Steps Toward Safe Cell Therapy Using Induced Pluripotent Stem Cells

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Abstract: The enthusiasm for producing patient-specific human embryonic stem cells using somatic nuclear transfer has somewhat abated in recent years because of ethical, technical, and political concerns. However, the interest in generating induced pluripotent stem cells (iPSCs), in which pluripotency can be obtained by transcription factor transduction of various somatic cells, has rapidly increased. Human iPSCs are anticipated to open enormous opportunities in the biomedical sciences in terms of cell therapies for regenerative medicine and stem cell modeling of human disease. On the other hand, recent reports have emphasized the pitfalls of iPSC technology, including the potential for genetic and epigenetic abnormalities, tumorigenicity, and immunogenicity of transplanted cells. These constitute serious safety-related concerns for iPSC-based cell therapy. However, preclinical data supporting the safety and efficacy of iPSCs are also accumulating. In this Review, recent achievements and future tasks for safe iPSC-based cell therapy are summarized, using regenerative medicine for repair strategies in the damaged central nervous system (CNS) as a model. Insights on safety and preclinical use of iPSCs in cardiovascular repair model are also discussed. (Circ Res. 2013;112:523-533.)

Key Words: induced pluripotent stem cell ▶ neural stem/progenitor cell ▶ transplantation ▶ spinal cord injury

Applications for the Use of Induced Pluripotent Stem Cells and Related Concerns: An Overview

Ever since pioneering reports introduced mouse1 and human-induced2–4 pluripotent stem cells (iPSCs) to the scientific community and the populace at large, there has been an increasing interest in applications for their use in the fields of biomedical research. These include cell therapy in regenerative medicine and modeling of human disease. By characterizing

Original received September 2, 2011; revision received October 31, 2011; accepted November 1, 2011. In November 2012, the average time from submission to first decision for all original research papers submitted to Circulation Research was 15.8 days.

From the Departments of Physiology (H.O., K.Y., Y.O., O.T., S.N., K.M.) and Orthopedic Surgery (M.N., O.T., S.N.) and Kanrinmaru Project (K.Y., Y.O.), School of Medicine, Keio University, Tokyo, Japan; Department of Pathology, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan (E.I.); Center for iPSC Cell Research and Application, Kyoto University, Kyoto, Japan (S.Y.); and Genomic Science Laboratory, Dainippon Sumitomo Pharma, Osaka, Japan (K.Y.).

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.111.256149

523
the in vitro phenotype of disease-specific iPSC-derived cells, researchers have gained new insights, not only into the pathophysiology of the particular disorder, but also into strategies for drug screening and the development of novel therapeutic agents. Although disease modeling is emerging as an extremely exciting research field, this issue will not be discussed here because of space limitations. In regard to the application of iPSCs for regenerative medicine, increasing experimental evidence supports their therapeutic benefits. However, some recent reports also indicate risk factors for the use of iPSCs, such as genetic and epigenetic abnormalities that could take place during reprogramming or maintenance in subsequent cell culture.

Of particular relevance is the potential tumorigenicity and immunogenicity associated with iPSC-based cell therapy. The purpose of this review article is to summarize previous efforts in the field, as well as the current status of iPSC-based cell therapy. Work from this group that uses iPSC-derived neural stem/progenitor cells (NS/PCs) in preclinical studies for repair of the damaged central nervous system (CNS) is discussed, with a special emphasis on spinal cord injury (SCI). These findings and those from other groups are presented in light of an important task for the future: how should researchers best address the implicit pitfalls of iPSC-based cell therapy?

Early Development of iPSC-Based Technologies

Mouse iPSCs were first established by Takahashi and Yamanaka in 2006, and continuous progress has been made in the methods for their production during recent years. This article does not attempt to fully cover the methodological details of iPSC production; however, the key findings that are related to safety issues of iPSC-based cell therapy are summarized herein.

Initially, iPSCs were generated from mouse fibroblasts by retroviral introduction of the transcription factors Oct4, Sox2, c-Myc, and Klf4. The actions of these 4 transcription factors are thought to reprogram somatic cells, including fibroblasts, into embryonic stem cell (ESC)-like pluripotent cells through multiple stochastic epigenetic events (eg, silencing of somatic genes and retroviral genes that putatively activate Dnmt3a and 3b) and activation of various pluripotent genes. The original mouse iPSCs were selected for the expression of Fbxo15, a marker of undifferentiated ES cells, and thus are called Fbxo15-iPSCs. These cells demonstrated in vitro and in vivo differentiation into various types of cells from all 3 germ layers, but their epigenetic and biological properties differed from ESCs. Oct4-iPSCs are currently understood to be partially reprogrammed iPSCs in which the retroviral transgenes are still expressed and the autoregulatory loops of endogenous Oct4/Sox2/Nanog genes are not completely established. In fact, our group could not obtain pups from Fbxo15-iPSC-derived chimeric mice, although these cells contributed to embryonic development.

In July 2007, 2 groups independently reported that selection for Nanog or Oct4 expression resulted in germline-competent mouse iPSCs with increased mouse ESC-like gene expression and DNA methylation patterns compared with Fbxo15-iPSCs. Nanog and Oct4 are known to be crucial for the maintenance of undifferentiated ESCs by forming autoregulatory loops of endogenous Oct4/Sox2/Nanog genes. Nanog-iPSCs were generated by Okita et al from fibroblasts of transgenic mice containing the Nanog-GFP-IRE-PURO reporter construct. Fibroblasts from this transgenic mouse do not express the endogenous Nanog gene; hence, they are puromycin sensitive. However, by the retroviral transduction of Oct4, Sox2, c-Myc, and Klf4, these fibroblasts are reprogrammed into ESC-like cells and start to express the protein products of the endogenous Nanog gene, as well as the Nanog-GFP-IRE-PURO reporter gene. As such, the cells acquire puromycin resistance and green fluorescent protein expression after transduction. Thus, through selection of puromycin-resistant and green fluorescent protein-positive colonies, iPSC clones with high endogenous Nanog expression can be obtained. These reprogrammed Nanog-iPSCs, as well as Oct4-iPSCs, are likely to be at a more advanced state of development than Fbxo15-iPSCs, with a phenotype characterized by activated Dnmt3a and 3b, silenced retroviral transgenes, and established autoregulatory loops of the Oct4/Sox2/Nanog genes. However, there is no guarantee that Nanog-selected iPSCs or Oct4-selected iPSCs are completely equivalent to mouse ESCs. In fact, reactivation of the c-Myc retroviral transgene increased tumorigenicity in chimeric mice obtained via blastocyst injection of Nanog-selected iPSCs.

In January 2008, Nakagawa et al reported a modified protocol for the generation of mouse iPSCs that did not require the c-Myc transgene. Importantly, chimeric mice derived from the c-Myc-minus iPSCs did not develop tumors during the study period. Furthermore, the omission of the c-Myc transgene resulted in the efficient isolation of iPSCs without drug selection (eg, Nanog-puromycin selection). This finding is advantageous for the generation of human iPSCs (hiPSCs), because the human genome does not accommodate the Nanog-green fluorescent protein-IRE-PURO reporter gene (Figure 1).

From April 2008 to February 2009, 3 articles were published reporting that mouse iPSCs could be generated from cells of various adult somatic origins, including terminally differentiated B lymphocytes, liver and stomach cells, and neural stem cells, although the required combinations of transgenes were different depending on the somatic origin. Thus, the epigenetic state of the somatic cells affected the efficacy of their reprogramming into iPSCs.
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 or Oct4, Sox2, Nanog, and Lin28. 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. 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 and NS/PCs. 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. 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.

On these grounds, soon after the first publication that introduced mouse iPSCs and the successful establishment of mouse iPSCs, our group began preclinical investigations of iPSC-based cell therapy for SCI. 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-iPSC 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 with either Noggin or a low concentration of retinoic acid, with subsequent neurosphere formation. 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. 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. 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.
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. 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 II 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.
Influence of the Somatic Origin of iPSCs for Safety Issues of Cell Therapy Using iPSC-Derived NS/PCs.

As a next step, mouse iPS cells 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 200821) 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 SNSs. For example, SNSs derived from iPSCs that were generated from adult tail-tip fibroblasts 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 iPSCs 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 brains,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 regions42 or mouse ESCs.43 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.
Preclinical SCI models Mouse+Primate

Mouse/Human iPSC cell

Neural Differentiation

Transplantation iPSC-derived Neural stem cell

Elimination of Tumor-Initiating cells!

- Appropriate somatic origin
- Integration-free iPSCs
- Appropriate clone
- Flowcytometric analysis
- Gene expression
- Epigenetics and Reprogramming
- Pre-evaluation of tumorigenesis

Figure 3. Therapeutic effects of secondary neurospheres (SNs) 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) 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 5x10^5 SNSs derived from mouse iPSCs (38C2 line; n=19); mouse EScs (EB3 line; n=15); or dissociated striatal regions derived from the brains of fetal mice (embryonic day 14 C57BL/6J mice; n=8). Other mice were injected with adult fibroblasts instead of SNSs (n=13). Negative control mice were injected with phosphate buffered saline (n=12). 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 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.

Transplantation of iPSC-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 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) 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 neurosphere-grafted mice during an observation period of ≤112 days. Furthermore, continuous functional recovery 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. 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. The neurospheres promoted significant functional recovery with no tumor formation for ≤3 months after transplantation.

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).

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. 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.

Potential Immunogenicity in iPSC-Based Cell Therapy

A recent study indicated that iPSCs are potentially immunogenic. 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 in a similar way to ESC-derived NS/PCs and fetal CNS-derived NS/PCs.

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
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.\textsuperscript{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,\textsuperscript{45–47} adenovirus vectors,\textsuperscript{48} transposons,\textsuperscript{49,50} plasmids,\textsuperscript{51} recombinant proteins,\textsuperscript{52,53} episomal vectors,\textsuperscript{54,55} Sendai virus vectors,\textsuperscript{56–59} and modified RNA.\textsuperscript{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\textsuperscript{61} and protein kinases (mitogen activated protein kinase and glycogen synthase kinase-3)\textsuperscript{62} and the replacement of c-Myc with L-Myc.\textsuperscript{14} Reprogramming

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**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 Mass5 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 (33314 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 (33314 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.
The rapid progress of stem cell technologies described above has triggered an increasing interest in the use of 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.

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, in-vehicle 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 cardiovascular development. Yuasa et al70 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,83 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.71–73

Selective Expansion and/or Survival of Cells of Cardiomyocyte Lineage

Precise regulation of the bone morphogenetic protein,30,76 Wnt,77–79 hedgehog,80 and Notch pathways71,81 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 hESC/iPSCs 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 cells68 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,33–35 although this method requires genetic modification of the cells. Recently, Hattori et al13 developed a nongenetic...
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, hESCs-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.

**Acknowledgments**

We thank Dr S. Shibata, H. Naka, Dr Francois Renault-Mihara, S. Ishii, Dr M. Nakamura, Dr T. Shimazaki, Dr W. Akamatsu, and Prof K. Fukuda for technical assistance and scientific discussion and all the members of Dr Okano’s laboratory and Dr Yamanaka’s laboratory for encouragement and kind support.

**Sources of Funding**

This work was supported by Grants-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science and the Ministry of Education, Culture, Sports, Science and Technology of Japan (to H.O. and S.Y.); the Project for Realization of Regenerative Medicine with support for the core institutes for iPSC cell research from Ministry of Education, Culture, Sports, Science and Technology of Japan (to S.Y.); the Program for World-Leading Innovative R&D on Science and Technology (FIRST Program) of the Japanese Society for the Promotion of Science (to S.Y. and H.O.); the Ministry of Health, Labor, and Welfare (to H.O. and S.Y.); and by a Grant-in-Aid for the Global Center of Excellence program from Ministry of Education, Culture, Sports, Science and Technology of Japan to Keio University.

**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 iPSC Academia Japan. The remaining authors report no conflicts.

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Circ Res. 2013;112:523-533
doi: 10.1161/CIRCRESAHA.111.256149

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/112/3/523

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