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REVIEW

Cell transplantation therapies for spinal cord injury focusing on induced pluripotent stem cells

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Stimulated by the 2012 Nobel Prize in Physiology or Medicine awarded for Shinya Yamanaka and Sir John Gurdon, there is an increasing interest in the induced pluripotent stem (iPS) cells and reprograming technologies in medical science. While iPS cells are expected to open a new era providing enormous opportunities in biomedical sciences in terms of cell therapies and regenerative medicine, safety-related concerns for iPS cell-based cell therapy should be resolved prior to the clinical application of iPS cells. In this review, the pre-clinical investigations of cell therapy for spinal cord injury (SCI) using neural stem/progenitor cells derived from iPS cells, and their safety issues *in vivo*, are outlined. We also wish to discuss the strategy for the first human trails of iPS cell-based cell therapy for SCI patients.
***Keywords*:** neural stem/progenitor cell; induced pluripotnet stem cell; spinal cord injury; transplantation
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Introduction

The central nervous system (CNS), including the brain and spinal cord, has been considered a representative example of organs in which regeneration is difficult. However, this commonly accepted theory is being disproved by recent progress in the field of stem cell biology. Neural stem/progenitor cells (NS/PCs) were experimentally identified by Reynolds and Weiss [1] in 1992, and subsequently, methods for culturing NS/PCs in mammals, including humans, have been established [1-5], which has allowed elucidation of the molecular biological characteristics of NS/PCs in the developmental process and in the CNS of adult mammals [6-10]. A number of studies have paid attention to the self-renewal capacity and multipotency of NS/PCs and tried to regenerate neural tissues lost as a result of neurodegenerative diseases and injuries. Particularly in the research field of spinal cord injury (SCI), mouse embryonic stem (ES) cell-derived NS/PCs [11] and rat embryonic spinal cord-derived NS/PCs [12] have been transplanted into the injured spinal cord of rats, and human embryo-derived NS/PCs have been transplanted into the injured spinal cord of the common marmoset in preclinical studies, aiming at clinical application [13, 14]. Safety of the cells and functional recovery were reported in all of the aforementioned studies. These results strongly suggest that application of *In vitro* passaged human NS/PCs for neural regeneration may be promising. However, clinical application has not yet been realized in Japan due to ethical issues with the use of NS/PCs derived from surplus embryos or aborted fetal tissues. In fact, the revised version of the Japanese Ministry of Health, Labor and Welfare's Guidelines for Clinical Research Using Human Stem Cells was launched on 1 November 2010. That appears to be a major step in terms of the future development of regenerative medicine in Japan. However, fetal and ES cell-derived NS/PCs are still not covered by the guidelines.

Under such circumstances, Yamanaka *et al*. [15-17] have introduced several genes into somatic cells to create induced pluripotent stem (iPS) cells with ES cell-like pluripotency and proliferative capacity. If adult somatic cells, such as blood cells or skin fibroblasts, can be converted into NS/PCs via iPS cells for transplantation into injured spinal cord, the aforementioned problems, such as ethical issues and rejection in transplantation, could be circumvented. This review outlines the history of research using fetal-derived NS/PCs, *In vitro* methods for inducing the differentiation of ES and iPS cells

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into NS/PCs that can possibly serve as the cell sources in place of fetal-derived NS/PCs, and furthermore, the safety of transplantation of iPS cell-derived NS/PCs and of their transplantation into SCI models.

Regenerative medicine for SCI using fetal-derived NS/PCs

Transplantation of *in vitro* propagated NS/PCs into the injured spinal cord began to be studied, since previous studies demonstrated the efficacy of transplantation of a fetal spinal cord tissue in a rat model of SCI. However, unfortunately, its efficacy could not be appreciated at first. It was considered that when NS/PCs are transplanted immediately after injury, the transplanted NS/PCs mostly differentiate into astrocytes and do not contribute to functional recovery of the injured spinal cord [18]. Ogawa *et al*. [12] reported that the transplantation of rat fetal spinal cord-derived NS/PCs into a rat model of cervical spinal cord contusion injury 9 days after injury resulted in significant functional recovery of the upper extremities as compared with that in the control group. It was revealed that the grafted cells differentiated into neurons, astrocytes and oligodendrocytes, and that in particular, the transplanted cell-derived neurons formed functional synapses with the host neurons. These results suggest that allowance of a therapeutic time window after injury is important before transplantation of NS/PCs into the injured spinal cord. Namely, the acute phase of SCI corresponds to the "inflammatory phase" due to the upregulation of inflammatory cytokines, excitatory neurotransmitters and free radicals, and is not suitable for transplantation, whereas in the chronic phase, about 2 weeks or more after injury, the injury enters the stage of glial scar formation, which prevents axonal regeneration. Therefore, the subacute phase of SCI is considered as the optimal time window for NS/PC transplantation in a rat SCI model [10, 12, 19] (Figure 1). However, we have to realize that the anatomy and functions of the spinal cord are considerably different between rodents and primates. Thus, it is very important to test proof of concept on the effectiveness of fetal NS/PCs transplantation for SCI in non-human primates. For this purpose, Iwanami *et al*. [13, 14] established a SCI model of a non-human primate, the common marmoset (*Callithrix jacchus*), and transplanted human aborted fetal forebrain-derived NS/PCs into this

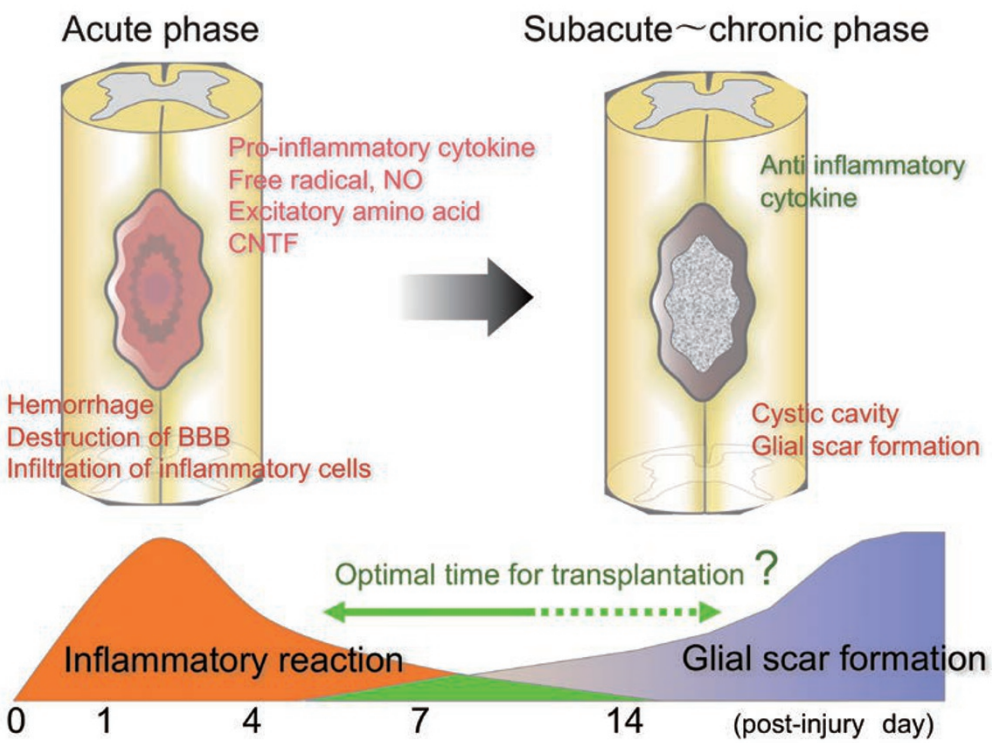

Figure 1 Microenvironment of the injured spinal cord. Because the immediately post-traumatic microenvironment of the spinal cord is in an acute inflammatory stage, it is not favorable for the survival and differentiation of NS/PC transplants. On the other hand, in the chronic stage after injury, glial scars form in the injured site that inhibit the regeneration of neuronal axons. Thus, we believe that the optimal timing of transplantation is 1-2 weeks after injury.

model. The transplanted cells survived and differentiated into neurons, astrocytes and oligodendrocytes, thereby promoting functional recovery as compared with that in the control group, and also no tumorigenesis from the transplanted cells was observed during the observation period (3 months after transplantation) [13]. Reconstruction of neural circuits by synapse formation between the transplanted cells and host neurons, and remyelination and trophic support provided by the transplanted cells are considered as being among the important mechanisms of the motor function recovery [20]. These results suggest that fetal tissue-derived NS/PCs are very useful as cell sources for transplantation. However, as described above, clinical application of these cells has not yet been realized in Japan due to the ethical issues associated with the use of aborted fetal tissues.

ES cell-derived NS/PCs

In vitro models of neural development and mouse ES cell-derived NS/PCs

NS/PCs are defined as cells that have self-renewal capacity and multipotency. However, their differentiation and proliferative capacity is strictly regulated according to the stage at which these cells are produced and their localization, and not all NS/PCs have identical characteristics. NS/PCs are known to already exist around embryonic day 5 (E5.5), and the NS/PCs at this stage can be cultured in the presence of leukemia inhibitory factor (LIF) [21]. On embryonic days E8.5-12.5, NS/PCs can be cultured *In vitro* in the presence of fibroblast growth factor-2 (FGF-2). From this stage to the late embryonic stages, radial glial cells around the ventricles can serve as NS/PCs, and these cells self-renew by symmetric divisions and generate neurons by asymmetric divisions [22, 23]. From the late developmental to the neonatal stage and in the adult brain, NS/PCs exist mainly around the ventricles, and differentiate not only into neurons but also into glial cells (astrocytes and oligodendrocytes) [24]. During and after the late embryonic stages, NS/PCs are stimulated to proliferate not only by FGF-2 but also by epidermal growth factor (EGF). However, the NS/PCs that appear during and after the late embryonic stages cannot generate early-born projection neurons, such as forebrain cholinergic neurons, dopaminergic neurons and motor neurons. Okada *et al*. [25] induced the development of highly plastic NS/PCs existing at relatively early developmental stages using mouse inner cell mass-derived ES cells, and succeeded in constructing a culture system mimicking the temporal and spatial specificity of the developmental process (Figure 2). In this culture system, first LIF, which is required to maintain the undifferentiated state, is removed and ES cells are cultured in suspension to induce the formation of embryoid bodies (EBs) containing cells derived from the three germ layers. These EBs contain relatively early-stage NS/PCs, which can be selectively cultured as neurospheres by suspended culture in serum-free medium for NS/PCs in the presence of FGF-2. Furthermore, the addition of Noggin, which inhibits BMP to promote differentiation into the neuroepithelium, thereby playing an important role in forebrain formation, or retinoic acid (RA), which is known to play an important role in neural induction and also in the development of the hindbrain and anterior spinal cord, at a low concentration during EB formation increased the proportion of NS/PCs in the EBs and increased the efficiency of neurosphere formation. Primary neurospheres thus formed can be passaged to generate secondary and tertiary neurospheres. Interestingly, almost only neurons are induced from primary neurospheres, whereas not only neurons but also glial cells, such as astrocytes and oligodendrocytes, are induced from secondary and tertiary neurospheres. These mouse ES cell-derived neurospheres (ES-NS) can be repeatedly passaged and have the ability to generate neurons, astrocytes and oligodendrocytes, indicating that these neurospheres contain NS/PCs with self-renewal capacity and multipotency. In addition, such a change in the differentiation capacity with culture passage well reflects the developmental process of the CNS, in that only neurons are generated in the early developmental stages, whereas glial cells are generated for the first time during and after the mid-gestation stages. Furthermore, the regional specificity of the induced NS/PCs along the anteroposterior axis can be regulated by changing the concentration of Noggin and RA added during EB formation. Moreover, the regional specificity along the dorsoventral axis has also been successfully regulated by the addition of the ventralizing factor, Sonic Hedgehog, and dorsalizing factors, BMP4 and Wnt3a, during the formation of the primary neurospheres [25]. These results suggest that the regional specificity of NS/PCs could be regulated by the addition of appropriate factors at appropriate stages during induction of differentiation.

Transplantation of mouse ES cell-derived NS/PCs into the injured spinal cord

Methods of inducing mouse ES cells to differentiate into neurons have been widely studied [26], and ES cell-derived NS/PCs (ES-NS/PCs) are ideal cell sources for transplantation. The differentiation stages at which ES-NS/PCs are transplanted are variable, and could range from undifferentiated ES cells to EBs and differentiated neurons. However, it is known that the lower the degree

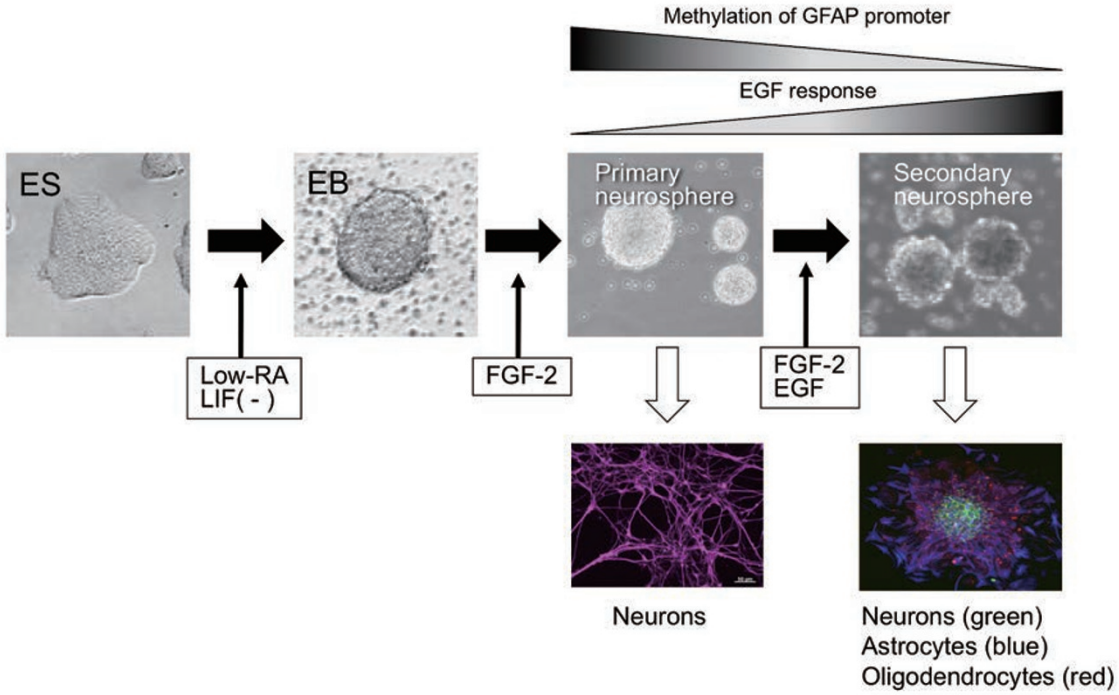

Figure 2 Neural induction of mouse ES cells through EBs. After the removal of LIF, ES cells are cultured in suspension to induce the formation of EBs. EBs contain relatively early-stage NS/PCs, which can be selectively cultured as neurospheres by suspended culture in serum-free medium for NS/PCs in the presence of FGF-2. Primary neurospheres can be passaged to generate secondary and tertiary neurospheres. Interestingly, almost only neurons are induced from primary neurospheres, whereas not only neurons but also glial cells, such as astrocytes and oligodendrocytes, are induced from secondary and tertiary neurospheres.

of differentiation, the higher the incidence of teratomas derived from the transplanted cells [27]. As regards the efficacy of ES-NS/PC transplantation into the injured spinal cord, McDonald *et al.* [11] demonstrated that the transplantation of EBs generated from mouse ES cells into the injured spinal cord of rats resulted in good functional recovery. However, the risk of tumorigenesis in the long term associated with transplantation of EBs at lesser stages of differentiation cannot be ignored. Keirstead *et al.* [28] established an effective method of inducing human ES cells to differentiate into highly pure populations of oligodendrocyte progenitors by using a culture medium containing factors promoting differentiation into oligodendrocytes, such as insulin and thyroid hormones, and reported that the transplantation of these oligodendrocyte progenitors into the injured spinal cord of rats resulted in remyelination of demyelinated axons and recovery of motor function. Subsequently, Yamada *et al.* [29] used electrically stimulated mouse EBs generated from ES cells to induce selective differentiation into neurons for transplantation into the injured spinal cord. They found that while the transplanted EBs differentiated into neurons in the injured spinal cord at a higher frequency than the transplanted EBs without electrical stimulation, the proliferative capacity of the electrically stimulated EBs was lower than that of the non-stimulated EBs. Thus, this method was effective from the aspect of safety, but did not lead to satisfactory recovery of motor function after SCI [29]. None of these studies has clarified exactly which stage of induction from ES cells to NS/PCs contained in the neurospheres would be the most suitable for transplantation therapy at the subacute phase of SCI.

To determine this issue, Kumagai *et al.* [30] transplanted primary neurospheres induced from mouse ES cells via EB formation using the above-described culture system, and secondary neurospheres, obtained after one passage, into a mouse SCI model to evaluate their efficacy. As described above, most of the primary neurospheres differentiated into neurons, whereas the secondary neurospheres differentiated into three types of cells, astrocytes, oligodendrocytes and neurons [25]. The engraftment rate was ~20% for both transplanted primary and secondary neurospheres. It is of interest that primary

neurospheres showed neuron-dominant differentiation, whereas the secondary neurospheres differentiated into neurons and glial cells, consistent with their *In vitro* characteristics. The transplantation of secondary neurospheres significantly prevented the atrophy and demyelination of the injured spinal crod and enhanced the axonal regrowth and angiogenesis as compared with the primary neurospheres transplantation group. Interestingly, behavioral analysis using the Basso Mouse Scale (BMS) revealed that only the secondary neurospheres transplantation group showed statistically significant functional recovery as compared to the vehicle control group. These results suggest that in the application of ES-derived NS/PCs to the treatment of SCI, it would be more desirable to transplant NS/PCs generating both neurons and glial cells than to transplant those differentiating dominantly into neurons.

However, as in the case of embryo-derived NS/PCs, ethical issues need to be confronted before attempts at clinical application of ES-derived NS/PCs in the future, because surplus embryos are used for establishing human ES cells.

iPS cell-derived neurospheres

Safety assessment of mouse iPS cell-derived neurospheres

iPS cells established by Yamanaka *et al.* have paved the way for the development of solutions for the above-mentioned ethical issues related to the use of embryos and aborted tissues [15, 16]. iPS cells are pluripotent stem cells that are generated by introducing *Oct4*, *Sox2*, *Klf4* and *c-Myc* genes into mouse/human fibroblasts to reprogram somatic cells, and exhibit proliferative and differentiation capacity almost equivalent to that of ES cells. iPS cells are expected to be a solution to problems such as the ethical issues and immunological rejection, because these cells can be established from the somatic cells of each patient. On the other hand, iPS cells may be associated with a greater risk of tumorigenesis than ES cells, because (1) foreign genes are introduced into the chromosome, and (2) there is the possibility that the reprograming is not necessarily complete. Miura *et al.* [31] have revealed that the responsiveness of mouse iPS cells to induction of neural differentiation and their safety after transplantation vary greatly depending on the somatic cells from which the iPS cells are derived. Thirty-six mouse iPS cell lines established in our laboratory were induced to differentiate into neural lineages and transplanted into the striata of the brains of immunodeficient NOD/severe combined immunodeficiency (SCID) mice as neurospheres to evaluate their *In vivo* differentiation capacity and safety after transplantation. All of the iPS cell line-derived neurospheres (iPS-neurospheres) were analyzed in detail by flow cytometry, and it was found that the proportion of *Nanog*-EGFP-positive undifferentiated cells remaining in the neurospheres varied greatly depending on the type of somatic cells from which the iPS cells were derived [31]. Mouse embryonic fibroblast (MEF)-derived iPS-neurospheres showed responsiveness to differentiation induction equivalent to that of ES cells, and almost no undifferentiated cells remained in the neurospheres. The frequency of post-transplantation teratoma formation in the MEF-iPS-neurosphere-transplanted mouse groups was as low as that in the ES-neurosphere-transplanted group. No teratoma formation was observed during the 16-week observation period in the groups transplanted with neurospheres induced from either of the two iPS cell lines established from adult gastric epithelial cells. On the other hand, adult tail tip fibroblast (TTF)-derived iPS cell lines showed significant resistance to differentiation, so that many undifferentiated cells remained in the neurospheres after the induction of differentiation. Teratoma formation was observed at a high frequency in the mouse groups transplanted with these neurospheres, and many mice became weak or died within a short time. The responsiveness of adult hepatocyte (Hep)-derived iPS cell lines to differentiation induction and their tumorigenicity were intermediate between those of the MEF-iPS cell lines and TTF-iPS cell lines [31]. Introduction/non-introduction of c-Myc and selection/non-selection of reprogramed cells by reporters at the time of establishment of the cell line did not affect the responsiveness of the iPS cells to differentiation induction or their safety after transplantation. The differences in the differentiation capacity of iPS cells depending on the somatic cells from which the iPS cells were derived may be caused by the epigenetic memory, i.e., the profiles of the remaining expressed genes from the somatic cells, and it is an urgent future task to further analyze the nature of these cells in greater detail.

Treatment of SCI using "safe" mouse iPS cell clone-derived secondary neurospheres

Secondary neurospheres were induced from the mouse iPS cells (clone 38C2) established from the MEF (hereafter referred to as 38C2-iPS-secondary neurospheres) and transplanted into a mouse SCI model, after their safety was confirmed by the above-described transplantation experiments into immunodeficient mouse brain [32]. Contusion SCI was induced at T10 level, and 9 days after the injury, 38C2-iPS-secondary neurospheres were transplanted into the injured spinal cord. The gene for the luminescent enzyme luciferase, *CBRluc*, and a red

fluorescent protein gene, *mRFP*, were introduced into the cells to be transplanted using a lentivirus, and the survival of the transplanted cells was sequentially monitored by bioimaging [33] for 6 weeks after the injury. Thereafter, quantitative assessment using bioimaging revealed that ~20% of the transplanted cells were engrafted with no apparent increase in the amount of luminescence, and histological analysis revealed no tumor formation up to at least 5 weeks after the transplantation (Figure 3). The transplanted cells differentiated into Hu-positive neurons, GFAP-positive astrocytes and GST-π-positive oligodendrocytes, at efficiencies of ~30%, 50% and 15%, respectively. Behavioral analysis using the BMS revealed that the 38C2-iPS-secondary neurospheres transplantation group showed functional recovery almost equivalent to that observed in the mouse ES-secondary neurospheres transplantation group, and significantly greater recovery of hindlimb motor function as compared to the vehicle control group injected with culture medium alone (Figure 4A). Analysis of the mechanism of this functional recovery revealed that the transplanted 38C2-iPS-secondary neurospheres differentiated into MBP-positive mature oligodendrocytes, which remyelinated the nerve fibers demyelinated by the injury (Figure 4B). As a result, the myelin sheath area, which was positively stained with Luxol fast blue (LFB), significantly increased in the 38C2-iPS-secondary neurospheres transplantation group as compared with that in the vehicle control group (Figure 4C and 4D). Furthermore, it is possible that the transplanted cells differentiated into immature astrocytes with bipolar processes in the injured spinal cord and that these immature astrocytes played a role in the guidance of

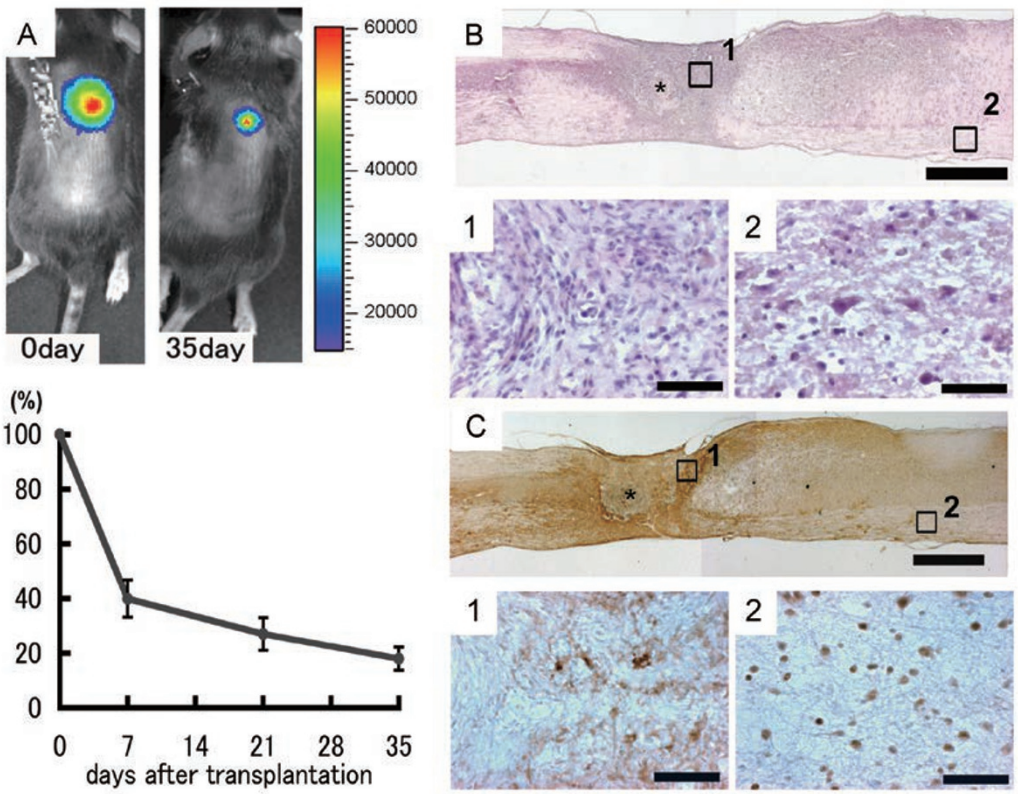

Figure 3 Transplanted safe 38C2-iPS-secondary neurospheres survive without any evidence of tumorigenesis [32]. **(A)** Representative BLI images of a mouse in which CBRluc-expressing 38C2-iPS secondary neurospheres were transplanted into the injured spinal cord (left, immediately after transplantation; right, 42 days after transplantation). Quantification of the photon intensity revealed that ~60% of the grafted cells were lost within 7 days after transplantation, and ~20% of the cells survived 35 days after transplantation. **(B)** HE and **(C)** anti-RFP DAB staining of sagittal sections of the spinal cord 42 days after injury (38C2-iPS-secondary neurospheres transplanted). There was no evidence of tumorigenesis **(B)**. No significant nuclear atypia was observed in magnified images of the boxed areas showing the lesion epicenter **(B-1)** or white matter caudal to the transplantation site **(B-2)**. Grafted cells survived and were diffusely distributed rostral and caudal to the lesion site **(C)**. Higher-magnification images of the boxed areas showing the lesion site **(C-1)** and white matter caudal to the lesion site **(C-2)**. *Lesion epicenter. Images are reproduced from reference [32].

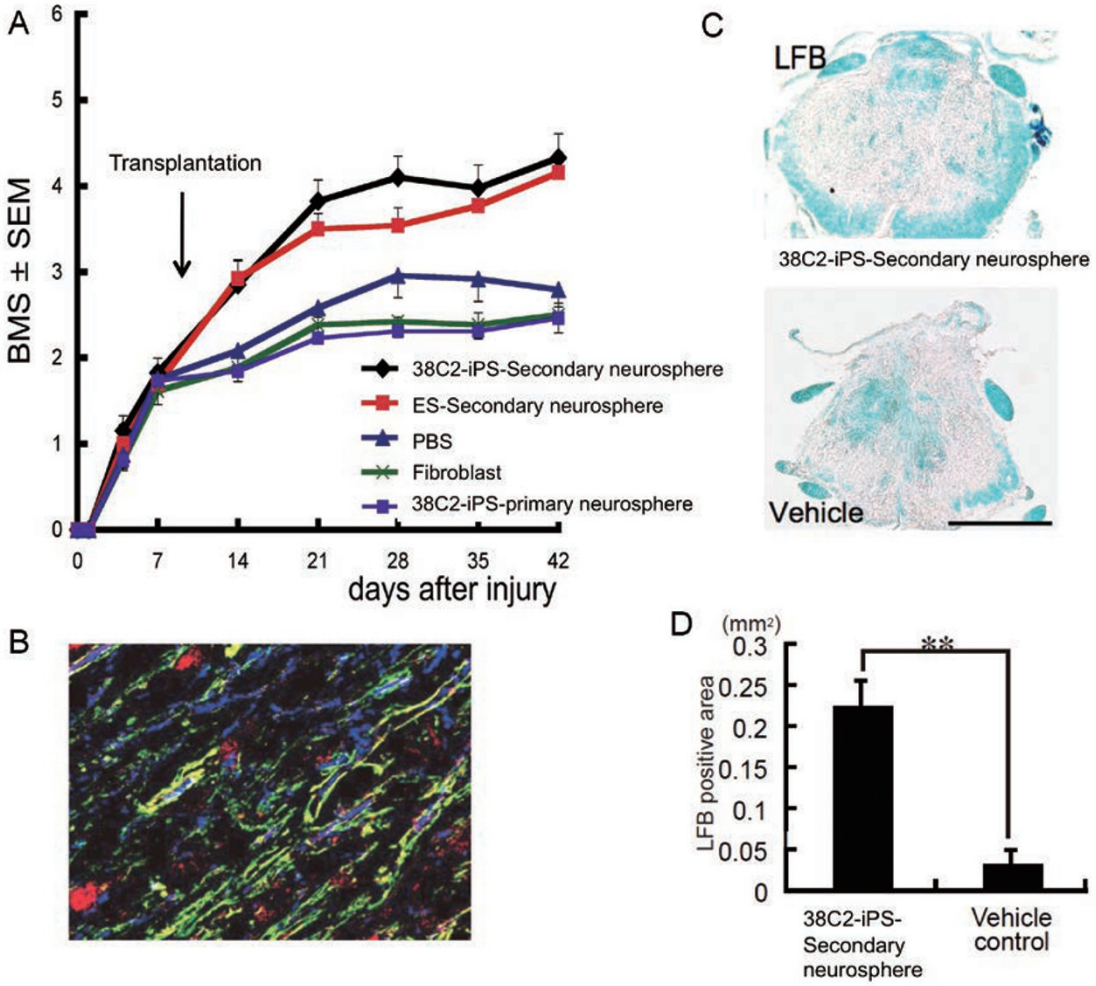

Figure 4 Transplanted secondary neurospheres derived from safe MEF-iPS clones contributed to the remyelination, thereby promoting functional recovery [32]. **(A)** 38C2-iPS-derived secondary neurosphere-transplanted mice showed significantly better functional recovery compared to the PBS and fibroblast control mice. **(B)** Immunohistochemistry of grafted secondary neurosphere-derived mature oligodendrocytes (MBP, green; RFP, red and NF200kD, blue). Grafted cells were integrated into myelin sheath (yellow). **(C)** LFB staining of axial sections of the spinal cord at the lesion epicenter 42 days after injury; 38C2-iPS-derived secondary neurospheres transplanted and vehicle control animals. **(D)** Quantification of LFB-positive areas at the lesion epicenter 42 days after injury (**P < 0.01). Images are reproduced from reference [32].

regenerating axons. In fact, 5-HT-positive raphe-spinal fibers, which are considered to play a great role in motor function in rodents, have been reported to be present in large numbers in the vicinity of these immature astrocytes, and quantification of these 5-HT-positive fibers at a distance of 4 mm from the site of injury revealed a significant increase of their number in the transplantation group (Figure 5). These results indicate that remyelination and glial support for the raphe-spinal fibers provided by the transplanted cells are the major mechanisms of recovery of the hindlimb function by 38C2-iPS-secondary neurospheres transplantation [32].

"Safe" and "dangerous" mouse iPS cell clones derived from adult tissues

Next, we performed similar transplantation experiments using adult tissue (TTF)-derived iPS cells, aiming at clinical application in autologous cell transplantation. Of the 36 mouse iPS cell clones used in the above-described safety assessment, 6 clones were derived from TTF, of which, only the 335D1 clone was confirmed to be safe [31]. This 335D1 clone and two TTF-derived "dangerous" clones with tumorigenicity (256H13 and 256H18) were induced to form neurospheres (335D1-iPS-secondary neurospheres, 256H13-iPS-secondary neurospheres and 256H18-iPS-secondary neurospheres,

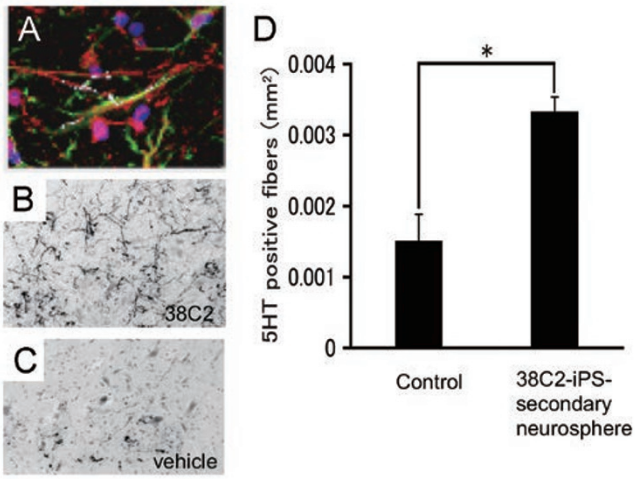

Figure 5 Transplantation of safe MEF-iPS-secondary neurospheres promoted serotonergic innervation of the dorsal cord [32]. **(A)** Immunohistochemistry of 38C2-iPS-secondary neurosphere-derived astrocytes closely associated with 5HT+ serotonergic fibers. (RFP, red; GFAP, green and 5-HT, white) **(B**, **C)** Transplantation of 38C2-iPS-derived secondary neurospheres promoted the growth of 5HT+ serotonergic fibers in the distal spinal cord. Axial sections of 38C2-iPS-derived secondary neurospheres transplanted **(B)** and vehicle control mice **(C)**. **(D)** Quantitative analysis of 5HT+ serotonergic fibers of distal cord in the vehicle control and 38C2-iPS-derived secondary neurospheres transplantation groups (6 weeks post injury) (*P < 0.01). Images are reproduced from reference [32].

respectively) and transplanted into a mouse SCI model. Although functional recovery was obtained in all groups transplanted with these neurospheres, the recovery was transient and was suddenly lost about 6 weeks after the injury, and the majority of the mice subsequently died in both the 256H13- and 256H18-iPS-secondary neurospheres transplantation groups due to the formation of teratomas. In contrast, in the 335D1-iPS-secondary neurospheres transplantation group, no tumor formation was observed in any of the mice, and the functional recovery was significantly better than that in the vehicle control group and equivalent to that in the ES-secondary neurospheres transplantation group. These results indicate that adult tissue-derived iPS cell clones could serve as useful cell sources for the treatment of SCI provided that their safety is strictly evaluated in advance [32].

Treatment of SCI using human iPS cell-derived neurospheres

Aiming for the eventual clinical application, we moved on to the transplantation of human iPS-neurospheres into injured spinal cord of NOD-SCID mice. *Oct4*, *Sox2*, *Klf4* and *c-Myc* were introduced into adult facial skin-derived fibroblasts using a retrovirus to create the cell line 201B7 [15], which was used as the cell source. The 201B7 clone was induced to differentiate into neurospheres (201B7-iPS-neurospheres) using a method similar to that used for

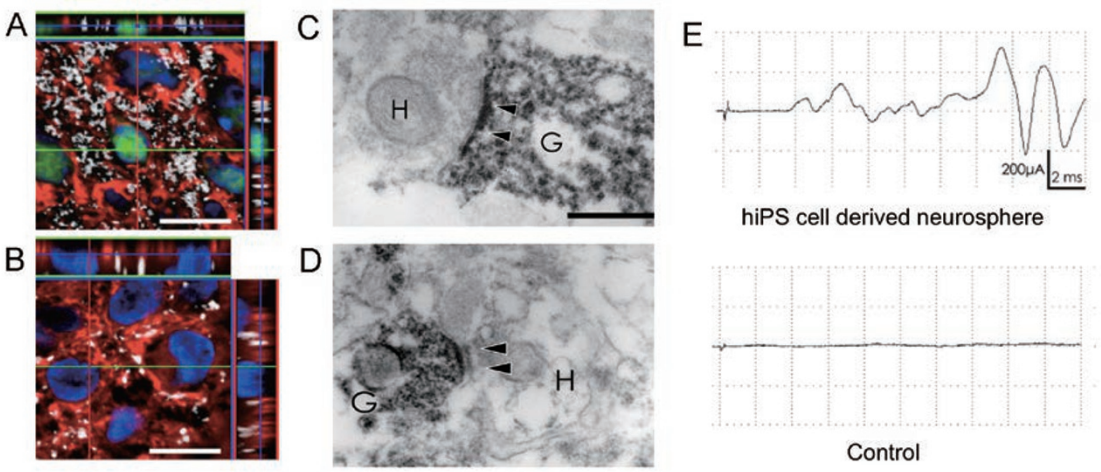

Figure 6 Evidence for synapse formation between human iPS cell (201B7)-derived neurons and host mouse spinal cord neurons [34]. **(A)** Sections were triple-stained with HNu (green), βIII tubulin (red) and the presynaptic marker Bassoon (Bsn, white). The Bsn antibody used here recognized the rat and mouse, but not human, protein. **(B)** Sections triple-stained for HNu (blue), βIII tubulin (red) and the human-specific presynaptic marker hSyn (white). **(C**, **D)** Electron microscopy showing synapse formation between host mouse neurons and graft-derived Venus+ (black) human neurons: the pre- and postsynaptic structures indicated transmission from a host neuron to a graft-derived neuron **(C)** and from a graft-derived neuron to a host neuron **(D)**. H, host neuron; G, graft-derived neuron; arrowheads, postsynaptic density. **(E)** Electrophysiological analysis performed 112 days after SCI. MEP waves were detected in most of the transplantation group (14 out of 17), whereas they were not detected in the control group (0 out of 15). Images are reproduced from reference [34].

mouse iPS cells. The safety of 201B7-iPS-neurospheres has already been confirmed by our analyses. 201B7-iPS-neurospheres were transplanted into the injured spinal cord of NOD-SCID mice to evaluate their therapeutic effect [34]. The transplanted cells were well engrafted in the mouse spinal cord and differentiated into NeuN- and βIII tubulin-positive neurons, GFAP-positive astrocytes and APC-positive oligodendrocytes. Approximately 50% of the transplanted cells differentiated into βIII tubulin-positive neurons, and of these, ~70% differentiated into GAD67-positive γ-aminobutyric acid (GABA)-ergic neurons. In addition, immunohistochemical and electron-microscopic analyses confirmed that the neurons derived from the transplanted cells formed synapses with the host neurons (Figure 6A-6D). Motor evoked potential (MEP) waveforms could be detected in the 201B7-iPS-neurospheres transplantation group, but not in the vehicle control group, suggesting that the neurons derived from the transplanted cells functioned as interneurons in the mouse spinal cord, contributing to the reconstruction of neural circuits (Figure 6E and 6F). Furthermore, angiogenesis, nerve regeneration and tissue protection, which were likely to be mediated by paracrine actions of graft-derived astrocytes, were also observed. BMS, rotarod test and gait analysis using a treadmill showed good improvement in the lower extremity motor function in the 201B7-iPS-neurospheres transplantation group (Figure 7). Furthermore, to confirm the long-term safety of 201B7-iPS-neurospheres transplantation, follow-up was continued for ~4 months after the SCI, which revealed that the functional recovery was maintained without tumor formation [34]. Consistently, Fujimoto *et al.* [35] also confirmed the efficacy and safety of human iPS-NS/PC transplantation for SCI treatment in immunodeficient mice.

Based on these findings, we moved on to 201B7-neurospheres transplantation for treatment of SCI in common marmosets as previously reported [13, 14]. Grafted hiPS-neurospheres survived and differentiated into NeuN-positive neurons, GFAP-positive astrocytes and Olig1-positive oligodendrocyte progenitor cells. hiPS-neurospheres transplantation enhanced axonal regrowth, myelination and angiogenesis, thereby promoting functional recovery after SCI. It was noteworthy that there was no tumor formation at least for 12 weeks after transplantation [36]. Taken together, pre-evaluated safe hiPSC-derived neurospheres could be a potential cell source for SCI treatment in clinic.

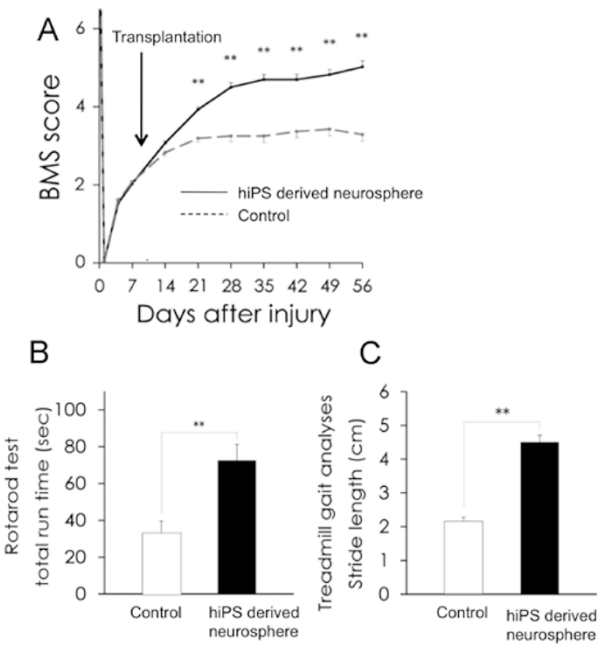

Figure 7 Transplanted human iPS cell (201B7)-derived neurospheres promoted motor functional recovery after SCI [34]. **(A)** Motor function in the hindlimbs was assessed weekly by the BMS score for 56 days. Values are means ± SEM. **(B)** Rotarod test 56 days after SCI. Graph shows the total run time. Values are means ± SEM. **(C)** Treadmill gait analysis using the DigiGait system 56 days after SCI. Graph shows stride length. Values are means ± SEM. (***P* < 0.01). Images are reproduced from reference [34].

Future problems and perspective

As described above, iPS cells could offer great promise as the cell source for autologous transplantation. Recently, it has become possible to establish human iPS cells from skin fibroblasts and also drops of blood [15, 37, 38]; thus, research on iPS cells has been rapidly advancing. However, retroviruses and lentiviruses are commonly used when reprograming factors are introduced in the establishment of iPS cells; these viruses are often integrated near the gene promoters, increasing the risk of tumorigenesis by changing the expression of endogenous genes in the vicinity. In fact, according to one study, 2 out of 10 patients with X-linked SCID (X-SCID) who received gene therapy with a retroviral vector developed leukemia [39]. Recently, research on this problem has rapidly progressed, and many studies have proposed solutions [40], including establishment of iPS cells with transient gene expression instead of using retroviruses or lentiviruses [38, 41-43], by introducing proteins [44-46], by substituting some genes with drugs [44, 46] and by using minicircle vectors that enable longer-term gene expression than plasmid vectors [47]. According to the results of our previous studies, transgene reactivation

and incomplete reprograming are considered as the main causes of tumorigenesis, and we propose to use integration-free iPS cells reprogramed using episomal vectors in the future to overcome the first of these problems [40, 48]. Furthermore, among our most important tasks before successful clinical application will be to induce Glis-1-transduced iPS cells, developed by Yamanaka *et al*. [49], which are reprogramed more completely, this allowing them to differentiate reliably into NS/PCs, and to accurately evaluate the safety of this final product. In addition to these improvements of the iPS cells, the safety issues must be validated through the intensive quality examination of iPS cell-derived NS/PCs in terms of genetic and epigenetic status, and their differentiation, proliferation and tumorigenicity *In vivo*, prior to the first human trials [40]. Finally, another important challenge before attempting clinical application pertains to the use of agents and cells derived from xenogeneic sources. Currently, animal-derived serum (bovine serum) is used for establishing iPS cells and inducing neural differentiation; however, a method to establish iPS cells without using such serum has also been reported [50], although these iPS cells will have to be characterized from the beginning. To accelerate these preclinical studies in the future, traceability of animal-derived agents should be ensured and methods of clean-up at the stage of the final product, namely, iPS-NS/PCs, according to GMP should be established as a practical strategy.

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