World Journal of Stem Cells

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DOI: 10.4252/wjsc.v7.i1.116

World J Stem Cells 2015 January 26; 7(1): 116-125
ISSN 1948-0210 (online)

REVIEW

Methods of induced pluripotent stem cells for clinical application

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Author contributions: Seki T prepared most of the paper; Fukuda K provided advice and proofread the paper.
Supported by The Support Program to break the bottlenecks at R&D Systems for accelerating the practical use of Health Research Outcome, the Highway Program for the Realization of Regenerative Medicine, and Health Labour Sciences Research Grant.

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Received: July 27, 2014
Peer-review started: July 28, 2014
First decision: September 4, 2014
Revised: September 18, 2014
Accepted: October 23, 2014
Article in press: December 16, 2014
Published online: January 26, 2015

Abstract

Reprograming somatic cells using exogenetic gene expression represents a groundbreaking step in regenerative medicine. Induced pluripotent stem cells (iPSCs) are expected to yield novel therapies with the potential to solve many issues involving incurable diseases. In particular, applying iPSCs clinically holds the promise of addressing the problems of immune rejection and ethics that have hampered the clinical applications of embryonic stem cells. However, as iPSC research has progressed, new problems have emerged that need to be solved before the routine clinical application of iPSCs can become established. In this review, we discuss the current technologies and future problems of human iPSC generation methods for clinical use.

Key words: Induced pluripotent stem cells; Cell reprogramming

Core tip: Each induced pluripotent stem cells methodology has advantages and disadvantages, as in the case of autologous *vs* allogenic transplantation, and the choice of appropriate strategy may vary depending on the intended use. Additionally, to avoid tumorigenesis and to establish effective differentiation into the intended cells, further investigation is needed to identify the most suitable iPSC line and how these lines should be selected.

Seki T, Fukuda K. Methods of induced pluripotent stem cells for clinical application. *World J Stem Cells* 2015; 7(1): 116-125 Available from: URL: http://www.wjgnet.com/1948-0210/full/v7/i1/116.htm DOI: http://dx.doi.org/10.4252/wjsc.v7.i1.116

INTRODUCTION

In 2006, Takahashi *et al*[1] established a novel method of reprogramming mouse somatic cells using exogenetic expression of genes related to pluripotency. The cell lines established by this group were named induced pluripotent stem cells (iPSCs) and demonstrated the same pluripotency and self-renewal properties that are characteristic of embryonic stem cells (ESCs). The following year, the

same group also succeeded in generating iPSCs from human somatic cells[2]. These groundbreaking steps have been expected to lead to novel regenerative cell therapies with the potential to solve many problems surrounding incurable diseases. In particular, the clinical application of iPSCs is expected to solve the problems of immune rejection and ethics that are currently key obstacles in the clinical use of ESCs. However, as research into iPSCs has progressed, new problems to solve have emerged before iPSCs can be established as cell sources for patients. In this review, we discuss the current technology and future problems surrounding human iPSC generation methods for clinical applications.

BENEFITS AND PROBLEMS OF ESCS AND IPSCS IN CLINICAL APPLICATION

For treating diseases that lack self-repairing cells, the transplantation of artificially generated cells is one attractive means for curing the diseases. In fact, regenerative cell transplantation therapies have been expected to treat incurable diseases, such as spinal cord injury[3], neurodegenerative disease[4], heart failure[5,6], diabetes[7], and retinal disease[8].

ESCs have the capacity to self-renew and differentiate into cells of the three germ layers. The development of suitable cultivation systems for maintaining the pluripotency of ESCs marked their promise as a cell source for regenerative medicine since human ESCs were first generated in 1998[9]. Numerous efforts have been made since then to realize the promise of making specific differentiated cells from ESCs. However, the ethical problem of needing human zygotes to generate human ESCs has remained unsolved. Additionally, immune rejection remains an issue because of the limited number of ESC cell lines and the ability of the cell lines to match the huge number of human leukocyte antigen (HLA) type combinations found in patients.

These problems with ESCs and the ongoing need for regenerative therapies drove further research efforts, such as Dr. Yamanaka's 2006 success in generating iPSCs from somatic mouse cells[1] and reports in 2007 by Takahashi *et al*[2] and Yu *et al*[10] of the successful generation of human iPSCs. In the original mouse work, 24 transcription factors showing high expression in ESCs were chosen as candidate reprogramming triggers[1]. Finally, the forced expression of *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC* together with a retrovirus evoked mouse somatic cell reprogramming into the pluripotent state, and the same combination of four factors forced human fibroblasts into iPSCs[2]. At the same time, Yu *et al*[10] successfully reprogrammed human fibroblasts using the combination of *OCT4*, *SOX2*, *NANOG*, and *LIN28*. These methods attracted much attention because these iPSC lines could potentially overcome the immune rejection and ethical issues hampering the development of ESCs for clinical use. Therefore, iPSCs showed promise as the breakthrough technology in regenerative medicine.

COMBINATION OF REPROGRAMMING FACTORS FOR IPSC GENERATION

Since iPSC generation methods were first reported, numerous efforts have been made to adapt them to clinical applications[5,11]. The reported generation methods vary in the combinations of reprogramming factors, vehicles for exogenous genes, and cell types to generate the iPSCs. Therefore, current discussions in the literature focus on selecting the most appropriate iPSC generation method for clinical use.

In relation to the combination of reprogramming factors for iPSC generation, the first reported combination of *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC*, known as the Yamanaka factors, are generally used for iPSC generation. Combining only three of these factors and omitting *C-MYC* was also reported to achieve successful iPSC generation, although with reduced reprogramming efficiency[12,13]. These alternative methods arose following concerns about using *C-MYC*, which is a known oncogene in human cells. Another group also reported the generation of mouse iPSCs, which showed more efficient germline transmission in chimeric formation experiments using the combination of *OCT3/4*, *SOX2*, *KLF4*, and *TBX3* as reprogramming factors[14]. Subsequently, iPSC generation using *L-MYC* instead of *C-MYC* was reported with improved efficiency in both reprogramming somatic cells and germline transmission of generated iPSCs[15]. Furthermore, *GLIS1* was also reported as a candidate alternate factor for *C-MYC* that showed high reprogramming efficiency, less incomplete reprogramming, and reduced tumor formation in iPSC-derived mice[16]. Recently, improved iPSC quality was also achieved by introducing *Zscan4,* which is highly expressed at the zygotic genome activation stage. Forced expression of *Zscan4* in combination with the Yamanaka factors improved iPSC quality as demonstrated by tetraploid complementation[17].

Recently, lineage-specific genes were substituted for *OCT3/4* or *SOX2*[18]. In this report, *OCT3/4* was replaced with an early mesendodermal lineage marker, such as *GATA3*, and *SOX2* was replaced with an early ectodermal lineage marker, such as *ZNF521,* in reprogramming using the Yamanaka factors. This finding raised the possibility that *OCT3/4* and *SOX2* might act as lineage specifiers for cell reprogramming and showed that reprogramming factors are not limited to genes associated with pluripotency[18].

Although methods for checking the quality of iPSCs such as germline transmission experiments are not applicable to human iPSCs, such studies can be useful for seeking the best combination of reprogramming factors to generate human iPSCs that are of sufficient quality for clinical use.

GENE-DELIVERY VEHICLES FOR IPSC GENERATION

Gene-delivery vehicles are also an important for selecting a suitable method of iPSC generation for clinical use, and to date, many gene delivery vehicles have proven to be applicable to iPSC generation. Initially, retrovirus vectors, such as pMXs[2,19,20], pLib[21] or pMSCV[22,23], were used for the delivery of reprogramming factors into somatic cells. Importantly, transgenes introduced with retrovirus vectors have been known to be silenced in pluripotent states[22,24], and therefore, silencing of transgene expression in iPSCs has been thought to be an important result of successful reprogramming[25]. Lentiviral vectors have also been used for successful iPSC generation because lentiviral vectors achieve a higher efficiency of infection than retrovirus vectors[10,26]. Importantly, transgenes introduced by lentiviral vectors are more resistant to being silenced in pluripotent states than those transtenes introduced by retrovirus vectors[27].

The genomic integration of transgenes that occurs as a result of these virus vectors was thought to be a problem for the clinical use of iPSCs because the delivered transgenes have the potential to be reactivated after cell reprogramming and thus drive oncogenesis in the iPSC-derived cells[28]. Furthermore, these transgenes have the potential to disrupt functional genes, even if they are silenced and not expressed. Therefore, many efforts have been made to generate iPSCs without the genomic insertions. For example adenovirus vectors are routinely used to introduce transient gene expression in target cells. Furthermore, a replication-defective pHIHG-Ad2 vector, was used to successfully reprogram somatic cells into iPSCs[29]. Therefore, although adenoviral vectors still integrate into the genome of target cells at extremely low frequencies[30] and the reprogramming efficiency is significantly lower than that with retrovirus, this method generates iPSCs that do not transfer residual transgenes into the host genomes.

More recently, a Cre-deletable lentivirus system was used for the successful generation of iPSCs[31]. However, although these systems can avoid transgene reactivation, the risk of gene breaks being introduced near the insertion site is present because the LoxP sequence remains in the host genome after removing the insert sequence by Cre recombinase[32].

In addition to virus vectors, the Sendai virus was also successfully used to generate iPSCs[33,34]. The Sendai virus genome is negative-sense single-stranded RNA. Because replication occurs in the cytoplasm, this virus vector does not pose a danger of genome insertion. Therefore, this method solves both problems of gene disruption near the insertion site and reactivation of transgenes. Additionally, the residual Sendai virus RNA can be removed from the infected cells using siRNA[35], and temperature-sensitive mutations[36] were also reported, improving the potential clinical suitability of this iPSC generation method.

As another approach for safe iPSC generation, a virus-free reprogramming method has received attention. For example, an iPSC generation method using episomal vectors has also been developed[37-40]. The early attempts using this method yielded lower efficiencies of successful reprogramming than those achieved using retrovirus, and only a low percentage of iPSC lines generated using this method had no plasmid integration. However, later modifications of the episomal vector method yielded a higher reprogramming efficiency using a combination of plasmids encoding *OCT3/4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28*, and shRNA for *TP53*[41,42].

Generating iPSCs using a piggyBac transposon was reported as another method to avoid the reactivation of residual transgenes and gene breaks in the host genome[43-45]. The piggyBac transposon is a moth-derived DNA transposon[46] that is highly active in mammalian cells and able to be completely eliminated from the host genome using the piggyBac transposase[47]. Despite generating integration-free iPSCs by this method, the reported reprogramming efficiency was lower than that with retrovirus, and thus further improvements are needed[43-45].

As another way to avoid introducing genetic material, introducing reprogramming factors such as RNAs or proteins has attracted much attention. Indeed, the direct delivery of synthetic mRNAs has been shown to successfully reprogram somatic cells to a pluripotent state[48]. In this study, *in vitro* transcribed RNAs were modified to avoid the endogenous antiviral cell defense. As a result, this method achieved a higher iPSC generation efficiency than the original retrovirus system[48]. Successful reprogramming of somatic cells has also been achieved using microRNAs[49], whereby expression of the *miR302/367* cluster containing five different miRNAs, *miR302a/b/c/d* and *miR367*, reprogrammed human fibroblasts more efficiently than previous retrovirus systems[49]. Such RNA-based reprogramming avoids both breaks in existing genes and the reactivation of transgenes. Therefore, these methods hold much promise as novel iPSC generation methods that could be applicable for clinical use.

Similarly, recombinant proteins were also reported as a successful means of gene introduction for generating iPSCs[50,51]. These protein-based methods are also attractive for clinical application because of the absence of breaks in existing host genes and the reactivation of transgenes; however, the generation efficiencies remain lower compared to those in the existing retrovirus systems[50,51].

Finally, small-molecule drugs have been investigated for establishing safe methods of iPSC generation for clinical application because they are nonimmunogenic, cost-effective, and easy to handle[52]. Recently, successful reprogramming of mouse somatic cells without transgene introduction was achieved with small-molecule drug combinations[53]. This strategy has many merits for applying the iPSC generation method for clinical use, and therefore, further research into applying this method to human somatic cells is expected in the near future.

TYPES OF DONOR CELLS FOR IPSC GENERATION

Generating iPSCs in clinical practice also requires the consideration of the most appropriate type of donor cells. At first, iPSCs were generated from mouse fibroblasts[1], followed by successful reprogramming of mouse hepatocytes and gastric epithelial cells[54]. Subsequently, terminally differentiated somatic cells have also been reprogrammed, including mouse B lymphocytes[55] and pancreatic beta cells[56]. With respect to human cells, iPSCs have been generated from human dermal fibroblasts[2,10] and many types of human somatic cells[5].

As a matter of course, clinical applications of cell therapies require that tissue collection from patients be as minimally invasive as possible, and harvesting human dermal fibroblasts by biopsy leaves a small scar on the patient's body. Recently, iPSCs were generated from human keratinocytes induced from plucked hair, a process that is much less invasive than biopsy[23,57]. However, several hairs are needed to obtain the successful cell outgrowth of keratinocytes in some cases, and therefore, a more stable protocol for primary culture is needed for routine clinical practice.

Dental tissue has also been proposed as a unique cell source for iPSC generation. Dental stem cells[58,59] and mesenchymal stromal cells derived from human third molars[60] were successfully reprogrammed and thought to be potentially useful material for clinical iPSC generation. Oral gingival[61] and oral mucosa fibroblasts[62], which can be obtained less invasively, were also investigated for iPSC generation. These methods are advantageous for clinical application because they involve a minimally invasive approach for the patients.

Cord blood was also reported as another cell source for generating iPSCs[63,64]. Early studies with cord blood yielded lower reprogramming efficiencies than those achieved with lentivirus or retrovirus systems. However, a modified method involving the knockout of p53, which was previously shown to increase the efficiency of reprogramming[65-70] for iPSCs, increased the efficiency of generating iPSCs from CD34-positive cells which were sorted from cord blood cells[71]. Additionally, gene introduction using Sendai virus vectors successfully reprogrammed CD34-positive cord blood cells more efficiently than lentiviral or retroviral vector used to reprogram cord blood cells[36]. Cord blood cells have attracted much attention because cord blood-derived cells do not require laborious mobilization or an invasive biopsy before introducing reprogramming factors. Using cord blood cells for iPSC generation also avoids the risk of transferring over accumulated genetic mutations into iPSC. Additionally, banked cord blood cells are relatively uncomplicated for use in iPSC generation because their immunological information is already available in cord blood banks[72].

Peripheral blood cells are also an attractive cell source because the method for cell sampling from patients is less invasive. Peripheral blood cells are more easily accessible as a cell source than the dermal fibroblasts obtained by skin biopsy. In the first study to generate iPSCs from human blood cells, the donor needed to be injected for 3 days with G-CSF to mobilize the CD34-positive cells, and the reprogramming efficiency was not higher than that of previous studies[73]; however, since then there have been many efforts to effectively generate iPSCs from peripheral blood cells. For example, less invasive methods have since been reported for generating iPSCs from peripheral mononuclear blood cells[34,74-77], whereby mononuclear blood cells from donors or frozen samples were induced using the Yamanaka factors with a retrovirus[74,77], lentivirus[75,76], or Sendai virus[34]. In these experiments, the majority of iPSCs generated from mononuclear cells had *TCR* gene rearrangements, indicating that these cell lines were derived from T lymphocytes, and the reprogramming efficiencies with the Sendai virus were similar to those with the previous retrovirus system[34]. Additionally, to avoid generating iPSCs containing genome rearrangements, methods were developed in which iPSCs were generated from peripheral mononuclear cells cultured under conditions that inhibit the proliferation of lymphocytes[78,79] and from CD34-positive cells that were mobilized without additional drug administration to the donors[80]. Sampling of peripheral blood is one of the least invasive procedures available, and therefore, generating iPSCs from peripheral blood could be one of the most appropriate methods for the clinical applications of iPSCs.

REMOVING ANIMAL PRODUCTS FROM CULTURE SYSTEMS

One of the most important issues to address in applying iPSCs to clinical therapy is that the culture systems for generating iPSCs contain animal-derived products with potential and unpredictable risks to patients[81]. The initial culture system for ESC generation contained fetal calf serum in the culture medium and mouse embryonic fibroblasts as a feeder layer. These animal-derived products conferred a risk of transferring exogenous antigens, unknown viruses, or zoonotic pathogens to the generated cell populations[9]. Thus, many investigations have be conducted to reduce such risks by establishing animal product-free culture systems for human iPSCs.

First, human-derived feeder cells have proven to be a useful alternative to mouse cells for human iPSC generation[82]. However, these feeder cell preparations need significant time and effort, and in the case of clinical therapies requiring the mass culture of human iPSCs in some situations, using human-derived feeder cells for culturing human iPSCs is not an optimal strategy. As an option for culturing human iPSCs without feeder cells, Matrigel has proven to be a useful alternative that enables the stable culture of human pluripotent stem cells[83-85]. Although Matrigel allows the generation of human iPSCs without animal-derived feeder cells[86,87], Matrigel itself was derived from Engelbreth-Holm-Swarm mouse

sarcoma cells[88]. Therefore, other types of matrices, such as CellStart[89,90], recombinant proteins[91-93], and synthetic polymers[94,95], which do not contain animal-derived agents, have been tested and used as feeder-cell substitutes for the successful maintenance and generation of human pluripotent cells.

Developing animal product-free medium for iPSC generation is also an important practice for achieving safe therapy using iPSCs. The culture media used in the early generation of human ESCs contained fetal bovine serum[9]. To remove unpredictable agents that might cause the differentiation of human ESCs, knockout serum replacement (KSR) has now been established as a defined material for maintaining human ESCs[96] and is also used for human iPSC generation[2]. Additionally, mTeSR1 medium was developed as a chemically defined medium for maintaining human pluripotent cells and is used for defined condition cultures of human pluripotent stem cells[97]. However, because KSR and mTeSR1 also contain animal-derived products, new media have now been commercially developed as xeno-free media for maintaining human pluripotent stem cells and have already been used successfully for iPSC generation; these media include TeSR2[98], NutriStem[99], Essential E8[91], and StemFit[100].

SELECTING THE MOST APPROPRIATE IPSC LINE FOR CLINICAL USE

One of the most intractable problems for applying iPSCs to clinical therapy is the variety of iPSC lines with respect to differentiation tendency and tumorigenic risk. Additionally, the laboratory in which the iPSCs are generated could influence global gene expression patterns of those iPSCs due to small possible differences in the culture conditions[101]. Therefore, how to select iPSC lines that are appropriate for a specific clinical use in terms of safety and differentiation ability remains a topic of intense discussion.

As described above, in contrast to generating iPSCs with Yamanaka factors, introducing *TBX3*[14], *L-MYC*[15], or *GLIS1*[16] instead of *C-MYC* has yielded high-quality iPSCs with respect to the efficiency of germ line transmission and prognosis of iPSC-derived mouse cells. These reports implied that selecting suitable combinations of reprogramming factors was an important consideration for clinical therapy using iPSCs. However, these reports were derived from mouse experiments, and how these findings translate to human iPSCs remains unknown. Of course, the quality index of iPSCs using chimeric formation is not applicable to human iPSCs. Therefore, another index of iPSC quality that is applicable to human iPSCs is required.

With respect to assessing the effects of the type of donor cell on the quality of generated iPSCs, the teratoma-forming propensity of neural stem and progenitor cells derived from mouse iPSCs was shown to differ depending on the donor cell type[102]. This finding suggested that the quality of the iPSCs should be considered when the type of donor cell is selected. Since that early report, an epigenetic memory of tissue of origin donor cell type that affected the differentiation tendency of iPSCs was reported[103,104]. Importantly, the effect of epigenetic memory was not demonstrated when pluripotent stem cells generated by nuclear transfer were used. Additionally, another recent study showed that human iPSCs contain more CpG sites that retain the DNA methylation pattern of the parental donor cells than human ESCs generated by nuclear transfer[105]. Although the fact that the differentiation tendency derived from epigenetic memory will not remain after long-term culture[103], these reports also suggested the importance of selecting the donor cell type when applying iPSCs clinically.

The effect of donor cell type on differentiation tendency was also reported in human iPSCs[106], but experiments that compared donor cell type, gene vehicle type, and volunteers on hepatic differentiation tendencies of iPSCs showed that the differentiation tendency of iPSCs depended on the volunteer from which the iPSCs were generated[107]. Interestingly, in this latter study, hepatic differentiation tendencies that were derived from donor cells were not observed, implying that such differences derive from the volunteer cell donor when epigenetic memory does not remain after long-term culture.

With respect to the tumorigenic tendencies of human iPSCs, a study of gene expression and DNA methylation in 21 human iPSC lines and 2 human ESC lines showed activated expression of genes containing specific LTR7 sequences in some human iPSC clones that showed a neural differentiation-defective phenotype and formation of teratomas when they were differentiated into dopaminergic neurons and transplanted in mouse brains[108]. Another report on 21 human iPSC lines and 6 human ESC lines indicated that certain human iPSC clones were in a pro-oncogenic state, as shown by the ectopic presence of secretory tumor tissue during *in vitro* cartilage differentiation[109]. These reports together implied that a marker foreseeing pro-oncogenic differences in iPSC lines would be required for establishing safe therapy using iPSCs.

AUTO-TRANSPLANTATION AND ALLO-TRANSPLANTATION OF IPSCS

One of the most important advantages expected of iPSCs for transplantation therapy is avoiding immune rejection and therefore avoiding combination immunosuppressive treatment. Indeed, autologous iPSCs were used in the first human clinical trial of iPSCs started by Dr. Masayo Takahashi at the RIKEN Center for Developmental Biology in Kobe in 2013 for treating age-related macular degeneration with iPSC-derived sheets of retinal pigment epithelium. With respect to the immunogenicity of autologous iPSCs, in the first report that aroused discussion on its credibility, immunogenicity showed in the case of iPSCs was not observed in the case of ESCs[110,111]. Subsequently, experiments with isogenic transplantation of mouse iPSCs revealed the successful engraftment of iPSC-derived tissue without immunosuppressive treatment and

verified the advantages of using auto-transplantation of iPSCs for avoiding immunosuppressive treatment[112,113]. However, a recent study demonstrated immune rejection upon transplanting autologous undifferentiated mouse iPSCs *in vivo*, and rejection was imperceptible upon transplanting autologous terminally differentiated mouse iPSCs[114]. Although this report supports the presence of immunogenicity in undifferentiated iPSCs[91] and conflicts with the successful autologous engraftment of undifferentiated iPSCs in other reports[93,94], it supports the advantages of using auto-transplantation of iPSC-derived terminally differentiated cells for avoiding immunosuppressive treatment. On another front, whether the immunogenicity in undifferentiated iPSCs contributes to removing contaminated undifferentiated cells from iPSC-derived cell populations and avoiding tumorigenesis after transplantation in the same manner as removing undifferentiated cells from cell populations before transplantation[115-117] remains to be clarified.

Allo-transplantation of iPSC-derived cells was also expected to provide a useful strategy for transplantation therapy using iPSCs due to saving cost and time in generating autologous iPSC lines for transplantation. Furthermore, as described above, selecting appropriate iPSC lines on a patient-by-patient basis will require significant numbers of studies for verification. Therefore, although the transplantation of iPSC-derived cells from an allogenic donor with foreign HLA requires lifelong immunosuppressive treatment of the recipient, the concept of homozygous HLA-typed iPSC banks may be feasible for achieving generalized therapy using iPSCs[41,118,119] and, indeed, this type of approach is already progressing[120].

CONCLUSION

The invention of iPSCs was groundbreaking for novel regenerative medicine and has been expected to lead to regenerative therapies with the potential to advance the treatment and management of incurable diseases. Importantly, iPSCs could overcome the problems of immune rejection and ethics that remain with ESCs. However, each strategy of using autologous or allogenic iPSCs has advantages and disadvantages, and the choice of appropriate strategy may vary depending on the intended use. Additionally, there remain many factors that affect establishing transplantation therapy using iPSCs. To avoid tumorigenesis and establish effective differentiation into the intended cells, further investigation is needed to clarify which iPSC line is the most suitable and how these lines can be best selected.

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P- Reviewer: Chen F, Chhabra A, Patanè S **S- Editor**: Tian YL
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