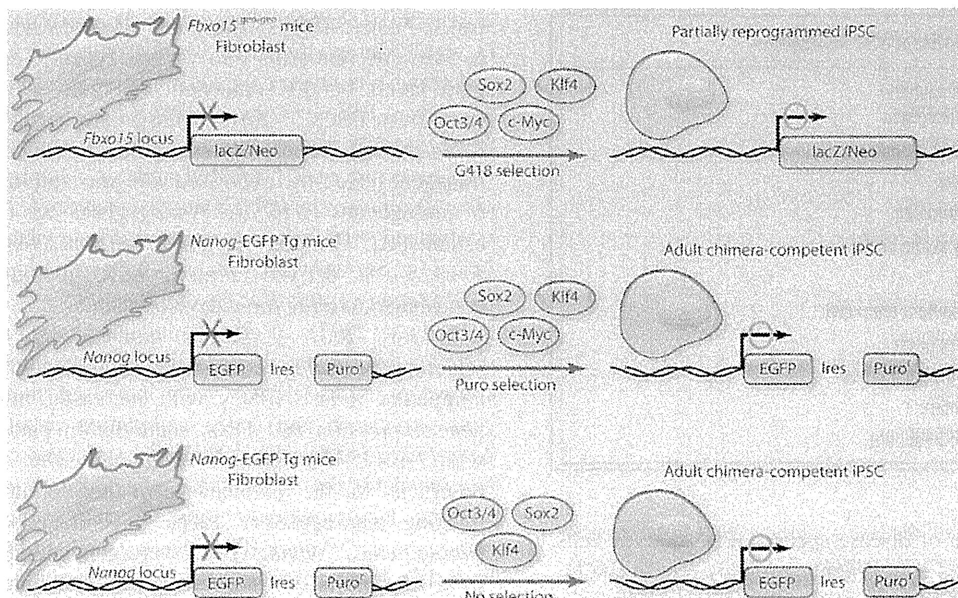

Figure 1. Evolution of methods for the generation of mouse iPSCs. Top, Generation of Fbxo15 induced pluripotent stem cells (iPSCs).[1] Four factors including *c-Myc* were transduced into fibroblasts from *Fbxo15*$^{geo/geo}$ mice. After G418 selection, partially reprogrammed *Fbxo15*-iPSCs were obtained. **Middle**, Generation of *Nanog*-iPSCs.[17] Four transcription factors including *c-Myc* were transduced into fibroblasts derived from transgenic mice carrying the *Nanog*-GFP-IRES-Puro^r reporter gene to generate *Nanog*-enhanced GFP (EGFP) transgenic mice. After puromycin selection, adult chimera-competent *Nanog*-iPSCs were obtained. **Bottom**, Generation of iPSCs with no drug selection.[19] Three transcription factors excluding *c-Myc* were transduced into fibroblasts derived from *Nanog*-EGFP transgenic mice or mice that did not have selection markers. Adult chimera-competent iPSCs were obtained without drug selection. Illustration credit: Ben Smith.

In November 2007, 2 groups independently reported the generation of hiPSCs from adult somatic cells by a retroviral/lentiviral-mediated gene transfer method that used a combination of *Oct4, Sox2, c-Myc*, and *Klf4*[2] or *Oct4, Sox2, Nanog*, and *Lin28*.[3] These reports stimulated enormous interest in iPSC research for cell therapy applications in human regenerative medicine, as well as human disease modeling, such as creating models of neurological diseases. In particular, there have been continuous efforts toward the establishment of well-characterized iPSCs that are both safe and efficacious for cell therapies. These efforts have involved improving the methods for iPSC generation and iPSC assay systems, as discussed later in this Review. However, a critical question remains, and that is how to actually test for the safety and efficacy of iPSC-based cell therapy. In the following paragraphs, lessons from previous investigations that include studies from this group will be introduced to address this issue.

Partial Reprogramming of Mouse iPSCs Is a High-Risk Factor for iPSC-Based Cell Therapy

Our group has been studying stem cell-based therapy for repair of the damaged CNS and, in particular, repair after SCI.[23–26] This Review now discusses the applicability of iPSC-based cell therapy for regenerating the contused spinal cord. Recent studies have revealed that ESCs have the potential to generate neural cells, including oligodendrocyte precursor cells[27,28] and NS/PCs.[29,30] Notably, clinical trials of human ESC therapies have finally been initiated for SCI patients at the subacute phase of injury after the primary mechanical trauma.[31] However, the use of human ESCs for SCI repair is complicated by both ethical and immunologic concerns, which could be overcome if pluripotent stem cells were derived directly from the patients' own somatic cells.[32–34]

On these grounds, soon after the first publication that introduced mouse iPSCs[1] and the successful establishment of mouse iPSCs, our group began preclinical investigations of iPSC-based cell therapy for SCI.[6,34] As a first step in the preclinical study, NS/PCs were induced from iPSCs and expanded in the form of neurospheres from various types of mouse iPSCs. The neural differentiation capability of the NS/PCs was then examined in vitro. The safety and differentiation potential of neurospheres derived from each iPSC clone were assessed through a series of transplantation experiments. In the initial experiments, 2 *Fbxo15*-iPSCs clones and 3 mouse ESCs (used as controls) were induced to differentiate into NS/PCs according to standard methods. These methods involved the treatment of neutrally biased embryoid bodies (EBs) with either Noggin or a low concentration of retinoic acid, with subsequent neurosphere formation.[35,36] The temporal changes in the differentiation potential of CNS stem cells in vivo were thereby mimicked, including the differentiation of newly generated neurons and gain of gliogenic competency that take place during fetal development.[37]

NS/PCs that were induced during EB formation from mouse ESCs were then expanded to form primary neurospheres in the presence of fibroblast growth factor-2. These primary neurospheres gave rise exclusively to early born neurons. However, after the passage of the primary neurospheres, the resultant secondary neurospheres gave rise to both neurons (mostly interneurons) and glial cells, including astrocytes and oligodendrocytes, because of the epigenetic modifications of genes involved in glial cell development.[36,37] By contrast,

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Fbxo15-iPSC-derived secondary neurospheres (SNSs) differentiated into neurons and astrocytes but not into oligodendrocytes, suggesting that the differentiation capability of NS/PCs derived from *Fbxo15*-iPSCs was somehow compromised (Figure 2A and 2B). It is relevant to note that both undifferentiated *Fbxo15*-iPS cells and *Fbxo15*-iPSC-derived SNSs exhibited high expression of all 4 transgenes (*Oct4*, *Sox2*, *c-Myc*, and *Klf4*) that were used to generate the *Fbxo15*-iPSCs (Figure 2C). This suggests that continuous expression of the transgenes restricted the differentiation potential of the *Fbxo15*-iPSC-derived SNSs and rendered them highly tumorigenic. To this point, upregulation of *Oct4* and *c-Myc* are reported in naturally occurring tumors.[38,39] Notably, when *Fbxo15*-iPSC-derived secondary neurospheres were transplanted into the brains (striatal region) of nonobese diabetic (NOD)/severe combined immunodeficient (SCID) mutant mice, they showed robust teratoma formation (Figure 2D). Taken together, these results suggest that partially reprogrammed iPSCs are not suitable for cell therapy, because somatic cells (eg, NS/PCs) induced from these iPSCs still show high teratoma-forming propensities attributed, at least in part, to their incomplete suppression of transgenes encoding *Oct3/4*, *Sox2*, *c-Myc*, and *Klf4*.

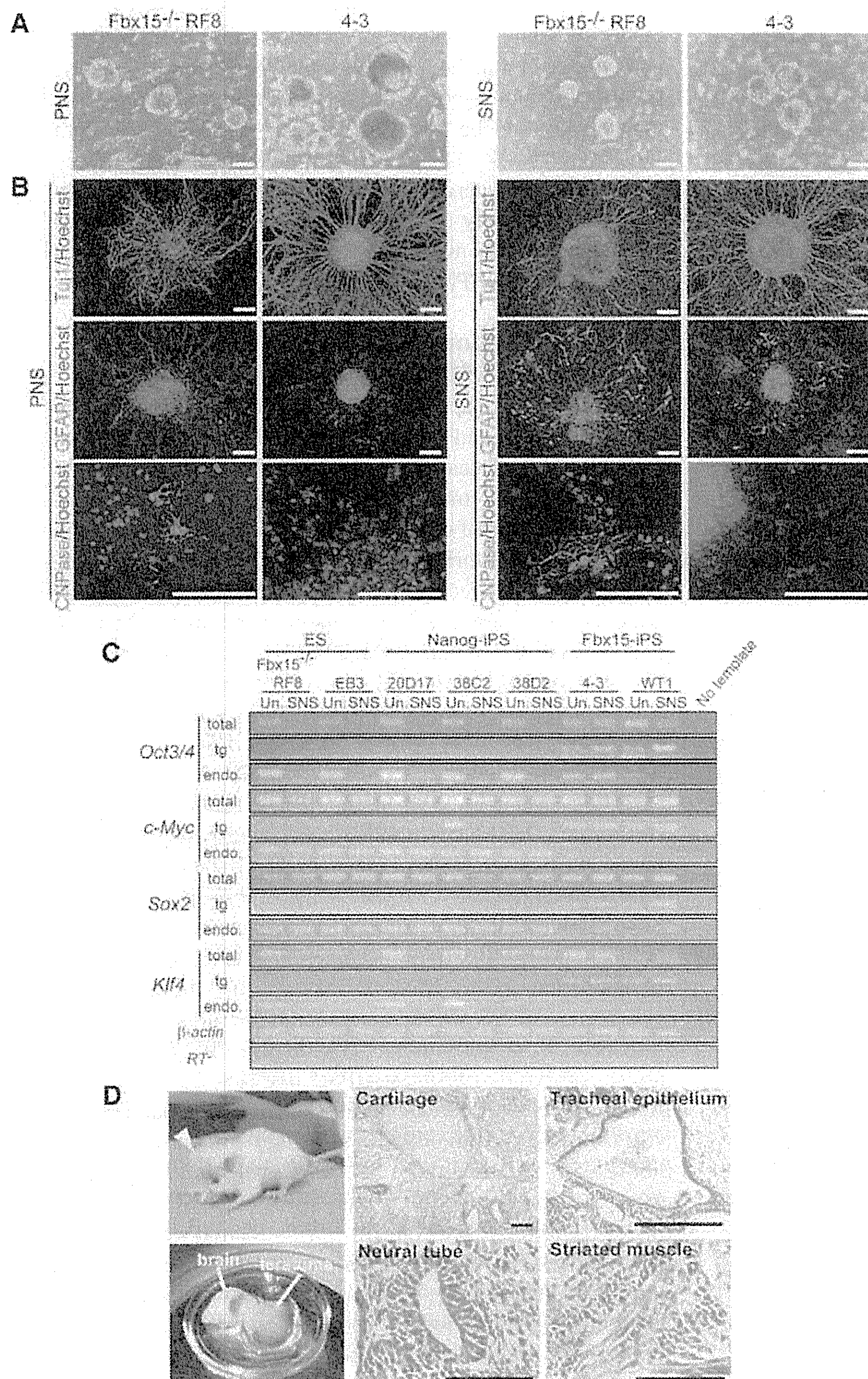

Figure 2. Characterization of neural stem/progenitor cells (NS/PCs) derived from *Fbxo15* induced pluripotent stem cells (iPSCs) in vitro. A, Neurosphere-like cell aggregates derived from *Fbxo15*-iPSCs (line 4-3). Scale bar, 200 μm. **B**, Immunocytochemical analyses of Tuj1 (class III β-tubulin), glial fibrillary acidic protein (GFAP), and 2′,3′-cyclic nucleotide 3[prime]-phosphodiesterase (CNPase) in differentiated primary neurospheres (PNSs) or secondary neurospheres (SNSs) derived from *Fbxo15*-iPSCs (line 4-3). Scale bar, 100 μm. **C**, Total RNA was isolated from undifferentiated cells (Un.) or SNSs of each cell clone and processed for reverse transcription polymerase chain reaction (RT-PCR) analysis with primers amplifying the coding regions of the 4 transgenes (total), endogenous transcripts only (endo.), or transgene transcripts only (tg). **D**, Immature teratomas derived from *Fbxo15*-iPSC (line 4-3)-derived SNSs. Large tumors were observed 4 weeks after transplantation of *Fbxo15*-iPSC (line 4-3)-derived SNSs (2 left images). These tumors were examined by histological methods using hematoxylin-eosin staining (right). Scale bar, 100 μm.

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Influence of the Somatic Origin of iPSCs for Safety Issues of Cell Therapy Using iPSC-Derived NS/PCs.

As a next step, mouse iPSCs generated with more advanced reprogramming (as reported by Okita et al in 2007,[17] Nakagawa et al in 2008, [19] and Aoi et al in 2008[21]) were examined instead of *Fbxo15*-iPSCs for their neural differentiation abilities and tumor-forming propensities. As described above, adult chimera-competent mouse iPSCs have been isolated by drug selection for the expression of pluripotency-associated genes such as *Nanog* and *Oct4*; and more recent approaches have allowed their generation in the absence of drug selection.[19] In these reports, tumor formation but not teratoma formation was observed to varying degrees and was ascribed to transgene (especially *c-Myc*) activation. However, this tumor-forming propensity in adult chimera mice might not necessarily correlate with tumorigenic risks of iPSC-based cell therapy in humans because of the different species-specific mechanisms underlying tumor formation. However, considering the variations in reprogramming methods reported to date, the safety and therapeutic implications of these variations must be thoroughly evaluated before iPSCs are used in cell therapies for human patients.

The teratoma-forming propensity of SNSs derived from advanced reprogramming mouse iPSC lines was next evaluated.[12] Mouse iPSC lines differ in terms of their somatic origin, as well as the method originally used for iPSC generation, that is, drug selection and/or use of *c-Myc* transgenes. However, the mouse iPSC lines examined in this study were all established with retroviral transgenes. Surprisingly, the presence or absence of the *c-Myc* transgenes used in the generation of the iPSCs did not affect the tendency of the SNSs to form teratomas. This differs from the tumorigenic inclinations of the adult chimeric mice, which are attributable to the reactivation of the *c-Myc* retrovirus.[16] However, in the case of iPSC-derived SNSs, reactivation of *c-Myc* or other transgenes was not observed, nor did the SNSs go on to form teratomas on transplantation. Furthermore, the use of drug selection did not affect the teratoma-forming propensity of the SNSs.

On the other hand, tumorigenic tendencies varied significantly depending on the somatic tissue of origin of the parent iPSC, showing good correlation with the persistence of undifferentiated cells within the SNS. For example, SNSs derived from iPSCs that were generated from adult tail-tip fibroblast iPSCs showed the highest teratoma-forming propensity. Those derived from iPSCs generated from mouse embryonic fibroblasts and stomach tissues showed the lowest propensity and were comparable to SNSs derived from ESCs. In fact, only 1 of 11 tail-tip fibroblast-iPSC lines (line 335D1) was free from teratomas after transplantation into the striatum. Our current hypothesis is that undifferentiated cells that are continually present within the SNS could act as a source of differentiation-resistant and teratoma-initiating cells. It will be of great interest to examine whether the presence of undifferentiated cells also correlates with the teratoma-forming propensity of iPSCs generated from other somatic cells, such as cardiomyocytes. Moreover, transplantation applications for human patients will necessitate the examination of the teratoma-forming properties of NS/PCs derived from hiPSCs established from various somatic origins.

The mechanisms underlying the different teratoma-forming propensities of SNSs derived from various mouse iPSC lines remain to be determined. However, the fact that the somatic origin of the iPSCs significantly influences this propensity might suggest the involvement of epigenetic mechanisms, although the involvement of genetic changes cannot be excluded. Genes that are differentially expressed between ESCs and iPSCs are termed reprogramming-resistant genes. These genes resist the induction of a transcriptional state in iPSCs that is identical to that seen in ESCs.[40] Resistance of reprogramming might result from any of the following 3 mechanisms: (1) insufficient induction of ESC-specific genes; (2) insufficient suppression of somatic cell-specific genes; and (3) excess induction of iPSC-specific genes. In the case of teratoma formation by SNSs, it seems likely that the second mechanism could be involved, because the tissue of origin of the parent iPSC is tightly associated with the tendency of the SNS to generate teratomas. It is therefore crucially important to identify the reprogramming-resistant genes that are associated with tumor formation, from the viewpoint of safety concerns for iPSC-based cell therapy.

Transplantation of Safe Mouse iPSC Clone-Derived NS/PCs for the Repair of SCI

As described above, many types of iPSCs have thus far been established, and each type exhibits different biological properties (eg, the capacity to form teratomas after neural differentiation and transplantation, discussed above). Thus, detailed evaluation of each iPSC line, including differentiation potential and tumorigenic activity in different contexts, should be investigated to establish the safety of that line and its effectiveness for cell transplantation therapies. On these grounds, this group examined the therapeutic potential of NS/PCs derived from mouse iPSCs in an SCI model.[6]

Neurospheres from safe iPSCs (38C2 mouse embryonic fibroblasts- and 335D1 tail-tip fibroblasts-iPSC lines), which had been pre-evaluated as nontumorigenic after their transplantation into the NOD/SCID mouse brain,[12] were first considered. These neurospheres were transplanted into the spinal cord 9 days after a contusion injury. The neurospheres differentiated into all 3 neural lineages (neurons, astrocytes, and oligodendrocytes) without forming teratomas or other tumors. The graft-derived oligodendrocytes participated in remyelination and induced axonal regrowth of host 5HT(+) serotonergic fibers, which are associated with the locomotor functions of the hindlimbs.[41] The therapeutic effects of the mouse embryonic fibroblasts-iPSC line (38C2)-derived NS/PCs were very similar in their regenerative capabilities to NS/PCs derived from either mouse fetal striatal regions[42] or mouse ESCs.[30] The 3 types of NS/PCs all stimulated recovery of locomotor function in the same mouse SCI contusion model (Figure 3). By contrast, iPSC-derived neurospheres pre-evaluated as unsafe showed robust teratoma formation on transplantation and sudden locomotor functional loss after preliminary functional recovery, putatively because of a tumor mass effect in the SCI model.

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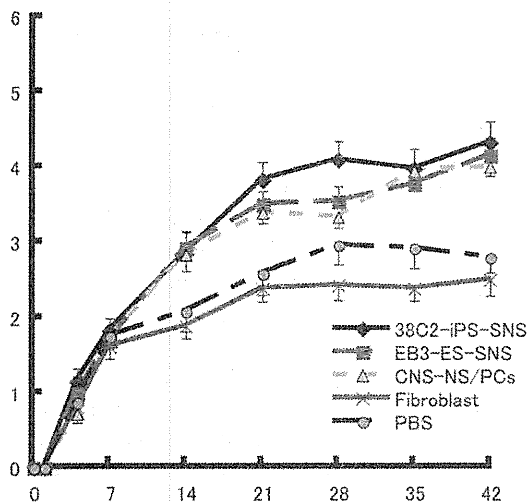

Figure 3. Therapeutic effects of secondary neurospheres (SNSs) derived from 3 independent sources (fetal mouse brains, mouse embryonic stem cells [ESCs], and mouse induced pluripotent stem cells [iPSCs]) in a mouse spinal cord injury (SCI) model. Summary of previous studies showing the time course of functional recovery of hindlimbs evaluated by the locomotor rating of the Basso Mouse Scale (BMS)[87] in SCI mice (adult female C57BL/6J mice) with a contusion injury at thoracic level 10 induced using an Infinite Horizon impactor (60 kdyn; Precision Systems, Lexington, KY). These mice were transplanted with 5×10^5 SNSs derived from mouse iPSCs (38C2 line; n=19)[6]; mouse ESCs (EB3 line; n=15)[6]; or dissociated striatal regions derived from the brains of fetal mice (embryonic day 14 C57BL/6J mice; n=8).[42] Other mice were injected with adult fibroblasts instead of SNSs (n=13).[6] Negative control mice were injected with phosphate buffered saline (n=12).[6] Cells were injected into the lesion epicenter at 9 days after the injury. As shown here, the therapeutic effects of neural stem/progenitor cells (NS/PCs) derived from 3 independent sources were very similar to one another. *P<0.05, **P<0.01.

In summary, pre-evaluated safe iPSC-derived NS/PCs and ESC-derived NS/PCs[30] showed similar therapeutic effects in an in vivo SCI model, suggesting that the availability of the human equivalent of these cells would provide a promising cell source for transplantation therapy after CNS damage.[34]

Transplantation of hiPSC-Derived NS/PCs Promoted Functional Recovery After SCI in NOD/SCID Mice

As discussed in the preceding section, once the safety issue is overcome, hiPSCs could be a potential cell source for regenerative medicine in human patients. Therefore, the therapeutic potential of transplantation of hiPSC-derived neurospheres for SCI in NOD/SCID mice was next investigated. The hiPSC clone (201B7 line)[2] used in this work was established using 4 reprogramming factors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) through retroviral transduction. The grafted hiPSC-derived neurospheres survived, migrated, and differentiated into all 3 neural lineages within the injured spinal cord. The transplanted hiPSC-derived neurospheres resulted in significant functional recovery through cell-autonomous, as well as nonautonomous, mechanisms. No tumor formation was observed in the hiPSC-derived neurosphere-grafted mice during an observation period of ≤112 days. Furthermore, continuous functional recovery

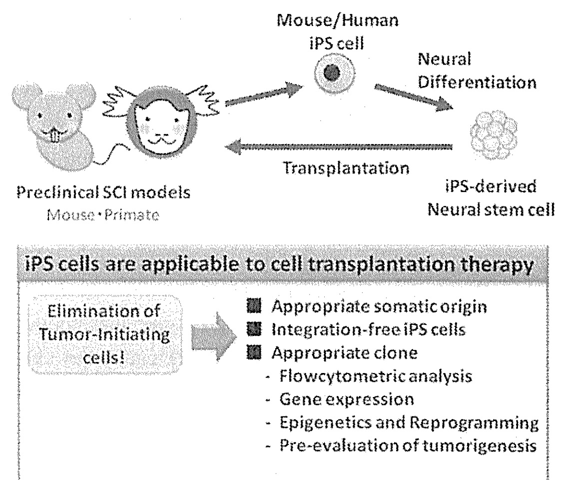

Figure 4. Preclinical studies for spinal cord injury (SCI) repair using induced pluripotent stem cell (iPSC)-derived neural stem/progenitor cells (NS/PCs). NS/PCs were generated from mouse or human iPSCs. The NS/PCs were subsequently transplanted into SCI models that used mice or nonhuman primates.[44] Appropriate selection of the iPSCs resulted in significant functional recovery without tumor formation. For future clinical applications, intensive validation of safety issues will be necessary, as indicated in the Figure.

was observed during this time period. Hence, NS/PCs derived from hiPSCs were an effective cell source for transplantation therapy in a murine model of SCI.[34] Recently, hiPSC (201B7 line) neurospheres were also successfully transplanted into a nonhuman primate SCI injury model, resulting in a functional recovery with no sign of tumor formation.[43,44] The neurospheres promoted significant functional recovery with no tumor formation for ≤3 months after transplantation.[43,44]

In summary, NS/PCs were induced from mouse and hiPSCs and transplanted into mouse and/or nonhuman primate SCI models. The NS/PCs stimulated functional locomotor recovery without forming detectable tumors. These results were accomplished via selection of the appropriate safe iPSC lines (Figure 4).

Potential Immunogenicity in iPSC-Based Cell Therapy

A recent study indicated that iPSCs are potentially immunogenic.[15] This conclusion was made after transplantation of undifferentiated mouse iPSCs and assays for teratoma formation. However, in the case of the work from this group, transplantation of predifferentiated NS/PCs from appropriately pre-evaluated mouse iPSCs into the damaged mouse spinal cord showed no evidence of tumorigenesis or immunogenicity. Furthermore, the NC/PCs differentiated into normal trilineage neural cells in the injured spinal cord[6] in a similar way to ESC-derived NS/PCs[30] and fetal CNS-derived NS/PCs.[42]

To further address the concern of immunogenicity, global gene expression profiles were compared among undifferentiated mouse ESCs (EB3 line), mouse iPSCs (38C2 line), and predifferentiated ESC/iPSC-derived NS/PCs. Principal component analysis and hierarchical clustering analysis revealed that predifferentiated NS/PCs were clustered closely with

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mouse fetal NS/PCs and separated completely from undifferentiated ESCs/iPSCs (Figure 5A and 5B). Moreover, expression levels of the representative pluripotent markers *Nanog* and *Oct4* were drastically downregulated and neuronal markers were strongly upregulated during neural differentiation (Figure 5C). Zhao et al[15] suggested that the potential immunogens *Hormad1* (a tumor antigen) and *Spt1* (a tissue-specific antigen) were abnormally upregulated in undifferentiated iPSCs and/or iPSC-derived teratomas. However, in this work, the expression levels of *Hormad1* and *Spt1* were undetectable in iPSC-derived NS/PCs, and the expression levels were very low even in undifferentiated mouse iPSCs (Figure 5D). These results strongly indicate that predifferentiated NS/PCs possess entirely different properties than undifferentiated ESCs/iPSCs and that selection of appropriate iPSC lines and proper differentiation of iPSCs will greatly reduce the potential risk of immunogenicity.

Recent Progress for the Generation of Safer hiPSCs

The above-mentioned results indicate that the safety of iPSC-based cell therapy depends on appropriate selection of iPSC lines. On the other hand, retroviral transgene activation and/or retroviral insertion mutagenesis are admittedly risk factors for the tumorigenesis of iPSC-derived cells. For this reason, increasing efforts have been directed recently toward the generation of insertion-less or insertion-free iPSCs using chemical compounds,[45–47] adenovirus vectors,[48] transposons,[49,50] plasmids,[51] recombinant proteins,[52,53] episomal vectors,[54,55] Sendai virus vectors,[56–59] and modified RNA.[60] Efforts are also underway to modify the transgenes and/or chemical compounds used in an attempt to both improve the quality of iPSCs and the efficiency of iPSC generation. These strategies include the development of inhibitors for histone deacetylase[61] and protein kinases (mitogen activated protein kinase and glycogen synthase kinase-3)[62] and the replacement of *c-Myc* with *L-Myc*.[14] Reprogramming

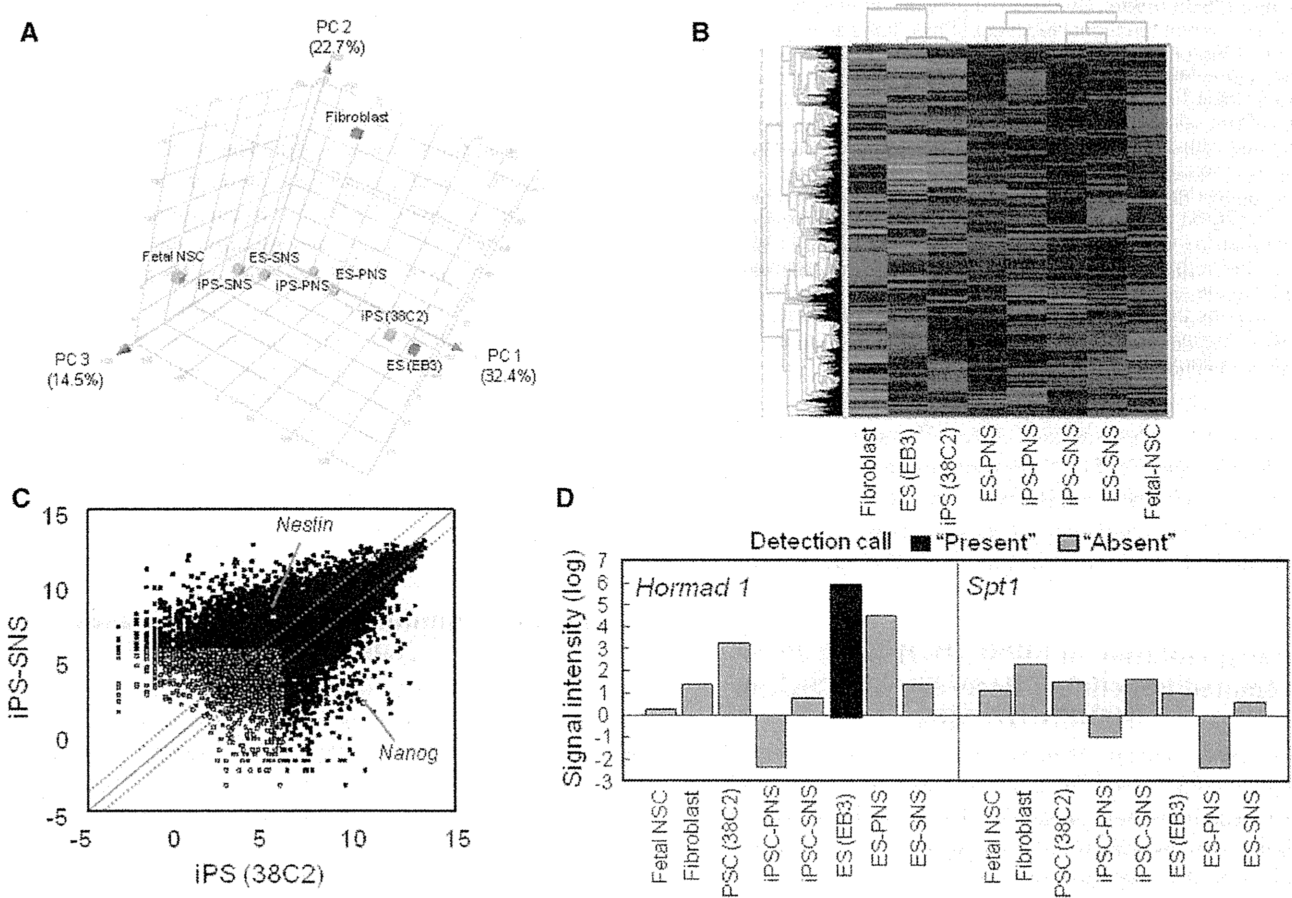

Figure 5. Global gene expression analysis of induced pluripotent stem cell (iPSC)-derived neural stem/progenitor cells (NS/PCs) and expression levels of genes encoding potential immunogens. Global gene expression analysis was carried out using Affymetrix GeneChip technology with standard protocols (accession No. GSE31725). Signal detection and quantification were performed using the MAS5 algorithm, and global normalization was performed so that the average signal intensity of all probe sets was equal to 100. **A**, Principal component analysis of gene expression data (33 314 probe sets). Color key: blue cube, fibroblast; green cubes, neurospheres; red cube, embryonic stem cells (ESCs; EB3 line); pink cube, iPSCs (38C2 line). **B**, Hierarchical clustering analysis of gene expression data (33 314 probe sets). The signal intensity of each gene was normalized and calculated for visualization. **C**, Scatter plot using microarray data of iPSCs and iPSC-derived secondary neurospheres (SNSs). Values represent the logarithmical signal intensity. Red dotted lines indicate a 2-fold increase or decrease between 2 samples. A signal intensity of 50 was set as the cutoff line for gene expression. Black squares, Expression in at least one sample; gray squares, no expression in either sample. **D**, Expression of genes encoding potential immunogens in undifferentiated ESCs/iPSCs and ESC/iPSC-derived neurospheres. Values represent the logarithmical signal intensity.

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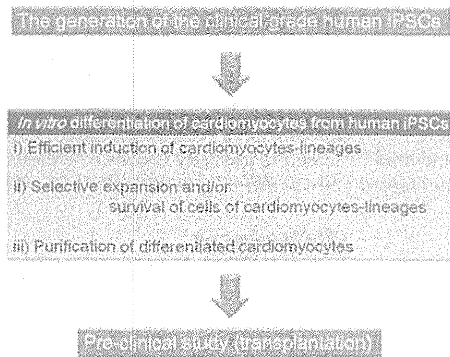

Figure 6. Safety and preclinical use of human induced pluripotent stem cells (iPSCs) in cardiovascular repair model. The steps toward safe human iPSC-based cell therapy for severe heart diseases, such as myocardial infarction, would include efforts on the generation of clinical-grade human iPSCs, in vitro differentiation of cardiomyocytes from human iPSCs, and preclinical study (transplantation) for cardiac diseases models to assess the safety and effectiveness.

of somatic cells is also being explored using mature microRNAs[63,64] and maternal transcription factor *Glis1*.[65] Another method to enhance reprogramming is by reducing p53-activity via induction of the dominant-negative form of *p53* or its short hairpin RNA.[55] The rationale behind this approach is that p53 suppresses the generation of iPSCs through the p53-p21 pathway.[66] Despite this rapid evolution of iPSC technology, fail-safe approaches for the generation of clinical-grade iPSCs have not yet been established and are part of an ongoing process.[67]

Safety and Preclinical Use of iPSCs in a Cardiovascular Repair Model

The rapid progress of stem cell technologies described above has triggered an increasing interest in the use of pluripotent stem cells including ESCs and iPSCs in cardiovascular repair. On the generation of clinical-grade hiPSCs, how can these cells be applied for regenerative medicine of cardiovascular diseases? One of the strongest advantages of using pluripotent stem cells for cardiovascular repair will be the highly expandable and self-renewal nature of these cells, which could provide an unlimited source of particular types of cardiovascular cells, including cardiomyocytes for cell therapy of severe heart diseases, such as myocardial infarction. On the other hand, as is the case in cell therapy for CNS disorders,[6,33] the potential pitfall of pluripotent stem cell-based therapy for the treatment of severe heart diseases is teratoma-forming propensity,[68] which is associated with the contamination of undifferentiated pluripotent stem cells and/or differentiation-resistant cells. Furthermore, a significantly larger number of cells would be required for cell therapy of severe heart diseases compared with that of SCI. Considering these issues, the large-scale preparation of clinical-grade cardiomyocytes would require addressing the following issues after the generation of clinical-grade human ESCs/iPSCs: (1) efficient induction of cardiomyocyte lineages from pluripotent stem cells; (2) selective expansion and/or survival of cells of cardiomyocyte lineages derived from the pluripotent stem cells; and (3) purification of differentiated cardiomyocytes derived from pluripotent stem cells and elimination of residual undifferentiated pluripotent stem cells (Figure 6). These steps are likely to be common for hESCs and hiPSCs and should be followed by performing preclinical testing for safety and effectiveness. Here, we introduce each strategy one by one.

Efficient Induction of Cardiomyocyte Lineages

As in neural differentiation,[35–37] the efficient induction of cardiomyocyte lineages from pluripotent stem cells in vitro requires the recapitulation of the microenvironmental factors that play a role during mesodermal and cardiac development. In fact, multiple steps are involved in cardiomyocyte development, including initial mesodermal differentiation, emergence of the cardiac myoblast, cardiac myoblast proliferation, and cardiomyocyte maturation.[69] In mouse embryonic development, the bone morphogenetic protein antagonist Noggin is transiently but strongly expressed in the heart-forming region during gastrulation and induces the mesendoderm for cardiogenic development. Yuasa et al[70] took advantage of this fact to develop an effective protocol for obtaining cardiomyocytes from mouse ESCs by inhibition of bone morphogenetic protein signaling. In contrast to the in vitro neural differentiation protocol of mouse ES cells, in which Noggin is administered for a prolonged period during EB formation,[35] transient Noggin treatment (from 3 days before to 1 day after EB formation) is important for the induction of cardiomyocytes. In fact, the transient Noggin treatment protocol is one of the most efficient protocols for cardiomyocyte differentiation, yielding an ≈100-fold increase in the number of cardiomyocytes compared with the control. In addition to the Noggin protocol, various techniques have been developed for the efficient induction of cardiomyocytes from pluripotent stem cells including iPS cells and human cells.[71–75]

Selective Expansion and/or Survival of Cells of Cardiomyocyte Lineage

Precise regulation of the bone morphogenetic protein,[70,76] Wnt,[77–79] hedgehog,[80] and Notch pathways[71,81] has been reported to play an important role in particular steps of cardiac development.[69] On the other hand, granulocyte colony-stimulating factor was identified as a humoral factor that uniquely promotes the proliferation of cardiomyocytes derived from mouse ESCs,[82] consistent with the expression profile of granulocyte colony-stimulating factor and its receptor in embryonic cardiac development. Administration of extrinsic granulocyte colony-stimulating factor was also found to promote the proliferation of hiPSC-derived cardiomyocytes, indicating that granulocyte colony-stimulating factor can be used to obtain high yields of cardiomyocytes from hESCs/hiPSCs for their potential application in regenerative medicine of heart diseases.

Purification of Differentiated Cardiomyocytes

The formation of teratomas in response to transplantation of undifferentiated pluripotent stem cells[68] implies that the purification of pluripotent stem cell-derived cardiomyocytes before transplantation is essential. For this purpose, various combinations of cardiomyocyte-specific reporters have been used to obtain highly pure cardiomyocytes from pluripotent stem cells,[83–87] although this method requires genetic modification of the cells. Recently, Hattori et al[13] developed a nongenetic

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cardiomyocyte purification method (>99% purity) based on the fact that differentiated cardiomyocytes are extremely enriched in mitochondria. In this method, a mitochondria-selective fluorescent dye and a flow cytometer (a mitochondrial method) are used for the purification of hiPSC-derived cardiomyocytes. Notably, hESC-derived cardiomyocytes purified by this method did not induce teratoma formation after transplantation into NOD/SCID mice. Thus, this mitochondrial method could potentially contribute to the safety of hiPSC-based cell therapy for severe heart diseases, although high-speed flow cytometry of clinical grade is required for the application of this method to the treatment of human patients. Thus, further technological improvements would be required to purify large amounts of clinical-grade cardiomyocytes.

Although the above-mentioned steps are essential for the large-scale preparation of clinical-grade purified cardiomyocytes derived from human ESCs/iPSCs, their safety and effectiveness should be assessed, and methods developed for the administration of therapeutic cells into damaged hearts should be optimized using large animals as preclinical models (Figure 6).

Conclusions: Perspectives for Safe iPSC-Based Cell Therapy

Despite some precautionary data and critical attitudes, accumulating preclinical evidence supports the effectiveness of iPSC-based cell therapy on the selection of appropriate iPSC clones. Continuous development of safer iPSCs has resulted from insertion-free systems and the use of new transgenes. Nevertheless, before clinical application of iPSC-based cell therapies is achieved, these safety concerns must be assuaged through a thorough examination of the quality of both iPSCs and iPSC-derived cells, in terms of genetic and epigenetic status, differentiation capability both in vitro and in vivo, and tumorigenicity. Initial studies will require transplantation of these cells into immune-deficient animals, with subsequent long-term observation.

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Disclosures

H. Okano is the scientific consultant of San Bio, Inc; Eisai Co Ltd; and Daiichi Sankyo Co Ltd. K. Yoshida is employed by Dainippon Sumitomo Pharma Co Ltd; he worked in Dr Okano's laboratory in Keio University for 1 year as a collaborative research fellow. S. Yamanaka is a member of scientific advisory boards of iPearian Inc and iPS Academia Japan. The remaining authors report no conflicts.

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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

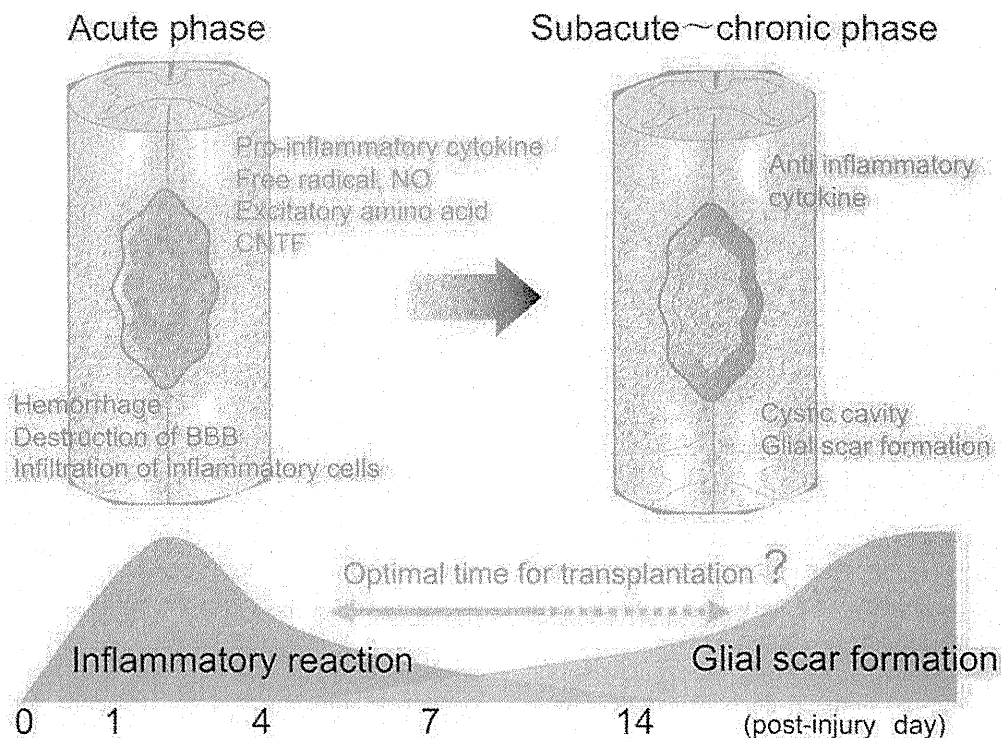

Figure 1 Microenvironment of the injured spinal cord. Because the immediately post-traumatic microenvironment of the spinal cord is in an acute inflammatory stage, it is not favorable for the survival and differentiation of NS/PC transplants. On the other hand, in the chronic stage after injury, glial 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

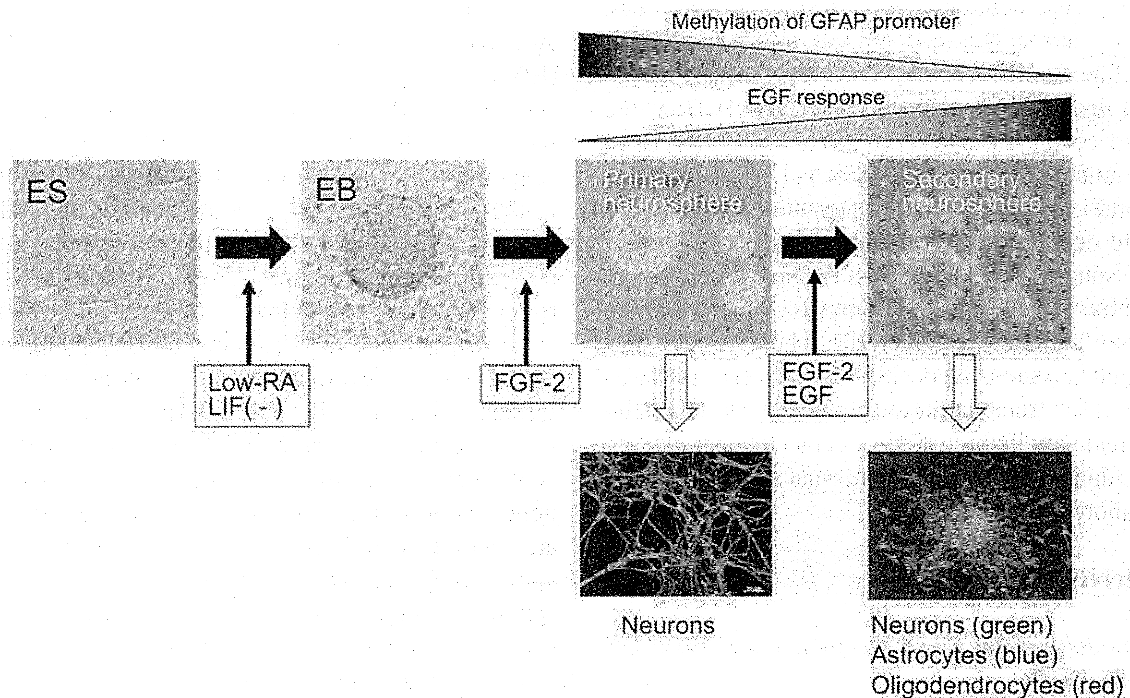

Figure 2 Neural induction of mouse ES cells through EBs. After the removal of LIF, ES cells are cultured in suspension to induce the formation of EBs. EBs contain relatively early-stage NS/PCs, which can be selectively cultured as neurospheres by suspended culture in serum-free medium for NS/PCs in the 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

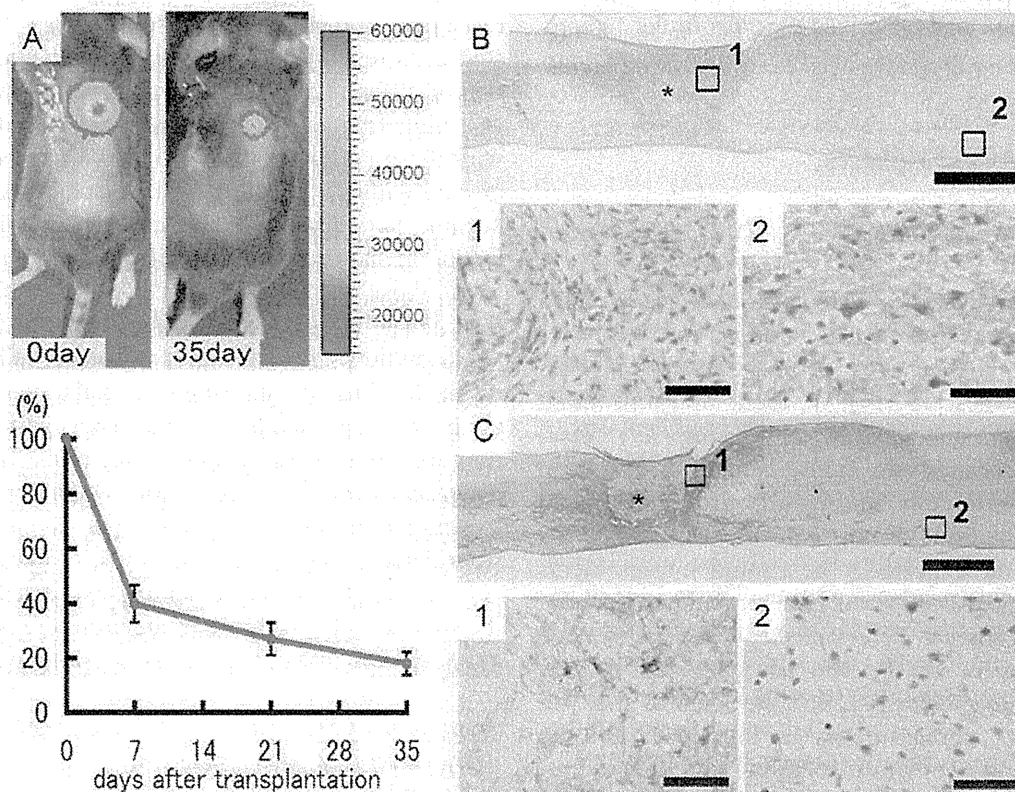

Figure 3 Transplanted safe 38C2-iPS-secondary neurospheres survive without any evidence of tumorigenesis [32]. **(A)** Representative BLI images of a mouse in which CBRluc-expressing 38C2-iPS secondary neurospheres were transplanted into the injured spinal cord (left, immediately after transplantation; 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].

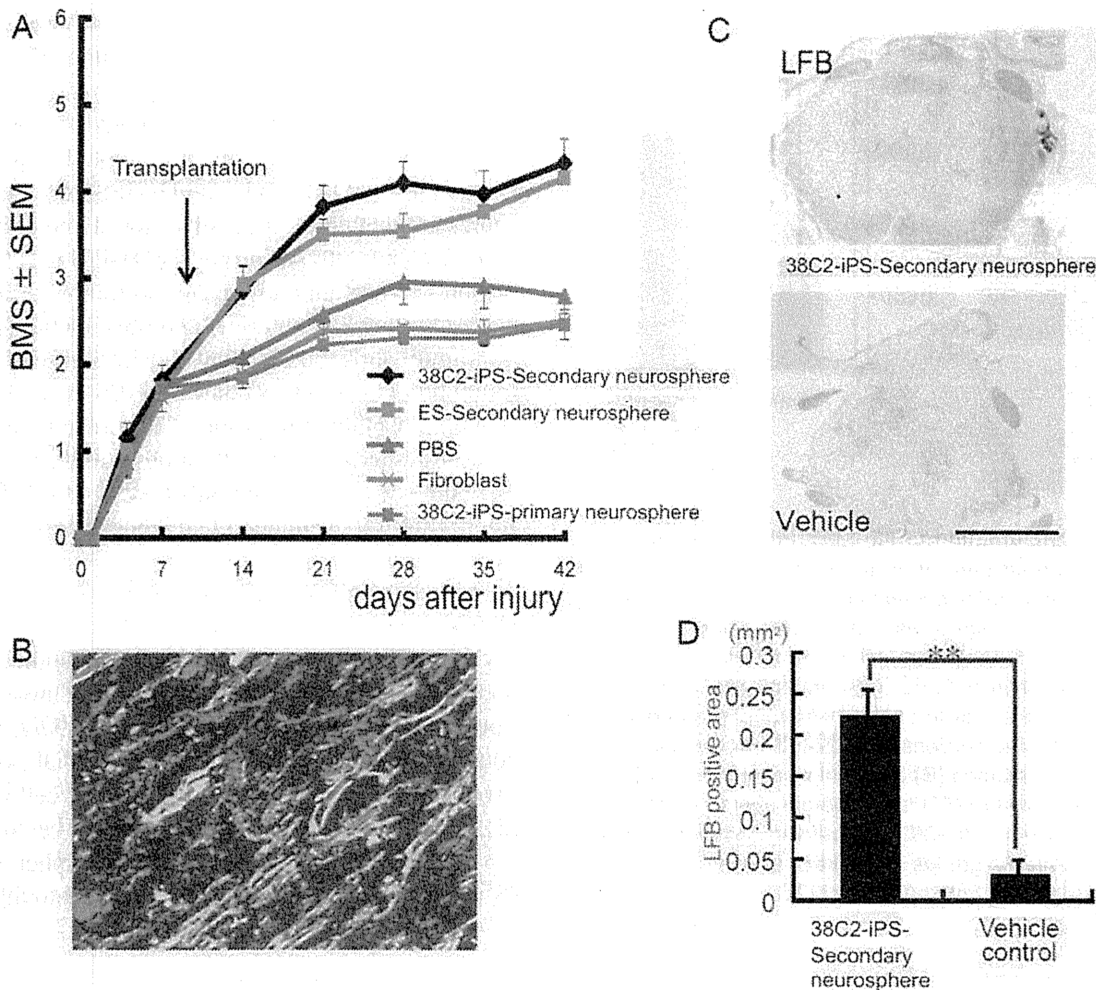

Figure 4 Transplanted secondary neurospheres derived from safe MEF-iPS clones contributed to the remyelination, thereby promoting functional recovery [32]. **(A)** 38C2-iPS-derived secondary neurosphere-transplanted mice showed significantly better functional recovery compared to the PBS and fibroblast 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,

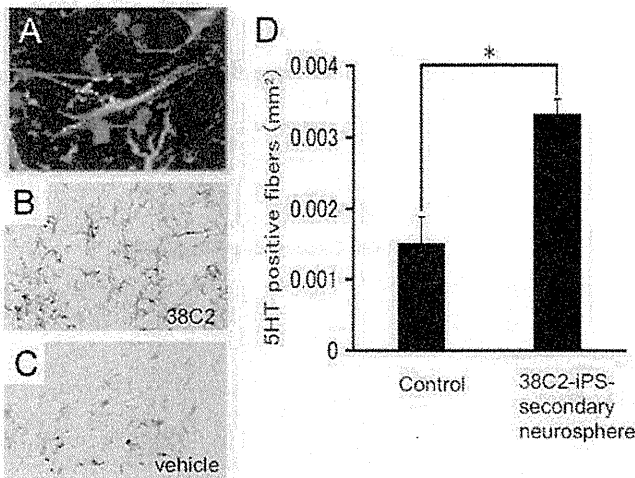

Figure 5 Transplantation of safe MEF-iPS-secondary neurospheres promoted serotonergic innervation of the dorsal cord [32]. **(A)** Immunohistochemistry of 38C2-iPS-secondary neurosphere-derived astrocytes closely associated with 5HT+ serotonergic fibers. (RFP, red; GFAP, green and 5-HT, white) **(B, 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

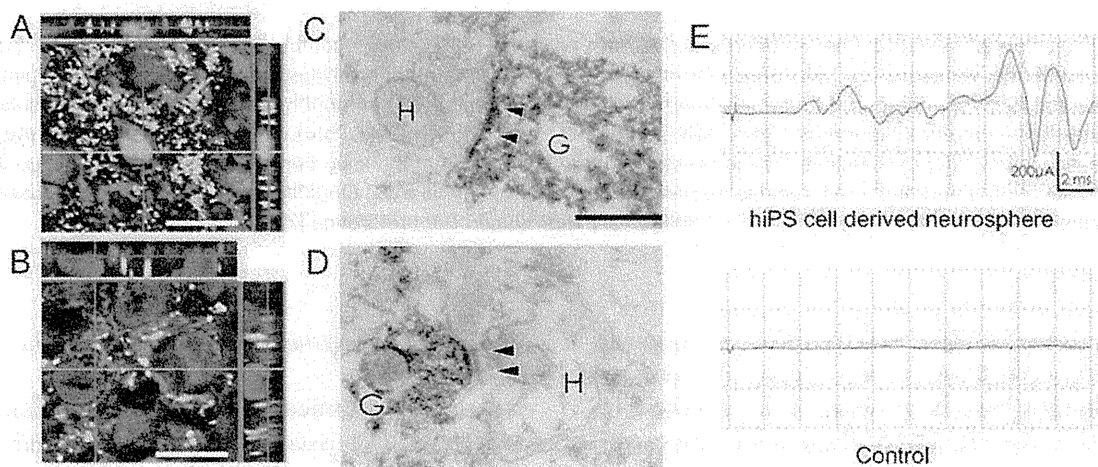

Figure 6 Evidence for synapse formation between human iPS cell (201B7)-derived neurons and host mouse spinal cord neurons [34]. **(A)** Sections were triple-stained with HNu (green), βIII tubulin (red) and the presynaptic marker Bassoon (Bsn, white). The Bsn antibody used here recognized the rat and 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.

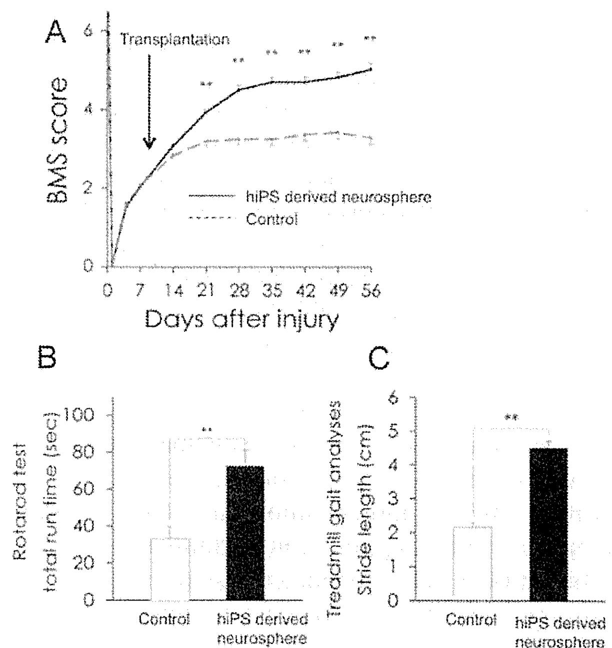

Figure 7 Transplanted human iPS cell (201B7)-derived neurospheres promoted motor functional recovery after SCI [34]. **(A)** Motor function in the hindlimbs was assessed weekly by the BMS score for 56 days. Values are means ± SEM. **(B)** Rotarod test 56 days after SCI. Graph shows the total run time. 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].

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.

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.

Acknowledgments

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