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主論文

Transplantation of neurons derived from human iPS cells cultured on collagen matrix into guinea pig cochleae

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Running headline: Human iPS cell transplantation into cochleae.

Abstract

The present study examined the efficacy of the neural induction method for human induced pluripotent stem (iPS) cells to eliminate undifferentiated cells and to determine the feasibility of transplanting neurally induced cells into guinea pig cochleae for replacement of spiral ganglion neurons (SGNs). A stepwise method for differentiation of human iPS cells into neurons was used. Firstly, a neural induction method was established on Matrigel-coated plates. Characteristics of cell populations at each differentiation step were assessed. Secondly, neural stem cells were differentiated into neurons on a three-dimensional (3D) collagen matrix using the same protocol of culture on Matrigel-coated plates. Neuron subtypes in differentiated cells on a 3D collagen matrix were examined. Then human iPS cell-derived neurons cultured on a 3D collagen matrix were transplanted into intact guinea pig cochleae, followed by histological analysis. *In vitro* analyses revealed successful induction of neural stem cells from human iPS cells with no retention of undifferentiated cells expressing OCT3/4. After neural differentiation of neural stem cells, approximately 70% of cells expressed a neuronal marker, 90% of which were positive for vesicular glutamate transporter 1 (VGLUT1). The expression pattern of neuron subtypes in differentiated cells on a 3D collagen matrix was identical to the differentiated cells on Matrigel-coated plates. In addition, the survival of transplant-derived neurons was achieved when inflammatory responses were appropriately controlled. Our preparation method for human iPS cell-derived neurons efficiently eliminated undifferentiated cells and contributed to the settlement of transplant-derived neurons expressing VGLUT1 in guinea pig cochleae.

Key words

Pluripotent stem cell, neural induction, glutamatergic neuron, spiral ganglion neuron, hearing, scaffold, inner ear, regeneration.

1. Introduction

Sensorineural hearing loss (SNHL) is a common disease affecting hundreds of millions of people worldwide. SNHL is largely caused by dysfunction of the cochlea, a sensory organ in the inner ear. Spiral ganglion neurons (SGNs) are primary auditory neurons in the mammalian cochleae. SGNs are localized in Rosenthal's canal of the cochlear modiolus and transmit sound stimulation from cochlear hair cells to the central auditory system. At present, a cochlear implant (CI), which directly stimulates SGNs through electrodes, is the only therapeutic option for patients with severe to profound SHNL. Several clinical studies have indicated poor speech recognition in patients with poor preservation of SGNs (Starr *et al*., 1996; Valero *et al*., 2012). If functional restoration of SGNs can be achieved by cell transplantation, then the number of patients who get clinical benefits from CIs will increase. In addition, SGN degeneration is also one cause of SNHL (Keithley *et al*., 1989). In such cases, the efficacy of SGN regeneration for hearing recovery will be more direct.

Previous studies have demonstrated that cell transplantation is a potential method for SGN regeneration (Chen *et al*., 2012; Coleman *et al*., 2006; Corrales *et al*., 2006; Hu *et al*., 2005; Hu and Ulfendahl *et al*., 2005; Iguchi *et al*., 2003; Matsumoto *et al*., 2008; Nishimura *et al*., 2009; Nishimura *et al*., 2012; Ogita *et al*., 2009; Okano *et al*., 2005; Palmgren *et al*., 2012; Reyes *et al*., 2008; Tamura *et al*., 2004). Embryonic stem cells (ESCs) have been identified in these studies as the most promising source of transplants because of their ability to generate auditory nerve-like glutamatergic neurons (Reyes *et al*., 2008) and to form synaptic connections with cochlear hair cells (Corrales *et al*., 2006; Matsumoto *et al*., 2008) or the central auditory path (Chen *et al*., 2012). Furthermore, a few studies have demonstrated functional restoration of SGNs via

transplantation of neural progenitors derived from mouse (Okano *et al*., 2005) or human (Chen *et al*., 2012) ESCs in animal models with SNHL. These findings indicated SGNs to be the primary target for cell-based therapies in the treatment of hearing disorders.

The establishment of human (Takahashi *et al*., 2007; Yu *et al*., 2007) induced pluripotent stem cells (iPS cells) has had a significant impact on regenerative medicine. iPS cells enable one's own cells to be used as transplants, which gives iPS cells a substantial advantage over ESCs. Similar to ESCs, several neural induction methods have been established for iPS cells (Nikoletopoulou and Tavernarakis 2012). These neural induction methods have contributed to functional restoration of the central nervous system in animal models of spinal cord injury (Fujimoto *et al*., 2012; Kobayashi *et al*., 2012). Previous studies also have reported that iPS cells and ESCs have an equivalent potential for SGN regeneration (Nishimura *et al*., 2009). However, using iPS cells carries a risk of tumorigenesis (Miura *et al*., 2009). Therefore, researchers have attempted to reduce the risk of tumorigenesis by establishing integration-free methods for generating iPS cells (Fusaki *et al*., 2009; Okita *et al*., 2008; Stadtfeld *et al*., 2008) and using selected cell lines (Koyanagi-Aoi *et al*., 2013). One solution may be to eliminate undifferentiated cells from transplants, and the use of terminally differentiated cells is desirable (Doi *et al*., 2012).

Our ultimate goal is to replace SGNs in patients with SGN degeneration by transplanting human iPS cell-derived neurons. To achieve successful clinical application of human iPS cell-derived cells, we must focus on eliminating undifferentiated cells from transplants and using terminally differentiated neurons. The present study aimed to examine the efficacy of our method for neural induction of human iPS cells to eliminate undifferentiated cells and to determine the feasibility of

transplanting differentiated neurons derived from human iPS cells into guinea pig cochleae. In this study, we established neural induction method for human iPS cell-derived neurons, and prepared transplants by differentiating neural stem cells into neurons on a three-dimensional (3D) matrix. Finally, we transplanted these cells into guinea pig cochleae and examined their fate.

2. Materials and Methods

2.1. Human iPS cells

The human iPS cell line 201B7 (Takahashi *et al*., 2007) was used in this study. Human iPS cells were maintained according to the method of Ohnuki et al. (Ohnuki *et al*., 2009). In brief, human iPS cells were cultured on SNL feeder cells treated with mitomycin-C (Kyowa Hakko Kirin Co, Ltd, Tokyo, Japan) using human iPS cell medium, Dulbecco's modified Eagle's medium (DMEM)/F-12 (Nacalai Tesque, Kyoto, Japan) supplemented with 20% knockout serum replacement (KSR; Invitrogen, Carlsbad, CA), 1% Glutamax (Invitrogen), 1% minimum essential medium nonessential amino acid solution (Wako, Osaka, Japan), 0.1 mM 2-mercaptoethanol (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 ng/mL recombinant human basic fibroblast growth factor (bFGF; Wako). Passages were performed using a dissociation solution, phosphate-buffered saline (PBS) containing 0.25% trypsin (Invitrogen), 1 mg/mL collagenase type IV (Invitrogen), 1 mM $CaCl_2$ (Wako), and 20% KSR.

To label human iPS cells with green fluorescent protein (GFP), 201B7 iPS cells were transfected with PB-EF1a-EiP piggyBac vector, which expresses EGFP-IRES-PuroR under the control of human EF1α promoter, and PBaseII plasmid, which expresses piggyBac transposase, using a FuGENE HD (Promega, Tokyo, Japan) lipofection reagent. After 3 days of plasmid transfection, stably transduced cells were selected by 1 μg/mL of puromycin. The established cell line was designated as 201B7-GFP and was maintained similarly to 201B7.

2.2. Neural stem cell induction

Induction of neural stem cells from human iPS cells was performed according to a

modified method of Li et al (Li *et al*., 2011). Human iPS cells were cultured in SNL-conditioned medium on Matrigel (BD Biosciences, San Jose, CA)-coated multiwell culture plates (Matrigel-coated plates) prior to neural induction. SNL-conditioned medium was the culture supernatant of the SNL feeder cell culture using human iPS cell medium for 16 h. Two small molecules were used to induce neural stem cells from human iPS cells: CHIR99021, an inhibitor of glycogen synthase kinase 3β, and SB431542, a transforming growth factor-β-receptor antagonist. Human iPS cells were cultured on Matrigel-coated plates for 7 days in DMEM/F12, which included neurobasal medium (Invitrogen; 1:1) supplemented with 1xN2 (Invitrogen), 1xB27 (Invitrogen), 1% Glutamax, 3 μM CHIR99021 (CHIR; Cayman Chemical, Ann Arbor, MI), 2μM SB431542 (SB; Sigma, St. Louis, MO), and 10^3 U/mL human leukemia inhibitory factor (LIF; Nacalai Tesque). Induced cells were maintained in the same medium and were passaged using Accumax (ICT, San Diego, CA). Overnight treatment with a ROCK inhibitor (10 μM Y-27632; Wako, Osaka, Japan) was performed during the initial few passages.

2.3. Neural differentiation of neural stem cells

Neural stem cells derived from human iPS cells were differentiated into neurons on a Matrigel-coated plate, and the differentiated cells were used for reverse transcription-polymerase chain reaction (RT-PCR) and immunocytochemistry analyses to determine the characteristics.

SGNs are glutamatergic neurons. Previous studies have demonstrated neural stem cells can differentiate into glutamatergic neurons under conditions without exogenous morphogens (Li *et al*., 2009; Liu and Zhang 2011). Therefore, neural stem cells derived

from human iPS cells were cultured in N2B27 medium for differentiation into neurons.

Neural stem cells were seeded on Matrigel-coated plates at a density of 5×10^4 cells/cm^2 and cultured in neural stem cell medium for 2 days. Thereafter, the medium was changed to N2B27 medium containing DMEM/F12: neurobasal medium (1:1), supplemented with 1xN2, 1xB27, and 1% Glutamax for 7 or 14 days for neural differentiation. After neural differentiation, the cells were identified by immunocytochemistry and quantitative analyses of each marker were performed. The number of each marker-positive cell was counted in three representative images, and then the average number was calculated from three independent experiments.

2.4. Preparation of transplants on a 3D collagen matrix

Neural stem cells derived from human iPS cells were differentiated into neurons on a 3D collagen matrix for preparation of transplants. An atelocollagen Honeycomb sponge (Koken, Tokyo, Japan) served as the 3D collagen matrix. The 3D collagen matrix is cubical in shape (length times width times height; 2 mm x 2 mm x 3 mm). For cell attachment, the 3D collagen matrix was immersed in 200 μl of neural stem cell suspension at a density of 2×10^6 cells/mL in neural stem cell medium in 96-well culture plate. On the following day, the 3D collagen matrix was transferred to fresh neural stem cell medium. Induction with N2B27 medium was initiated on the second day after attachment and maintained in N2B27 medium for 7 days. Immediately after 7-day-differentiation of neural stem cells on a 3D collagen matrix, transplantation into cochleae was performed. After 7-day-differentiation of neural stem cells on a 3D collagen matrix, the number of differentiated cells was counted by NucleoCounter NC-100^{TM} (Chemometec, Allerod, Denmark), which is an automated cell counting

device using fluorescence of Propidium iodide (Shah *et al*., 2006). For quantification of cell numbers on a 3D collagen matrix, the number of differentiated cells on a 3D collagen matrix was counted using three samples per experiment. Three independent experiments were conducted for calculating the average number. For quantification of marker expression in cells cultured on a 3D collagen matrix, the ratio of each marker-positive areas for βIII TUBULIN expressing areas was employed, because counting numbers of each marker-positive cells on a 3D collagen matrix after immunostaining was impossible. For measurement of each marker-positive area on a 3D collagen matrix, images were taken with a BioRevo fluorescent microscope, BZ-9000 (Keyence, Osaka, Japan). Images with a focus on the bottom surface of a 3D collagen matrix were used. The area was analyzed by application of BZ-9000, Hybrid Cell Count BZ-H2C. We used two samples per experiment, and repeated the procedures in three independent experiments for calculating the average value.

2.5. RT-PCR

Total RNA was extracted using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA) and reverse transcribed with 200 ng RNA using TaqMan Reverse Transcription Reagents and oligo (dT) 16 primer (Applied Biosystems, Foster City, CA). PCR amplification was performed using an ExTaq HS^{TM} Kit (Takara Bio, Shiga, Japan). The primer sequences are shown in Table 1.

2.6. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (PFA) for 15 min. After permeabilization with 0.2% Triton X-100 for 5 min, cells were treated with 1% bovine

serum albumin (BSA; Wako) for 10 min at room temperature. Cells were then incubated with primary antibodies (Table 2) overnight at 4°C. A 4-day incubation period was required for anti-vesicular glutamate transporter 1 (VGLUT1) antibody. The specimens were rinsed with PBS and incubated with fluorescent-labeled secondary antibodies for 1 h at room temperature. Nuclear labeling was performed with 4', 6-diamino-2-phenyl-indole (DAPI) staining (Invitrogen). Signals were visualized with an Olympus BX50 microscope equipped with fluorescent filters (Olympus, Tokyo, Japan) or a BioRevo fluorescent microscope, BZ-9000 (Keyence, Osaka, Japan).

2.7. Animals

The animal experimental protocol described in this study was approved by the Animal Experimentation Committee of Kyoto University. Female Hartley guinea pigs (purchased from Japan SLC Inc, Hamamatsu, Japan) were used in transplantation experiments and for mesenchymal stromal cell (MSC) preparation. Transplant recipients were four-week-old guinea pigs weighting 300-350 g; 8-12-week-old guinea pigs were used for MSC preparation.

2.8. Preparation of guinea pig MSC

Bone marrow cells were obtained from guinea pig femoral bones following euthanasia with an overdose of midazolam and xylazine. Their femurs were crushed with a hammer and suspended in MSC medium, which consisted of DMEM low glucose (Invitrogen), 10% fetal bovine serum, and antibiotics (100,000 mg/L sodium penicillin G, 100 mg/L streptomycin sulfate; Wako). Bone marrow cells were seeded on a tissue culture dish at a density of 1×10^7 cells/mL. Nonadherent cells were removed at

each medium change, which was performed every other day. After two passages, adherent cells were collected for intravenous injection at a density of 1×10^7 cells/mL in PBS to reduce immune responses in guinea pig cochleae.

2.9. Surgical procedures for transplantation

Animals were anesthetized with an intramuscular injection of midazolam (10 mg/kg) and xylazine (0.01 mg/kg). After local anesthesia with 0.5% lidocaine, the left otic bulla was exposed via a postauricular incision, and a small hole was made in the otic bulla to identify the round window and basal turn of the cochlea. Cochleostomy was performed to access to the scala tympani in the basal turn of the cochlea. A 3D collagen matrix alone or containing human iPS cell-derived cells was implanted into the scala tympani of the cochlear basal turn. The cochleostomy site was closed with the temporal fascia and a pedicled temporal muscle, which was covered with fibrin glue. During the survival period, all animals received daily intramuscular injections of an immunesuppressant (FK506, 1 mg/kg; Astellas, Tokyo, Japan).

2.10. Experimental groups

Normal guinea pigs (n = 4) received implantation of a 3D collagen matrix alone into cochleae, followed by histological analysis 1 week later. Then, the animals were used as sham-operated animals to evaluate whether surgical procedures using a 3D collagen matrix cause cochlear inflammation or not. A 3D collagen matrix containing human iPS cell-derive cells was transplanted into cochleae (n = 21). MSCs were administered through the right jugular vein in animals immediately after transplantation (n = 14). And, animals were further divided into two groups according to their survival

periods, which was either 1 (n = 7) or 2 weeks (n = 7). As a control for MSC administration, the remaining seven animals received an injection of PBS instead of MSC, followed by histological analysis 1 week after transplantation.

2.11. Histological analysis of transplanted cochleae

One or 2 weeks after transplantation, animals were anesthetized with an overdose of midazolam and xylazine and were intracardially perfused with PBS, followed by 4% PFA. The left cochlea was collected and immersed in 4% PFA for 4 h at 4°C. After decalcification with 0.1 M ethylenediaminetetraacetic acid for 2 weeks at 4°C, the cochlea was immersed in 30% sucrose overnight and embedded in the Tissue-Tek O.C.T. Compound (Sakura Finetek Japan, Tokyo, Japan). Cryostat sections (12-μm thick) were affixed to Matsunami adhesive slide (MAS)-coated glass slides (Matsunami Glass, Osaka, Japan).

Hematoxylin and eosin (HE) staining and immunostaining were used in histological evaluation. HE staining was used to evaluate the gross morphology and determine cell infiltration into the cochlea. For immunohistochemistry, the sections were permeabilized and blocked with PBT1 solution (0.1% Triton X-100, 1% BSA, 0.02% sodium azide, 5% heat-inactivated goat serum in PBS) for 30 min. Primary antibodies diluted in PBT1 solution were applied to samples for 16 h at 4°C, followed by washing three times and incubation of secondary antibodies diluted in PBT2 solution (0.1% Triton X-100, 0.1% BSA, 0.02% sodium azide in PBS) for 1 h at room temperature. After a final rinse with PBS, nuclei were stained using DAPI.

Surviving transplanted cells were identified by the expression of both GFP and

human nuclear antigen (HNA), with normal nuclear morphology demonstrated by DAPI staining. The number of surviving cells was counted in five mid-modiolus sections of each cochlea. Each of the average number per section was used for calculating the total number of surviving transplanted cells in a cochlea.

2.12. Statistical analyses

The proportion of surviving transplanted cells per transplanted cells was compared between the two groups using a Mann–Whitney U-test. All statistical analyses were performed using a commercially available software package (GraphPad Prism, GraphPad Software Inc, La Jolla, CA). Data are presented as mean ± standard deviation; a p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Neural stem cell induction from human iPS cells

In the current study, we used a stepwise method for neural induction of human iPS cells (Figure 1). The primary step was induction of neural stem cells using the combination of glycogen synthase kinase 3β inhibitor and a transforming growth factor-β-receptor from human iPS cells.

Human iPS cells expressed the pluripotent marker OCT3/4 (Figure 2A and Figure 2C). After the primary induction step, 201B7 and 201B7-GFP lines did not express OCT3/4 (Figure 2B and Figure 2C), which indicated that no undifferentiated iPS cells remained after the primary induction step. Instead, the remaining cells expressed the neural stem cell markers *NESTIN*, *SOX1*, and *PAX6* in RNA levels (Figure 2C), as well

as NESTIN and SOX2 in protein levels (Figure 2B). In addition, the obtained neural stem cells allowed passages more than 20 times. These findings demonstrate the primary induction step successfully eliminated undifferentiated iPS cells and generated neural stem cells from human iPS cells.

3.2. Neural differentiation of neural stem cells derived from human iPS cells

In the second induction step, we aimed to differentiate neural stem cells into neurons, particularly glutamatergic neurons, which are consistent with neurons in the auditory nerve. Differentiated cells that had been cultured in N2/B27 medium on Matrigel-coated plates for 7 or 14 days were examined to determine the characteristics. In samples of 14-day cultured cells, RT-PCR analysis revealed that a variety neuron types were generated (Figure 3A). We found no difference in the expression patterns between 201B7- and 201B7-GFP cells; both expressed the immature neuronal marker *doublecortin* (*DCX*), neuronal marker *MAP2* and *SYNAPSIN1*, thus indicating the neurons were in various stages of differentiation. In immunocytochemistry, both 7- and 14-day cultured cells exhibited the expression of NESTIN, βIII TUBULIN and Neurofilament (NF) (Figure 3B). The proportions of βIII TUBULIN-positive cells in 7- and 14-day cultured cells were 69.1 ± 7.9% and 68.7 ± 4.8%, respectively, which is almost identical to previous findings in non-human ES cells (Shimada et al., 2012) (Figure 3Da). Regarding NESTIN expression, the proportions in 7- and 14-day cultured cells were 36.8 ± 2.9% and 30.3 ± 3.9%, respectively. The proportions of NF-positive cells in 7- and 14-day cultured cells were 6.8 ± 2.9% and 18.3 ± 1.1%, respectively. GFAP-positive glia were present in 14-day cultured cells, but not in 7-day cultured cells (Figure 3B). RT-PCR also revealed *GFAP* expression in 14-day cultured specimens

(Figure 3A). These findings demonstrated that neural stem cells derived from human iPS cells had the potential for differentiation into neurons or glia.

For neuron subtypes, RT-PCR analysis revealed the GABAergic neuronal marker *glutamate decarboxylase 67* (*GAD67*), glutamatergic neuronal marker *T-box brain 1* (*TBR1*), dopaminergic neuronal marker *tyrosine hydroxylase* (*TH*), or cholinergic neuronal marker *choline acetyltransferase* (*CHAT*) (Figure 3A). These findings indicated that various types of neurons were present in 14-day cultured cells. Immunocytochemistry demonstrated that various types of neurons were present in both 7- and 14-day cultured cells, including VGLUT1-, TH-, and GABA-positive cells (Figure 3C). The proportions of VGLUT1-positive cells in βIII TUBULIN- and DAPI-positive cells were 96.3 ± 1.4% and 95.0 ± 3.4% in 7- and 14-day cultured cells, respectively (Figure 3Db). The proportions of TH-positive cells were 3.0 ± 1.6% and 3.2 ± 1.9% in 7- and 14-day cultured cells, respectively. The proportions of GABA-positive cells were 0.8 ± 0.3% and 1.7 ± 2.3% in 7- and 14-day cultured cells, respectively. Overall, the cell population in 7-day cultured cells was almost identical to that in 14-day cultured cells for the proportion of neural subtypes. Based on these findings, we adopted 7-day-differantiation of neural stem cells for the preparation of transplants.

3.3. Preparation of transplants on a 3D collagen matrix

From the viewpoint that neurons are greatly damaged by detachment from culture surface, differentiation of neural stem cells on a scaffold can be effective for the feasibility of transplanting neurons into a cochlea. We, therefore, differentiated neural stem cells into neurons on a 3D collagen matrix using the same protocol of culture on

Matrigel-coated plates. After 7-day-differentiation of neural stem cells on a 3D collagen matrix, we harvested $9.2 \pm 1.9 \times 10^4$ cells on a 3D collagen matrix (n = 3). Immunocytochemistry revealed that differentiated cells on a 3D collagen matrix contained VGLUT1-, TH- or GABA-positive neurons (Figure 4A). The proportion of VGLUT1-positive areas in βIII TUBULIN-positive areas was 77.0 ± 25.7% (Figure 4B). The proportions of TH- and GABA-positive areas in βIII TUBULIN-positive areas were 8.6 ± 14.2% and 5.7 ± 2.1%, respectively. The expression pattern of VGLUT1, TH or GABA in differentiated cells was almost similar to that in cultured cells on a Matrigel-coated plate (Figure 3Db). In the following transplantation experiments, we used the matrix containing human iPS cell-derived cells (9.2×10^4 cells) as a transplant, which contained several types of neuronal cells at various stages of maturation.

3.4. Inflammatory response in guinea pig cochleae after transplantation and its inhibition

To evaluate whether surgical procedures using a 3D collagen matrix causes cochlear inflammation, four animals received implantation of a 3D collagen matrix alone into cochleae as sham-operated animals, followed by histological analysis one week after implantation. As a result, cochleae of sham-operated animals exhibited limited infiltration of inflammatory cells, indicating that the implantation of a 3D collagen matrix induced virtually no inflammation in guinea pig cochleae (Figure 5A).

Transplantation of human iPS cell-derived cells into guinea pig cochleae is xenografting. Therefore, we expected a severe immunoreaction in guinea pig cochleae following transplantation, which is an obstacle to survival of transplanted cells. Our preliminary experiment using conventional immunosuppression (intramuscular injection

of FK506) demonstrated that any transplanted cells were not survived one week after transplantation, indicating the necessity of additional treatments to reduce immune rejection. Therefore, we examined whether MSCs were capable to reduce the immune response leading to promotion of the survival of transplanted cells in guinea pig cochleae (Aggarwal and Pittenger 2005; Bernardo et al., 2012; Le Blanc et al, 2008). In total, 14 animals received transplantation, and thereafter were divided into two groups. Seven animals received an intravenous injection of MSCs, whereas the remaining seven animals received a PBS injection (Figure 1). One week after transplantation, we compared the survival rate of transplanted cells and inflammatory cell infiltration between MSC- and PBS-treated animals. PBS-treated animals exhibited severe infiltration of inflammatory cells within and surrounding the transplants, whereas MSC-treated animals showed limited inflammatory cell infiltration in their cochleae (Figure 5A). Immunohistochemistry revealed that the inflammatory cells were positive for CD45, a leukocyte marker (Figure 5B). The survival of transplanted cells was rarely found in PBS-treated animals but was identified in all MSC-treated animals. The proportions of surviving GFP-, HNA-, and DAPI-positive cells per transplanted cells in a cochlea were 14.8 ± 13.1% for MSC-treated animals and 0.6 ± 1.6% for PBS-treated animals, respectively. The difference in the survival number of transplanted cells between the two groups was statistically significant ($P = 0.0034$; Figure 5C). Based on these findings, we administered MSCs in the following transplantation experiments.

3.5. Survival of human iPS cell-derived neurons in guinea pig cochleae 1 week and 2 weeks after transplantation

To determine the survival period of transplanted cells after transplantation, the

survival number of transplanted cells in the 1-week-survival group was compared with that in the 2-week-survival group (Figure 1). Survival of transplanted cells was found in all seven animals 1 week after transplantation, but in only three of seven animals 2 weeks after transplantation (Figure 6A). The proportions of surviving GFP-, HNA-, and DAPI-positive cells per transplanted cells in the 1-week-survival group was 14.8 ± 13.1%, which decreased significantly in the 2-week-survival group at 2.0 ± 3.5% ($P = 0.0141$, Figure 6B). The proportions of βIII TUBULIN-positive transplanted cells in the 1- and the 2-week-survival group were 64.3 ± 25.4% and 90.7 ± 5.4%, respectively. And VGLUT1-positive neurons derived from transplanted cells were observed in both groups (Figure 6C). The proportions of VGLUT1-positive cells in βIII TUBULIN-positive transplanted cells in the 1- and the 2-week-survival group were 90.7 ± 5.4% and 94.3 ± 7.0%, respectively. These results revealed that human iPS cell-derived cells survived as neurons in guinea pig cochleae for 2 weeks following transplantation. Furthermore, the majority of the surviving neurons exhibited a glutamatergic phenotype. However, the number of surviving transplanted cells in the 2-week-survival group was decreased in comparison with that in the 1-week-survival group.

4. Discussion

The current study had two aims. The first was the complete removal of undifferentiated cells from transplants; the second was the settlement of VGLUT1-expressing neurons in the cochlea by transplanting differentiated neurons derived from human iPS cells. For the former aim, we succeeded in generating human iPS cell-derived neurons without contamination from undifferentiated cells expressing

OCT3/4. For the later aim, the settlement of human iPS cell-derived neurons expressing VGLUT1 was achieved by implanting cultured cells on a 3D collagen matrix. However, the present findings revealed that there was a problem in investigating the potential of human iPS cells when xenografting into the cochlea. Conventional immunosuppression was not effective in repressing inflammatory responses due to xenografting in the cochlea. MSC application was required for the survival of human iPS cell-derived neurons in guinea pig cochleae. Even with MSC application, the long-term survival of human iPS cell-derived neurons in guinea pig cochleae was still challenging. Therefore, we should use immune-deficient animals for evaluation of the functionality after transplantation of human iPS cell-derived neurons.

Recent studies have indicated the necessity of eliminating pluripotent stem cells from transplants to prevent tumor formation (Chung *et al*., 2006; Fukuda *et al*., 2006). Furthermore, stem cells must be induced to differentiate into neurons expressing a SGN-like phenotype for better functional restoration of SGNs. In our previous studies with iPS cells, we used a stromal cell-derived inducing activity (SDIA) method for neural induction (Nishimura *et al*., 2009; Nishimura *et al*., 2012). After 6 or 7 days of neural induction, the SDIA method successfully induced neural differentiation. However, after neural induction of three different iPS cell lines, the proportions of residual undifferentiated cells expressing OCT3/4 were over 50% (Nishimura *et al*., 2012). Furthermore, the SDIA method was originally developed as a differentiation method for dopaminergic neurons, and the proportion of VGLUT1-positive neurons was small after one week transplantation (Nishimura *et al*., 2009). Our present method for neural induction of human iPS cells eliminated undifferentiated iPS cells and expanded over 20 passages. In addition, the high efficiency of our method for induction of

VGLUT1-positive neurons from human iPS cells is comparable with that of other neural induction methods with neurons that may closely resemble SGNs (Lee *et al*., 2012; Purcell *et al*., 2013; Reyes *et al*., 2008).

In terms of future clinical applications that combine CIs and cell therapy, simple and safe transplantation procedures are included in critical issues. Furthermore, if tumor formation occurs in the cochlea, transplant-derived cells should be easily removed. Therefore, we prepared transplants using a 3D collagen matrix and implanted them into a cochlea. One or 2 weeks after transplantation, transplant-derived cells survived in 10 of 14 cochleae. Regarding the short-term survival within 2 weeks of transplantation, the high survival ratio of transplanted cells indicates the stability of our transplantation procedure using a 3D collagen matrix, which is comparable with that of other transplantation procedures described previously (Corrales *et al*., 2006; Nishimura *et al*., 2009; Ogita *et al*., 2009). To obtain functional restoration of SGNs, survival for at least 4 weeks post-transplantation is crucial (Chen *et al*., 2012; Okano *et al*., 2005). To achieve the long-term survival of transplant-derived cells, proper neurotrophic support and/or forming suitable environments may be indispensable. For this purpose, cografting of growth factor-releasing cells or consistent infusion of growth factors to transplanted sites should be utilized (Hu *et al*., 2005; Palmgren *et al*., 2012).

Cell transplantations aiming at functional restoration of SGNs face several challenges. The differentiation of surviving transplanted cells into SGN-like neurons, projection to the central auditory system, and formation of synaptic connections with central auditory neurons are all major challenges. Although we did not observe neurite outgrowth from transplanted cells toward SGNs in our present study, previous studies have demonstrated that neuritis from transplanted cells that were settled in the scala

tympani penetrated into Rosenthal's canal via tiny holes (Coleman et al., 2006, Hu and Ulfendahl et al., 2005). The tiny holes, Schuknecht's canaliculae perforantes, are present in the osseous spiral lamina, lining the medial wall of the scala tympani (Sando et al., 1971, Lim and Kim et al, 1983, Lim, 1986). Therefore, Schuknecht's canaliculae perforante might be a corridor for neurites from transplanted neurons in the scala tympani extending to host auditory neurons. We previously demonstrated the potential of mouse ESC-derived cells for transplantation into the cochlea for functional restoration of SGNs (Okano *et al*., 2005). In addition, a recent report has shown restoration of auditory function by transplanting human ESC-derived cells (Chen *et al*., 2012). In those studies, neural progenitors were directly injected into the cochlear nerve trunk. As a result, transplanted cells survived as neurons with neurite elongation migrated towards the brainstem and formed synaptic connections in the cochlear nucleus. In previous studies, functionality improvement initiated 4 weeks after transplantation. Regarding transplantation of neural stem cells into the cochlea, our previous study demonstrated the majority of the surviving transplanted cells differentiated into glia (Tamura *et al*., 2004) and produced BDNF and glial cell line-derived neurotrophic factor (GDNF) (Iguchi *et al*., 2003), which are known to have trophic effects on SGNs (Shinohara *et al*., 2002; Yagi *et al*., 2000; Endo *et al.*, 2005). To achieve functional restoration of SGNs through transplantation of human iPS cell-derived cells, additional treatments, such as trophic support, may be required.

Conclusions

The present findings demonstrated that our method for neural induction of human iPS cells efficiently eliminated undifferentiated cells and enabled the settlement of

human iPS cell-derived VGLUT-1-expressing neurons in guinea pig cochleae. However, the long-term survival of transplant-derived cells was a future consideration. For future functional restoration of SGNs with human iPS cell-derived cells, optimization of the differential stages of iPS cells and the establishment of methods for promoting the survival of transplanted cells are required. In addition, use of immune-deficient animals including severe combined immune deficient (SCID) mice should be taken into consideration.

Acknowledgments

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Author Disclosure Statement

All authors declare that no competing financial interests exist.

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Figure legends

Figure 1. Flowcharts for experimental design of the present study. The efficacy of neural induction used in the present study was validated *in vitro*. As a primary step, human iPS cells were differentiated into neural stem cells on Matrigel-coated plates with N2/B27 medium containing two small molecules, SB431542 (SB) and CHIR99021 (CHIR), and leukemia inhibitory factor (LIF). As a second step, neural stem cells were differentiated into neurons for 7 of 14 days. For preparation of transplants, 7-day-differentiation of neural stem cells was performed on a three-dimensional (3D) collagen matrix. In transplantation experiments, transplants were implanted into guinea pig cochleae, and fates of transplanted cells were evaluated 1 or 2 weeks after transplantation. Effects of mesenchymal stromal cells (MSC) application on the survival of transplanted cells and inflammation in cochleae were examined in the 1-week-survival group.

Figure 2. Neural stem cell induction from human iPS cells. (A) Immunostaining demonstrated the expression of GFP (a, d), human nuclear antigen (HNA) (b) and the pluripotent marker OCT3/4 (e) in human 201B7-GFP iPS cells before neural induction. DAPI showed nuclei (c, f). Scale bars, 200 μm. (B) Immunostaining revealed the expression of NESTIN (a, d) and SOX2 (e), but no expression of OCT3/4 (b) after neural induction. DAPI shows nuclei (c, f). Scale bars, 200 μm. (C) RT-PCR analyses of 201B7 and 201B7-GFP human iPS cell-derived cells showed the expression of the neural stem cell marker genes *NESTIN*, *SOX1* and *PAX6*, but no expression of the pluripotent marker gene *OCT3/4*. Undifferentiated iPS cells were used as controls.

Figure 3. Neural differentiation of neural stem cells derived from human iPS cells. Neural stem cells were cultured on Matrigel-coated plates for 7 or 14 days. (A) RT-PCR analysis showed the expression of marker genes for neuronal cells at several stages and several neuronal subtypes in 14-day cultured cells. Undifferentiated human iPS cells were used as the negative controls. (B) Immunostaining images after 7- and 14-day culture. The expression of βIII TUBULIN (βIII TUB) (a, f), NESTIN (b, g) and Neurofilament (NF) (d, i) were observed in both samples. GFAP-positive glia were only observed in 14-day cultured cells (e, j). In both of 7- and 14-day cultured cells, nuclei were labeled with DAPI (blue). Scale bars, 50 μm. (C) Examination of neuronal subtype by immunostaining. In both 7- and 14-day cultured cells, the expression of VGLUT1 (a-c, j-l), TH (d-f, m-o) or GABA (g-i, p-r) was observed. Scale bars, 100 μm. (D) The proportion of βIII TUB-positive cells in DAPI-positive cells was over 65% in both samples (a). Quantitative analysis showed that over 90% of both βIII TUB- and DAPI-positive cells expressed VGLUT1 in both 7- and 14-day cultured cells, only a few cells were positive for TH or GABA (b). Data are presented as mean ± SD (n = 3).

Figure 4. Preparation of transplants on a 3D collagen matrix. (A) Immunostaining images of differentiated cells on a collagen matrix. The expression of VGLUT1 (a), TH (d), GABA (g), βIII TUB (b, e, h) was observed. Bright field images are shown (c, f, i). Scale bars, 500 μm. (B) Quantitative analysis showed that over 75% of βIII TUB-positive area exhibited VGLUT1 expression. Data are presented as mean ± SD (n = 3).

Figure 5. Inflammatory response in guinea pig cochleae after transplantation and its

inhibition. (A) A schema of a mid-modiolus section of a guinea pig cochlea (a). Transplants were implanted into the scala tympani (ST) of the basal portion of the cochlea. Hematoxylin and eosin staining images of sham-operated (b), PBS-treated (d) and MSC-treated cochleae (f). Higher-magnification views of boxed areas in (b), (d) and (f) show severe infiltration of inflammatory cells in PBS-treated animals (e) and limited infiltration of inflammatory cells in sham-operated (c) and MSC-treated animals (g). Arrow heads indicate collagen-matrix. Dotted lines indicate Rosenthal's canal (RC) with spiral ganglion neurons. Scale bars, 400 μm. (b, d, f) and 100 μm. (c, e, g). (B) Immunostaining images of a cochlea in a PBS-treated animal at low- (a) and high-magnification (b) show CD45-positive cell infiltration. Scale bars, 1 mm (a) and 50 μm. (b). (C) Quantitative analysis in the proportions of GFP-, HNA- and DAPI-positive cells per transplanted cells revealed significant difference between MSC- and PBS-treated animals (**$P < 0.01$ with Mann-Whitney U-test). Data are presented as mean ± SD (n = 7). AN, auditory nerve.

Figure 6. Survival of human iPS cell-derived neurons in guinea pig cochleae 1 week and 2 weeks after transplantation. (A) A schema of a mid-modiolus section of a guinea pig cochlea (a). Immunostaining images of GFP expression in 7-day cultured cells on Matrigel-coated plates (b-d). Immunostaining images of the whole cochleae in 1- (e) and 2-week-survival (f) groups. Red arrows show surviving transplanted cells. In high-magnification images of the scala tympani (ST) of the basal portion of cochleae, DAPI- (g, k), HNA- (h, l) and, GFP- (i, m) positive cells exhibited the expression of βIII TUB (j, n). Insets show magnified images of boxed area. Scale bars, 100 μm. (B) Quantitative analysis in the proportions of GFP-, HNA- and DAPI-positive cells per

transplanted cells demonstrated a significant difference between 1- and 2-week-survival groups (*P < 0.05 with Mann-Whitney U-test). Data are presented as mean ± SD (n = 7). (C) In both 1- (a-d) and 2-week-survival groups (e-h), DAPI- and GFP-positive cells expressed βIII TUB and VGLUT1. Insets show magnified images of boxed area. Scale bars, 100 μm. AN, auditory nerve; RC, Rosenthal's canal.

Figure 1

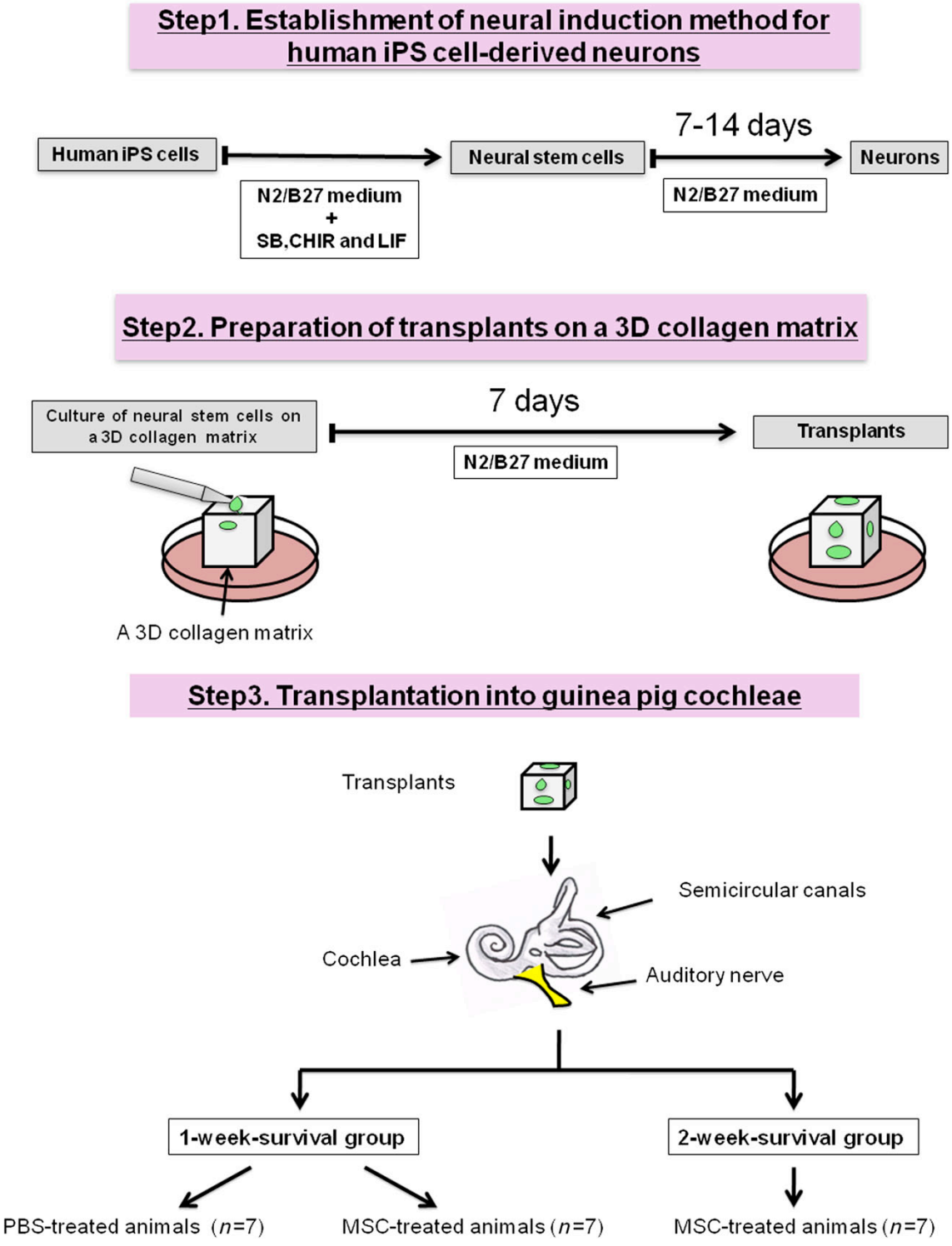

Step1. Establishment of neural induction method for human iPS cell-derived neurons
Human iPS cells
N2/B27 medium + SB,CHIR and LIF
Neural stem cells
7-14 days
N2/B27 medium
Neurons
Step2. Preparation of transplants on a 3D collagen matrix
Culture of neural stem cells on a 3D collagen matrix
7 days
N2/B27 medium
Transplants
A 3D collagen matrix
Step3. Transplantation into guinea pig cochleae
Transplants
Semicircular canals
Cochlea
Auditory nerve
1-week-survival group
2-week-survival group
PBS-treated animals (n=7)
MSC-treated animals (n=7)
MSC-treated animals (n=7)

Figure 2

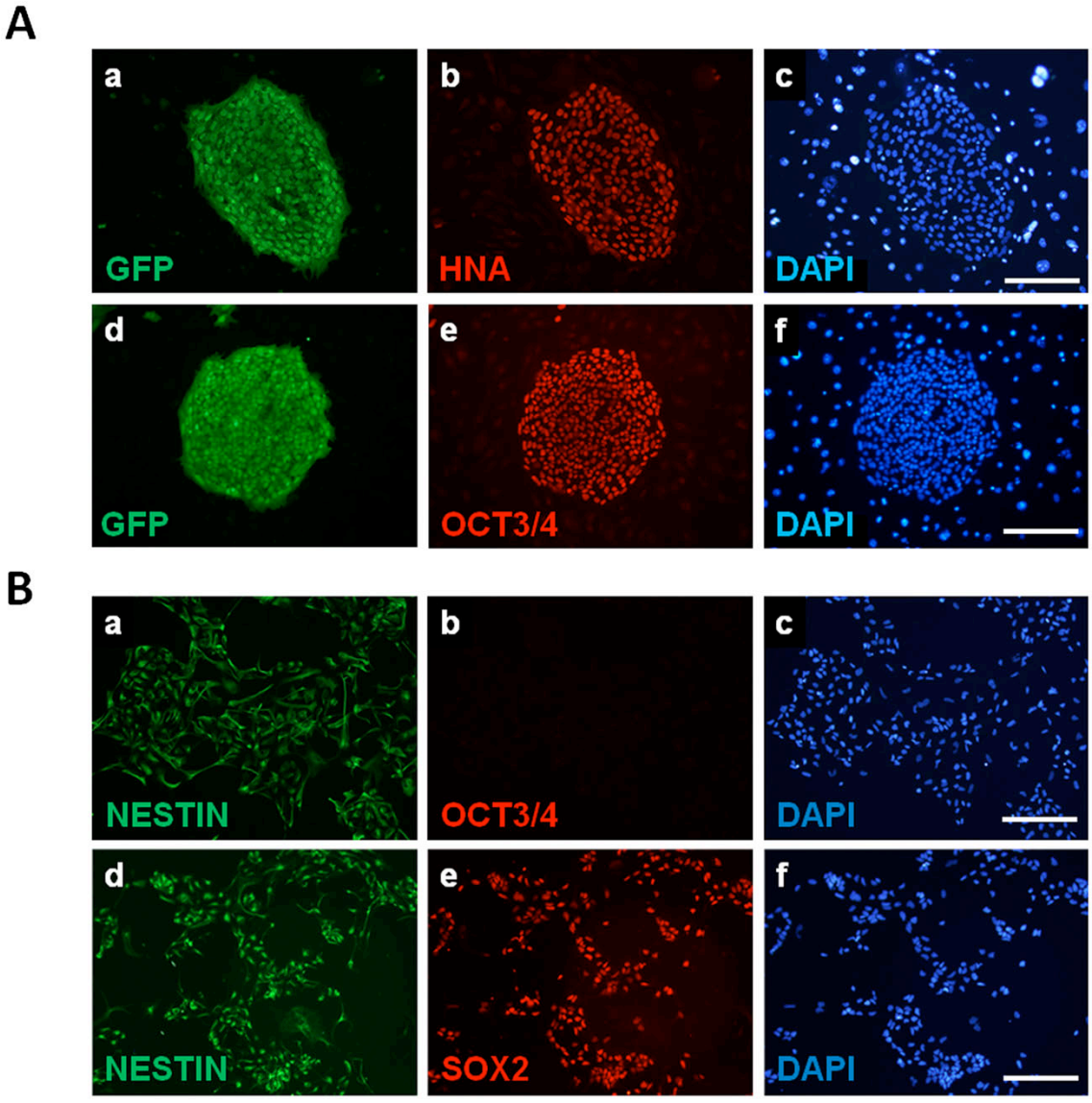

A
a
GFP
b
HNA
c
DAPI
d
GFP
e
OCT3/4
f
DAPI
B
a
NESTIN
b
OCT3/4
c
DAPI
d
NESTIN
e
SOX2
f
DAPI
C
Undifferentiated iPS cells
Induced cells from 201B7
Induced cells from 201B7-GFP
NESTIN
SOX1
PAX6
OCT3/4
GAPDH RT-
GAPDH RT+

Figure 3

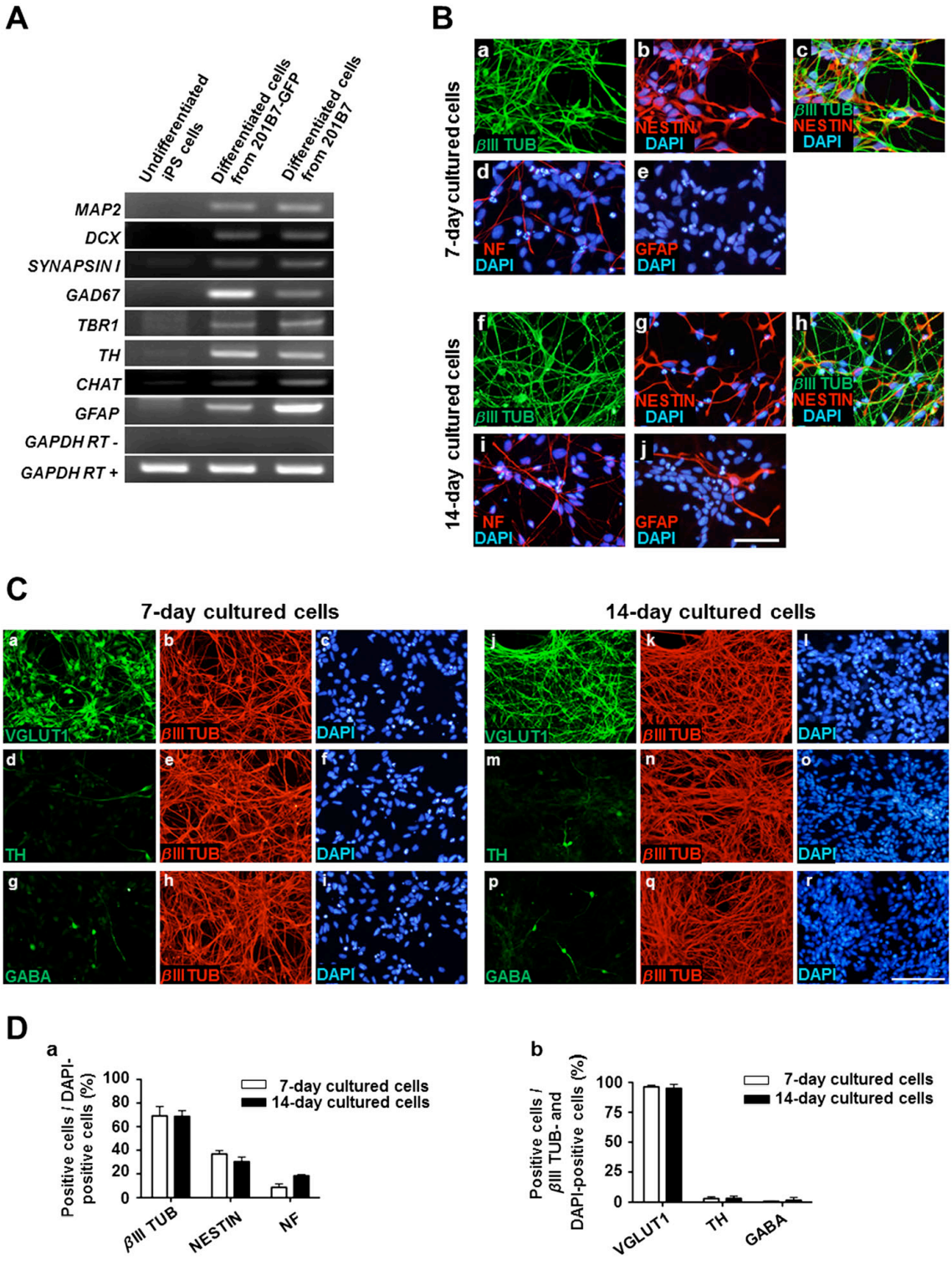

A
Undifferentiated iPS cells
Differentiated cells from 201B7-GFP
Differentiated cells from 201B7
MAP2
DCX
SYNAPSIN I
GAD67
TBR1
TH
CHAT
GFAP
GAPDH RT -
GAPDH RT +
B
7-day cultured cells
14-day cultured cells
βIII TUB
NESTIN
DAPI
NF
GFAP
C
7-day cultured cells
14-day cultured cells
VGLUT1
TH
GABA
βIII TUB
DAPI
D
a
Positive cells / DAPI-positive cells (%)
7-day cultured cells
14-day cultured cells
βIII TUB
NESTIN
NF
b
Positive cells / βIII TUB- and DAPI-positive cells (%)
VGLUT1
TH
GABA

Figure 4

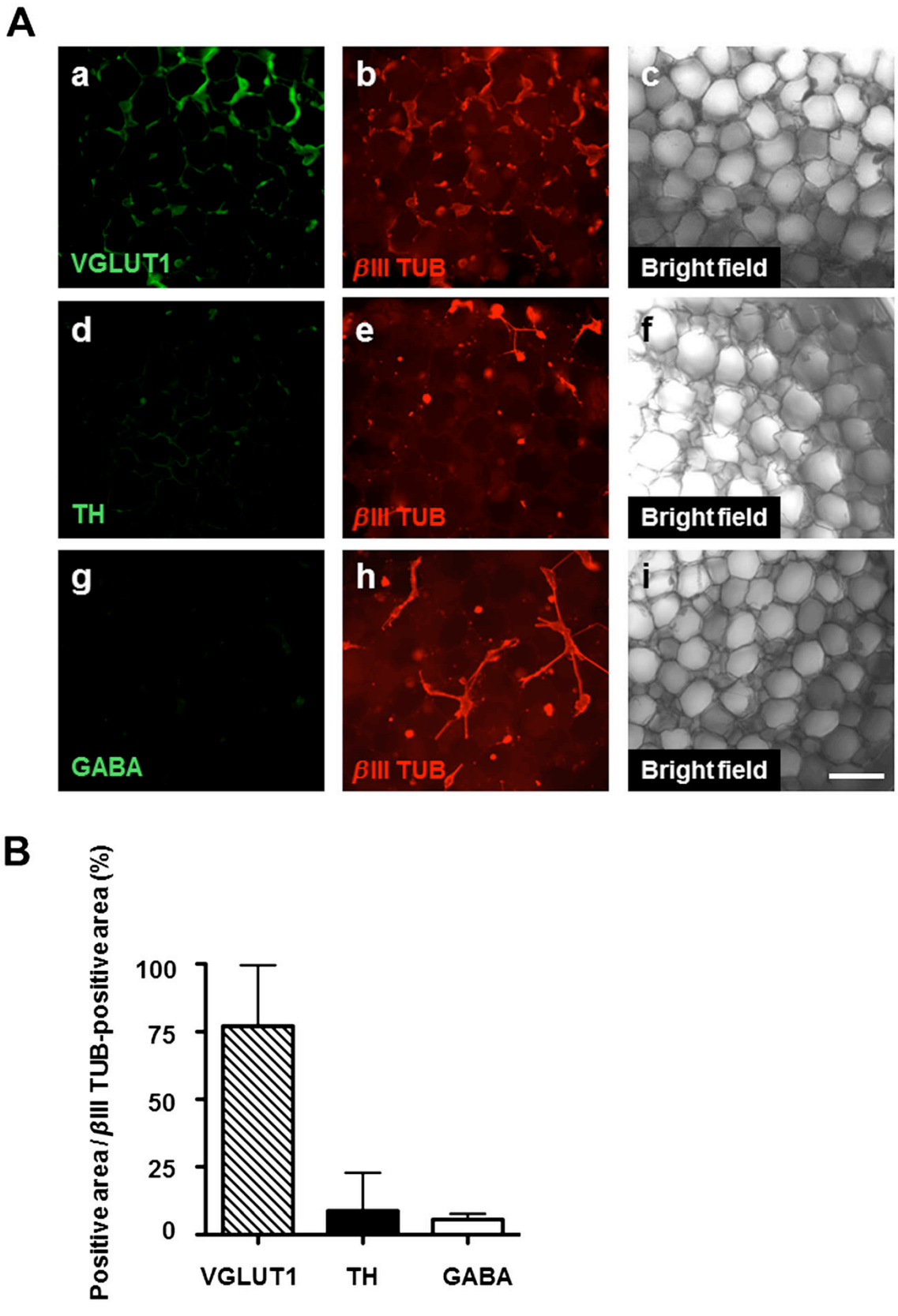

A
a
VGLUT1
b
βIII TUB
c
Bright field
d
TH
e
βIII TUB
f
Bright field
g
GABA
h
βIII TUB
i
Bright field
B
Positive area / βIII TUB-positive area (%)
100
75
50
25
0
VGLUT1
TH
GABA

Figure 5

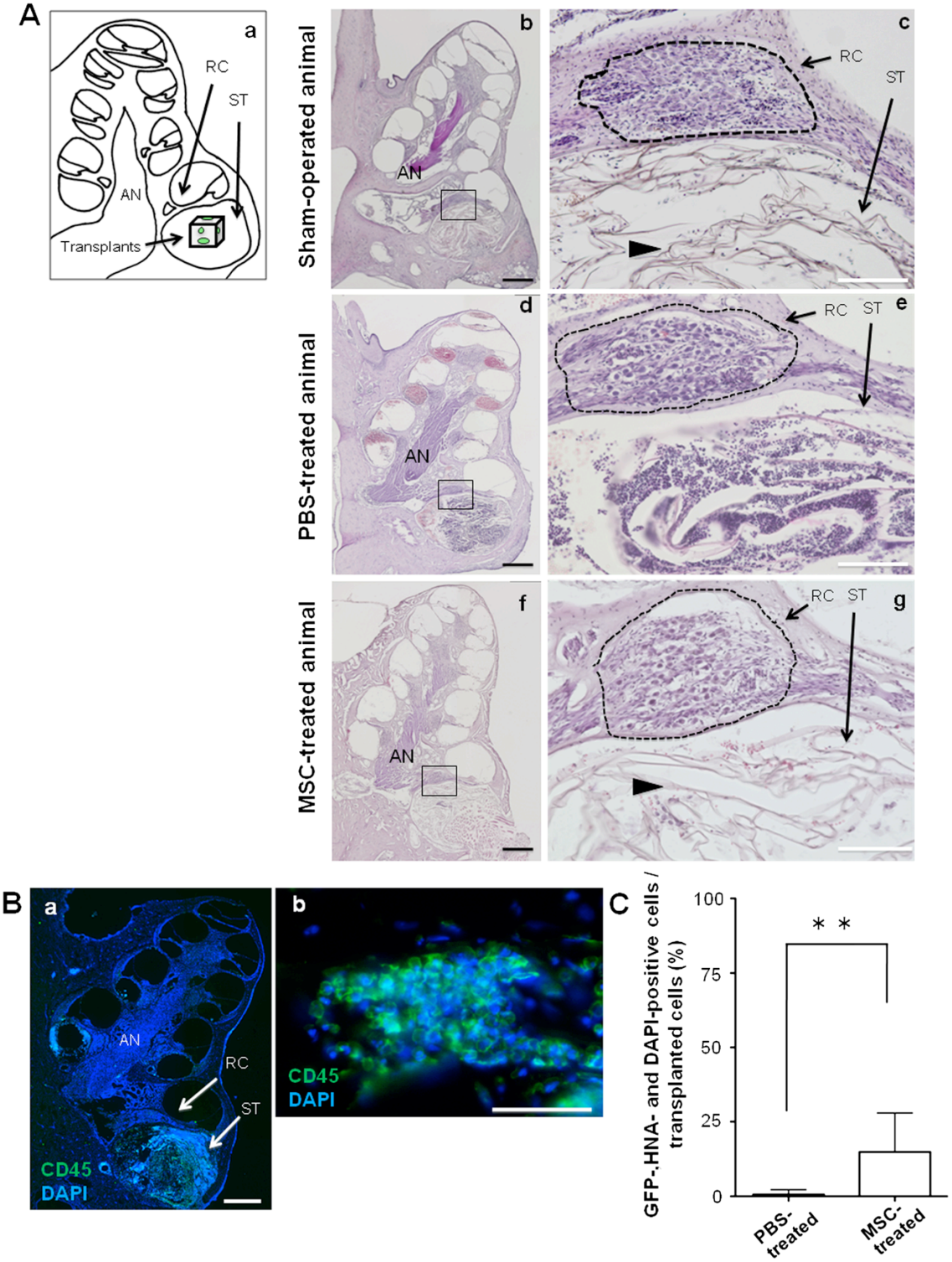
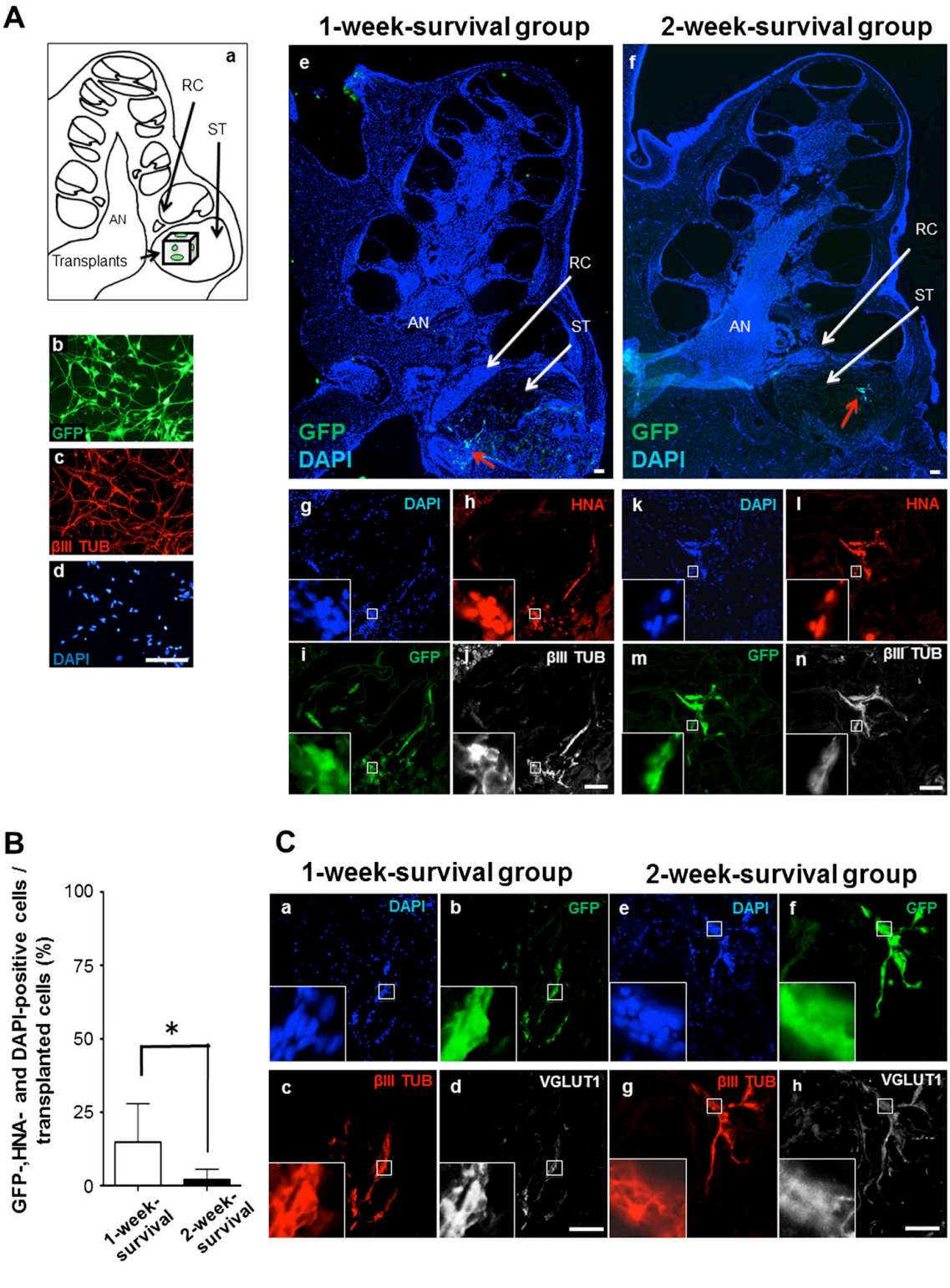

A
a
RC
ST
AN
Transplants
Sham-operated animal
b
c
AN
RC
ST
PBS-treated animal
d
e
AN
RC
ST
MSC-treated animal
f
g
AN
RC
ST
B
a
AN
RC
ST
CD45
DAPI
b
CD45
DAPI
C
GFP-,HNA- and DAPI-positive cells / transplanted cells (%)
100
75
50
25
0
* *
PBS-treated
MSC-treated

Figure 6


A
1-week-survival group
2-week-survival group
a
RC
ST
AN
Transplants
b
GFP
c
βIII TUB
d
DAPI
e
f
RC
ST
AN
GFP
DAPI
g
DAPI
h
HNA
k
DAPI
l
HNA
i
GFP
j
βIII TUB
m
GFP
n
βIII TUB
B
GFP-,HNA- and DAPI-positive cells / transplanted cells (%)
100
75
50
25
0
*
1-week-survival
2-week-survival
C
1-week-survival group
2-week-survival group
a
DAPI
b
GFP
e
DAPI
f
GFP
c
βIII TUB
d
VGLUT1
g
βIII TUB
h
VGLUT1