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REVIEW

Integration-free Methods for Generating Induced Pluripotent Stem Cells

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Abstract   Induced pluripotent stem (iPS) cells can be generated from mouse or human fibroblasts by exogenous expression of four factors, Oct4, Sox2, Klf4 and c-Myc, and hold great potential for transplantation therapies and regenerative medicine. However, use of retroviral vectors during iPS cell generation has limited the technique's clinical application due to the potential risks resulting from genome integration of transgenes, including insertional mutations and altered differentiation potentials of the target cells, which may lead to pathologies such as tumorigenesis. Here we review recent progress in generating safer transgene-free or integration-free iPS cells, including the use of non-integrating vectors, excision of vectors after integration, DNA-free delivery of factors and chemical induction of pluripotency.

Introduction

In 2006, induced pluripotent stem (iPS) cells were first generated from mouse fibroblasts by forced expression of four protein factors, Oct4, Klf4, Sox2 and c-Myc, mediated by retrovirus [1]. Human cells can also be reprogrammed using the same set of four factors [2] or Oct4, Sox2, Lin28 and Nanog [3]. iPS cells were proven to exhibit pluripotency at levels similar to embryonic stem (ES) cells. Thus patient-specific pluripotent stem cells can be produced without the problems of immune rejection for disease models, transplantation therapies and regenerative medicine.

However, iPS technology is complicated by the potential risks posed by genome-integrating viruses. The transgenes are randomly but permanently integrated into the host genome at multiple sites together with viral vector backbone. The expression of exogenous transgenes is silenced after reprogramming is achieved, and this silencing is indispensable for maintaining pluripotency. Such genome-integrating viral vectors can produce insertional mutations which may influence differentiation potential, or even result in tumorigenesis due to reactivation of the c-Myc oncogene [4]. Reprogramming methods that utilize viral vectors are therefore judged too risky to be used in clinical therapies. Recently, several approaches have been developed to generate safer transgene-free or integration-free iPS cells, as listed in Table 1.

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Table 1 Summary of iPS cell induction strategies

Species	Vector or method	Reprogramming efficiency	Year	Ref
Mouse	Retrovirus	10^{-4}	2006	[1]
Non-integrating vector				
Mouse	Adenovirus	10^{-6}–10^{-5}	2008	[5]
Human	Adenovirus	2×10^{-6}	2009	[6]
Human	Sendai virus	10^{-3}–10^{-2}	2009	[7]
Mouse	Expression plasmids	10^{-6}–$2 \times 10^{-5*}$	2008	[8]
Human	Episomal vectors	3–6×10^{-6}	2009	[9]
Human	Episomal plasmid vectors	1×10^{-5}–3×10^{-4}	2011	[10]
Human	Minicircle vector	5×10^{-5}	2010	[11]
Mouse	Liposomal magnetofection	4×10^{-4}	2012	[12]
Deletion after integration				
Human	Retroviral transfection plus Cre recombinase	–	2012	[13]
Mouse/human	*piggyBac* transposon	3×10^{-4}	2009	[14]
Mouse/human	*piggyBac* transposon	–	2009	[15]
DNA-free delivery				
Mouse	Fusion protein transduction	$6 \times 10^{-5*}$	2009	[16]
Human	Fusion protein transduction	10^{-5*}	2009	[17]
Mouse/human	mRNA transduction	$1 \times 10^{-2*}$	2010	[18]
Chemical induction				
Mouse	Small molecule compounds	2×10^{-3}	2013	[20]

Note: *Repeated transfections were conducted.

Non-integrating vectors

A series of non-integrating vectors, including viral vectors and non-viral vectors, have been used successfully for generating iPS cells.

Adenovirus

Replication-incompetent adenoviral vectors were used to generate iPS cells from mouse [5] and human [6] somatic cells. These vectors allow for transient expression of exogenous genes without integration into the host genome, although with a low efficiency compared to integrating vectors [5].

Sendai virus

Sendai virus (SeV) is thought to be able to replace retrovirus, since it is also an RNA virus and can be easily removed by antibody-mediated negative selection. SeV replicates in the form of single-stranded RNA in the cytoplasm of infected cells, therefore DNA would be neither produced nor integrated into the host genome during transduction. Thus it has been used as a safe vector in the field of gene therapy. SeV-derived transgenes were expressed three days after transduction. The resulting iPS cells were generated with a high efficiency [7], and the SeV vectors were then diluted by cell passages [7].

Expression plasmids

Several classes of vectors have been used as viral substitutes to produce transgene-free iPS cells without integration, and expression plasmids were among the first to be investigated. Repeated transfection of the plasmids containing the four Yamanaka factors into mouse embryonic fibroblasts (MEF) resulted in iPS cells without integration, although with a much lower efficiency than viral vectors [8].

Episomal vectors

Other types of non-integrating vectors were explored to introduce reprogramming factors into mouse or human somatic cells. For example, portions of the Epstein-Barr human herpesvirus were used to create a vector that can be transfected without viral packaging. The resulting extrachromosomal oriP/EBNA1 (Epstein-Barr nuclear antigen-1) episomal vector also features a drug selection mechanism for removing the vector from cells. The oriP/EBNA1 vector undergoes stable extrachromosomal replication only once per cell cycle, without integrating into the host genome. However, efficiency for iPS generation using episomal vector was very low (3–6×10^{-6}) [9]. Okita et al. [10] further improved the episomal vector by using p53 suppression and non-transforming L-Myc instead of c-Myc, resulting in markedly enhanced iPS generation (1×10^{-5}–3×10^{-4}).

Minicircle vector

Minicircle expression vectors (*i.e.*, supercoiled DNA molecules that lack a bacterial origin of replication) have higher transfection efficiencies and longer-term transgene expression compared to plasmids due to reduced silencing for exogenous genes. A single minicircle vector containing four reprogramming factors was introduced into human ES cells by nucleofection, and the minicircle plasmid backbone was then excised and degraded using the PhiC31-based intramolecular recombination system [11].

Liposomal magnetofection

For liposomal magnetofection (LMF), cationic lipids mediate the self-assembly of complexes containing plasmids and nanoparticles of superparamagnetic iron. These "CombiMag-DNA" ternary complexes can be concentrated at cell surfaces using a strong magnetic field to transfect vectors into the

targeted cells. An optimized LMF protocol for generating iPS cells achieved short reprogramming times of 8 days or less. In addition, out of seven independently-generated iPS lines, two iPS lines that had no integrated vector were produced [12].

Deletion after integration

Two systems have been applied to remove integrated transgenes from mouse or human iPS cells.

Cre-loxP system

Cre/loxP recombination was used to excise integrated transgenes from iPS cells. Human iPS cells were produced using a single retroviral vector carrying *OCT4*, *SOX2*, *KLF4* and *c-MYC* linked via picornaviral 2A plasmids. This cassette was removed by transfection of Cre recombinase after reprogramming was achieved. However, residual vector sequences were left behind, therefore insertional mutations remain a risk [13].

piggyBac transposon

Another system used seamless excision of *piggyBac* (PB) transposons to produce vector- and transgene-free mouse iPS cells. Inverted terminal repeats derived from the PB transposon are used to flank a transgene with recognition sequences for a transposase enzyme. Insertions and excisions can then be triggered by regulated, transient expression of the transposase. The four factors were transferred into the PB transposon plasmid under the transcriptional control of the tetO2 tetracycline/doxycycline inducible promoter. MEF were transfected with circular PB transposon plasmid together with a PB transposase expression plasmid, and then maintained in the presence of doxycycline.

After complete reprogramming, excision of the *piggyBac* vector and its induction factors from iPS cell lines was achieved with a pulse of PB transposase expression. In 10 out of 11 subclone lines, the sequence at the transposon integration site reverted to wild type after vector excision [14,15].

DNA-free delivery

Proteins

Alternative methods to avoid introducing genetic modifications include delivery of the reprogramming proteins or mRNA directly into cells, rather than their expression from DNA. These methods have been successfully demonstrated but can be much more complicated to perform.

To generate recombinant proteins that can penetrate and cross the plasma membrane of somatic cells, the carboxy termini of four reprogramming factors were fused to a poly-arginine protein transduction domain. After being added to the cell culture medium, the recombinant transcription factors readily entered cells within 6 h and could translocate to the nucleus. iPS cells were obtained after four repeated protein transductions at 8 mg/ml with 1 mM valproic acid (VPA), a histone deacetylase inhibitor that can significantly improve reprogramming efficiency [16].

Similar protein transduction was also accomplished by Kim et al. [17]. Human iPS cell-like colonies were established within

8 weeks after six rounds of transduction using four reprogramming factors fused with 9-arginine and myc tags. The resulting induction efficiency was approximately twice as high as that using viral transduction.

mRNAs

The use of synthetic RNAs for reprogramming was reported by Warren et al. with surprisingly high reprogramming efficiencies [18]. mRNAs including 3′ and 5′ untranslated regions were synthesized via *in vitro* transcription. RNA transfection with a cationic vehicle into MEF and human somatic cells revealed a high, dose–dependent cytotoxicity. Modified ribonucleotides and phosphatase treatment, in conjunction with media supplemented with the interferon inhibitor B18R, were used to optimize the synthetic mRNA approach and improve cell viability. Maximal protein expression was observed 12–18 h after transfection, followed by a rapid turnover that necessitates subsequent daily transfections. A five-factor cocktail including a modified LIN28 RNA produced reprogramming efficiencies of 1.4%, much higher than using the virus-based approach (10^{-4}), and with a shorter time period (around 18 days) [18].

Chemical induction

Small molecules have advantages because they are non-immunogenic, and can be more easily administered and standardized. Moreover, their effects on inhibiting or activating specific proteins are often reversible and dose–dependent. Small molecule libraries and combinations of compounds have been screened to identify substitutes for transgenic DNA delivery. Initially, iPS cells were generated from mouse fibroblasts using a single gene, Oct4, with a small molecule combination termed "VC6T" [VPA, CHIR99021 (CHIR), 616452 and tranylcypromine] [19]. Next, chemical substitutes for Oct4 were screened as supplements to VC6T to obtain transgene-free iPS cells. In the *Oct4* promoter-driven GFP expression (OG) system, some GFP-positive clusters were induced by VC6T plus forskolin (VC6TF), however they lacked expression of Oct4 and Nanog, indicating incomplete reprogramming [20]. To identify the chemicals critical for late reprogramming, a doxycycline (DOX)-inducible Oct4 expression system was used in which DOX was added only in the first 4–8 days. Several cAMP agonists including forskolin, prostaglandin E2 and rolipram, and epigenetic modulators such as 3-deazaneplanocin A (DZNep), 5-azacytidine, sodium butyrate and RG108 were identified [20]. Among all the candidates, DZNep was proven to work well. DZNep was added 16 days after treatment with VC6TF, followed by a day 28 switch to 2i-medium for dual inhibition (2i) of glycogen synthase kinase-3 (GSK-3) and mitogen-activated protein kinase (MAPK) signaling. The resulting chemically induced pluripotent stem cells (CiPSCs) developed into colonies with ES-like properties. Efficiency can be boosted by a synthetic retinoic acid receptor ligand, TTNPB, for up to 0.2% cells induced [20].

DZNep is critical for activating endogenous Oct4. As an S-adenosyl homocysteine (SAH) hydrolase inhibitor, DZNep may repress the SAM-dependent cellular methylation process and significantly decrease DNA and H3K9 methylation at the *Oct4* promoter, which may contribute to Oct4 activation. These findings indicate that appropriate combinations of inducing chemicals and previously characterized drugs can

provide attractive mechanisms and applications for potential clinical utility, bypassing nucleic acid manipulations and the need to deploy protein factors.

Perspective

The efficiency and safety of generating and using iPS cells seem to show a negative correlation, and thus clinical applications of iPS technology await the validation of a mature protocol that better balances these two important elements. 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. Recent reports point out that activation of toll-like receptor-3 (TLR3) leads to epigenetic modification and facilitates nuclear reprogramming by the four inducing factors [21]. Therefore, TLR3 manipulation may support nuclear reprogramming with a much higher efficiency compared to using retrovirally encoded proteins alone. Additionally, mRNA transduction was reported to have high efficiency, but the rapid turnover of mRNA complicates this approach and necessitates repeated, daily transfections. The newly reported chemical induction method is promising for clinical use, owing to its high safety and feasibility. Moreover, the chimeric mice generated from CiPSCs were 100% viable and apparently healthy, unlike those generated from retrovirus-induced iPS cells. Chemical library screening for TLR3 activators could lead to refinements in the CiPSC induction cocktail and protocol that further boost efficiency. Additional verification of CiPSCs including tetraploid complementation is needed to confirm stem cell function before its extensive application. Nonetheless, CiPSCs provide a new tool to study the mechanisms of reprogramming that may lead to more improvements in iPS cell production and safety, and eventually powerful iPS cell therapeutics.

Competing interests

The authors declared that no competing interests exist.

Acknowledgements

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