Neuronal Cell Sheets of Cortical Motor Neuron Phenotype Derived from Human iPSCs

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Abstract
Transplantation of stem cells that differentiate into more mature neural cells brings about functional improvement in preclinical studies of stroke. Previous transplant approaches in the diseased brain utilized injection of the cells in a cell suspension. In addition, neural stem cells were preferentially used for grafting. However, these cells had no specific relationship to the damaged tissue of stroke and brain injury patients. The injection of cells in a suspension destroyed the cell–cell interactions that are suggested to be important for promoting functional integrity of cortical motor neurons. In order to obtain suitable cell types for grafting in patients with stroke and brain damage, a protocol was modified for differentiating human induced pluripotent stem cells from cells phenotypically related to cortical motor neurons. Moreover, cell sheet technology was applied to neural cell transplantation, as maintaining the cell–cell communications is regarded important for the repair of host brain architecture. Accordingly, neuronal cell sheets that were positive Forebrain Embryonic Zinc Finger (Fez) family zinc finger 2 (FEZF2), COUP-TF-interacting protein 2, insulin-like growth factor–binding protein 4 (IGFBP4), cysteine-rich motor neuron 1 protein precursor (CRIM1), and forkhead box p2 (FOXP2) were developed. These markers are associated with cortical motoneurons that are appropriate for the transplant location in the lesions. The sheets allowed preservation of cell–cell interactions shown by synapsin1 staining after transplantation to damaged mouse brains. The sheet transplantation brought about partial structural restoration and the improvement of motor functions in hemiplegic mice. Collectively, the novel neuronal cell sheets were transplanted into damaged motor cortices; the cell sheets maintained cell–cell interactions and improved the motor functions in the hemiplegic model mice. The motoneuron cell sheets are possibly applicable for stroke patients and patients with brain damage by using patient-specific induced pluripotent stem cells.

Keywords
neural regeneration, human iPSCs, cortical motor neurons, hemiplegic mice, COUP-TF-interacting protein 2 (CTIP2), forebrain embryonic zinc finger family zinc finger 2 (Fezf2), forkhead box p2 (Foxp2)

Introduction
Transplantation of embryonic stem cells and induced pluripotent stem cells (iPSCs) differentiated into neural precursors brings about functional improvement in preclinical studies of stroke.1-4 The transplanted cells differentiate into neurons, astrocytes, and oligodendrocytes and secrete various molecules associated with functional improvement.5-7

In a majority of the studies, iPSC-derived neurons were transplanted into the diseased brain by injecting cell suspensions.8 The transplanted cells survived, differentiated, and migrated to the stroke lesion and extended axons.9-11,12-16 Behavioral

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The hiPSC lines, 201B7 and 253G1,24 were purchased from Neuronal Cell Sheets.

Materials and Methods

Induction of Neural Differentiation of hiPSCs and Neuronal Cell Sheets

The hiPSC lines, 201B7 and 253G1,24 were purchased from RIKEN BioResource Center (Ibaraki, Japan) through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. Because both cell lines demonstrated essentially the same results in this study, only the results obtained using 253G1 (reprogrammed by octamer-binding transcription factor 3/4 [Oct3/4], sex-determining region Y-box 2 (SOX2), and Kruppel-like factor 4 [KLF4]) were presented. The hiPSC lines were maintained according to the RIKEN cell preparation manual. Cells were cultured in differentiation medium consisting of Dulbecco’s modified eagle medium (DMEM)/F12 with N2 supplement (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA).

Undifferentiated hiPSCs (253G1) were maintained in a growth medium on a feeder layer consisting of mitomycin C-treated mouse embryonic fibroblasts generated from ICR mouse embryos (Japan SLC, Shizuoka, Japan), as previously described.25,26

Embryoid bodies were developed in a floating condition for 4 d (from day 0 to day 4). Then the cells were cultured in fibronectin-coated dishes (Corning Inc., Corning, NY, USA) for 4 d (from day 4 to day 8). RA of 1 μM (Sigma-Aldrich, Tokyo, Japan), 10 nM NOG (R&D Systems, Minneapolis, MN, USA), and 2.5 pm CyP (Enzo Life Sciences, Farmingdale, NY, USA) were introduced into the dishes twice on days 5 and 7. The cells were harvested on day 8, and the cell suspensions were transplanted into mice as transplantation controls (neural stem/progenitor cells).

An aliquot of the cells was cultured in 24-well culture plates for 12–16 d, which were then harvested using trypsin/ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Inc.) solution for transplantation. The remaining cells were cultured on temperature-responsive gelation polymer-coated plate (temp. resp. polymer; Upcell@, Cell-Seed, Tokyo, Japan) for 12–16 d (average 14 d) depending on the neuronal maturation, where they extended axon-like processes, leading to the formation of neuronal cell sheets. To initiate cell sheet formation, 1.0 × 10^6 cells/well were introduced in the 24-well plates, and the resulting cell sheets contained 0.4–1.0 × 10^6 cells/sheet.

The plates were transferred to another incubator, set at 22°C, for 1 h to release the cultured cells as intact cell sheets. Under this protocol, confluent neuronal cell sheets were spontaneously detached from the plates (Fig. 1B–G).

Real-time Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from cells with an RNase kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer’s instructions. Briefly, relative messenger RNA (mRNA) levels were measured by quantitative RT-PCR. TaqMan probes specific for the targeted gene as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control (Life Technologies, see for TaqMan probe IDs) were used.

Experimental Brain Injury and Transplantation

To use genetically normal mice, female C57BL/6 mice (6 to 8 weeks old; Japan SLC) were used as transplant recipients.28,29 All experimental procedures were performed in accordance with the Guide for the Care and Use of

analyses showed improved scores in the neuron-grafted mice compared with those in vehicle-injected control mice.

To generate the cell suspension, the cells were detached from the culture dishes, resulting in the loss of cell–cell interactions and possibly influencing the functional integrity of cortical motor neurons. Human iPS (hiPSC)-derived neural progenitor cells were transplanted into hemiplegic model mice and the motor dysfunction was ameliorated.10 Within several months after the transplantation, the grafted cells decreased remarkably in number. Withdrawal of immunosuppressants accelerated the intracranial cell loss.10

In addition, neural stem/progenitor cells derived from iPSCs had proliferative potential with an intrinsic risk of tumor development.17

In order to obtain suitable cell types for grafting in patients with stroke and brain damage, a protocol was modified for differentiating hiPSCs into cells phenotypically related to cortical motor neurons.18-20

Neuronal differentiation of hiPSCs was induced in a way mimicking cortical motor neuron development in embryos by using retinoic acid (RA), noggin (NOG), and cyclopa mine (CyP). RA is an efficient inducer of neural differentiation of various cell types.21 NOG is an essential protein that induces the neural fate of stem cells as a bone morphogenetic protein (BMP) inhibitor,22 and CyP plays an important role in the regulation of neural stem/progenitor cell proliferation and differentiation.23 The hiPSC-derived neurons on day 19 of in vitro culture resembled more mature cortical motor neurons of humans because they expressed forkhead box p2 (Foxp2), forebrain embryonic zinc finger family zinc finger 2 (Fezf2), and cysteine-rich motor neuron 1 protein precursor (CRIM1) simultaneously.

We hypothesized that hiPSC-derived neuronal cell sheets had enhanced neuronal differentiation and survived and functioned well after the transplantation as neuronal clusters consisting of differentiating cells. We expected that transplanted neuronal cell sheets formed a new cortical layer beyond the lesion including damaged cortex. In this study, we conducted histopathological and functional assessments in mice with injured brains after cell or sheet transplantation and compared the data between the 2 groups.
The cells on day 8 were used as neural stem cells; CRIM1 mRNA was expressed throughout the culture period. Thus, the cell sheet expressed mRNAs of cortical motoneuron–associated proteins. A white horizontal bar represents 200 μm in panel D, 100 μm in panels E and F, and 50 μm in panel G. iPSCs, undifferentiated-induced pluripotent stem cells; CRIM1, cysteine-rich motoneuron 1 protein precursor; MEF, mouse embryonic fibroblasts; floating indicates floating condition; Foxp2, forkhead box P2; CRIP1, crimp1; Isl1, islet 1; NFM, neurofilament medium; Bactin, β-actin.

**Figure 1. (continued)** Neuronal cell sheet. For comparison, neuronal cells on day 8 (neural cells were cultured in the same manner except that SHH was added instead of CyP and were harvested on day 8 before making cell sheet) were included. The cell sheet expressed Foxp2, Fezf2, Igfbp4, CTIP2, and synaptophysin mRNA predominantly. CRIP1 mRNA was expressed throughout the culture period. Thus, the cell sheet expressed mRNAs of cortical motoneuron–associated proteins. A white horizontal bar represents 200 μm in panel D, 100 μm in panels E and F, and 50 μm in panel G. iPSCs, undifferentiated-induced pluripotent stem cells; CRIP1, cysteine-rich motor neuron 1 protein precursor; MEF, mouse embryonic fibroblasts; floating indicates floating condition; Foxp2, forkhead box P2; Fezf2, forebrain embryonic zinc finger family zinc finger 2; Igfbp4, insulin-like growth factor-binding protein 4; CTIP2, COUP-TF-interacting protein 2; mRNA, messenger RNA.

**Figure 1.** In vitro characterization of neuronal cell sheet derived from human induced pluripotent stem cells (hiPSCs). (A) A schematic representation of neuronal sheet formation from hiPSCs and transplantation. Embryoid bodies were developed from undifferentiated hiPSCs in a floating condition for 4 d. Then the cells were cultured in fibronectin-coated dishes for 4 d during which retinoic acid (RA), noggin (NOG), and cycloamine (CyP; 3 factors) were introduced twice (on days 5 and 7). In some experiments, sonic hedgehog (SHH) were introduced instead of adding CyP, together with RA and NOG in order to compare the effects of CyP with those of SHH. Thereafter, the cells cultured with RA, NOG, and CyP were harvested on day 8. An aliquot of the cells was cultured in 24-well culture plates for 12 to 16 d and then harvested using trypsin/ethylenediaminetetraacetic acid (EDTA) solution for transplantation. The remaining cells were cultured on a temperature-responsive gelation polymer-coated plate (temp. resp. polymer) for 12 to 16 d depending on the neuronal maturation, where they extended axon-like processes, leading to the formation of neuronal cell sheet. The neuronal cell sheet was recovered by lowering the temperature of culture plate below 22°C. Schedule for brain injury, transplantation, and motor function test was reported previously.10,20,28,29 The cells on day 8 were used as neural stem/progenitor cells for comparison. (B) Stereomicroscopic view of the neuronal cell sheet cultured in a 48-well (diameter 12 mm) culture plate. Right lower part of the sheet detached from the bottom of the plate. (C) Stereomicroscopic view of the sheet in a 10-cm culture dish (diameter 90 mm). (D) Hematoxylin and eosin staining of the neuronal cell sheet (D, lower; E, higher magnification). Flow cytometric analysis of the cells in the sheet treated with trypsin/EDTA revealed that more than 75% of the cells expressed human NCAM (data not shown). (F, G) The neuronal cell sheet at day 19 was stained with anti-human nuclei (F) and anti-β-tubulin antibodies (G). (H) Real-time polymerase chain reaction analyses of the
Committee (Institutional Animal Care and Use Committee (IACUC)).

Motor Function Analyses: Beam Walking Test and Rotarod Test

The beam walking test allows the assessment of refined fore-limb and hind limb locomotor activity and is used to assess the recovery of the hemiplegic model of brain injury.1,6,32,33 The animals were trained to walk along a narrow wooden beam of 6 mm wide and 120 mm length, which was suspended at 300 mm above a soft pad, and the number of foot faults for the contralateral (right) hind limb was recorded over 50 steps. Foot faults were directly observed in accordance with gait disturbance and were counted. Normal mice grasped the beam completely on every foot, and the foot fault was defined as that without complete grasp of the beam. A basal level of competence at this test was established before injury with an acceptance level of <5 faults per 50 steps.

The rotarod test allows the assessment of refined motor function and coordination and is used to assess the recovery of hemiplegic model of brain injury.6,32,33 For each mouse, the rotarod unit (Muromachi, Tokyo, Japan) consists of a rotating rod of 3.5 cm diameter and an individual compartment. Infrared beams were used to check whether the mouse had fallen onto the grid beneath the rotarod. Before brain injury, mice was trained on the rotarod and a basal level of competence was established at this test, with an acceptance level of >200 s at a speed of 20 rpm. After injury, the mice were placed on the rod and then run on the rotarod at 30 rpm for the maximum of 300 s. The system logged the total time running on the rod as well as the time of the fall, and all experimental set up parameters were recorded. The mice were given 5-min intervals in order to reduce stress and fatigue. Each animal received at least 2 consecutive trials, with the longer time on the rod used for analysis.

Immunofluorescence Staining

Immunofluorescence staining was conducted as reported previously.10,25,26,28,29 The following antibodies were used: rabbit anti-neurofilament middle chain (Millipore, Billerica, MA, USA), mouse anti-βIII tubulin (Promega, Madison, WI, USA), rabbit anti-CRIM1 (Sigma-Aldrich), rat anti-CTIP2 (Abcam, Cambridge, MA, USA), anti-Fezf2 (Abcam), rabbit anti-Foxp2 (Abcam), mouse anti-human Neural cell

Figure 2. (continued). maturation of neuronal cell sheets, the sheets were stained with several antibodies. Cells in the sheets lacked protein expressions of Nanog, Oct3/4, and Pax6, suggesting their differentiation. Undifferentiated human iPSCs expressed these antigens. Cells in the sheets expressed motor neuron–associated and positional antigens extensively, such as Fezf2, CTIP2, Foxp2, and CRIM1. Foxp2, forkhead box p2; Fezf2, forebrain embryonic zinc finger family zinc finger 2; Igfbp4, insulin-like growth factor-binding protein 4; CRIM1, cysteine-rich motor neuron 1 protein precursor; Oct3/4, octamer-binding transcription factor 3/4.
Statistical Analysis

All statistical analyses were performed using JMP 8.0.2 software (SAS Institute Japan, Tokyo, Japan). Each point in Fig. 3 represented mean ± standard error of the mean (SEM) of the mouse groups. Continuous variables subjected to repeated measurements over a period of time on the beam walking and rotarod test were analyzed using a multivariate analysis of variance (MANOVA), followed by Tukey’s honestly significant difference. P value less than 0.05 was considered significant.

Results

Induction of Neural Differentiation of hiPSCs and Formation of Neuronal Cell Sheets

Cell sheets composed of motor neurons were made in a laminar array, resembling anatomy of the cortical motor area (Fig. 1). Using a combination of RA, NOG, and CyP, substantially pure mature neurons were obtained from hiPSCs. RT-PCR analyses revealed that they were COUP-TF-interacting protein 2 (CTIP2) positive and Fezf2 positive (Fig. 1H), both of which were cortical motor neuron-specific transcription factors. They expressed insulin-like growth factor –binding protein 4 (IGFBP4) mRNA (Fig. 1H).

The neurons in the cell sheet (day 21) expressed 600 times more synaptophysin mRNA than those before making cell sheet (cells at day 8) using quantitative RT-PCR with $2^{-\Delta\Delta Ct}$ method of TaqMan primers and probe (data not shown).

By immunofluorescence, these cells were almost completely positive for human nuclear antigen and $\beta$III tubulin ($\beta$-tubulin; Fig. 1F and G).

To further characterize cells in the sheets, the sheets were stained with several antibodies (Fig. 2). Cells in the sheets lacked protein expressions of Nanog, Oct3/4, and Pax6, suggesting their differentiation. Undifferentiated hiPSCs expressed these antigens. Cells in the sheets were negative for alkaline phosphatase which had been positive on the undifferentiated hiPSCs (data not shown). They expressed motor neuron associated and positional antigens extensively, corresponding to the cortical motor area, such as Fezf2, CTIP2, Foxp2, and CRIM1. They were approximately 76% Fezf2 positive, 29% CTIP2 positive, 41% Foxp2 positive, and 72% CRIM1 positive.

After transplanting neuronal cell sheet with polymer coverage, the sheets were observed to be well attached to the brain surface of injured mice (Fig. 3).

Grafted Cell Sheet Significantly Improved Motor Function in Injured Hemiplegic Mice

The cells were cultured on a temperature-dependent gelation polymer-coated plate (Upcell CellSeed). The cell sheet was recovered by lowering the temperature to provoke sheet

adhesion molecule (NCAM) (hNCAM; Santa Cruz Biotechnology, Dallas, TX, USA), antisynapsin1 (Millipore), rabbit anti-Nanog (ReproCELL, Kanagawa, Japan), mouse anti-Oct3/4 (Santa Cruz Biotechnology), rabbit anti-Pax6 (BioLegend, San Diego, CA, USA), and mouse anti-human nuclei (Abnova, Taipei City, Taiwan).
detachment without using trypsin. The cell sheet was grafted to hemiplegic mice developed as previously reported. In the beam walking test, motor performance was measured as the number of foot faults in 50 steps. Although the scores were zero in normal control mice, the scores in the mice that had right hemiplegia caused by the injury at days 2 and 5 were almost 50, namely, 100% as in Fig. 4A. Thereafter, the scores were gradually improved in the cell sheet–transplanted mice. Even using the same neuronal cells cultured for 24 d, mice with sheet transplantation improved more efficiently than mice with injection of single-cell suspension. The scores between the mice injected with single-cell suspension of the neuronal cells cultured for 24 d and those transplanted with the neural cell sheet (cultured for 24 d) were statistically significant (MANOVA, P < 0.01; Fig. 4A). However, the scores of the sheet-transplanted mice remained higher than those of intact control mice, during the observation period (Fig. 4A).

To confirm recovery of motor function in the transplanted mice, a rotarod test was conducted to measure the duration a mouse can stay on the rotating rod, reflecting refined motor function and coordination. Most efficient recovery of the duration time in the rotarod test was observed in the mice transplanted with the neuronal cell sheet as compared with those treated with single-cell suspensions of 24-d cultured neurons and with PBS injection (Fig. 4B). It appeared that preservation of cell-to-cell interaction was effectively associated with the functional recovery of the hemiplegic mice.

Difference in the duration time of rotarod test and that of the score of beam walking test between cell sheet recipient mice and mice injected with single-cell suspension of neural stem/progenitor cells (neural cells at day 8) did not show the statistical significance.

Collectively, transplantation with the neuronal cell sheet restored motor function efficiently in the hemiplegia mouse to an extent similar to that with neural stem/progenitor cells.

**Neurons Derived from hiPSCs Migrated into the Damaged Cortex from the Cell Sheet and Formed a Cell Layer of Motoneuron Phenotype**

hNCAM+ and human nuclear+ cells resided in the cell sheet 21 d after transplanation (Fig. 5A, B, D). Some of them entered the damaged cortex and showed synapsin1 expression, suggesting possible neural connections between human and mouse neurons (Fig. 5A and B). Most of them were CTIP2, Fezf2, and Foxp2 positive located at both the grafted sheet and damaged cortex (Fig. 5B–E).

It was noteworthy that the neurons with motoneuron phenotype formed a cell layer underneath the cells of nonmotoneuron phenotype (Fig. 5A, B, C, D, and F), in contrast to the wide distribution of grafts across the cortex in cell-transplanted mice (Fig. 5G).

**Discussion**

We successfully generated cortical motor neurons in the cell sheet derived from hiPSCs. The cell sheet was suggested to be functional in the mouse intracranial space and to provide neurons to the damaged cortex (Fig. 5). Some cells entered the damaged cortex and showed expression of synapsin1 and
Figure 5. Immunohistochemical analyses of mouse brains grafted with human induced pluripotent stem cells–derived neuronal cell sheet of cortical motor neuron phenotype. The expressions of neuron-associated proteins in the brain sections were analyzed 21 d after the transplantation (at day 42 in Fig. 1A). (A1–A4) Anti-human NCAM staining (green) and antisynapsin I staining (red). Human NCAM+ cells showed expression of synapsin I, suggesting neural connections between human neurons and mouse neurons. Marginal detachment of the grafted sheet had occurred during staining procedure. (B1–B4) Anti-CTIP2 staining (green) and anti-hNuc staining (red). CTIP2-positive human neurons moved to and resided in the damaged cortex. CTIP2-positive human neurons located underneath the CTIP2-negative human neurons. (B5) Higher magnification of central area of panel B4. (C1–C4) Anti-Foxp2 staining (green) and anti-hNuc staining (red). Foxp2-positive human neurons located in the damaged cortex. (C5) Higher magnification of central area of panel C4. (D1–D4) Anti-Fezf2 staining (green) and anti-hNuc staining (red). Fezf2-positive human neurons made cell layer underneath the Fezf2-negative human neurons, resembling layered structure of motoneurons. (D6) H&E staining of the same cell sheet grafted on the damaged motor cortex. (E1–E4) Anti-CTIP2 staining (green) and anti-Foxp2 staining (red). Majority of CTIP2-positive human neurons in the damaged cortex coexpressed Foxp2. The CTIP2 and Foxp2 double-positive neurons existed underneath the double negative (nonmotoneuron phenotype) 4′,6-diamidino-2-phenylindole (DAPI)-positive neurons. (F1–F4) Anti-neurofilament middle chain (NFM, green) and anti-hNuc staining (red). (G1–G4) Anti-NFM (green) and anti-hNuc staining (red) in the brain of single-cell suspension transplanted mouse. (H1–H4) Anti-NFM (green) and anti-hNuc staining (red) in the brain of PBS-injected mouse. Panels of the left vertical row are a schematic representation of each DAPI staining. A white horizontal bar in panel A4 represents 50 μm in panels A1–A4, B1–B4, and D1–D4, F1–F4, G1–G4, H1–H4; 25 μm in panels B5, C1–C4, D5, and E1–E4; and 12.5 μm in panel C5. CTIP2, COUP-TF-interacting protein 2; Foxp2, forkhead box p2; Fezf2, forebrain embryonic zinc finger family zinc finger 2; hNuc, human nuclei; H&E, hematoxylin and eosin; PBS, phosphate-buffered saline.
human CRIM1 (Fig. 5A and data not shown). It was thus suggested that the human neurons having a cortical motor neuron phenotype survived and connected with the host neural circuit to bring about motor functional improvement.17 We have clearly shown that functional recovery of mice treated with the cell sheet transplantation method far exceeded that of mice treated by injection of dissociated neural stem/progenitor cells.27 We have clearly shown that functional recovery of neural circuit to bring about motor functional improvement was achieved in a large number of mice treated by the cell sheet transplantation method. We have also observed that the human neurons having a cortical motor neuron phenotype survived and connected with the host neural circuit to bring about motor functional improvement.17 Thus, cell sheets were less likely to form tumors and existed as a purer population of neurons, without the usual contaminating cell types in progenitor cell sources. Indeed, we did not observe any tumor formation in the brains of the sheet recipients.

We found that Fezf2-positive human neurons made a cell layer underneath the Fezf2-negative human neurons, resembling a layered structure of motoneurons (Fig. 5D). A similar layered structure was observed when CTIP2 and Foxp2 antibodies were employed (Fig. 5B and E). We hardly detected a layered structure of motoneurons when a single-cell suspension of motoneurons was injected into the brain.10,20,26,28

The histological restoration of the current cell sheet transplantation is far different from the layered structure of an intact motor cortex. Nonetheless, further modification of the sheet may bring about fine structural reorganization and efficient functional improvement in the damaged motor cortex.

**Ethical Approval**

This study was approved by the local Animal Care Committee (Animal Care and Use Committee, St. Marianna University School of Medicine).

**Statement of Human and Animal Rights**

This article does not contain any studies with human subjects. All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals, 8th edition (National Research Council) and were approved by the local Animal Care Committee (Animal Care and Use Committee, St. Marianna University School of Medicine).

**Statement of Informed Consent**

There are no human subjects in this article, and informed consent is not applicable.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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