

Induced pluripotent stem-cell lines in the clinic - still a long road ahead

Abstract

Discovery of induced Pluripotent stem cells (iPSCs) has revolutionized the fields of stem cell biology and regenerative medicine. iPSCs can now be generated through reprogramming of somatic cells from various tissues and mammalian species by ectopic expression of defined factors (Oct3/4, Sox2, c-Myc, Klf4). However, these methods of gene transduction, often involving viral vectors for delivery and genomic integration, may not be safe for clinical application owing to high rates of malignant transformation and tumor genesis. In this article we provide a comprehensive overview of various new strategies including but not limited to mRNA or protein based delivery and/or chemical induction of pluripotency that circumvent the use of viral vectors for gene delivery. These methodological advancements increase the ease and efficiency of reprogramming, avoiding genomic modification, thereby increasing the suitability for clinical and translational applications in humans.

Volume 2 Issue 3 - 2015

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Received: March 15, 2015 | **Published:** April 09, 2015

Introduction

The seminal work by Takahashi and Yamanaka in 2006 laid the foundation for what we today know as the induced Pluripotent stem-cell (iPSC) field.^{1,2} Through an elegant set of experiments they were the first to reveal that mature somatic cells can be forced to reprogram into an embryonic-like state, with a capacity for unrestrained growth. This reprogramming and turning back the clock required expression of four essential genes, namely, *Oct4*, *Sox2*, *c-Myc* and *Klf4*. Several studies have drawn and demonstrated a parallel between iPSCs and embryonic stem cells (ESCs) in terms of their Pluripotent potential and hence therapeutic value.³⁻⁹ In fact, iPSCs may actually be preferable to ESCs in some therapeutic context as they do not carry a risk of immune rejection due to their patient specific nature, and are not affected by the same ethical concerns as ESCs.¹⁰ But the methods of gene delivery to induce pluripotency while generating iPSCs have often come under scrutiny owing to the concerns on genomic alterations that some methods involve that renders them unsuitable for clinical applications in humans.¹¹⁻³⁵ While Yamanaka et al.¹ used the genome integrating viral vectors for gene delivery, the field has since seen an explosion of gene delivery methods including non-viral vectors, RNAs, proteins as well as reprogramming using small

molecules and/or chemical treatment, all of which circumvent issues of genomic integration.

Genetic and integrating methods of reprogramming

Pioneering work in the iPSCs field suggested the requirement for stable expression of reprogramming factors like Oct4, Sox2, c-Myc, Klf4, Lin28 to induce a state of pluripotency.³⁶⁻⁴⁶ This required genomic integration of these genes into the host genome using lentivirus or retrovirus vectors. While after the iPSC generation, the stable and high expression level of integrated exogenous genes gets down-regulated, yet, the possibility of tumorigenesis due to expression of oncogenes and inactivation of tumor suppressor genes cannot be ruled out. To circumvent the clinical drawbacks of genetic induction of reprogramming, owing to genomic integration, several methods have been reported for generating iPSCs cells through the use of non-integrating plasmid and adenovirus vectors (Table1). However, in such cases, poor reprogramming efficiency remains to be a challenge. Therefore, there is an imminent need for the establishment of new methods of reprogramming that can bypass genomic integration without compromising efficiency of reprogramming, thereby facilitating the use of iPSCs for therapeutic applications.

Table 1 Non-integrating methods of reprogramming to generate iPSCs

Reprogramming Method	Species	Reference
Plasmid	Mouse and Human	Okita K et al. 2008
Adenovirus vector	Mouse and Human	Stadtfeld M et al. 2008
Excisable poly cistronic lentiviral vector	Human	Chang CW et al. 2009; Sommer C et al. 2009
OriP/EBNA1-based episomal vector	Human	Yu J et al. 2009
PiggyBac transposition	Mouse and human	Woltjen K et al. 2009; Yusa K et al. 2009
Recombinant Proteins	Mouse and human	Zhou H et al. 2009 Kim D et al. 2009
Sendai virus	Human	Fusaki N et al. 2009; Lieu PT et al. 2013 Judson RL et al. 2009; Mallanna SK et al. 2010; Warren L et al. 2010; Anokye-Danso F et al. 2011; Miyoshi N et al. 2011; Yoshioka N et al. 2013; Liao B et al. 2011; Subramanyam D et al. 2011
mRNA and micro RNA	Mouse and human	
Magnetic Nano particles	Mouse and human	Lee CH et al. 2011
Small Molecules	Mouse	Pasha Z et al. 2011; Hou PP et al. 2013
Sleeping beauty transposon	Mouse and human	Davis RP et al. 2013

Non-integrating methods of reprogramming

To circumvent the clinical drawbacks of genetic induction of reprogramming, owing to genomic integration, several avenues of research have proposed alternate strategies that work with varied efficiency (Figure 1). A comprehensive review and comparison of the various alternatives available is presented below.

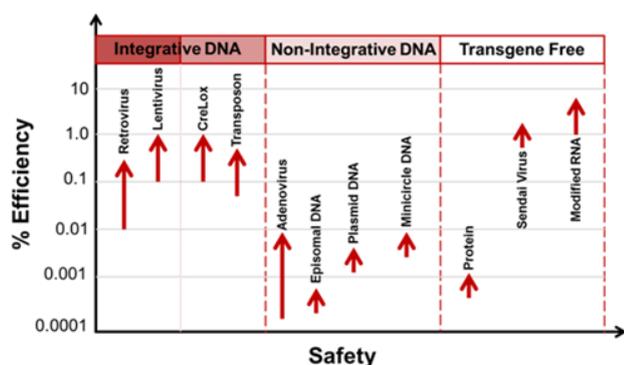


Figure 1 The Evolution of Reprogramming Technologies.

Viral vectors for generating Transgene-free iPSCs

The pioneering work for the derivation of iPSCs by Yamanaka and colleagues undertook integrative viral vectors for the delivery of reprogramming factors largely because of their obvious merits,

1. Delivery to a wide range of cell types
2. Stable and high levels of expression.

One equally obvious drawback of this methodology is the genomic integration of these exogenous gene sequences into the host genome that may lead to

1. Mutations in endogenous genes due to random integration
2. Inactivation of tumor suppressor genes
3. Hyper activation of oncogenes leading to cancer and tumorigenesis.

To circumvent these clinical drawbacks, several technical advancements have been suggested through research conducted in different parts of the world.^{47,48} Soldner et al. used dox-inducible lentiviral vectors that involved the use of cre-excisable lentiviruses that upon integration can subsequently be excised from the host genome.⁴⁹ The technology was later improvised by Sommer et al. generated a novel version of this single polycistronic vector containing a reporter fluorochrome to allow direct visualization of vector excision in live iPSCs in real-time.⁵⁰

While this approach equals retroviral transduction in terms of efficiency but has the drawback of the need for additional genetic manipulations for the excision of cre-lox cassette. Another concern is the possibility of introducing chromosomal translocations during cre-mediated recombination. Lastly, a small piece of the vector is left in the integration site even after excision, which again leaves the possibility of insertional mutagenesis. To further improvise, Papapetrou et al. developed a strategy that includes additional steps for mapping the integration site in the genome to allow for the selection of clones with a single appropriate integration site for the residual LTR, however there still remains a risk that insertional mutagenesis may arise from this genetically integrated exogenous DNA.⁵¹⁻⁵³

To circumvent the integration step altogether, several groups have resorted to non-integrating viruses such as adenoviruses for reprogramming factor delivery. However, adenoviral methods are highly inefficient and limited to permissive cell types. Stadtfeld et al. reported mouse iPSCs from fibroblasts and liver cells at extremely low efficiency (0.0001% to 0.001%), which is significantly lower than that obtained from integrating viruses (0.01% to 0.1%).^{18,54} Their low efficiency has been largely attributed to the failure to sustain factor expression due to a gradual loss of viral vectors in dividing cells. Sendai virus (SeV), is another type of non-integrating virus vector which has been successfully used to reprogram human cells.⁵⁵ SeV viruses are RNA viruses and hence do not integrate into host genome unlike other DNA viruses.⁵⁶⁻⁵⁸ Additionally, SeV viruses are replication competent and hence unlike the adenoviruses can sustain high-levels of factor expression, which can reach up to 10 times higher (efficiency ~1%) than those obtained by conventional retroviral vectors. An additional benefit of this system is the convenient removal of viruses by antibody mediated negative selection utilizing the cell surface marker, HN, expressed on SeV infected cells.

Plasmid DNA vectors for generating Transgene-free iPSCs

Several groups have successfully generated non-viral, transgene-free iPSCs using DNA-based expression vectors such as plasmid, mini circle vector, piggyBac transposon and episomal plasmid. But this transgene-free factor delivery comes at a price of low efficiency as transient transfection of DNA vectors is less efficient than viral transduction.⁵⁹ To this end, several groups have reportedly used polycistronic expression cassettes to ensure that every cell gets the full complement of factors.^{17,60} Alternately, mini circle vectors expressing Oct4, Sox2, Nanog and Lin28 from a single cassette of OSNL plus a GFP reporter gene, each separated by self-cleaving peptide 2A sequences, have been used to generate transgene-free iPSCs from human adipose stem cells.⁶¹ These vectors used circular expression cassettes produced from regular plasmids by removing the bacterial backbone through intra molecular recombination. Compared to their parental plasmids, mini circles are smaller in size and less prone to silencing, both of which make them highly sought after for reprogramming somatic cells, however, they are far less efficient compared to retroviral vectors (reprogramming efficiency ~0.005%).⁶²

Moreover, their usage requires cumbersome protocols with large number of starting cells and repeated transient transfections to compensate for the rapid loss of reprogramming factors in dividing cells. But compared to other methods of reprogramming mini circle approach has several advantages, including the use of a single vector without the need for subsequent drug selection, vector excision, or the inclusion of oncogenes such as SV40, making the derivation process free of foreign transgenes or chemicals. These unique features bagged it the FDA approval, making it a potentially significant approach from clinical and translational standpoint.

Some other groups designed a piggyBac transposon approach to deliver reprogramming factors to host cells.⁶³⁻⁶⁷ In this approach, a transient transposase expression leads to stable integration of the piggyBac transposon cassette into the host genome, thus ensuring a high level of expression of reprogramming factors. Interestingly, the piggyBac transposon cassette can be easily excised, taking advantage of the natural propensity of the system for seamless excision, by transiently expressing the transposase gene in iPSCs without any risk of mutations at the integration site.⁶⁸ However, the excision step is inefficient and in certain cases reversion of iPSCs to wild type phenotypes has been observed after piggyBac transposon excision.

An alternate strategy for stable expression of reprogramming factors is made available by the use of non-integrating oriP/EBNA1-based episomal vectors.^{69,70} Derived from the Epstein-Barr virus, oriP vectors can be easily transfected into human somatic cells, without the need for viral packaging, and can be subsequently removed from cells in the absence of drug selection without further genetic manipulation. In such vectors the cis element oriP and the EBNA1 gene product act in Trans and together control the replication and partition of the episomes during cell cycle. This process provides these vectors with self-sufficiency to function in a variety of cell types, eliminating the need for repeated transfections. Additionally, the removal of episomal transgenes is not only automatic and efficient, it required repeat application and also error proof, and thus making oriP episomal vectors a method of choice. However, the stable transfection efficiency of the oriP vectors is several orders of magnitude less than that of the lentiviral vectors.

DNA-free methods for generating Transgene-free iPSCs

To overcome the caveat of genome modification presented by DNA-based vectors, several groups have successfully attempted to deliver reprogramming factors as mRNAs or proteins. In such cases, instead of being produced by a transgene, recombinant proteins are directly delivered to cells by fusing them to cell-penetrating peptides such as poly-arginine.⁷¹⁻⁷³ Although such proteins make it to the nucleus successfully, but require repeated application for a successful reprogramming. Moreover, all reports of protein-based reprogramming suffer from low efficiencies (~0.001%). Additionally, it is technically challenging to produce large quantities of biochemically active recombinant proteins.

Worth highlighting also is the discrepancy in the various published reports. Compared to Zhou et al.²⁸ who show incapability in generating mouse iPSCs with only recombinant proteins, without the use of chemical supplements, Kim et al.²⁹ demonstrated successful derivation of human iPSCs with direct delivery of reprogramming proteins in the absence of any chemical treatment. The plausible scientific explanation for these observed differences could be the mode of expression of reprogramming proteins, which involved mammalian cells in case of Kim et al. while expression in *E.coli* followed by protein refolding for Zhou et al.²⁸ Taken together, direct protein based reprogramming approach still remains a cumbersome technique offering poor efficiencies compared to other known approaches, thereby lacking the competitive edge to be of value in clinical setting.

Schematic representation of the contrast in the efficiency and safety of reprogramming between various technologies reported till date. (Illustration adapted from *Cardiovasc. Trans. Res.* 2013, 6, 956-68) As an alternative, synthetic mRNAs have shown promise for fast and efficient reprogramming.⁷⁴⁻⁷⁸ Warren et al.⁷⁴ proposed a non-integrating strategy for cellular reprogramming by delivery of synthetic mRNA, modified to overcome innate anti-viral responses.^{74,79,80} A five-factor cocktail (OKSML) was able to reprogram human fibroblasts with an efficiency of 2%, which is two orders of magnitude higher than virus-based reprogramming. Additionally, microRNAs (miRNAs) have shown great potential in regulating pluripotency.⁸¹⁻⁸⁵ Intriguingly, five miRNAs (miR-302a-d and miR-367) have been shown to be sufficient in reprogramming human cells without any exogenous transcription factor.^{86,87} Later, another group reported Tg-free iPSCs in mouse and human systems by transfections of mature miRNAs (different combinations of miR-200c, 302s and 369s).⁸⁸ While their ease of production and small size ensuring efficient delivery makes mRNAs and miRNAs an attractive option for the production of transgene-free iPSCs but poor efficiency still remains a stumbling block.

The quest for reprogramming somatic cells solely with the use of chemical compounds ended when Hou et al.⁸⁹ reported the derivation of mouse iPSCs at a frequency as high as ~0.2% using a combination of seven small-molecule compounds.^{5,19,21-23,28,42,90-96} This discovery brought a paradigm shift to the technology of cellular reprogramming. Additionally, the use of small molecules alone brought with it several advantages like the molecules being cell permeable showing a promise of easy delivery, easily synthesized in large quantities and non-immunogenic. But while the ease of use and efficiency of these small molecules in mouse system has been well documented, their success in human cells is yet to be established.

Conclusion: Challenges ahead

Many roadblocks remain to be addressed before iPSCs reach the clinic.⁹⁷⁻¹⁰⁹ A major impediment to the use of iPSCs for therapeutic purposes remains the viral-based delivery of reprogramming factors. Additionally, for patient-specific autologous treatment, methods need to be improved able to generate iPSCs in sufficient quantity. Efficiency of reprogramming remains the biggest challenge, especially in the context of technologies that do not integrate transgenes into the host genome. Moreover, there still remains a risk of teratoma formation in case a subpopulation of iPSCs fails to terminally differentiate prior to transplantation.

Even upon differentiation it is difficult to control and predict events of spontaneous differentiation and dedifferentiation into other cell types and the clinical outcome of such a heterologous cell populations within a certain tissue. Current scientific and technical advancements are rapidly eliminating some of these fundamental issues in the iPSC field thereby reassuring the profound benefits of stem cell technology to regenerative medicine. But while a lot of ground has been covered, there might be a long way to go ahead before the full potential of iPSC technology may be harnessed.

Acknowledgements

We thank Mr. Daksh Khulbe for his constructive critiques and apologize to all colleagues whose work could not be cited due to space constraints. We are grateful to CSIR and JDRF for their generous support.

Conflicts of interest

None.

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