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Non-Viral Methods For Generating Integration-Free, Induced Pluripotent Stem Cells

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Abstract: Induced pluripotent stem (iPS) cells were created from mouse fibroblasts by induced expression of Yamanaka factors, Oct3/4, Sox2, Klf4, and c-Myc. This technique has quickly resulted in an exponential increase in the amount of pluripotency studies, and has provided a valuable tool in regenerative medicine. At the same time, many methodologies to generate iPS cells have been reported, and are comprised mainly of viral methods and non-viral methods. Although viral methods may not be applicable for clinical applications, various non-viral methods have been reported in recent years, including DNA vector-based approaches, transfection of mRNA, transduction of reprogramming proteins, and use of small molecule compounds. This review summarizes and evaluates these non-viral methods.

Keywords: DNA integration-free, induced pluripotent stem cells, non-viral methods.

INTRODUCTION

Induced pluripotent stem (iPS) cells, which are similar to embryonic stem (ES) cells in morphology, gene expression, epigenetic status, and *in vitro* differentiation, are a type of pluripotent stem cell directly generated from somatic cells by various synthetic methods [1]. Compared with ES cells, iPS cells possess indistinguishable pluripotent capabilities, and their specificity towards patients can bypass some of the risks of ES cells. They are therefore a potential alternative to ES cells in regenerative medicine. Also, they can circumvent ethical concerns. Because iPS cells were originally derived from mouse fibroblasts by retrovirus-mediated introduction of four factors, Oct3/4, Sox2, Klf4, and c-Myc [2], and then reprogrammed from human fibroblasts by the same four factors [3] or by Oct3/4, Sox2, Nanog, Lin28 [4], numerous methods for the generation of these cells have been developed.

Based upon different ways of transforming exogenous genes, the methodology for iPS cell generation can be divided into viral-based methods and non-viral methods. Both these methods may or may not involve integration of exogenous genes into the host genome. Because viral methods may result in gene reactivation and unusual phenotypic expression of iPS cells [5, 6], which could be valuable for further studies and clinical applications, studies using non-viral methods, especially without integration, have been frequently used.

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The following review presents a summary of methods for identification of iPS cells, discusses the current iPS cell generation strategies using non-viral delivery systems which result in DNA free of integration, and describes various applications of this methodology.

METHODS FOR IDENTIFICATION OF IPS CELLS

Compared with differentiated cells, iPS cells contain very different epigenetic signatures. With permissive chromatin, lower levels of heterochromatin, and the frequent appearance of bivalent domains, pluripotent cells are able to differentiate into various tissue types [7]. Currently, three different methods are used for identification.

First, preliminary identification of iPS cells can be based on morphology. Similar to early stage embryonic cells, the chief distinguishing features of iPS cells are small size, high nuclear/cytoplasm ratios, and one or more nuclei. Based upon microstructure, histochemistry, Forssman antigen, and protein synthesis, it has been reported that iPS cells are comprised of more euchromatin, unbound ribosome, and mitochondria, with less organelles and less complexities of cellular structures [8].

Second, immunocytochemistry staining and reverse transcription-polymerase chain reaction (RT-PCR) analysis are essential for identification of iPS cells. Immunological markers of iPS cells include alkaline phosphatase (AKP), stage-specific embryonic antigens (SSEA), Tra-1-60, Tra-1-81 and other molecular labeling techniques [9]. A number of studies have reported that expression of AKP was highly correlated with undifferentiated iPS cells, while negative expression was found in differentiated ES cells [10]. SSEA are glycoproteins expressed in early stage development,

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whose expression changes when cells differentiate [11]. The expression of the epitopes recognized by the monoclonal antibodies Tra-1-60 and Tra-1-81 is important for assessing the pluripotency status of iPS cells [12]. In addition, RT-PCR analyssis for stem cell markers, including Oct4, Sox2, Nanog, etc, is very essential [13].

Finally, common tests for pluripotency involve teratoma and chimera formation. Teratomas can develop when iPS cells are injected into immunodeficient animals, which consist of all three ectodermal, mesodermal, and endodermal embryonic germ layers [14]. Chimeras can be formed when iPS cells are microinjected into mouse blastocysts, which lead to differentiation into multiple cell types during the normal growing process [15, 16].

METHODS FOR DELIVERY OF REPROGRAMMING FACTORS

Delivery of Reprogramming Factors by DNA

Plasmids: Plasmids, the most common type of episomal vector, were first identified as a viable reprogramming vector using the canonical reprogramming factors detailed by Okita *et al*. [17]. Repeated transfection of expression plasmids into mouse embryonic fibroblasts produced iPS cells without evidence of plasmid integration, which caused teratomas when transplanted into immunodeficient mice. Gonzalez *et al.* designed and tested a single transcription cassette containing Oct4, Sox2, Klf4, and c-Myc, named pCAG-OSKM [18]. When pCAG-OSKM was delivered by nucleofection, it generated integration-free iPS cell lines, showing that iPS cell induction could be accomplished by transient expression using a single polycistronic cassette. By serially transfecting and encoding the four reprogramming factors (Oct4, Nanog, Sox2, and Lin28) independently, Si-Tayeb *et al.* were able to obtain human iPS cells from human foreskin fibroblasts [19]. Although the plasmid vectors used in their studies were almost the same as lentivirus vectors, the absence of packaging vectors precluded the possibility of emerging wild-type viruses and exogenous DNA, which would be lost from donor cells during cell division.

The use of plasmids, including non-episomal plasmids, requires fundamental programs that are easily available to any laboratory with even basic experience in molecular biology. However, further studies are necessary to improve the efficiency of this methodology.

Minicircle Vectors: Jia *et al.* used a single minicircle vector containing 4 reprogramming factors (Oct4, Sox2, Lin28, and Nanog), with a green fluorescent protein (GFP) reporter gene linked by self-cleaving peptide 2A sequences, to generate transgene-free iPS cells from adult human adipose stem cells without genomic integration [20]. By using integration-free oriP/Epstein-Barr virus nuclear antigen-1 (EBNA1) vectors to encode 7 factors, including Oct4, Sox2, Nanog, Lin28, Klf4, c-Myc, and SV40Tag, Yu *et al.* obtained human iPS cells from human foreskin fibroblasts, without vector integration after they were removed [21]. By transfecting a single multiprotein expression vector to encode c-Myc, Klf4, Oct4, and Sox2 linked with 2A sequences, Kaji *et al.* reprogrammed both mouse and human fibroblasts into iPS cells without integration. After they removed the exogenous factors when the reprogramming succeeded, the Cre-excised cell lines maintained the endogenous gene expression of c-Myc, Klf4, Sox2, and Oct4, indicating that the single vector system could completely eliminate exogenous genes without disturbing the iPS cell state [22].

Compared to other non-integrating reprogramming methods, the reprogramming efficiency with minicircle vectors is greater, but the technology is more complicated.

Delivery of Reprogramming Factors by RNA

By repeated application of synthetic mRNAs, using a simple integration-free strategy, Warren *et al.* efficiently reprogrammed multiple human cell types to pluripotency, and induced the differentiation of RNA-induced pluripotent stem cells (RiPSCs) into differentiated myogenic cells [23]. While the cytotoxicity of transfected mRNAs needed modifications, Rosa *et al*. adjusted the ribonucleotide bases of vector mRNAs by replacing 5-methylcytidine for cytidine and pseudouridine for uridine, to decrease the immunogenicity of the mRNAs. This effective method can also be used for directed differentiation of iPS cells, or even for transdifferentiation to create differentiated cell types for clinical use [24]. Furthermore, by increasing the sequence contexts amenable to RNA-directed genome editing, Hou *et al.* reported efficient targeting of an endogenous gene into 3 human iPS cell lines [25].

MicroRNAs (miRNA) have also played an important role in the control of pluripotent stem cells [26-28], and are also influenced by the pluripotency factors Sox2, Oct4, and Nanog [29, 30]. The miR-302s, -17s, -515s, and miR-371–373 clusters were increased in ES cells, but reduced when the cells differentiated [31, 32]. The mouse miR-291/294/295 homologous human counterpart, miR-302, was also found to be predominantly expressed in human embryonic stem (hES) and iPS cells, but not in differentiated cells [33, 34]. By integrating and introducing the miR-302 cluster into the genome, Lin *et al*. reprogrammed human hair follicle (hHF) cells into iPS cells [35]. Subramanyam *et al*. showed that miR-302 and miR-372 promoted the reprogramming of human fibroblasts to induced pluripotent stem cells [36] and Hu *et al.* demonstrated that miR-302 could increase reprogramming efficiency by repressing NR2F2 [37]. Recently, the use of miR-302/367 clusters has facilitated efficient generation of iPS cells [38], and knockdown or knockout of miR-302/367 clusters impaired the reprogramming [39]. Lee *et al*. showed that expression of exogenous miR-302 cluster could efficiently obtain reprogrammed iPS cells by relieving MBD2 (methyl-DNA binding domain protein 2) -mediated inhibition of NANOG expression [40]. Furthermore, Judson *et al*. reported that the effects of miRNA expression could also promote somatic cell reprogramming [41].

RNA-based methodology eliminates the risk of genomic integration as well as insertional mutagenesis, and has advantages regarding efficiency and kinetics. However, the modified mRNAs are more difficult to generate in the laboratory.

Delivery of Reprogramming Factors by Protein

Any DNA-based reprogramming methods cannot completely avoid random integration, so another optional method for the generation of transgene-free iPS cells involves deliv-

ery by proteins [42]. By delivering 4 reprogramming proteins, Oct4, Sox2, Klf4, and c-Myc, directly fused with a cell-penetrating peptide (CPP), Kim *et al.* obtained stable iPS cells from human fibroblasts [43]. Nemes *et al.* reported that transduction of the 4 reprogramming proteins, combined with a CPP consisted of the glutathione-S-transferase tag and a transcription-nuclear localization signal polypeptide, were capable of reprogramming mouse embryonic fibroblasts (MEFs) to iPS cells [44]. Furthermore, Zhou *et al.* reported the generation of mouse iPS cells by transduction involving 4 rounds of reprogramming proteins tagged with polyarginine in the presence of valproic acid (VPA) in *E. coli* [45]. Kwon *et al.* differentiated both mouse embryonic stem cells and protein-based iPS cells into midbrain dopaminergic (DA) neurons. By comparing the efficiency of DA neuron differentiation from the 2 cell types, protein-based reprogramming resulted in more stable and authentic DA neuron-specific marker expression [46]. Cho *et al.* reported that transfer of embryonic stem cell-derived proteins into adult mouse fibroblasts resulted in complete reprogramming to pluripotency without expression of ectopic transgenes [47]. Using the improved transactivator of transcription kappa (TATκ), a synthetic TAT-HIV, Nordin *et al.* recently described a strategy to generate 293T cells secreting the pluripotent factors Oct-3/4 or KLF4, which could be important in the generation of iPS cells for therapeutic purposes [48].

However, it is not definitively known whether protein transduction can be used for adult cells, which have proven to be more difficult to reprogram, compared to embryonic cells [49]. In addition, the difficult production and poor reprogramming efficiency have restricted the applicability of protein-based iPS cells. Using cell permeable reprogramming factor proteins, Lim *et al.* induced the outgrowth of stem cell-like colonies, which failed to expand into iPS cells or ES cell lines. They concluded that partial reprogramming was a common response to protein-based delivery of programming factors into somatic cells [50]. However, use of self-penetrating proteins and truncated proteins has been reported to be a more effective approach [51]. Harreither *et al.* reported that unmodified OCT-4 protein can be used as a self-penetrating pluripotency reprogramming factor, without the addition of a cationic fusion tag [52]. Thier *et al.* established optimized stabilization conditions for Oct4-TAT and Sox2-TAT proteins, which was a substitute for viral methods [53, 54]. Moreover, a truncated version of the transcription factor Nanog retained the ability to promote reprogramming [55], and versions of Sox proteins shortened to their DNA binding high mobility group (HMG) domain also retained reprogramming [56]. Because both the efficiency [57] and outcome [58] greatly depend on exposure time and the sequence of factor additions, the use of proteins for cellular reprogramming could be an excellent way to generate iPS cells, that could involve extensive control of the reprogramming procedure.

GENERATION OF IPS CELLS BY SMALL MOLECULE COMPOUNDS

For iPS cells to be used in clinical applications, several challenges remain, including possible risks and drawbacks of genetic manipulation and low efficiency. Among the emerging methods for reprogramming of iPS cells, one of the most ideal and practical ways to obtain reprogrammed iPS cells involves the introduction of small molecules instead of exogenous genes into somatic cells. These functional non-peptide or peptide small molecules and natural products are small in molecular mass, contain a simple structure, are inexpensive to produce, are easily absorbed, and are stable under physiological conditions.

Up to now, plenty of small molecules have been identified to substitute reprogramming factors for attaining iPS cells. Huangfu *et al.* reported that DNA methyltransferase and histone deacetylase (HDAC) inhibitors, especially VPA, improved reprogramming efficiency, even without the need for the oncogenes c-Myc or Klf4 [59, 60]. Shi *et al.* found that BIX-01294, a methyltransferase inhibitor, could replace Sox2 and c-Myc for reprogramming, and meanwhile improved the efficiency [61, 62]. Li *et al.* reported that a specific glycogen synthase kinase 3 (GSK-3) inhibitor, CHIR99021, was able to reprogram both mouse embryonic fibroblasts and human primary keratinocyte transduced by Oct4 and Klf4 [63]. Yuan *et al.* reported that, combined with transforming growth factor (TGF)-β inhibitor A-83-01, a protein arginine methyltransferase inhibitor AMI-5 enabled Oct4-induced reprogramming of mouse embryonic fibroblasts [64]. By integrative genomic analysis of reprogramming of mouse fibroblasts and B lymphocytes, Mikkelsen *et al.* suggested that both RNA inhibition of transcription factors and treatment with DNA methyltransferase inhibitors could improve the overall efficiency of the reprogramming process [65]. Li *et al.* reported that the small molecule, Oct4-activating compound 1 (OAC1), could enhance iPS cell reprogramming efficiency and accelerate the reprogramming process [66]. Kang *et al.* obtained iPS cells in two steps, generating stable intermediate cells from mouse astrocytes by Bmi1 and convensing them into iPS cells by treatment with MEK/ERK and GSK3 pathway inhibitors, which demonstrated that combinations of small molecules can directly reprogram mouse somatic cells into iPS cells [67].

Dozens of studies have managed to reprogram cells using small-molecule compounds, but Oct4 was still indispensable. Recently, Hou *et al.* generated pluripotent stem cells from mouse somatic cells at a frequency up to 0.2% using a combination of 7 small chemical molecules only, which were called chemically induced iPS cells (CiPSC) [68]. Though some of these substitutes may trigger unexpected pathways and a comprehensive comparison of the CiPS and ES cells is required for downstream applications, small molecules are still more advantageous for their availability, reversibility, cell-permeation and standardization.

APPLICATIONS AND EXPECTATIONS

In order to apply iPS cell technology for clinical use, efficiency and safety should be improved. Previous studies have shown that removing some obstacles [69-73] and activating innate immunity [74] could promote reprogramming efficiency, and using piggyBac (PB) transposition could eliminate the potential dangers of insertion [75-79]. Moreover, besides obtaining iPS cells from blood [80] and primary skin fibroblasts [81], generating a non-viral human iPS cell bank from donors has been achieved [82], based on previous virus-based methods [83-86]. Considering the rapid progress of this area, involving safer, more affordable, more efficient,

and more convenient protocols, it is conceivable that this methodology will in the near future be used clinically, for various human tissues.

The iPS cells provide a widely available, non-ethically disputed, and almost infinite source of pluripotent cells, which promise a new paradigm in regenerative medicine. In the present review, we described non-viral and integration-free technologies, as well as their potential applications in both therapeutic and research settings. Compared to viral methods, DNA transfection-based methods appear safer, yet they also entail some risks of genomic recombination or insertional mutagenesis. The RNA-based technology throughly evades the risks while the techniques to modify mRNAs are difficult. Generation of iPS cells by recombinant proteins is still worth considering for clinical use due to its high safety, despite being quite expensive and having very low efficiency. Since some small molecules have been identified as enhancing reprogramming efficiency and replacing certain reprogramming factors, reprogramming by defined chemical means may be achieved in the future. Since small molecule compounds have been identified as enhancing reprogramming efficiency and replacing certain reprogramming factors, complete chemical reprogramming approaches remain to be further developed to reprogram human somatic cells into iPS cells and eventually meet the needs of regenerative medicine.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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