury. *J Neurotrauma* 2010;27:411–421.
- Yan J, Welsh AM, Bora SH et al. Differentiation and tropic/trophic effects of exogenous neural precursors in the adult spinal cord. *J Comp Neurol* 2004;480:101–114.
- Nothias JM, Mitsui T, Shumsky JS et al. Combined effects of neurotrophin secreting transplants, exercise, and serotonergic drug challenge improve function in spinal rats. *Neurorehabil Neural Repair* 2005;19:296–312.
- Kim D, Murray M, Simansky KJ. The serotonergic 5-HT(2C) agonist *m*-chlorophenylpiperazine increases weight-supported locomotion without development of tolerance in rats with spinal transections. *Exp Neurol* 2001;169:496–500.

- 32 Courtine G, Song B, Roy RR et al. Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury. *Nat Med* 2008;14:69–74.
- 33 Hooshmand MJ, Sontag CJ, Uchida N et al. Analysis of host-mediated repair mechanisms after human CNS-stem cell transplantation for spinal cord injury: Correlation of engraftment with recovery. *PLoS One* 2009;4:e5871.
- 34 Yan J, Xu L, Welsh AM et al. Extensive neuronal differentiation of human neural stem cell grafts in adult rat spinal cord. *PLoS Med* 2007;4:e39.
- 35 Nakamura M, Houghtling RA, MacArthur L et al. Differences in cytokine gene expression profile between acute and secondary injury in adult rat spinal cord. *Exp Neurol* 2003;184:313–325.
- 36 Bareyre FM, Schwab ME. Inflammation, degeneration and regeneration in the injured spinal cord: insights from DNA microarrays. *Trends Neurosci* 2003;26:555–563.
- 37 Lu P, Tuszynski MH. Growth factors and combinatorial therapies for CNS regeneration. *Exp Neurol* 2008;209:313–320.
- 38 Okita K, Nakagawa M, Hyenjong H et al. Generation of mouse induced pluripotent stem cells without viral vectors. *Science* 2008;322:949–953.
- 39 Zhou H, Wu S, Joo JY et al. Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* 2009;4:381–384.
- 40 Kaji K, Norrby K, Paca A et al. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* 2009;458:771–775.
- 41 Lindvall O, Kokaia Z. Stem cells in human neurodegenerative disorders—time for clinical translation? *J Clin Invest* 2010;120:29–40.
- 42 Cao QL, Howard RM, Dennison JB et al. Differentiation of engrafted neuronal-restricted precursor cells is inhibited in the traumatically injured spinal cord. *Exp Neurol* 2002;177:349–359.
- 43 Han SS, Kang DY, Mujtaba T et al. Grafted lineage-restricted precursors differentiate exclusively into neurons in the adult spinal cord. *Exp Neurol* 2002;177:360–375.
- 44 Louro J, Pearse DD. Stem and progenitor cell therapies: recent progress for spinal cord injury repair. *Neurol Res* 2008;30:5–16.
- 45 Jain KK. Cell therapy for CNS trauma. *Mol Biotechnol* 2009;42:367–376.
- 46 Bareyre FM, Kerschensteiner M, Raineteau O et al. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. *Nat Neurosci* 2004;7:269–277.
- 47 Shumsky JS, Tobias CA, Tumolo M et al. Delayed transplantation of fibroblasts genetically modified to secrete BDNF and NT-3 into a spinal cord injury site is associated with limited recovery of function. *Exp Neurol* 2003;184:114–130.
- 48 Tobias CA, Shumsky JS, Shibata M et al. Delayed grafting of BDNF and NT-3 producing fibroblasts into the injured spinal cord stimulates sprouting, partially rescues axotomized red nucleus neurons from loss and atrophy, and provides limited regeneration. *Exp Neurol* 2003;184:97–113.
- 49 Erceg S, Ronaghi M, Oria M et al. Transplanted oligodendrocytes and motoneuron progenitors generated from human embryonic stem cells promote locomotor recovery after spinal cord transection. *Stem Cells* 2010;28:1541–1549.
- 50 Siegenthaler MM, Tu MK, Keirstead HS. The extent of myelin pathology differs following contusion and transection spinal cord injury. *J Neurotrauma* 2007;24:1631–1646.
- 51 Miura K, Okada Y, Aoi T et al. Variation in the safety of induced pluripotent stem cell lines. *Nat Biotechnol* 2009;27:743–745.
- 52 Tsuji O, Miura K, Okada Y et al. Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. *Proc Natl Acad Sci USA* 2010;107:12704–12709.
- 53 Jensen JB, Parmar M. Strengths and limitations of the neurosphere culture system. *Mol Neurobiol* 2006;34:153–161.



See www.StemCells.com for supporting information available online.

Insulin biosynthesis in neuronal progenitors derived from adult hippocampus and the olfactory bulb

Tomoko Kuwabara^{1*}, Mohamedi N. Kagalwala², Yasuko Onuma¹, Yuzuru Ito¹, Masaki Warashina^{1†}, Kazuyuki Terashima¹, Tsukasa Sanosaka³, Kinichi Nakashima³, Fred H. Gage², Makoto Asashima^{1**}

Keywords: diabetes; hippocampus; insulin; neural stem cell; olfactory bulb

DOI 10.1002/emmm.201100177

Received March 08, 2011
Revised August 04, 2011
Accepted August 08, 2011

→See accompanying article
<http://dx.doi.org/10.1002/emmm.201100178>

In the present study, we demonstrated that insulin is produced not only in the mammalian pancreas but also in adult neuronal cells derived from the hippocampus and olfactory bulb (OB). Paracrine Wnt3 plays an essential role in promoting the active expression of insulin in both hippocampal and OB-derived neural stem cells. Our analysis indicated that the balance between Wnt3, which triggers the expression of insulin via NeuroD1, and IGFBP-4, which inhibits the original Wnt3 action, is regulated depending on diabetic (DB) status. We also show that adult neural progenitors derived from DB animals retain the ability to give rise to insulin-producing cells and that grafting neuronal progenitors into the pancreas of DB animals reduces glucose levels. This study provides an example of a simple and direct use of adult stem cells from one organ to another, without introducing additional inductive genes.

INTRODUCTION

There are considerable similarities between the genes expressed in the developing brain and pancreas in mammals (Edlund, 2002; Habener et al, 2005; Hori et al, 2005). In *Drosophila*, insulin is produced by neurons within the brain (Brogiolo et al, 2001; Rulifson et al, 2002). Clusters of these insulin-producing cells in flies and vertebrate pancreatic islet β cells were considered functional analogues and evolved from a common ancestral insulin-producing neuron (Rulifson et al, 2002). Insulin signalling plays important roles in the proliferation and differentiation of cells in both developmental and adult stages (Hsieh et al, 2004; McMorris and Dubois-Delcq, 1986). Insulin, IGF-1 and IGF-2 provide instructive signals for

regulating cell fate choice of adult neural stem cells (NSCs) in the hippocampus (HPC) (Hsieh et al, 2004). Interestingly, diabetes impairs hippocampal learning and memory in diabetic (DB) rats and mice, thus indicating its detrimental effects on neurogenesis and synaptic neuronal plasticity (Stranahan et al, 2008). Moreover, diabetes increases the risk of clinical depression and dementia (Greenwood and Winocur, 2005; Messier 2005).

Mammalian neurogenesis occurs throughout adulthood in the subventricular zone (SVZ) of the forebrain lateral ventricles and the HPC. In the SVZ, NSCs migrate within the rostral extension of the SVZ along the rostral migratory stream, eventually reaching the olfactory bulb (OB). Multipotent NSCs can be isolated from the easily accessible OB (Bédard and Parent, 2004; Gritti et al, 2002; Hayakawa et al, 2007; Pagano et al, 2000). Extensive studies on adult hippocampal neurogenesis have revealed that it can be modulated by physiological and behavioural events, such as aging, stress, disease, seizures and learning. In the HPC, new neurons are continuously generated from self-renewing NSCs in the dentate gyrus (DG) (D'Amour and Gage, 2003; Gage, 2000; Suh et al, 2007), a process promoted by Wnt3 released from underlying astrocytes (Lie et al, 2005). Therefore, local inhibition of Wnt signalling in the DG reduces the number of newborn neurons (Jessberger et al, 2009; Kuwabara et al, 2009; Lie et al, 2005).

(1) Research Center for Stem Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Science City, Japan

(2) Laboratory of Genetics, The Salk Institute, La Jolla, CA, USA

(3) Laboratory of Molecular Neuroscience, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Takayama, Ikoma, Japan

*Corresponding author: Tel: +81-298-61-2534; Fax: +81-298-61-2987; E-mail: t.warashina@aist.go.jp

**Corresponding author: Tel: +81-298-61-2529; Fax: +81-298-61-2987; E-mail: m-asashima@aist.go.jp

† Present address: Genome Research Laboratories, Wako Pure Chemical Industries, Ltd., Amagasaki, Hyogo, Japan