Original Article

Induction of neural cells with spinal motoneuron phenotype from human iPS cells and the transplantation to totally transected spinal cords in mice

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Transplantation of neural cells is a promising therapeutic strategy for spinal cord injury (SCI). Here we generated neurons including those with spinal motoneuron phenotype from human induced pluripotent stem (hiPS) cells by leading to formation of embryoid bodies (EB) and subsequent adherent culture for 4 days during which retinoic acid (RA), noggin (NOG) and sonic hedgehog (SHH) were introduced twice. Spinal motoneuron specific HB9 mRNA expression remarkably increased in the presence of RA, NOG and SHH. With this culture condition, the neurons expressed neurofilament middle chain (NFM), βIII tubulin and HB9 proteins *in vitro*.

We then transplanted the hiPS derived human neurons into SCI mice with complete transection of Th11. Motor function of neuron transplanted SCI mice was significantly improved compared with those of vehicle injected SCI mice. Grafted cells survived and expressed βIII tubulin and HB9 diffusely 36 days after the transplantation. Galactocerebroside positive cells increased and glial fibrillary acidic protein (GFAP) expression reduced in the transection site of neuron transplanted SCI mice.

The grafted motoneurons elongated human neural cell adhesion molecule (hNCAM) positive axons over the L1 spinal level. NCAM positive axons at L1 carried a neuron tracer cholera toxin β subunit (CTβ), which had been injected into Th10 of spinal cord, cranial region of the SCI/motoneuron grafted site (Th11).

Collectively we succeeded in generating neurons which include those with HB9 positive spinal motoneuron phenotype from hiPS cells. The neurons brought about restoration of the motor function and histological improvement in an SCI.

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Introduction

Mouse induced pluripotent stem (miPS) cells were established in 2006[1] and then human iPS (hiPS) cells were similarly established[2]. hiPS cells have proliferative, self-renewal and differentiation abilities, almost the same features as human embryonic stem (ES) cells. It is becoming easy to establish human "patient specific" iPS cells from patient's somatic cells. Thus, they are expected to become new cell sources for neural cell transplantation without ethical problems and immune rejection[3].

Stem cell replacement therapy has been shown to be beneficial for recovery of patients with spinal cord injury (SCI) in several clinical trials using human ES cells[4], neural stem/progenitor (NSP) cells[4] and mesenchymal stem cells[5]. It was suggested that transplanted NSP cells replaced injured neurons, restored disrupted neural circuits and remyelinated axons in patients with SCI[4, 6, 7].

We previously reported that motoneurons were successfully induced from mouse ES cells. The neurons were functional *in vivo* even in the lesion of complete spinal cord transection model of mice 28 days after the cell transplantation, and brought about functional recovery of hind limbs of the mice[8, 9].

Then we tried to induce mouse spinal motoneurons from mouse iPS cells using retinoic acid (RA), noggin (NOG) and sonic hedgehog (SHH)[10]. RA is an efficient inducer of neural differentiation of various cell types[11]. NOG is an essential protein to induce neural fate in stem cells as a bone morphogenetic protein (BMP) inhibitor and SHH plays an important role in the regulation of neural stem cell proliferation and differentiation. Especially, SHH regulates dorsal/ventral fate determination during brain development[12]. We found that combination of the three factors induced neurons with spinal motor neuron phenotype efficiently from iPS cells[10].

We here attempted to induce neurons with spinal motor neuron phenotype effectively from human iPS cells, utilizing RA, NOG and SHH *in vitro* for future application to the patients. The cells expressed βIII tubulin, neurofilament middle chain (NFM), Islet1, Lim1 and HB9 *in vitro*, suggesting that they differentiated into cells with post-mitotic spinal motor neuron phenotype. After the transplantation of 3-factor induced neurons, SCI model mice showed gradual improvement of motor function with histological evidence of graft survival, cellular connection and remyelination.

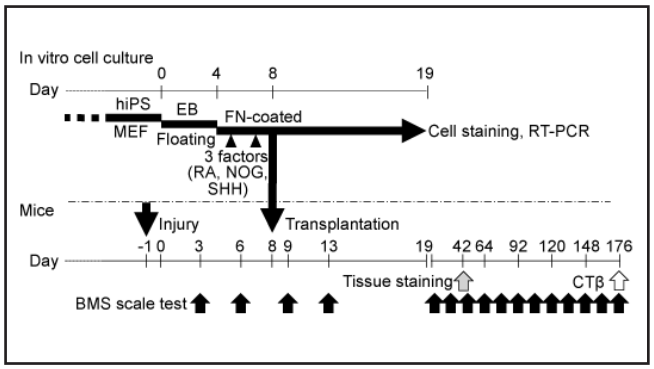

Fig. 1 A schematic representation of neural induction of hiPS cells and their transplantation to SCI mice

Undifferentiated hiPS cells were maintained in growth medium on a feeder layer consisting of mouse embryonic fibroblasts (MEF). Day 0 was the starting date of embryoid body (EB) formation.
We developed EB from the undifferentiated hiPS cells in a floating condition (Floating) for 4 days. Then the cells were cultured in fibronectin (FN)-coated dishes for 4 days. We introduced retinoic acid (RA), Noggin (NOG), and Sonic hedgehog (SHH) (3 factors) into the culture twice (at day 5 and day 7). The cells were disaggregated into single cell suspensions at day 8 for cell transplantation. The aliquots of the same cell suspensions were further cultured for subsequent characterization by immunocytochemical analysis and RT-PCR.
We conducted open-field Basso Mouse Scale (BMS) scoring on the first day, third day, and once every two weeks after injury for up to day 176. Spinal cord tissues were obtained from neuron transplanted SCI mice for histological analysis at day 42 and 176.

Materials and Methods

1)Induction of neural differentiation of human iPS cells

The hiPS cell lines (253G1 and 201B7, RIKEN, Tsukuba, Japan) were used in this study. Both cell lines gave essentially the same results, thus we presented the results obtained using 253G1 cell line.

Undifferentiated hiPS cells were maintained in growth medium consisting of DMEM/F12 supplemented with non-essential amino acids, pyruvate, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine (all purchased from Gibco, Grand Island, NY), 20 % knockout serum replacement (KSR, Gibco) and basic fibroblast growth factor (bFGF, R&D systems, Minneapolis, MN). The cells were cultured on mitomycin C treated mouse emryonic fibroblasts (MEF) as a feeder layer. The schedule for the neural cell induction was shown in Fig. 1. Day 0 was the starting date of embryoid body (EB) formation. We developed EB from undifferentiated hiPS cells in a floating condition for 4 days (from day 0 to day 4). Then the cells were cultured in fibronectin (FN, BD Biosciences, San Diego, CA) coated dishes for 4 days (from

day 4 to day 8). We introduced 1 μM RA (Sigma, Tokyo, Japan), 10 nM NOG (NOG-Fc, R&D systems) and 10 nM SHH (R&D systems) into the culture twice (at day 5 and day 7).

We have already tested a variety of culture conditions employing different culture media and growth factors, and found that medium containing the defined amounts of the three factors (RA, NOG and SHH) were most suitable for inducing the differentiation into neurons including those with spinal motoneuron phenotype[10].

The cells were disaggregated into single cell suspensions at day 8 for characterization and transplantation. The suspensions contained feeder cells in less than 0.01 %. For morphological and immunochemical analyses, the neurons in the aliquot of the same suspensions were further cultured in DMEM/F12 with N2 supplement (Gibco) on FN-coated dishes without mitogens nor growth factors for up to 11 days (from day 8 to day 19). We assessed proliferation potential of the cells using a tetrazolium bromide (MTT) assay kit (Promega, Madison, WI).

2) Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells with an RNeasy kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized with *Taq*Man reverse transcription reagents (Applied Biosystems, Foster City, CA) using random hexamers as primers, in accordance with the manufacturer's instructions. We designed the primers as follows:

β-actin, sense: TCCTGTGGCATCCACGAAAC,
antisense: GAAGCATTTGCGGTGGACGA;
Choline acetyltransferase (CAT),
sense: GGAACCGCTTCCTCCCCAAATTG,
antisense: TGCTGTAGTGGTCGAACTGGTTCTTC;
Islet1, sense: GTTACCAGCCACCTTGGAAA,
antisense: TGAATGTTCCTCATGCCTCA;
Lim1, sense: ATCCTGGACCGCTTTCTCTT,
antisense: GTACCGAAACACCGGAAGAA;
Nestin, sense: AGACTTCCCTCAGCTTTCAGG,
antisense: GCCTGGAGGAATTCTTGGTT;
NFM, sense: TAGCACATTTGCAGGAAGCA,
antisense: CGGCCAATTCCTCTGTAATG;
βIII tubulin, sense: CTCAGGGGCCTTTGGACATC,
antisense: CAGGCAGTCGCAGTTTTCAC.

In quantitative RT-PCR, eukaryotic 18S ribosomal RNA was used as an endogenous control. We studied seven combinations of TaqMan primers and probes for the following molecules: HB9, Islet1, Lim1, Nestin, βIII tubulin, synaptophysin and NFM (all from Applied Biosystems). Relative expression in differentiated cells was calculated by $2^{-\Delta\Delta Ct}$ method, and was compared to that in undifferentiated cells.

3) Experimental spinal cord injury (SCI)

Female C57BL/6 mice (5-12 weeks old, Japan SLC) were used as transplant recipients because we wanted to use genetically normal mice[13, 14]. Animals were housed 5/cage with mouse food and water adlib on a 12/12hr light/dark cycle. All surgical interventions, pre- and postsurgical animal care, and euthanasia followed the Guide for the Care and Use of Laboratory Animals, 8th edition (National Research Council) and were approved by the local Animal Care Committee (Institutional Animal Care and Use Committee).

The mice were anesthetized with an intraperitoneal injection of pentobarbital (Merck Animal Health, Summit, NJ) and xylazine (Bayel AG, Leverkusen, Germany). Laminectomy was performed at spinal level Th11. We slit the dura matter and exposed the spinal cord. We completely transected the entire depth of the spinal cord using No. 11 scalpel blade and probed the vertebral cavity several times with a fine spatula to ensure the complete spinal cord transection. After the injury, muscle layers over the laminectomy and skin on the back were sutured.

4) Cell transplantation

We transplanted the neurons with spinal motoneuron phenotype into the SCI mice. 9 days after the spinal cord injury, the spinal cord was reopened at the injury area. 2 μl of neural cell suspension (4.0×10^5 cells, n=35) and vehicle (phosphate buffered saline, PBS) (n=23) were placed into the transection pit with a 5-μl microsyringe. Immediately after injection, the neuron suspension and vehicle (PBS) were covered with thermo-reversible gelation polymer which had the reversible sol-gel process by temperature[15, 16] to retain the injected neurons. The polymer was then covered with a cement protector, and then muscle layers were sutured. We hardly found any direct effects of the polymer on the injured spinal cords and on the cell grafts in the current immunohistological analyses.

Both mouse groups (the neuron transplantation group and the PBS injection group) were administered 10 mg/Kg cyclosporine (Novartis Pharmaceuticals Japan, Tokyo,

Japan) and 0.2 mg/Kg dexamethasone (Sigma) one hour before the transplantation. 10 mg/Kg cyclosporine was given into the two groups of mice once a day from the next day of the transplantation until the mouse was sacrificed.

5)Behavioral analysis

Function recovery of mice after SCI was evaluated by open-field Basso Mouse Scale (BMS)[17]. We conducted the BMS scoring experiment on the first day, third day and once per two weeks after injury (Fig. 1). We compared the mean scores and standard error of the mean (s.e.m.) of both treatment groups for up to day 176.

6)Immunofluorescence staining

Immunofluorescence staining was conducted as reported previously[18, 19]. In brief, for all immunofluorescence procedures, consecutive tissue slices were made for immunohistochemical analysis of neuron associated proteins. Each of their negative controls underwent the same staining procedure without the respective primary antibody. For *in vitro* cell culture assay, cultured neurons were fixed in 4 % paraformaldehyde (Merck, Darmstadt, Germany) for 15 minutes after PBS washing. Cells and cryostat sections were blocked for 2 hours with or without mouse on mouse blocking reagent (Vector Laboratories, Burlingame, CA). The sections were incubated overnight with appropriate primary antibodies. The fixed cells and spinal cord tissues were stained with rat anti-Nestin (Millipore, Billerica, MA), rabbit anti-NFM (Millipore), mouse anti-βIII tubulin (Promega), mouse anti-HB9 (Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti-human Nuclei (hNuc, Abnova, Taipei City, Taiwan), rabbit anti-GFAP (Dako, Glostrup, Denmark), mouse anti-Galactocerebroside (Gal-C, Millipore), mouse anti-Synaptophysin (Dako) and mouse anti-human Neural Cell Adhesion Molecule (NCAM, Beckman Coulter, Brea, CA) antibodies. We used anti-hNuc antibody to detect human neurons in the recipient mouse spinal cord. As a control staining, adjacent tissue samples were stained essentially with the same procedure without addition of the primary antibody.

In the immunocytochemistry, we counted at least 200 cells in each experiment to calculate the number of cells expressing neural cell associated proteins *in vitro*. Each percentage of the antigen positive cells in immunofluorescence study was expressed as mean and (s. e. m.) of at least three independent experiments ($n \geqq 3$).

We stained the cryostat sections with hematoxylin-eosin (Wako, Osaka, Japan) to observe cell localization and morphological features.

7)Anterograde tracing of injured spinal cord with and without neuron transplantation

We performed a small laminectomy at Th10 of spine in the SCI mice. We injected 2 μl Alexa Fluor® 594 conjugate cholera toxin β-subunit (CTβ) (Life Technologies, Carlsbad, CA) into spinal cord using a glass capillary. 12, 24 and 48 hours after the injection, mice were sacrificed and spinal cords were processed for fluorescence analyses. Results shown in Fig. 5 were the representative of those sacrificed 24 hours after injection.

8)Statistical analysis

We performed all statistical analysis using JMP statistical software 8.0.2 (SAS, Cary, NC). Continuous variables throughout 176 days subjected to repeated measurements over a period of time on the BMS scoring and were analyzed using a repeated measures multivariate analysis of variance (MANOVA) followed by Tukey's post-test. We performed Kaplan-Meier analysis for percent survival of SCI mice. A p value less than 0.05 was considered significant.

Results

1)Expressions of motoneuron associated proteins on the neurons derived from hiPS cells

We cultured undifferentiated hiPS cells in a floating condition for 4 days to make embryoid bodies (EB). Thereafter, the cells were cultured on FN-coated dishes for 4 days.

On the next day we harvested the cells and the cells were disaggregated into single cell suspensions for characterization and transplantation. The aliquots of the same cell suspensions were further cultured for immunocytochemical analysis and RT-PCR.

We performed RT-PCR of hiPS cell derived neurons for several motor neuron associated molecules. We observed CAT, Nestin, Lim1, Islet1, NFM and βIII tubulin mRNA expressions in hiPS derived neurons at day 19 (data not shown). We conducted quantitative RT-PCR for Nestin, Lim1, Islet1, NFM and βIII tubulin in hiPS cell derived neurons at day 8 and day 19 (n=3). Relative gene expressions of Islet1 and HB9 in hiPS derived neurons were remarkably high compared with undifferentiated hiPS cells (Fig. 2A). The cells expressed mRNA of galactosylceramidase at day 19 by conventional as well as

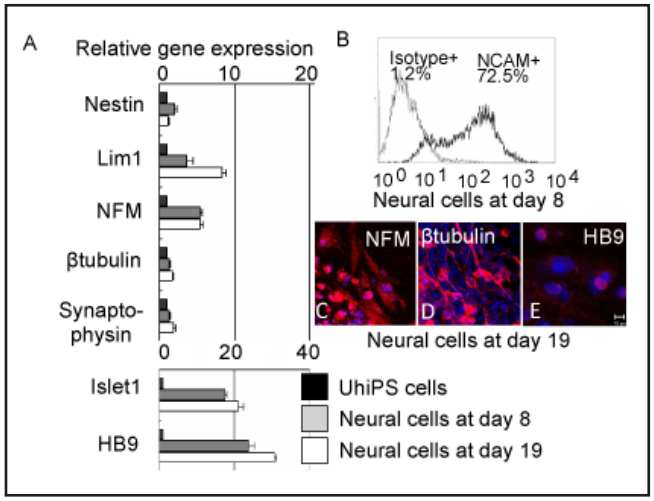

Fig. 2 Expressions of motoneuron associated proteins on the neurons derived from hiPS cells

We cultured undifferentiated hiPS cells in a floating condition for 4 days. Thereafter, the cells were cultured on fibronectin (FN)-coated dishes for 4 days, during which retinoic acid (RA), Noggin (NOG), and Sonic hedgehog (SHH) were introduced twice. After the cell culture, cells were disaggregated into single cell suspensions at day 8. Aliquots of the same cell suspensions were processed immediately and further cultured until day 19 for immunocytochemical analysis and RT-PCR.

(A)Gene expression analysis of neural cells derived from hiPS cells by quantitative RT-PCR for neuron associated molecules (n=3). We observed higher expressions of Islet1 and HB9 mRNA in hiPS cell derived neurons compared with those in undifferentiated hiPS cells. NFM: neurofilament middle chain, UhiPS: Undifferentiated hiPS

(B)Flow cytometric analysis of neural cells at day 8. Human neural cell adhesion molecule (NCAM) was expressed in approximately 72 % of the cells.

(C-E)Immunocytochemical analyses of the neural cells at day 19 (scale bar is 10 μm). The neurons expressed NFM (C, red) and the percentage of NFM positive cells was 88 ± 6.7 % at day 19 (mean ± s. e. m. of three independent experiments). The neurons expressed βIII tubulin (D, red) and the percentage of βIII tubulin positive cells was 80 ± 5.8 % at day 19. The neural cells expressed HB9 (E, red) and the percentage of HB9 positive cells was 7.0 ± 3.0 % at day 19. The cells were counterstained with DAPI (blue).

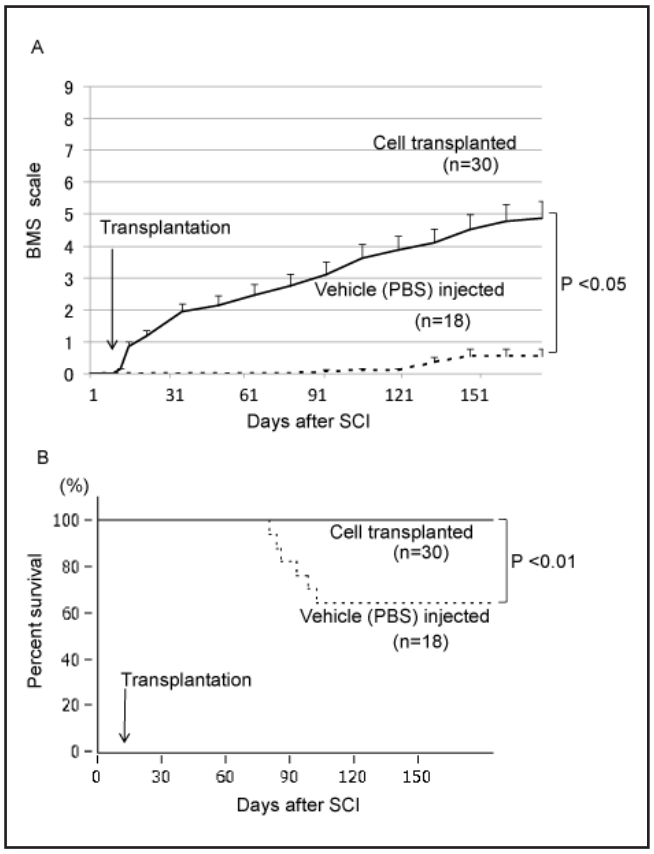

Fig. 3 Behavioral analysis of SCI mice with neuron transplantation

(A)Function recovery of SCI mice after transplantation was evaluated by open-field Basso Mouse Scale (BMS). We conducted the BMS scoring on the first day, third day and once per two weeks after injury. We compared the mean scores of both groups for up to day 176. Motor function in cell transplanted mice was significantly improved compared with that in vehicle (PBS) injected mice (p<0.05).

(B)The survival rate of the SCI mice with transplantation. The SCI mice grafted with the hiPS cell derived motoneurons survived much better as compared with vehicle injected SCI mice (p<0.01). PBS: phosphate buffered saline.

real time RT-PCR (data not shown).

Flow cytometric analysis of human NCAM expression showed that approximately 72 % of transplanted cells were human NCAM positive (Fig. 2B). We stained several neuron associated proteins on the neurons cultured in the FN-coated dishes at day 19 to confirm their neural differentiation.

The neural cells expressed NFM (Fig. 2C) and the percentage of NFM positive cells was 88 ± 6.7 % at day 19 (mean ± s. e. m. of three independent experiments). The neural cells expressed βIII tubulin (Fig. 2D) and the percentage of βIII tubulin positive cells was 80 ± 5.8 % at day 19. The neural cells expressed HB9 (Fig. 2E) and the percentage of HB9 positive cells was 7.0 ± 3.0 % at day 19. The cells expressed CAT and the percentage of CAT positive cells was 77 ± 8.2 % at day 19 (data not shown).

To assess the differences in cell proliferation potential between the cultured cells and the undifferentiated hiPS cells, we conducted a tetrazolium bromide (MTT) cell proliferation assay. We found that the both cell types were kept alive, and that proliferation potential of the cultured cells was approximately one-half of that of the

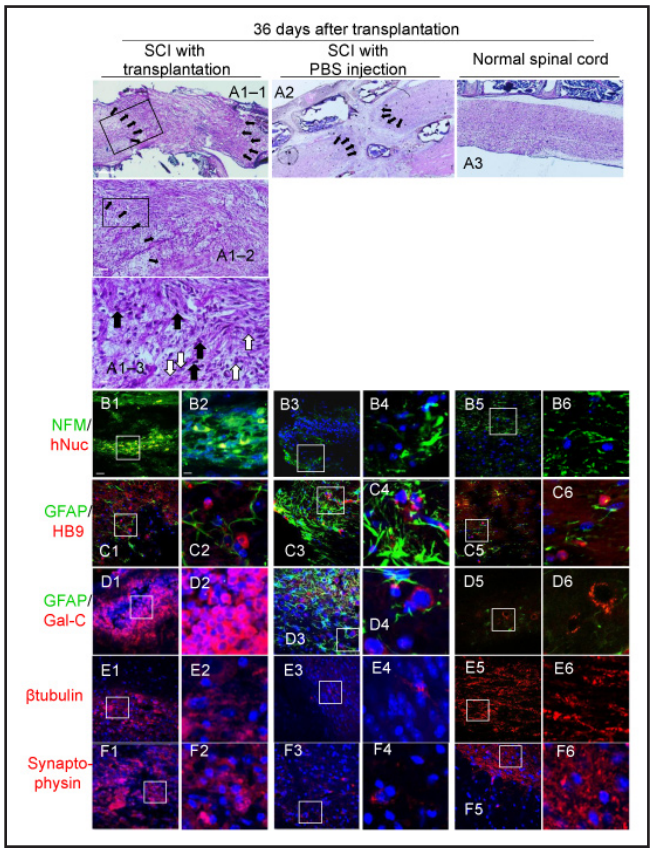

Fig. 4 Histological and immunohistochemical analyses

(A)Mouse spinal cords were processed for histological analysis 36 days after the transplantation by hematoxylin and eosin staining. (A1-1)Injured spinal cord with hiPS cell derived neuron transplantation. The grafted neurons connected the dissected spinal cord (arrows). Scale bar: 500 μm. (A1-2)Higher magnification of panel A1-1 inset. Mouse neural cells in the spinal cord have smaller and densely stained nuclei compared with larger nuclei of rather spindle shaped cells locating in the transected area. The boundary is indicated by arrows. Scale bar: 100 μm. (A1-3)Higher magnification of panel A1-2 inset. Both cell types located closely each other. The cells with larger nuclei (white arrows) moved beyond the boundary, suggesting possible interactions with mouse neural cells (black arrows) in the spinal cord. Scale bar: 20 μm. (A2)Injured spinal cord with PBS injection. Both cranial side and caudal side of injured spinal cord shrank and fibrous tissue emerged, leading to disruption of neural reconnection between cranial side and caudal side of injured spinal cord (arrows). (A3)Normal spinal cord.

(B-F)We analyzed the expressions of neuron associated proteins in the spinal cord 6 months after the transplantation by immunofluorescence with DAPI nuclear staining (blue). (B1)Anti-human Nuclei (hNuc) staining (red) and anti-Neurofilament middle chain (NFM) staining (green) of injured spinal cord with neuron transplantation. Scale bar: 20 μm. (B2)Higher magnification of panel B1 inset. Scale bar: 5 μm. (B3)Anti-hNuc staining (red) and anti-NFM staining (green) of injured spinal cord with PBS injection. (B4)Higher magnification of panel B3 inset. (B5)Anti-hNuc staining (red) and anti-NFM staining (green) of normal spinal cord. (B6)Higher magnification of panel B5 inset.

(C1)Anti-HB9 staining (red) and anti-Glial fibrillary acidic protein (GFAP) staining (green) of injured spinal cord with neuron transplantation. (C2)Higher magnification of panel C1 inset. (C3)Anti-HB9 staining (red) and anti-GFAP staining (green) of injured spinal cord with PBS injection. (C4)Higher magnification of panel C3 inset. (C5)Anti-HB9 staining (red) and anti-GFAP staining (green) of normal spinal cord. (C6)Higher magnification of panel C5 inset.

(D1)Anti-Galactocerebroside (Gal-C) staining (red) and anti-GFAP staining (green) of injured spinal cord with neuron transplantation. (D2)Higher magnification of panel D1 inset. (D3)Anti-Gal-C staining (red) and anti-GFAP staining (green) of injured spinal cord with PBS injection. (D4)Higher magnification of panel D3 inset. (D5)Anti-Gal-C staining (red) and anti-GFAP staining (green) of normal spinal cord. (D6)Higher magnification of panel D5 inset.

(E1)Anti-βIII tubulin (βtubulin) staining (red) of injured spinal cord with neuron transplantation. (E2)Higher magnification of panel E1 inset. (E3)Anti-βIII tubulin staining (red) of injured spinal cord with PBS injection. (E4)Higher magnification of panel E3 inset. (E5)Anti-βIII tubulin staining (red) of normal spinal cord. (E6)Higher magnification of panel E5 inset.

(F1)Anti-Synaptophysin staining (red) of injured spinal cord with hiPS cell derived neuron transplantation. (F2)Higher magnification of panel F1 inset. (F3)Anti-Synaptophysin staining (red) of injured spinal cord with PBS injection. (F4)Higher magnification of panel F3 inset. (F5)Anti-Synaptophysin staining (red) of normal spinal cord. (F6)Higher magnification of panel F5 inset.

For the control, normal spinal cord was stained with the same procedures. hNuc and NFM positive neurons located diffusely at the transection site of injured spinal cord with hiPS cell derived neuron transplantation (B1, B2).

HB9 positive neurons were more prevalent in injured spinal cord with hiPS cell derived neuron transplantation compared with those in injured spinal cord with PBS injection (C1, C3). The scarring area with GFAP expression was remarkably reduced in injured spinal cord with hiPS cell derived neuron transplantation compared with that in injured spinal cord with PBS injection (C1-C4).

Gal-C positive cells were more prevalent in injured spinal cord with hiPS cell derived neuron transplantation compared with those in injured spinal cord with PBS injection (D1-D4).

βIII tubulin and Synaptophysin expressions were widely observed in injured spinal cord with hiPS cell derived neuron transplantation compared with that in injured spinal cord with PBS injection (E1-E4, F1-F4).

undifferentiated hiPS cells (data not shown).

2)Behavioral analysis

Function recovery of mice after SCI was evaluated by BMS scoring. We compared the mean scores of both treatment groups for up to day 176. Motor function in cell transplanted mice improved significantly compared with that in vehicle injected mice (Fig. 3A, $p<0.05$). Indeed, some of the grafted mice bent their hind limbs to raise their hip (data not shown). The SCI mice grafted with the hiPS cell derived motoneurons survived much better, as compared with vehicle injected SCI mice (Fig. 3B, $p<0.01$).

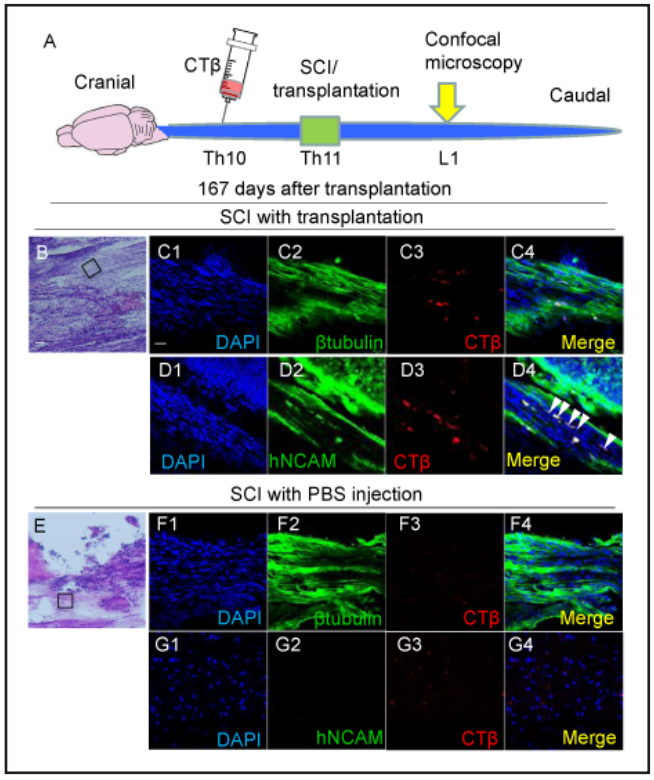

Fig. 5 Anterograde tracing of injured spinal cord after neuron transplantation

We conducted anterograde tracing of spinal cord using CTβ. We injected CTβ at spinal level Th10 (complete transection was performed at Th11). 24 hours later we conducted double immunostaining of CTβ and either anti-βIII tubulin antibody or anti-human specific NCAM antibody using adjacent sections of the injured spinal cords.

(A)Schematic representation of CTβ tracing experiment in mice.

(B-G) Mouse spinal cords with hiPS cell derived motoneuron transplantation were processed for histological and antegrade tracing analysis 176 days after the injury. (B)Hematoxylin and eosin staining of injured spinal cord with hiPS cell derived neuron transplantation. Scale bar: 100 μm.

(C1-4)Anti-βIII tubulin staining and CTβ expression of panel B inset, corresponding to L1 region of spinal cord. We found βIII tubulin and CTβ double positive axons at the spinal level L1. Scale bar: 20 μm. (C1)DAPI staining (blue). (C2)Anti-βIII tubulin staining (green). (C3)CTβ expression (red). (C4)Merged image of C1, C2, and C3.

(D1-4)Anti-hNCAM staining and CTβ expression of panel B inset. We found that hNCAM and CTβ double positive axons were identified at the spinal level L1, below the SCI/grafted region of Th11 (arrow heads). (D1)DAPI staining (blue). (D2)Anti-hNCAM staining (green). (D3)CTβ expression (red). (D4)Merged image of D1, D2, and D3.

(E)Hematoxylin and eosin staining of injured spinal cord with PBS injection.

(F1-4) Anti-βIII tubulin staining and CTβ expression of panel E inset, corresponding to L1 region of spinal cord. (F1)DAPI staining (blue). (F2)Anti-βIII tubulin staining (green). (F3)CTβ expression (red). (F4)Merged image of F1, F2, and F3.

(G1-4) Anti-hNCAM staining and CTβ expression of panel E inset. (G1)DAPI staining (blue). (G2)Anti-hNCAM staining (green). (G3)CTβ expression (red). (G4)Merged image of G1, G2, and G3.

3)Histological and immunohistochemical analyses

Mouse spinal cords were processed for histopathological analysis 36 days after the transplantation. It looked that the neurons derived from hiPS cells connected the transected spinal cord by hematoxylin-eosin staining (Fig. 4A1). For the control, normal spinal cord was stained with the same procedures.

Mouse neural cells in the spinal cord had smaller and densely stained nuclei compared with larger nuclei of rather spindle shaped human cells locating in the transected area (Fig. 4A1-2). Both cell types demarcated boundaries at the transection site and located close to each other. The human cells with larger nuclei moved beyond the boundary (Fig. 4A1-3), suggesting possible interactions of rather spindle shaped cells of larger nuclei with mouse neural cells of smaller nuclei in the spinal cord.

To further clarify the relationship of both cell types in the grafted area, we conducted immunohistochemical analysis. We found hNuc and NFM positive human neurons locating diffusely in the transection site of a neuron transplanted SCI mouse (Fig. 4B1, B2).

HB9 positive neurons were found in the transection site (18 ± 2.1 % positive of the nucleated cells, n=3, Fig. 4C1, 2) to a similar extent as normal spinal cord (26 ± 3.8 % positive, n=3, Fig. 4C5, 6). Scarring area with GFAP expression reduced remarkably in neuron transplanted SCI mice compared with that in PBS injected SCI mice (Fig. 4C1-C4). We found that Gal-C positive cells were prevalent in cell transplanted SCI mice (over 30 % positive of the nucleated cells, n=3, Fig. 4D1, 2) compared with those in PBS injected SCI mice (approximately 8 % positive, n=3, Fig. 4D3, 4) and normal spinal cord (approximately 15 % positive, n=3, Fig. 4D5, 6).

βIII tubulin positive neurons and synaptophysin expressions were widely observed in neuron transplanted SCI mice compared with that in PBS injected SCI mice (Fig. 4E1-E, F1-F4).

4)Anterograde tracing of injured spinal cord with or without neuron transplantation

We conducted anterograde tracing experiments of spinal cord using CTβ for detection of the CTβ anterograde

labeled axons over the cell transplanted site 167 days after the transplantation[20]. Spinal cord was exposed by a small laminectomy at spinal level Th10 and was injected with CTβ. 24 hours later, immunofluorescence of CTβ was examined throughout spinal cords including more caudal part of the injured/grafted site at Th11. We did not observe any of CTβ-labeled axons crossing the lesion in PBS injected SCI mice. To clearly identify the axons in SCI mice, we performed immunostaining with anti-βIII tubulin antibody, which reacted with both mouse and human neurons.

In addition, we employed anti-human specific NCAM antibody to detect axons of human iPS cell derived neurons using adjacent sections of the spinal cord. We found βIII tubulin and CTβ double positive axons at the spinal level L1 (Fig. 5C). Similarly, hNCAM and CTβ double positive axons were identified at the spinal level L1, below the SCI/grafted region of Th11 (Fig. 5D, arrow heads).

Discussion

Conventional treatment given to the SCI patients scarcely recovers their lost functions. Thus, a new efficient modality is strongly awaited[21, 22].

In mammalian development, it is thought that neuroepithelial cells in the dorsal neural tube differentiate into spinal motoneurons. The neural progenitor cell fate was strictly regulated by the emergence of SHH secreted from notochord[23]. The neuroepithelial cells responded well to the stimulation of RA[24] and RA deficit had close relationship with neural tube defects[25]. BMP secreted from neuroepithelial cells kept their ectodermal cell lineages and BMP inhibition by NOG promoted neural differentiation in the cells[26].

We have induced neural precursor cells enriched with motoneurons *in vitro* from ES cells using RA[13] and NOG[18]. In this study, to induce differentiation of human iPS cells into cells resembling spinal motoneurons, we cultured them in a way mimicking spinal development in embryos by using RA, NOG and SHH (Fig. 1). Without NOG and SHH, efficient spinal motoneuron induction was not observed in our previous study[10]. We transplanted the cells at day 8 as neural progenitor cells because they were Nestin positive (Fig. 2A). We confirmed that the neurons at day 19 *in vitro* resembled more mature spinal neurons of humans, because they expressed HB9, Islet1, Lim1 and NFM (Fig. 2A, E).

After transplantation, the cells found in the spine transection site clearly expressed HB9 almost to the same extent as the normal spinal cord (Fig. 4C). We thus induced neurons with spinal motoneuron phenotype from hiPS cells, applicable for transplantation.

Gal-C expressions were more prevalent in the transection cavities with grafted cells than those in the intact spinal cord portion of PBS injected SCI mice and normal mice (Fig. 4D). It was possible that transplanted neurons spared Gal-C positive cells in microenvironments of the SCI lesion, and the macroglial cells coated axons with their cell membrane, some of which were, to some extent, beneficial for neural remyelination.

Another potential beneficial effect of the neuron transplantation was to reduce the expression of GFAP inside the transection site. In SCI where only poor functional recovery occurred, it was caused by several myelin-derived inhibitory factors such as neurite outgrowth inhibitor (Nogo)-A, oligodendrocyte myelin glycoprotein, myelin associated glycoprotein and chondroitin sulfate proteoglycans[21].

We have reported that mouse ES cell derived neurons lacked Nogo receptor and survived in SCI lesion after cell transplantation[9]. In the chronic phase, glial scar was a major adverse event caused by inhibitory effects of astrocytes.

In case severe damage occurred in SCI, astrocytes in and near the lesion increased GFAP expression, continuously proliferated, distorted the tissue and formed a glial scar[27].

Transplantation in SCI models using NSP cells[28] and mesenchymal stem cells[29] reduced the size of glial scars. However, the transplantation was not so effective for the functional recovery probably due to insufficient neural reconnection by scar formation[30].

We observed that scarring area with GFAP expression was remarkably reduced in neuron transplanted mice 36 days after the transplantation (Fig. 4C, D). It is possible that the neurons grafted to the site exerted long term inhibitory effects on the scar formation. Further investigations are needed to disclose underlying molecular mechanisms. Several studies demonstrated that axonal growth and synaptic formation were limited in SCI models with stem cell transplantation and they needed neurotrophic factors after the transplantation[31, 32]. We showed tight connection between transplanted human cells and mouse neurons (Fig. 4A) and functional survival of the grafted cells throughout 176-day observation period (Fig. 5).

CTβ is a sensitive anterograde and retrograde tracer and is taken up and transported by a strictly limited group of damaged neurons whose body and axons were placed

around the injected site[33]. CTβ was first injected at Th10 of spinal cord, cranial site of injury/transplantation (Fig. 5A). It was carried down to caudal spinal cord beyond the injury/transplantation site of Th11. It reached L1 of detection site on βIII tubulin positive axons. We found that majority of CTβ positive axons were simultaneously hNCAM positive in the spinal cords grafted with the hiPS cell derived motoneurons (Fig. 5D). We suggest that damaged host neurons transported CTβ to the transection site and grafted cells took CTβ up and migrated to the caudal spinal cord.

We suggest that hiPS cells are a promising cell source of spinal motoneurons which bring about motor functional recovery in SCI, by extending axons and making functional connection of damaged spinal cord.

Conclusions

Neurons with spinal motoneuron phenotype were successfully induced from hiPS cells and may become a promising cell source for neural repair and regeneration in patients with SCI.

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Conflict of interests

The authors declare no competing financial interests.

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