

CHINESE MEDICAL SCIENCES JOURNAL

REVIEW

Generation of Transgene-free Induced Pluripotent Stem Cells with Non-viral Methods

Tao Wang^{1,2,3}, Hua-shan Zhao^{4,5}, Qiu-ling Zhang¹, Chang-lin Xu^{2,3},
and Chang-bai Liu^{1*}

¹Institute of Molecular Biology, ²Third Clinical Medical School, ³First Clinical Medical School,
⁴Department of Neurosurgery, Gezhouba Central Hospital, ⁵Yichang City Central Hospital,
China Three Georges University, Yichang 443002, China

Key words: induced pluripotent stem cells; non-viral methods; transcription factor; re-generative medicine

Abstract Induced pluripotent stem (iPS) cells were originally generated from mouse fibroblasts by enforced expression of Yamanaka factors (Oct3/4, Sox2, Klf4, and c-Myc). The technique was quickly reproduced with human fibroblasts or mesenchymal stem cells. Although having been showed therapeutic potential in animal models of sickle cell anemia and Parkinson's disease, iPS cells generated by viral methods do not suit all the clinical applications. Various non-viral methods have appeared in recent years for application of iPS cells in cell transplantation therapy. These methods mainly include DNA vector-based approaches, transfection of mRNA, and transduction of reprogramming proteins. This review summarized these non-viral methods and compare the advantages, disadvantages, efficiency, and safety of these methods.

Chin Med Sci J 2013; 28(1):50-54

MOUSE and human embryonic stem (ES) cells were derived 26 and 11 years ago, respectively.¹ ES cells are capable of infinite self-renewal *in vitro*, and of differentiation into derivatives of the three germ layers.² Therefore, ES cells have enormous potential of application in regenerative medicine. In the field of cell therapies, ES cells could be used for replacing injured organs. In tissue engineering, they could be employed for tissue repair and regeneration. However, the application of ES cells for replacement

therapies is restricted by ethical concerns and immune rejection. To overcome these obstacles, considerable effort has been made to derive ES-like cells by reprogramming somatic cells to embryonic state, such as somatic cell nuclear transfer and cell fusion.³ However, none of these methods is likely to become a common approach for producing patient-specific pluripotent cells for clinical use. In 2007, Yu et al⁴ and Takahashi et al⁵ reported the astonishing discovery that by retrovirus-mediated expression of four defined transcription factors (Oct3/4, Sox2, Klf4, and c-Myc), mouse fibroblasts could be reprogrammed back to pluripotent stem cells. These induced pluripotent stem (iPS) cells were morphologically indistinguishable from ES cells, capable of expressing ES cell marker genes,

Received for publication June 18, 2012.

*Corresponding author Tel: 86-717-6397179, E-mail: cblui@ctgu.edu.cn

with normal karyotype and the ability to generate teratomas-containing tissues derived from all the three germ layers when subcutaneously injected into immunodeficient mice. After minor improvement with the initial reprogramming protocol, iPS cells were shown to be germ-line-competent and capable of forming any cell types of the body.⁶ The fact that iPS cells can be derived from easily accessible somatic cells has opened up new horizons in the field of regenerative medicine.⁷ The therapeutic potential of iPS cells has been demonstrated in animal models of sickle cell anemia and Parkinson's disease.^{8,9} In addition, iPS cells could circumvent the ethical controversy that precludes the isolation of human ES cells from early embryos. Although the technology of iPS cells harbors the potential to revolutionize regenerative medicine, the use of viral vectors still hinder its translation into clinical implementation.¹⁰ This article reviewed reprogramming approaches that have been currently developed to generate transgene-free iPS cells with non-viral methods.

REPROGRAMMING BY DNA VECTOR-BASED APPROACHES

Compared with viral methods, nucleic acid-based approaches are much safer, but offering lower reprogramming efficiencies; compared with mRNA or protein approaches, nucleic acid-based approaches are simpler and more accessible.

By repeated transfection of two expression plasmids (one containing the cDNAs of Oct3/4, Sox2, and Klf4; the other containing the cDNA of c-Myc) into mouse embryonic fibroblasts, Okita et al¹¹ obtained iPS cells without evidence of plasmid DNA integration. For efficient expression of the 4 factors in one cell, they used the foot-and-mouth disease virus 2A self-cleaving peptide, which allows near-stoichiometric production of proteins *via* a ribosome "skipping" mechanism. Neither polymerase chain reaction nor Southern blot detected any integration of plasmid DNA in the iPS cells. Simple sequence length polymorphisms analysis excluded the possibility that the iPS cells were derived from contamination of ES cells. By sequential transfections with non-episomal plasmids that independently encoded the four reprogramming factors (Oct4, Nanog, Sox2, and Lin28), Si-Tayeb et al¹² successfully generated human iPS cells from human foreskin fibroblasts. The plasmid vectors used in that study were identical to those used to generate the lentivirus vectors. However, the absence of packaging vectors from the transfection prevented the possibility of generating wild-type virus. Since the plasmids they used to reprogram lacked sequences

required for self-replication in eukaryotic cells, exogenous DNA would be lost from donor cells during cell division. The use of plasmids or non-episomal plasmids only requires elementary procedures that are readily accessible to any laboratory with even basic experience in molecular biology, not as demanding as other methods, which entails substantial knowledge of virology or protein biochemistry. In their experiments, 1-29 positive colonies could be emerged from 1×10^6 transfected cells, indicating lower efficiency of their method compared with viral method, in which 100-1000 positive colonies could be obtained using retroviruses. Therefore, further studies are required to increase the efficiency of this method.

With single minicircle vectors, Wu et al¹³ obtained transgene-free iPS cells from human adipose stem cells. Compared with plasmids, minicircle DNA provides higher transfection efficiencies and longer ectopic expression. In that study, they constructed a plasmid containing a single cassette of four reprogramming factors (Oct4, Sox2, Lin28, and Nanog) plus a green fluorescent protein (GFP) reporter gene, each separated by self-cleaving peptide 2A sequences. After 14 days transfecting somatic cells with the minicircle vector, they observed GFP-positive clusters that were morphologically similar with human ES cells. Southern blot analysis did not detect genomic integration of the minicircle transgene in the subclones. The efficiency with minicircle DNA is higher than other plasmid-based transfection reprogramming methods, though in their study may be partially because of differences in donor cell types and the number of reprogramming factors that were used. In addition, this method requires only a single vector without the need of subsequent drug selection or vector excision.

Using non-integrating oriP/Epstein-Barr virus nuclear antigen-1 (EBNA1) vectors encoding 7 factors (Oct4, Sox2, Nanog, Lin28, Klf4, c-Myc, and SV40Tag), Yu et al¹⁴ derived human iPS cells from human foreskin fibroblasts. After removal of these vectors, iPS cells were completely free of vector and transgene sequences. The oriP/EBNA1 vectors are derived from Epstein-Barr virus which can be transfected without the need of viral packaging and subsequently removed from cells by culturing without drug selection. Stable extrachromosomal replication of oriP/EBNA1 vectors in mammalian cells requires only a cis-acting oriP element and a trans-acting EBNA1 gene. The oriP/EBNA1 vectors replicate only once per cell cycle, and with drug selection can be established as stable episomes in about 1% of the initial transfected cells. If drug selection is subsequently removed, the episomes will be lost in every cell generation due to defects in plasmid synthesis and partitioning. In their study, to counteract the

possible toxic effects of c-Myc expression, they included the SV40 large T gene (SV40LT) in some of the combinations. Three of these combinations, all of which included Oct4, Sox2, Nanog, Lin28, c-Myc, Klf4, and SV40LT, were successful in producing iPS cell colonies from human foreskin fibroblasts using oriP/EBNA1-based vectors. PCR analysis did not detect episomal vector integration in the genome of the iPS cells, and DNA fingerprinting confirmed that the iPS cells originated from foreskin fibroblasts. However, this technique required three individual plasmids carrying a total of seven factors, including the oncogene SV40. The expression of the EBNA1 protein, which was required for this technique, may increase immune cell recognition of transfected cells, thus potentially limiting clinical application of the technique if the transgene is not completely removed. Similar with other non-integrating reprogramming methods, the reprogramming efficiency with oriP/EBNA1 vectors is low. Since different cell types have different reprogramming frequencies, it might be possible to identify another accessible human cell type more easily reprogrammed with these episomal vectors than foreskin fibroblasts. Nevertheless, Yu et al's work is an important step toward studying patient-specific cells and associated disease as well as future application of iPS cell technology in regenerative medicine and other clinical usages.

Gonzalez et al¹⁵ obtained iPS cell lines with no evidence of integration of the reprogramming vector from mouse embryonic fibroblasts by nucleofection of a polycistronic construct co-expressing Oct4, Sox2, Klf4, and c-Myc. They also utilized the 2A peptide sequence to deliver all the 4 factors to one cell. They cloned a chicken beta actin-driven polycistronic plasmid expressing Oct4, Sox2, Klf4, and c-Myc to generate iPS cells. Reverse transcription-polymerase chain reaction and Western blot analysis confirmed that this plasmid could efficiently express the 4 reprogramming factors when transfecting mouse embryonic fibroblasts. iPS-like colonies appeared 12 days after the final seeding. That approach included all the necessary factors in a single construct and delivered them by nucleofection, showing that iPS cells induction is possible by transient expression of the 4 factors by a single polycistronic construct, therefore progressing toward a safe and simple method of deriving clinically relevant human iPS cells.

By transfection of a single multiprotein expression vector encoding c-Myc, Klf4, Oct4, and Sox2 separated with 2A sequences, Kaji et al¹⁶ reprogrammed both mouse and human fibroblasts to iPS cells. They used the transient Cre transfection to remove the exogenous reprogramming

factors once the reprogramming was achieved. The Cre-excised cell lines maintained the endogenous gene expression of c-Myc, Klf4, Sox2, and Oct4, indicating that their single-vector system enabled complete elimination of exogenous genes without disturbing maintenance of the iPS cells state. However, a part of the Cre vector backbone remained in the integration site. When combined with PiggyBac (PB) transposon, this reprogramming system can also reprogram human somatic cells effectively. In contrast to Cre transposons, PB transposons are completely removable from their integration site without any residual change in the original DNA sequence, making this reprogramming system an ideal method for generating non-genetically modified human iPS cells for regenerative medicine.

GENERATION OF IPS CELLS BY TRANSFECTION OF MRNA

By repeated administration of synthetic mRNAs incorporating modifications designed to bypass innate antiviral responses, Warren et al¹⁷ reprogrammed multiple human cell types to pluripotency with high conversion efficiencies and superior kinetics. They further showed that the same technique can also be used to direct the differentiation of RNA-iPS cells to terminally differentiated myogenic cells. They first constructed a template including genes of interest for RNA transcription reactions. Then these mRNAs were complexed with a cationic vehicle to facilitate uptake by mammalian cells. Daily transfection with these modified mRNAs gave rise to numerous hESC-like colonies. Although this method is technically complex, it has advantages over established reprogramming techniques in conversion efficiency and kinetics. More fundamentally, because this technique is RNA-based, it completely avoids the risk of genomic integration and insertional mutagenesis inherent to all DNA-based methods, including those that are ostensibly non-integrating. Moreover, this approach allows exquisite regulation of protein stoichiometry while avoiding the stochastic variation of expression typical of integrating vectors, as well as the uncontrollable effects of viral silencing. Warren et al¹⁷ demonstrated that the modified RNA-based technology enables highly efficient reprogramming and that it can equally be applied to redirect pluripotent cell to terminal differentiation without compromising genomic integrity. In light of these considerations, this method has the potential to develop into a major technique for cell-based therapies and regenerative medicine.

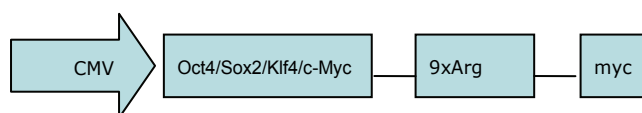


Figure 1. Schematic representation of the mammalian expression vector of the 4 reprogramming factors. The respective cDNA of Klf4, c-Myc, Oct4, and Sox2 was connected with 9R and the myc tagging peptide at the C-terminus.

GENERATION OF IPS CELLS BY TRANSDUCTION OF REPROGRAMMING PROTEINS

By directly delivering four reprogramming proteins (Oct4, Sox2, Klf4, and c-Myc) fused with a cell penetrating peptide (Fig.1), Kim et al¹⁸ obtained stable iPS cells from human fibroblasts. They confirmed at first that red fluorescent protein fused with a 9 arginine could penetrate into COS7 cells and human newborn fibroblasts (HNFs). Stable HEK293 cell lines that could express each of the four human reprogramming factors (Oct4, Sox2, Klf4, and c-Myc) fused with 9R and the myc tag were generated. Cell extracts from the HEK293 cell lines were used to generate iPS cells. After three or four rounds of protein treatment cycles, several colonies with iPS-like morphology were observed. This protocol is advantageous as it does not use genetic material to reprogram the somatic cells, therefore eliminates the potential risks associated with the use of viruses, DNA transfection, RNA transfection, and potentially harmful chemicals. In the future, Kim et al's method could provide a safe source of transgene-free iPS cells for regenerative medicine. However, the cell extracts containing reprogram factors have to be added once a week for 6 weeks, and the generation of protein-human iPS cells is very slow and inefficient, requiring further improvement. In particular, the whole protein extracts used in that study limited the concentrations of factors delivered into the target cells, suggesting that p-hiPS cells may be more efficiently generated using purified reprogramming proteins.

CONCLUSIONS

The generation of iPS cells 6 years ago has provided a unique platform to dissect the mechanisms of cellular reprogramming and enormous potential for regenerative medicine.¹⁹ As the reprogramming technology moves from basic science field towards translational medicine, efficient derivation of transgene-free iPS cells with non-viral methods is absolutely critical. Although viral transduction of the Yamanaka factors remains the most common strategy of producing iPS cells, many non-viral methods have emerged to improve the safety of iPS cells generation.

Compared to viral methods, DNA transfection-based methods appear safer, but they also entail some risk of genomic recombination or insertional mutagenesis. Although excisable vectors may prove suitable for most applications, and the transgenes can be excised by inducible gene expression once reprogramming is established, residual sequences and chromosomal disruptions may still result in harmful alterations, posing clinical risks. Compared with viral transduction, DNA transfection-based methods also has a major drawback-low reprogramming efficiency. However, DNA transfection-based methods are simple and highly accessible. The use of mRNA has higher efficiency but is technically complex. Methods that rely on protein delivery might become routine once their efficiency is enhanced because of complete elimination of the use of genetic material. However, the protein-based methods are challenging since the recombinant proteins have to be generated and purified to reach the quantities required for iPS cell generation, requiring either more than 4 rounds of protein treatment or chemical treatment (such as valproic acid). What is more, protein-based methods necessitate expertise in protein chemistry and handling-skills that many laboratories are lack of. Since some small molecules have been identified as enhancing reprogramming efficiency and replacing certain reprogramming factors,²⁰ reprogramming by defined chemical means may be achieved in the future. In this article, we compared different non-viral methods of iPS cell generation, elaborating the advantages, disadvantages, efficiency, and safety of those methods. Given the rapid progress of this field, further optimization of the methods will certainly facilitate their translation into clinical application.

REFERENCES

1. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981; 292:154-6.
2. Smith AG. Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol* 2001;17:435-62.
3. Jaenisch R, Young R. Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell* 2008; 132: 567-82.
4. Yu J, Smuga-Otto K, Thomson JA, et al. Induced pluri-

- potent cell lines derived from human somatic cells. *Science* 2007; 318:1917-20.
5. Takahashi K, Tomoda K, Yamanaka S et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;131:861-72.
 6. Hochedlinger K, Plath K. Epigenetic reprogramming and induced pluripotency. *Development* 2009;136:509-23.
 7. Yamanaka S. Ekiden to iPS cells. *Nat Med* 2009;15: 1145-8.
 8. Hanna J, Wernig M, Markoulaki S, et al. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science* 2007;318:1920-3.
 9. Wernig M, Zhao JP, Pruszak J, et al. Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. *Proc Natl Acad Sci U S A* 2008;105:5856-61.
 10. Lengner CJ. iPS cell technology in regenerative medicine. *Ann N Y Acad Sci* 2010;1192:38-44.
 11. Okita K, Nakagawa M, Yamanaka S, et al. Generation of mouse induced pluripotent stem cells without viral vectors. *Science* 2008;322:949-53.
 12. Si-Tayeb K, Noto FK, Duncan SA, et al. Generation of human induced pluripotent stem cells by simple transient transfection of plasmid DNA encoding reprogramming factors. *BMC Dev Biol* 2010;10:81.
 13. Wu JC, Jia F, Wu JC, et al. A nonviral minicircle vector for deriving human iPS cells. *Nat Methods* 2010;7:197-9.
 14. Yu J, Hu K, Tian S, et al. Human induced pluripotent stem cells free of vector and transgene sequences. *Science* 2009; 324:797-801.
 15. Gonzalez F, Barragan Monasterio M, Izpisua Belmonte JC, et al. Generation of mouse-induced pluripotent stem cells by transient expression of a single nonviral polycistronic vector. *Proc Natl Acad Sci U S A* 2009;106:8918-22.
 16. Kaji K, Norrby K, Paca A, et al. Virus-free induction of pluripotency and subsequent excise on of reprogramming factors. *Nature* 2009;458:771-5.
 17. Warren L, Manos PD, Rossi DJ, et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* 2010;7:618-30.
 18. Kim D, Kim CH, Moon JI, et al. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell* 2009;4:472-6.
 19. Yamanaka S. A fresh look at IPS cells. *Cell* 2009; 137: 13-7.
 20. Huangfu D, Maehr R, Melton DA, et al. Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat Biotechnol* 2008;26:795-7.