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# Induced Pluripotent Stem (iPS) Cell Research Overview

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Stem cells are capable of self-renewal and differentiation into a wide range of cell types with multiple clinical therapeutic applications. The two most important issues associated with embryonic stem (ES) cells are immune rejection and medical ethics. In 2006, induced pluripotent (iPS) cells were generated from somatic cells via the introduction of four transcriptional factors: OCT4, SOX2, c-MYC, and KLF4. Researchers found that iPS cell morphology, proliferation, surface antigens, gene expression, telomerase activity, and the epigenetic status of pluripotent cell-specific genes were similar to the same characteristics in ES cells. iPS cells are capable of overcoming hurdles associated with ES cells due to their generation from mature somatic cells (e.g., fibroblasts). For this reason, iPS cells are considered an increasingly important cell therapy technology. iPS cell production entails the use of retroviruses, lentiviruses, adenoviruses, plasmid transfections, transposons, or recombinant proteins. In this article we discuss the advantages and limitations of each strategy and address issues associated with clinical trials, including the potential for liver tumor formation and low generation efficiency.

Key words: Induced pluripotent stem cells; Embryonic stem cells; Cell therapy

## INTRODUCTION

In 2006, Takahashi and Yamanaka became the first researchers to successfully produce induced pluripotent (iPS) cells (23). They reported that both embryonic and adult mouse fibroblasts acquired capabilities similar to embryonic stem (ES) cells following treatment with four transcriptional factors (selected from 24 candidates): Oct3/4, Sox2, c-Myc, and Klf4 (23,27). The following year, two separate sets of four factors were shown to reprogram human somatic cells to pluripotency at similar efficiency levels: OCT4, SOX2, C-MYC, and KLF4 by Takahashi et al. (22) and OCT4, SOX2, NANOG, and LIN28 by Yu et al. (30). iPS cell morphology, proliferation, surface antigens, gene expression, telomerase activity, and the epigenetic status of pluripotent cell-specific genes are similar to the same characteristics in ES cells (22,30). Also similar to ES cells, iPS cells are capable of differentiating into three germ layer cell types—ectoderm, endoderm, and mesoderm—in vitro and in teratomas (23,27). Accordingly, iPS cells hold great promise for medicine due to their potential for generating patient-specific cell types for cell replacement therapy and producing in vitro disease models without embryonic tissues or oocytes. To date, at least six strategies for making iPS cells have been identified: retrovirus, lentivirus, adenovirus, plasmid transfection, transposon, and recombinant protein. Each strategy has advantages and limitations, which is the focus of this overview article.

## iPS CELL IMPORTANCE AND LIMITATIONS

Both ES and iPS cells are pluripotent and capable of differentiating into three primary germ layer deriva-

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tives—an important characteristic for producing healthy cells for therapeutic purposes. While there are reports of ES cell applications for therapeutic approaches in animal models (9,13,16,17,28), at least two important roadblocks to their use in humans must be overcome: post-transplantation immune rejection and ethical issues. iPS cell technology addresses both concerns: it is possible to use a patient's own somatic cells to generate therapeutic iPS cells (thus eliminating the potential for immune rejection), and they represent an acceptable alternative to the use of human embryos for stem cell production. An added benefit is that patient-specific iPS cells can be used for drug and regenerative medical research.

At least three significant hurdles remain: (a) the low efficiency of primary human cell reprogramming, which makes it difficult to generate patient-specific iPS cells from small initial cell populations; (b) the integration of viral transgenes into the somatic genome, especially oncogenes such as c-MYC and KLF4 (note that c-MYC retrovirus reactivation contributes to tumor formation in chimeric mice derived from iPS cells) (27); and (c) iPS cell teratoma formation can lead to tumor formation. Because even a small number of undifferentiated cells can result in teratoma formation, a key goal is inducing iPS differentiation into target cell types without producing large numbers of undifferentiated cells. Some chimeric mice success has been achieved using iPS cells, but tumor formation potential has not been completely eliminated.

## VIRUS-GENERATED iPS CELLS

Currently the majority of iPS cells are generated by retrovirus transduction, through which gene expression levels are higher and silenced after cells turn into iPS cells. These characteristics are important for cell therapy applications because they indicate that reduced abnormal gene expression occurs during stem cell stages. When Takahashi et al. generated the first iPS cells from mouse embryonic fibroblasts, reported efficiency was 0.1% (23). The following year they used the same technique to obtain human iPS cells, and efficiency decreased to 0.01% (22). To reduce c-Myc oncogene generation potential, the same group used only three factors (Oct4, Sox2, and Klf4) to generate mouse and human iPS cells, but efficiencies fell to 0.001% and 0.01%, respectively (Table 1) (18). iPS cell clone formation did not occur when only two transcriptional factors (Oct4 and Sox2) were used to generate mouse iPS cells (12). An alternative strategy tested by Huangfu et al. entailed using Oct4, Sox2, and valproic acid (VPA, a histone deacetylase inhibitor that supports primary human fibroblast reprogramming with two factors) to generate human iPS cells from human fibroblasts (8). Their results support the possibility of reprogramming through purely chemical means, which would increase both cell safety and the potential for therapeutic use. However, this strategy still carries the risk of viruses being integrated into chromosomes and altering endogenous genome sequences. In 2009, Kim et al. generated mouse iPS cells from neural stem cells after introducing a single factor (Oct4) (11). Although their work did not entail the use of somatic cells, their results are still considered important because they indicate that iPS cells with only one transcriptional factor are capable of generating c-MYC and KLF4 oncogenes.

Lentiviruses have also been used to generate human iPS cells from somatic cells. In 2007, Yu et al. became the first team to generate human iPS cells using a lentivirus (overexpressing 4 of 14 candidate genes: OCT4, SOX2, NANOG, and LIN28) (30) at an efficiency of 0.01%—the same as that reported by Takahashi et al. for OCT4, SOX2, c-MYC, and KLF4 (22). The following year Liao et al. used the transcriptional factors OCT4, SOX2, C-MYC, KLF4, NANOG, and LIN28 to generate iPS cells at an efficiency level of 0.1%—10 times higher than previously reported (14). They speculated that the addition of c-MYC and/or KLF4 either prevented apoptosis or regulated the cell cycle. The use of a lentivirus to introduce genes for iPS cell generation supported the integration of genes sequences into the genome. Unlike retroviruses, the gene expression levels of lentivirus transduction do not shut down following transformation into iPS cells, meaning that iPS cells generated via lentiviruses cannot be used for clinical trials. To prevent viral integration into genome sequences, Stadtfeld et al. (21) used an adenovirus carrying OCT4, SOX2, c-MYC, and KLF4 to generate mouse iPS cells from hepatocytes—the first instance of iPS cell generation without virus integration, a more acceptable method for clinical applications. However, because the efficiency of this strategy is very low (0.0006%), it may never be practical for generating human iPS cells.

There is no definitive answer to the question of how many transcriptional factors are required to generate iPS cells, but generating them from somatic cells treated with only one factor is an important research goal. The most important factor appears to be OCT4, which by itself is sufficient for generating iPS cells from neural stem cells (11). An interesting possibility is using a single transcriptional factor to induce OCT4, SOX2, C-MYC, and KLF4; that is, having it serve as a substitute for those four factors. If successful, this factor will both simplify the generation process for iPS cells and support their use in clinical trials.

## GENERATING iPS CELLS WITH PLASMID TRANSFECTIONS

In 2008, Okita et al. (the same Japanese group that generated the first iPS cells) reported that they had suc-

**Table 1.** The Many Ways of Reprogramming Methods to Make iPS Cells

| Genes | Strategy | Species | Cell Type | Efficiency | Years | Reference |
|---|---|---|---|---|---|---|
| Retrovirus | | | | | | |
| Four factors (Oct4, Sox2, Klf4, c-Myc) | retrovirus | mouse | embryonic fibroblasts | 0.1% | 2006 | 23 |
| Four factors (Oct4, Sox2, Klf4, c-Myc) | retrovirus | human | fibroblasts | 0.01% | 2007 | 22 |
| Three factors (Oct4, Sox2, Klf4) | retrovirus | mouse | fibroblasts | 0.01% | 2008 | 18 |
| | | human | fibroblasts | 0.001% | | |
| Two factors (Oct4, Klf4 or c-Myc) | retrovirus | mouse | neural stem cells | 0.14% | 2008 | 12 |
| Mir-302 | retrovirus | human | skin cancer cells | NA | 2008, Aug | 15 |
| Two factors (Oct4, SOX2) + VPA | retrovirus | human | fibroblasts | 0.001% | 2008, Oct | 8 |
| One factors (Oct4) | retrovirus | mouse | neural stem cells | 0.1% | 2009 | 11 |
| Adenovirus | | | | | | |
| Four factors (Oct4, Sox2, Klf4, c-Myc) | adenovirus | mouse | hepatocyte | 0.0006% | 2008, Nov | 21 |
| Lentivirus | | | | | | |
| Four factors (Oct4, Sox2, NANOG, and LIN28) | lentivirus | human | fibroblasts | 0.01% | 2007 | 30 |
| Six factors (Oct4, Sox2, Klf4, c-Myc, NANOG, and LIN28) | lentivirus | human | newborn foreskin fibroblasts | 0.1% | 2008 | 14 |
| Plasmid transfection | | | | | | |
| Four factors (Oct4, Sox2, Klf4+c-Myc) | plasmid transfection | mouse | embryonic fibroblasts | 0.0015% | 2008, Oct | 19 |
| Six factors (Oct4, Sox2, Klf4, c-Myc, NANOG, and LIN28) | plasmid transfection | human | human foreskin fibroblasts | 0.1% | 2009, May | 29 |
| Transposon | | | | | | |
| Four factors (Oct4, Sox2, Klf4, c-Myc) | piggyBac transposition | mouse | embryonic fibroblasts | NA | 2009, Apr | 25 |
| Recombinant protein | | | | | | |
| Four factors (Oct4, Sox2, Klf4, c-Myc) | recombinant protein | human | fibroblasts | 0.001% | 2009, Jun | 10 |

cessfully generated mouse iPS cells without viral vectors (19). The repeated transfection of two expression plasmids (one containing the cDNAs of Oct3/4, Sox2, and Klf4 and the other containing c-Myc cDNA) into mouse embryonic fibroblasts resulted in iPS cells with no indications of plasmid integration. When transplanted into mice, these cells produced teratomas and contributed to adult chimeras (19). This production of virus-free iPS cells addressed a critical safety concern for their use in regenerative medicine. Still, this method is very inefficient (0.0015%); therefore, it remains to be seen whether it can be used to generate human iPS cells from human fibroblasts.

In 2009, Yu et al. used the same nonviral vector transfection strategy to generate human iPS cells, utilizing three plasmids containing six transcriptional factors (OCT4, SOX2, C-MYC, KLF4, NANOG, and LIN28); this method achieved a much higher efficiency level of ~0.1% (29). They also determined that the generated human iPS cells were (a) completely free of vector and transgene sequences, and (b) similar to human ES cells in terms of proliferative and developmental potential. Their results demonstrate that human somatic cell reprogramming does not require genomic integration or the continued presence of exogenous reprogramming factors, and thus removes one obstacle to clinical applications of human iPS cells. If this strategy does not trigger the c-MYC and KLF4 oncogenes while still achieving higher efficiencies, it may emerge as the best method for generating iPS cells.

## GENERATING iPS CELLS BY MICRO-RNA

To prevent the generation of oncogenes such as c-MYC and KLF4, researchers are searching for alternative approaches to generating iPS cells by looking at factors that are abundant in stem cells and lacking in somatic cells. In 2008, Lin et al. (15) reported that mir-302 reprograms human skin cancer cells into a pluripotent ES cell-like state. The mir-302 micro-RNA family (referred to as mir-302s) is expressed most abundantly in slow-growing human ES cells and quickly decreases after cell differentiation and proliferation (20). In addition to reprogramming cancer cells into an induced pluripotent state, mir-302s maintain this state under a feeder-free cultural condition that may offer opportunities for therapeutic intervention, making them a focus of interest as a potential key factor in ES cell renewal and pluripotency maintenance (15). Lin et al. were the first to use

micro-RNA to generate iPS cells, but they used a human skin cancer cell line instead of human somatic cells such as fibroblasts. If micro-RNAs can be used to generate iPS cells from human somatic cells, it will help prevent the use of oncogenes such as c-MYC and KLF4.

## GENERATING iPS CELLS BY TRANSPOSONS

In 2008, a new approach to generating iPS cells without vector integration was reported by Woltjen et al., who used piggyBac (PB) transposition to insert OCT4, SOX2, C-MYC, and KLF4 into mouse embryonic fibroblasts in order to generate iPS cells (25). Several researchers have demonstrated the functionality of PB transposition (which is host factor independent) in various human and mouse cell lines (2,3,24,26). PB transposon/transposase technology only requires (a) the inverted terminal repeats that flank the targeted transgene, and (b) the transient expression of the transposase enzyme to catalyze insertion or excision events (7). Individual PB insertions can be removed from established iPS cells in order to prevent gene insertions and oncogene overexpression. Although this technique does not utilize the virus system, it still carries the risk of transgenes remaining in the genome.

## GENERATING iPS CELLS BY RECOMBINANT PROTEINS

In 2009, Kim et al. (10) generated stable DNA-free iPS cells from human fibroblasts by directly delivering four reprogramming proteins (Oct4, Sox2, Klf4, and c-Myc) and fusing them with a cell-penetrating peptide (CPP). A major challenge to the intracellular delivery of proteins and other macromolecules is their limited ability to cross cellular membranes (1). In 1988, Frankel and Pabo found that the HIV transactivator of transcription (HIV-TAT) has a short basic segment residing at amino acids 48–60 that supports its penetration into cell membranes and subsequent activation of HIV-specific genes (5,6). This and other naturally occurring CPPs that are capable of overcoming cell membrane barriers contain high proportions of basic amino acids (e.g., arginine or lysine) (4,31). Kim et al.'s DNA-free iPS cells (protein-based iPS) are similar to ES cells in terms of morphology, proliferation, surface antigens, gene expression, telomerase activity, and the epigenetic status of pluripotent cell-specific genes (10). They are capable of differentiating into ectoderm, endoderm, and mesoderm layer cell types in vitro and in teratomas. Protein-based iPS technology represents a new and potentially safe method for generating patient-specific stem cells, one that does not require ex utero embryos. However, at present DNA-free iPS cells generation efficiency is significantly lower compared to virus-based protocols (approximately 0.001% vs. 0.01% of input cells) (10).

## CONCLUSION AND RESEARCH DIRECTIONS

Still in its infancy (27), iPS technology should eventually make cell transplantation therapies possible for a wide variety of diseases and injuries, while circumventing ethical issues and immune rejection challenges. Researchers must evaluate different types of original cells and induction methods to determine the best combination for generating the safest iPS cells. At minimum, researchers need to focus on four limitations to using iPS cells in clinical applications: (a) unacceptably low efficiency; (b) complexity, with the ideal process reduced to a single step; (c) safety in light of the potential for cancer formation, with all iPS cells generated by any method from any cell source submitted to vigorous examination prior to use in clinical applications (27); and (d) the use of animal feeders, noting that iPS cell culturing technology currently uses mouse embryonic fibroblasts as the feeder layer, meaning there is potential for the secretion of factors that might change iPS cell characteristics.

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