Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury

Osahiko Tsuchiab,c,1 Kyoko Miuraa,c,1 Yohei Okadaa,d KANEHIRO FUJIOSHIa,b, MASAHIKO MUKAINOa,e, NARIHITO NAGOSHIB, f, KAZUYA KITAMURAa,b, GENTARO KUMAGIA, MAKOTO NISHINOb, SHUTA TOMISATOb, HISANOBU HIGASHIB, TOSHIHIRO NAGAIB, HIROYUKI KATOHb, f, KAZUHISA KOHDAb, YUMI MATSUZAKIA, MICHISUKE YUZAKIA, EIJI IKEADB, YOSHIAKI TOYAMA b, MASAYA NAKAMURA b, 2, SHINYA YAMANAKA, and HIDEYUKI OKANOb, 2

Departments of aPhysiology and Orthopedic Surgery, School of Medicine, Keio University, Shinjuku, Tokyo 160-8582, Japan; bCenter for Induced Pluripotent Stem Cell Research and Application, Kyoto University, Kyoto 606-8507, Japan; cKanrinmaru-Project and Departments of Rehabilitation Medicine, dElectron Microscope Laboratory, and ePathology, School of Medicine, Keio University, Tokyo 160-8582, Japan; gStem Cell Research and Application, Kyoto University, Kyoto 606-8507, Japan; hElectron Microscope Laboratory, and iPathology, School of Medicine, Keio University, Tokyo 160-8582, Japan; dKanrinmaru-Project and Departments of eRehabilitation Medicine, hElectron Microscope Laboratory, and iPathology, School of Medicine, Keio University, Tokyo 160-8582, Japan; fDepartment of Orthopedic Surgery, National Hospital Organization, Murayama Medical Center, Tokyo 208-0011, Japan; gDepartment of Orthopedic Surgery, Graduate School of Medicine, Hirosaki University, Aomori 036-8560, Japan; and hDepartment of Pathology, Graduate School of Medicine, Yamaguchi University, Yamaguchi 755-8505, Japan

Edited by Fred Gage, Salk Institute, San Diego, CA, and approved June 3, 2010 (received for review September 3, 2009)

Various types of induced pluripotent stem (iPS) cells have been established by different methods, and each type exhibits different biological properties. Before iPS cell-based clinical applications can be initiated, detailed evaluations of the cells, including their differentiation potentials and tumorigenic activities in different contexts, should be investigated to establish their safety and effectiveness for cell transplantation therapies. Here we show the directed neural differentiation of murine iPS cells and examine their therapeutic potential in a mouse spinal cord injury (SCI) model. “Safe” iPS-derived neurospheres, which had been pre-evaluated as nontumorigenic by their transplantation into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse brain, produced electrophysiologically functional neurons, astrocytes, and oligodendrocytes in vitro. Furthermore, when the safe iPS-derived neurospheres were transplanted into the spinal cord 9 d after contusive injury, they differentiated into all three neural lineages without forming teratomas or other tumors. They also participated in remyelination and induced the axonal regrowth of host 5HT+ serotonergic fibers, promoting locomotor function recovery. However, the transplantation of iPS-derived neurospheres pre-evaluated as “unsafe” showed robust teratoma formation and sudden locomotor functional loss after functional recovery in the SCI model. These findings suggest that pre-evaluated safe iPS clone-derived neural stem/progenitor cells may be a promising cell source for transplantation therapy for SCI.

Given their ability to generate all types of neural cells, neural stem/progenitor cells (NS/PCs) are a promising source for cell replacement therapy for various intractable CNS disorders (reviewed in refs. 1–6). Notably, ES cells have great developmental plasticity and can be induced to become NS/PCs with specific differentiation potentials (7–11), making them a major candidate for cell replacement therapies for CNS disorders (12–16). The clinical use of ES cells is complicated, however, by ethical and immunological concerns, both of which might be overcome by using pluripotent stem cells derived directly from a patient’s own somatic cells (17).

We recently reported the establishment of induced pluripotent stem (iPS) cells from mouse fibroblasts by the retroviral introduction of four factors (Oct3/4, Sox2, Klf4, and c-Myc) with selection for Flk15 expression (18) and Nanog expression (19, 20). Compared with Flk15-selected iPS cells, Nanog-selected iPS cells more closely resembled ES cells’ gene-expression pattern and could contribute to germine-competent adult chimeras (19–21). More recently, we and others (22, 23) generated iPS cells without using c-Myc retroviruses, albeit with lower efficiency. The successful establishment of these iPS cell lines, along with initial reports showing efficacy in the therapeutic use of iPS cells in rodent models of sickle cell anemia (24) and Parkinson disease (25), led us to examine the use of iPS cells as a treatment for spinal cord injury (SCI).

A number of important issues need to be addressed before a clinical trial using iPS cells as a cell-therapy source for SCI is initiated. First, a detailed evaluation of iPS cells’ potential to generate neural cells compared with ES cells is required. Second, iPS cells are likely to carry a higher risk of tumorigenicity than ES cells, due to the inappropriate reprogramming of these somatic cells, the activation of exogenous transcription factors, or other reasons (25–27). Thus, it is essential to confirm the safety of grafted iPS-derived NS/PCs. Finally, the effectiveness of iPS-derived NS/PC transplantation as a treatment for SCI must be evaluated.

In the previous study, we pre-evaluated iPS clones for safety by transplanting iPS-derived neurospheres into the NOD/SCID mouse brain (27). Here, we show that the transplantation of neurospheres derived from safe iPS cell clones into the injured spinal cord promoted functional recovery without any tumor formation. In contrast, the transplantation of neurospheres derived from unsafe iPS cells, showing robust teratoma formation in the NOD/SCID mouse brain, also resulted in initial functional recovery, but was later followed by teratoma formation and deterioration of locomotor function. These data suggest that the evaluation of in vitro differentiation and in vivo tumorigenicity are important for identifying safe iPS clones for cell therapy, and that the NS/PCs derived from IPS cells deemed safe by such pre-evaluation are a promising source for cell therapy for SCI.

Results

Pre-Evaluated Safe MEF-iPS Cells Exhibit ES-Like Neural Differentiation Potentials in Vitro. We previously reported the neural differentiation of 36 independent murine iPS cell clones (27). The results of this study led us to classify several iPS clones as safe or unsafe
clones, according to the teratoma-forming activity of the iPSC-derived neurospheres after transplantation into the NOD/SCID mouse brain.

Here, we first performed a detailed examination of the neural differentiation potential of a safe iPSC clone, 38C2, which was established from mouse embryonic fibroblasts (MEFs) by the introduction of four factors, including c-Myc, and by the selection for Nanog expression (19, 28), and compared them with mouse ES cells (EB3) (29, 30). 38C2 iPSCs and EB3 ES cells were induced into embryoid bodies (EBs) in medium containing a low concentration of retinoic acid, then dissociated and cultured in suspension in serum-free medium with FGF-2 for 7 or 8 d to form primary neurospheres (PNS) (38C2 iPSCs/EB3 ES-PNS) (29). These PNSs were dissociated and formed secondary neurospheres (38C2 iPSC/EB3 ES-SNSs) under the same conditions (Fig. 1D). To induce further differentiation, 38C2 iPSC-SNSs were adherently cultured in the absence of FGF-2, resulting in the generation of Tuj1+ neurons after 21 d of differentiation of 38C2 iPSC cells, similar to EB3 ES cells (Fig. 1D).[16, 17].[16, 17] Such markers in the progeny of the 38C2 iPSCs showed drastic decrease of the expression of undifferentiated ES cell marker genes, such as Nanog, Eras, and Oct3/4, and the up-regulation of neural markers such as Sox1, βIII-tubulin, and GFAP during the neural differentiation of 38C2 iPSCs, similar to EB3 ES cells (Fig. 1D).

Moreover, electrophysiological analysis using whole-cell patch clamping in both the 38C2 iPSC-PNSs and EB ES-PNSs-derived neurons after 21–28 d of adherent differentiation showed tetrodotoxin (TTX; 1 μM)-sensitive repetitive action potentials in the current-clamp mode [38C2 iPSC-PNS (n = 11 of 16) and EB3 ES-PNS (n = 5 of 7)] (Fig. S24) and very rapid inward currents immediately followed by transient outward currents in voltage-clamp mode (Fig. S2B 1 and 2). Steady outward currents, similar to those mediated by delayed-rectifier K⁺ channels, were also observed (Fig. S2B 1 and D). These findings suggest that 38C2 iPSC-PNSs produced neuronal cells equipped with functional channels that could generate and modify action potentials (SI Text).

Safe MEF-iPS Cells Can Differentiate into Trilineage Neural Cells in the Injured Spinal Cord Without Tumorigenesis. Previously, we confirmed that SNSs from the safe 38C2 MEF-iPS cell clone survived and showed no teratoma-forming activity in the NOD/SCID mouse brain for 24 wk after transplantation (27) (Fig. S3). 38C2 iPSC-SNSs that were transplanted into the intact spinal cord survived and differentiated into trilineage neural cells without any tumorigenesis (Fig. S4). Next, to evaluate their therapeutic effects in the mouse SCI model, we transplanted 38C2 iPSC-SNSs into the contused spinal cord 9 d after injury and compared them with EB3 ES-SNSs, using adult fibroblasts and PBS as controls. We also made a comparison with 38C2 iPSC-PNSs, because we recently confirmed that the transplantation of ES cell-derived SNSs, but not PNSs, provides therapeutic benefit after SCI (31). We transplanted 38C2 iPSC-SNSs that had been prelabelled by lentivirus to express both CBRluc and mRFP (32, 33) into the lesion epicenter 9 d after the injury. Bioluminescence imaging (BLI) analysis (34), which detects luciferase photon signals only from living cells, revealed an approximate graft survival rate of 18% at 35 d after transplantation (Fig. 2A). We also histologically confirmed that the grafted cells survived and exhibited no apparent evidence of tumorigenesis (Fig. 2B), and that there were no Nanog⁺ cells (Fig. S5), at least during our observation period.

Fig. 1. Neural differentiation of pre-evaluated safe MEF-iPS cells in vitro. (A) Neurospheres derived from EB3 ES cells and 38C2 iPSC cells. (Scale bar: 200 μm.) (B) Immunocytochemical analysis of neural cell marker proteins in the differentiated SNSs derived from EB3 ES and 38C2 iPSC cells. (Scale bar: 100 μm.) (C) Neural differentiation efficiencies of neurospheres derived from EB3 ES and 38C2 iPSCs. (n = 5, n.s.) (D) RT-PCR analysis of undifferentiated cells (Un.), EBs, PNSs, SNSs, differentiated PNSs (PNS diff.), and SNSs (SNS diff.) of the EB3-ES and 38C2 iPSCs.
period. Graded RFP+ cells were located mainly around the lesion epicenter, whereas some cells had migrated as far as 4 mm rostral and caudal to the graft site (Fig. 2C). In the injured spinal cord, the grafted 38C2 iPS-SNSs differentiated into three types of neural cells, including Hu+ neurons (31.4 ± 1.1%), GFAP+ astrocytes (49.3 ± 4.5%), and n-GST+ oligodendrocytes (14.4 ± 3.0%), whereas 38C2 iPS-PNSs differentiated dominantly into neurons—that is, Hu+ neurons (50.4 ± 3.8%), GFAP+ astrocytes (14.9 ± 0.6%), and n-GST+ oligodendrocytes (4.6 ± 1.8%) (Fig. 2D and E and Fig. S6).

Transplantation of SNSs Derived from Safe MEF-iPS Cells into the Injured Spinal Cord Promotes Functional Recovery. The contusive SCI initially caused complete paralysis, followed by gradual recovery that reached a plateau. There were statistically significant differences in Basso mouse scale (BMS) between the 38C2 iPS-SNS and PBS groups at 21, 28, 35, and 42 d after injury, whereas no significant difference was observed between the 38C2 iPS-SNS and EB3 ES-SNS groups. Forty-two days after injury, the 38C2 iPS-SNS–grafted animals could lift their trunks and had significantly better BMS than the PBS control or adult fibroblast-treated animals, which were unable to support their body weight with their hindlimbs (Fig. 3A). To reveal the potential mechanism of functional recovery after 38C2 iPS-SNS transplantation, we conducted further histological analyses. By Luxol Fast Blue (LFB) staining, 38C2 iPS-SNS–grafted mice showed a significantly larger myelinated area at the lesion epicenter than the PBS control mice at 42 d after injury (Fig. 3B). We also found that grafted 38C2 iPS-SNS–derived cells myelinated NF200+ host neuronal fibers, confirmed by the positive staining of RFP and myelin basic protein (MBP; Fig. 3C), indicating that graft cell-derived oligodendrocytes were capable of remyelination. For further confirmation of the myelinating ability of 38C2 iPS-SNSs, we transplanted 38C2 iPS-SNSs into the injured spinal cord of MBP-null shiverer mice, a severely hypomyelinating mutant mouse that lacks the major dense line of CNS myelin (35). Myelinating potential of the grafted 38C2 iPS-SNSs–derived cells was confirmed, exhibiting MBP+ deposits (Fig. 3D) and the major dense line, by electron microscopic analysis (Fig. 3E).

To determine the effect of the grafted 38C2 iPS-SNSs on serotonergic nerve fibers, which are important for the motor functional recovery of hind limbs (36, 37), we immunostained for 5HT and quantified the positive area at the distal cord 1, 2, and 6 wk after injury. Some of the nerve fibers associated with graft cell-derived Hu+ neurons were identified as 5HT+ serotonergic fibers, and were prominent at the distal cord compared with the PBS control group (Fig. 4A–C). Quantitative analysis of the serotonergic innervation of the distal cord revealed a significant difference between the 38C2 iPS-SNS and PBS control groups (Fig. 4B). The contusive injury (60 kDyn) resulted in a significant decrease in the number of 5HT+ fibers at the distal cord, followed by a slight recovery, which is the nature of contusive SCI. The injection of PBS in the PBS control group did not induce any additional increase in the number of 5HT+ fibers at the distal cord. In contrast, innervation of the distal cord by these 5HT fibers was enhanced by the grafted 38C2 iPS-SNS 6 wk after SCI (Fig. 4B). Moreover, 38C2 iPS-SNS–derived astrocytes, which exhibited a bipolar morphology with long processes, were observed closely associated with the 5HT+ serotonergic fibers (Fig. 4D).

Transplantation of Neurospheres Derived from Pre-Evaluated Safe or Unsafe TTF-iPS Cells into the Injured Spinal Cord. Toward the goal of clinical application, we next examined the therapeutic potential...
of adult tissue-derived iPS cells. Among six TTF-iPS clones pre-evaluated in our previous study (27), we used the safe 335D1 TTF-iPS clone, which was generated with Nanog selection and without the transduction of c-Myc. We also used the unsafe 256H13 and 256H18 TTF-iPS clones (22, 27), which were generated without genetic selection or the transduction of c-Myc, and were originally established from CAG-EGFP mice (22). A subclone of RF8 ES cells carrying the Nanog-EGFP reporter (1A2) (19) was used as control. All of the TTF-iPS clones formed PNSs and SNSs (Fig. 5A), and generated cells of all three neural lineages, similar to those derived from 1A2 ES cells (Fig. 5B). We transplanted these TTF-iPS-derived SNSs into injured spinal cords 9 d after injury. Transplantation of the safe 335D1 iPS-SNS (prelabeled with RFP lentivirally) resulted in better functional recovery compared with the PBS control group, without any apparent tumorigenesis during our observation period (Fig. 5C and D). Grafted and survived RFP+ 335D1 iPS-SNS-derived cells could differentiate into neural lineages (Fig. S7A and B). Furthermore, LFB staining revealed that 335D1 iPS-SNS-grafted mice had a significantly larger myelinated area at the lesion epicenter than the PBS control mice at 42 d after injury (Fig. S8A and B), and grafted RFP+ 335D1 iPS-SNS-derived cells differentiated into MBP+ oligodendrocytes (Fig. S8C). However, all unsafe 256H18 iPS-SNS-grafted mice and one of 256H13 iPS-SNS-grafted mice formed teratomas containing EGFP+ donor cells within the injured spinal cord (Fig. 5E and F and Fig. S7C). Histological analyses revealed that these teratomas contained epithelial and smooth muscle tissue (Fig. S9A′), and also exhibited Nanog immunoreactivity (Fig. 5G). Although the motor functions gradually recovered in both groups to the same extent as in the safe 335D1 iPS-SNS recipients until 35 d after injury, the 256H18 iPS-SNS-grafted animals exhibited a sudden deterioration of motor function 42 d after injury. In contrast, the 256H13 iPS-SNS–grafted animals maintained their functional recovery at 42 d after injury (Fig. 5C). Notably, in most mice of the 256H13 iPS-SNS group, scattered small clusters of Nanog+ cells were observed in the spinal cords without obvious teratoma formation (Fig. S9B and C). Thus, we speculate that teratoma formation and subsequent deterioration of function would occur in the 256H13 group if a longer observation period was set.

Discussion
In the present study, we showed that the pre-evaluated safe iPS cells could produce neurospheres containing NS/PCs (Fig. 1A) that give rise to trilineage neural cells, including several types of neurons (Fig. 1B and C), and that the neurons were electrophysiologically functional in vitro similar to ES cells (Fig. S2). Based on these safety assessments and in vitro findings, we performed an in vivo study using the safe 38C2 MEF-iPS cell clone. Grafted 38C2 iPS-SNSs differentiated into neurons, astrocytes, and oligodendrocytes without forming teratomas or other tumors, and promoted functional recovery after SCI, whereas 38C2 iPS-PNSs did not show any therapeutic effects (Fig. 3A). These findings were compatible with our recent data on mouse ES cell-derived neurosphere transplantation into an identical mouse SCI model (31). Transplantation of ES-derived SNSs, which can differentiate into neural trilineages, promoted remyelination, axonal regrowth and tissue sparing, leading to improved function. In contrast, predominantly neurogenic PNSs showed no therapeutic effects on SCI (31). Thus, we elected to use iPS-SNSs and not iPS-PNSs for this study. In fact, the grafted 38C2 iPS-SNSs formed MBP+ myelin sheaths within the injured spinal cord. We also confirmed the myelination potential of 38C2 iPS-SNS–derived cells in the spinal cord of the MBP-null shiverer mouse by electron microscopy (Fig. 3D).
astrocytes derived from cells grafted into the injured spinal cord. These progeny of iPS cells. These needed to exclude undifferentiated cells from the differentiated virus-free (43) and transgene-free (44) systems, a new strategy is establishing iPS cells are constantly being developed, including important hurdles must still be overcome. Though new methods for functional recovery after SCI without teratoma formation, like the SNSs derived from a pre-evaluated safe TTF-iPS clone promoted protection, axon sprouting and remyelination) and other effects, functional recovery (39, 40). The tissue sparing (e.g., neuro- and brain-derived neurotrophic factor (BDNF), were expressed in iPS-SNSs highly correlated with teratoma-forming propensity, (27). In fact, we recently observed grafted 38C2 iPS-SNS derived glial cells probably contributed to locomotor function recovery.

For clinical applications, the findings with TTF-iPS cells were promising, as most SCI patients are adults. The transplantation of SNSs derived from a pre-evaluated safe TTF-iPS cell promoted functional recovery after SCI without teratoma formation, like the SNSs from safe MEF-iPS clone did (Fig. 5D). However, the transplantation of SNSs derived from the unsafe TTF-iPS cells resulted in teratoma formation and functional deterioration. The teratoma-forming activity of TTF-iPS-SNSs could be caused by the presence of undifferentiated cells that might be resistant to differentiation signals within the SNSs (27). In fact, we recently reported that persistent presence of undifferentiated cells within iPS-SNSs highly correlated with teratoma-forming propensity, assayed by flow cytometric analysis using Nanog-EGFP reporter and transplanation into the brains of immunodefferent (NOD/SCID) mice (27). Further purification of the SNSs can be used clinically, though new methods for establishing iPS cells are constantly being developed, including virus-free (43) and transgene-free (44) systems, a new strategy is needed to exclude undifferentiated cells from the differentiated progeny of iPS cells. These findings show that the pre-evaluation of iPS cells’ in vitro differentiation potential could play a critical role in terms of their safety and therapeutic effects on the mouse SCI model. Thus, iPS-derived neursphere transplantation has potential therapeutic use in SCI, when the iPS cell clones are carefully pre-evaluated.

From a clinical viewpoint, it is particularly encouraging that delaying the iPS-derived NS/PC transplantation (to 9 d after injury) enhanced both the survival of the grafted cells and functional recovery, the therapeutic effects of which is almost comparable to those of fetal CNS-derived NS/PCs transplantation (refs. 34 and 45). This finding may also be applicable to the treatment of patients with SCI. Since our first report of iPS cells (18), there has been increasing interest in their characteristics and therapeutic potential. Our present study demonstrates the therapeutic potential of iPS-derived NS/PCs for SCI repair. Before any clinical trial of human CNS disorders using iPS cells, it will be essential to pre-evaluate each iPS cell clone carefully to guarantee a safety level equal to other types of cells, such as Schwann cells (46, 47) and fetal-derived neurosphere cells (NS/PCs) (3), and to conduct preclinical transplantation studies using appropriate primate models (48, 49).

**Methods**

Reverse-Transcription and RT-PCR. RNA was isolated with TRIzol (Invitrogen) according to the manufacturer’s instructions. Total RNA (0.5 μg) was treated with TURBO DNase (Ambion) and then reverse-transcribed with oligo (dt) primer and SuperScript III (Invitrogen). The primers and PCR conditions used in this study are listed in Table S1.

Cell Culture, Neural Induction, and Immunocytochemistry. Mouse ES and iPS cells were cultured as described previously (19, 28, 29). Mouse ES and iPS cells were differentiated into neurospheres in EBs treated with 10⁻⁸ M retinoic acid (Sigma), as described previously with minor modification (28, 29). (Detailed differentiation protocol is described in SI Text) ES and iPS cell-derived neurospheres were dissociated and differentiated on poly-L-ornithine/fibronectin-coated coverslips for 5 d and subjected to immunocytochemical analysis. The number of cells immunoreactive for each marker was counted and shown as the percentage of the total number of cells counterstained with Hoechst 33258. The antibodies used in this study are listed in Table S2.

**Lentivirus Production and Infection of Secondary Neurospheres.** For BLI tracing of grafted 38C2 iPS-SNSs, we generated a modified lentivirus vector encoding both the click beetle red luciferase (C8Rluc; Promega) and mRFP, pC2i-EF-C8Rluc-iRES2-mRFP (32, 33). For lentivirus preparation, HEK-293T cells were transfected with pC2i-EF-C8Rluc-iRES2-mRFP, pCAG-HIVgP, and pCMV-HSV-G-RSV-Rev, and the conditioned medium containing virus particles was concentrated and used for viral transduction.

**Spinal Cord Injury Model and Transplantation.** Adult female C5BL/6J mice (20–22 g) were anesthetized via an i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). A contusive spinal cord injury using an In Vivo Impactor (60 kdy; Precision Systems) was induced at the Th10 level as reported previously (34). For transplantation, 5 × 10⁵ cells of mouse ES/iPS cell-derived neurospheres, adult dermal fibroblasts in 2 μL of cell suspension, or PBS was injected into the lesion epicenter. Hindlimb motor function was evaluated by the locomotor rating of the Basso mouse scale (BMS) (50) for 42 d after injury. For BLI tracing of intact and injured spinal cords after the transplantation, a XenogenIVIS 100 cooled CCD optical macroscopic imaging system (SC Bioscience) was used for BLI, as reported previously (34) (SI Text). All procedures were approved by the ethics committee of Keio University, and were in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). Grafted animals were deeply anesthetized and intracardially perfused with 4% paraformaldehyde (PFA; pH 7.4). The dissected spinal cords were sectioned into 20-μm axial/sagittal sections using a cryostat and processed for histological analyses. Detailed conditions for histological analyses are described in SI Text.

**Statistical Analysis.** All data are reported as the mean ± SEM. An unpaired two-tailed Student’s t-test was used for the analyses of in vitro and in vivo 38C2 iPS-SNS and ES-SNS differentiation efficiency (Figs. 1C and 2E), SHT+ areas (Fig. 4B), and LFB+ areas (Fig. 2B). Repeated-measures two-way ANOVA, followed by the Tukey–Kramer test, was used for BMS analysis. *P < 0.05, **P < 0.01.

**Acknowledgments.** We thank Drs. H. Abe, T. Sunabolr, F. Renault-Mihara, W. Akamatu, S. Shibata, T. Harada, S. Miyao, and H. J. Okano (Keio University) for technical assistance and scientific discussions, and all the members of Dr. Okano’s and Dr. Yamanaka’s laboratories for encouragement and generous support. We also thank Drs. K. Okita, M. Koyanagi, and K. Tanabe (Kyoto University) for the undifferentiated iPSCs, Dr. H. Niwa (Riken CDB) for the ES/iPS cells, Dr. R. Farese (University of California-San Francisco) for the shiverer mice, and Dr. H. Miyoshi (Riken BRC) for the lentiviral vectors. We especially thank Drs. S. Okada (Kyusyu University), A. Iwanami (University of California-San Francisco and Keio University), and J. Yamane (Keio University) for scientific discussions, technical advice, and encouragement. This work was supported by grants from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), a grant from Uehara Memorial Foundation, and Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) and the Ministry of Education, Culture, Sports, Science and Technology (MEXT), the project for realization of regenerative medicine and support for the core institutes for iPS cell research from MEXT; Japan Science and Technology Agency (SORST); the Ministry of Health, Labor, and Welfare; the General Insurance Association of Japan; Research Fellowship for Young Scientists from the Japan Society for the Promotion of Science; Keio Gijuku Academic Development Funds; and a Grant-in-aid for the Global COE program from MEXT to Keio University.
Supporting Information

Tsuji et al. 10.1073/pnas.0910106107

SI Text

Electrophysiological Analysis of Safe MEF-iPS–Derived Neurons. We examined the electrophysiological properties of the 38C2 iPS-derived neurons. After 21–28 d of differentiation, 38C2 iPS-PNS– and EB3 ES-PNS–derived neurons were examined electrophysiologically using whole-cell patch clamping. In the current-clamp mode, depolarizing current injections triggered repetitive action potentials in both the 38C2 PNS– (n = 11 of 16) and EB3 ES-PNS– (n = 5 of 7) derived neurons. These action potentials were completely blocked by tetrodotoxin (TTX; 1 μM; Fig. S1A), indicating that they were mediated by voltage-gated Na+ channels. In voltage clamp at −60 mV, when step voltage commands were applied to 38C2 PNS-derived neurons, very rapid inward currents were observed from around −40 mV (Fig. S1 B-1 and B-2). The transient inward currents were immediately followed by transient outward currents (Fig. S1B-1), observed from about −20 mV. These K+ channel-like transient currents may be responsible for the reduction in the amplitudes of the all-or-none Na+ currents (Fig. S1C). Steady outward currents, similar to those mediated by delayed-rectifier K+ channels, were also observed (Fig. S1 B-1 and D). Although we have not yet identified the channels that were responsible for these currents, our findings showed that the 38C2 PNSs produced neuronal cells equipped with functional channels that could generate and modify action potentials.

SI Materials and Methods

Cell Culture and Neural Induction. To culture PNSs, day 6 EBs were dissociated and cultured in suspension at 5 × 10^5 cells/mL in media hormone mix (MHM) supplemented with B27 and 20 ng/mL FGF-2 (Peprotech) for 7–8 d (1, 2). To culture SNSs, PNSs were dissociated and cultured in the same culture medium for 7–8 d. For further differentiation, neurospheres were plated onto poly-l-ornithine/bronectin-coated coverslips and cultured without FGF-2 for 5 d. To examine the differentiation efficiency, neurospheres dissociated by TrypLE Select (Invitrogen) were plated onto poly-l-ornithine/bronectin-coated coverslips at 5 × 10^4 cells/cm², cultured without FGF-2 for 5 d, and processed for immunocytochemistry as described previously (1, 2).

Lentivirus Production and Infection of Secondary Neurospheres. High-titer, concentrated stocks prepared by ultracentrifugation and resuspension in Dulbecco’s PBS (2.68 mM KCl, 1.47 mM KH2PO4, 136.89 mM NaCl, and 8.1 mM Na2HPO4) were added to the culture medium in which SNSs were formed from EB3 ES, 38C2 PNSs (multiplicity of infection, MOI = 1.0). To prelabel the grafted cells in the case of 335D1 TTF-iPS-SNSs, we used lentivirus to transduce mRFP into 335D1 PNSs by modified lentivirus vectors pCSH-EF-mRFP (3).

Electrophysiology. For electrophysiological analysis, EB3 ES or 38C2 PNSs were plated on poly-l-ornithine/bronectin-coated coverslips and allowed to differentiate for 21–28 d. The cells with a neuron-like morphology were identified under an inverted microscope (Diaphot-TMD 200; Nikon), and whole-cell patch clamp recording was performed at room temperature using an Axopatch 200B (Axon Instruments). The cultured cells were continuously perfused with an external solution containing (in mM) 150 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 10 r-glucose, and 10 Hepes (pH 7.4 adjusted with NaOH, 310 mOsm). Patch pipettes had a resistance of 10–15 MΩ when filled with pipette solution composed of (in mM) 130 K-glucurate, 1 CaCl2, 1 MgCl2, 10 EGTA, 10 sucrose, and 10 Hepes (pH 7.4 adjusted with KOH, 280 mOsm). Capacitance and series resistance were compensated, and the liquid junction potential was corrected. Cultured cells were held at −60 mV, and voltage steps were applied (10 mV, 150 ms) from −90 mV to 40 mV to elicit voltage-activated currents. The peak amplitudes of the inward currents and the steady-state current amplitudes were plotted against the step-voltage commands. Action potentials were evoked by injecting step currents (25 pA, 150 ms) in the current-clamp mode. TTX (Sankyo Co. Ltd.) was added to the external solution to block action potentials. Signals were filtered at 5 kHz and digitized at 20 kHz (Digidata 1200; Axon Instruments).

Transplantation into Intact and Injured Spinal Cord. The transplantation of lentivirally transduced iPSC- and ES-SNSs was performed using a glass micropipette configured to a stereotaxic injector (KDS 310; Muromachi Kikai). Transplantation into the intact spinal cord of C57BL/6 female mice was conducted after laminectomy without the induction of spinal cord injury (Fig. S3; 38C2 SNS, n = 2; EB3 SNS, n = 2). For transplantation into the injured spinal cord, the injured site was again exposed 9 d after the injury, and 5 × 10^5 cells of 38C2 PNSs/SNSs; 335D1, 256H113, or 256H15 iPSC-SNSs; EB3 or 1A2 ES-SNSs; or adult dural fibroblasts in 2 μL of cell suspension were transplanted into the lesion epicenter at a rate of 0.5 μL/min. In the vehicle-control group, PBS was injected instead of SNSs into the lesion site 9 d after the injury.

Bioluminescent Imaging. A Xenogen-IVIS 100 cooled CCD optical macroscopic imaging system (SC BioScience) was used for BLI, as reported previously (4). To quantify the photon counts, we defined a ROI over the cell-implanted area and examined the same ROI in all of the animals. The obtained photon count intensity was expressed as a percentage of the initial value.

Histological Analyses. Grafted animals were deeply anesthetized and intracardially perfused with 4% paraformaldehyde (pH 7.4). The dissected spinal cords were postfixed for 3 h in 4% PFA, soaked overnight in 10% followed by 30% sucrose, embedded in optimal cutting temperature (OCT) compound, and sectioned into 20-μm axial/sagittal sections using a cryostat. Hematoxylin and eosin (H&E) staining was carried out for general histological examination. To examine the distribution of 38C2 SNSs grafted into intact or injured spinal cords, the sections were immunostained with an anti-RFP antibody and visualized with diaminobenzidine (DAB) solution. The phenotypes of the grafted cells were assessed by fluorescent double-immunostaining with antibodies against RFP and one of the cell-type-specific markers listed in Table S2. Images were obtained by fluorescence microscopy (Axioskop 2 plus; Carl Zeiss, and BZ-9000; KEYENCE Co.) or confocal microscopy (LSM510; Carl Zeiss). To quantify the proportion of each phenotype in vivo, we selected five representative mid sagittal sections and captured five regions within 500-μm rostral and caudal to the lesion epicenter randomly at 200x magnification. RFP-positive engrafted cells as well as each phenotypic marker-positive cells were counted in each section. To quantify the 5HT+ fibers after 38C2 SNS transplantation, we selected five representative axial sections from each animal (38C2 SNS group, n = 3; PBS control group, n = 3), randomly captured ten regions in each axial section 4 mm caudal to the epicenter at 200x magnification, and quantified the total 5HT+ area using the MCID system equipped with a CCD camera (DXC-390; Sony).

Transplantation into the Brain of NOD/SCID Mice. The transplantation of neurospheres lentivirally transduced with Venus (pCSH-EF-MCS-1RES2-Venus) (3) was performed using a glass micropipette.

Tsuji et al. www.pnas.org/cgi/content/short/0910106107 1 of 8
fitted to a stereotaxic injector as described previously (5). The tip of the micropipette was inserted into the right striatum (2 mm lateral, 1 mm rostral to bregma; depth, 3 mm from dura) of female NOD/SCID mice, and 3 μL of 38C2 SNS cell suspension (2 × 10⁵ cells) was injected. Twenty-four weeks after transplantation, the treated mice were cardially perfused, processed for immunohistochemical analysis, and analyzed by confocal laser scanning microscopy (LSM 510 META; Carl Zeiss). To distinguish Venus from EGFP, the LSM 510 META system was used.

**Preparation of Mouse Whole-Dermal Fibroblasts.** The whole dermis was collected from adult female C57BL/6J mice and cultured in MF-start medium (Toyobo). After lentiviral transduction with Venus (3), these cells were used for transplantation.


![Fig. S1. Neural differentiation of pre-evaluated safe MEF-iPS cells in vitro. Immunocytochemical analyses of tyrosine hydroxylase (TH), 5-hydroxytryptamine (5HT), and glutamic acid decarboxylase 67 (GAD67) proteins in the differentiated PNSs derived from EB3 ES and 38C2 iPS cells. (Scale bar: 100 μm.)](./image.png)
Fig. S2. Action potentials evoked in safe 38C2 iPS-derived neurons. (A) Positive step-current injections (25 pA, 150 ms) evoked repetitive TTX-sensitive action potentials in both 38C2 iPS- and EB3 ES-derived neurons by current clamp analyses. (B) Representative voltage-activated currents obtained from 38C2 iPS-derived neurons by voltage clamp analyses. (B-1) Currents observed when voltage steps (10 mV, 150 ms) were applied from −90 mV to 40 mV. (B-2) Na⁺ currents observed during the initial 15 msec of the voltage steps. (C) I–V relationship for the peaks of the inward Na⁺ currents in 38C2 iPS-derived neurons. (D) The steady-state current amplitudes plotted as the I–V relationship for K⁺ currents in 38C2 iPS-derived neurons.
**Fig. S3.** No evidence of tumorigenesis in the brain of NOD/SCID mice 24 wk after the transplantation of safe 38C2 SNSs. (A) EGFP expression examined by META analysis. No Nanog EGFP+ cell was detected. (B) Venus+-grafted 38C2 SNSs survived. (C) Merged image of A and B. (D and E) H&E staining of NOD/SCID mouse brains 24 wk after 38C2 SNS transplantation. No sign of tumorigenesis was observed (E is magnified image of D). (Scale bar: A, 100 μm; D and E, 250 μm.) Arrowheads indicate graft sites.

**Fig. S4.** Safe MEF-iPS-SNSs transplanted into intact spinal cord survive without any evidence of tumorigenesis and differentiate into three neural lineages. (A) Representative BLI images of a mouse transplanted with CBR/luc-expressing 38C2 SNSs. (Left) Immediately after transplantation. (Right) Forty-two days after transplantation. (B) Time course of BLI photon intensity showing the viability of 38C2 SNSs transplanted into the intact spinal cord. There was a drastic reduction in signal intensity within the first 7 d after transplantation, which was followed by a relatively stable bioluminescent signal for the following 42 d. Values are means ± SEM (n = 2). (C) H&E and (D) anti-RFP diaminobenzidine staining of sagittal section of the spinal cord 42 d after transplantation. There was no evidence of tumorigenesis (C). Higher-magnification images of the boxed areas show the injection epicenter (C-1), and the white matter caudal to the injection site (C-2). There existed the grafted RFP+ cell around the injection site (D). Higher-magnification images of the boxed areas reveal RFP+ grafted cells in both the gray (D-1) and white matter (D-2) with no evidence of tumorigenesis. *Graft site. (E) Immunohistochemical analyses of the 38C2 SNSs grafted into the intact spinal cord 42 d after transplantation show RFP+ grafted cells that were also positive for markers of neural lineages: Hu+ neurons, GFAP+ astrocytes, and γ-GST+ oligodendrocytes. (Scale bar: C and D, lower magnification 1 mm; C and D, higher magnification, 50 μm; E, 50 μm.)
Fig. S5. No tumor formation in the 38C2 SNSs grafted spinal cords. Histological analysis of 38C2 iPS-SNS grafted spinal cord using anti-Nanog antibody. No Nanog$^+$ cells were observed in the injured spinal cord 42 d after the transplantation. (Lower) Higher-magnification view of the boxed area of upper panel. (Scale bar: A, 1 mm; B, 100 μm.) See Fig. 5G for comparison.

Fig. S6. Immunohistochemical images in x-y-z planes of the injured spinal cords after 38C2 SNSs transplantation. Reconstructed images showed that the RFP$^+$ graft-derived cells are also positive for markers of neural lineages: Hu$^+$ neurons, GFAP$^+$ astrocytes, and GST-$\alpha$ $^+$ oligodendrocytes. (Scale bar: 50 μm.)
Fig. S7. Immunohistochemical analyses of the injured spinal cords after safe or unsafe TTF-IPS-SNSs transplantation. (A) anti-RFP DAB staining of sagittal sections of the spinal cord 42 d after injury (safe 335D1 SNS transplanted). There was no evidence of tumorigenesis. (B) Immunohistochemical analyses of 335D1-SNSs grafted into spinal cord 42 d after injury, revealing grafted cells double-positive for RFP and markers of neural lineages. (C) anti-GFP staining of sagittal section of the spinal cord 42 d after injury (unsafe 256H18 SNS transplanted). Teratomas containing EGFP* cells were observed.

Fig. S8. Quantitative analysis of LFB* area after 335D1 SNSs transplantation. (A) Quantification of LFB* areas at the lesion epicenter 42 d after injury (n = 3 each; **P < 0.01). (B) LFB staining of axial sections of the spinal cord at the lesion epicenter 42 d after injury; PBS control (Left) and 335D1-iPS-SNS–transplanted (Right) animals. (Scale bar: 500 μm.) (C) Immunohistochemistry of 335D1-iPS-SNS–derived mature oligodendrocytes (MBP*). Grafted cells were integrated into myelin sheath. (Scale bar: 50 μm.)
Fig. S9. (A) Teratoma formation in injured spinal cords after unsafe 256H18 SNSs transplantation. Higher-magnification views of Fig. 5E. Stratified squamous epithelia with keratinization (Left) and smooth muscle tissues around the dilated cyst (Right) were observed. (B and C) Scattered small cell clusters of Nanog\textsuperscript{+} undifferentiated cells in injured spinal cords after unsafe 256H13 SNSs transplantation. Only one mouse in the 256H13 group showed teratoma formation at 6 wk after injury through H&E staining. No tumor formation was observed in the other mice in 256H13 group (B). However, scattered small clusters of Nanog\textsuperscript{+} undifferentiated cells were observed in these mice without obvious teratoma formation (C). (Scale bar: A and C, 100 μm; B, 1 mm.)
Table S1. RT-PCR primers in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanog</td>
<td>AGGGTCTGCTACTGAGATGCTCTG</td>
<td>CAACCACTGGTTTTTCGCAACCC</td>
</tr>
<tr>
<td>ERas</td>
<td>ACTGCCCCCTCATCAGACTGCTACT</td>
<td>CACTGCTTTGATCTGGGTAGTG</td>
</tr>
<tr>
<td>Oct3/4</td>
<td>CTGAGGCCAGGGCAGGAGCAGCAG</td>
<td>CTGAGGCCAGGGCAGGAGCAGCAG</td>
</tr>
<tr>
<td>Sox2</td>
<td>GGTTCACATTCTCTCCCCACTCCAG</td>
<td>TCACATGTGCAAGAGGAGCGAG</td>
</tr>
<tr>
<td>c-Myc</td>
<td>CAGAGGAGGAGAAGCTGGAAGGC</td>
<td>TTATGCACCAAGGTTCAGTTTTTC</td>
</tr>
<tr>
<td>Klf4</td>
<td>CACACGGCAGGGCGTGGCGTCCAGAAA</td>
<td>TTACGCTGTCCTTTCCGAGGGCAG</td>
</tr>
<tr>
<td>Sox1</td>
<td>AGTACAGCATGATAGTGGAGACC</td>
<td>ACGAGGTACTTGCTTCTTGAGC</td>
</tr>
<tr>
<td>Tuj1</td>
<td>AGATCCGCTGAGGAGGCCATCAG</td>
<td>CAGCTCCGCGCTTTCTGATAGT</td>
</tr>
<tr>
<td>GFAP</td>
<td>GCAAATGATGCGGTCTCTACAGC</td>
<td>TCAGCCACGGCAGAGGCAGCAAC</td>
</tr>
<tr>
<td>Brachury (T)</td>
<td>ATGCCAAAGAAAGAAACGAC AGAGGCTGTAGAACATGATT</td>
<td></td>
</tr>
<tr>
<td>EGFP</td>
<td>CGACTTCTTCAAGTCCGCCATGCCG</td>
<td>CCAGCAGGACCAGTAGTGATCGGCGCTTC</td>
</tr>
<tr>
<td>b-actin</td>
<td>CGTGGGCCGGCCTAGGCAACCA</td>
<td>TTGCGCTTAGGCTTCCAGGGG</td>
</tr>
</tbody>
</table>

Table S2. Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody type</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>For immunocytochemistry</td>
<td></td>
</tr>
<tr>
<td>Tuj1 (Sigma)</td>
<td>Mouse IgG, 1:1,000</td>
</tr>
<tr>
<td>GFAP (DAKO)</td>
<td>Rabbit IgG, 1:4,000</td>
</tr>
<tr>
<td>O4 (Chemicon)</td>
<td>Mouse IgM, 1:5,000</td>
</tr>
<tr>
<td>CNPase (Sigma)</td>
<td>Mouse IgG, 1:400</td>
</tr>
<tr>
<td>5-HT (Sigma)</td>
<td>Rabbit IgG, 1:20,000</td>
</tr>
<tr>
<td>TH (Chemicon)</td>
<td>Rabbit IgG, 1:100</td>
</tr>
<tr>
<td>GAD67 (Chemicon)</td>
<td>Mouse IgG, 1:2,500</td>
</tr>
<tr>
<td>For immunohistochemistry of NOD/SCID mice analyses</td>
<td></td>
</tr>
<tr>
<td>NeuN (Chemicon)</td>
<td>Mouse IgG, 1:500</td>
</tr>
<tr>
<td>GFAP (DAKO)</td>
<td>Rabbit IgG, 1:400</td>
</tr>
<tr>
<td>APC (Oncogene)</td>
<td>Mouse IgG, 1:100</td>
</tr>
<tr>
<td>GFP (MBL)</td>
<td>Rabbit IgG, 1:500</td>
</tr>
<tr>
<td>For immunohistochemistry of intact/injured spinal cord</td>
<td></td>
</tr>
<tr>
<td>Hu* (Human serum)</td>
<td>Human serum, 1:1,000</td>
</tr>
<tr>
<td>GFAP (Zymed)</td>
<td>Rat IgG, 1:200</td>
</tr>
<tr>
<td>α-GST (BD Biosciences)</td>
<td>Mouse IgG, 1:500</td>
</tr>
<tr>
<td>5-HT (Immunostar)</td>
<td>Goat IgG, 1:200</td>
</tr>
<tr>
<td>NF200 (Chemicon)</td>
<td>Mouse IgG, 1:500</td>
</tr>
<tr>
<td>MBP (Aves Labs)</td>
<td>Chick IgY, 1:200</td>
</tr>
<tr>
<td>RFP (MBL)</td>
<td>Rabbit, 1:200</td>
</tr>
<tr>
<td>Nanog (ReproCELL)</td>
<td>Rabbit, 1:200</td>
</tr>
</tbody>
</table>

*A gift from Dr. Robert Darnell, Rockefeller University.*