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Review Article

No factor left behind: generation of transgene-free induced pluripotent stem cells

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Abstract: Induced pluripotent stem cells (iPSCs) hold great potential for regenerative medicine, yet their implementation in the clinic still seems far out of reach due to concerns about their safety. The development of safe and efficient reprogramming methods is a critical step towards clinical application of iPSCs. Recently, much progress has been made in the technology of generating iPSCs that are free of integrated transgenes. The newly developed methods improve genome integrity of iPSCs and will likely replace virus-based reprogramming in the future. Here, we review the recent technological advancements of transgene-free reprogramming and discuss the challenges of ensuring genome integrity of iPSCs.

Keywords: iPS cell, reprogramming, episomal vector, minicircle, excisable lentiviral vector, piggyBac transposon, Adenoviral vector, Sandai viral vector, miRNA

Introduction

The potential of induced pluripotent stem cells (iPSCs) in regenerative medicine was evident immediately after Yamanaka and colleagues discovered that ectopic expression of defined transcription factors could reprogram somatic cells to pluripotent stem cells. Soon after, what also became obvious was that the original virus-based reprogramming methods had many side effects that greatly limit the usefulness of iPSCs in mechanistic studies and therapeutic applications. Particularly, randomly integrated transgenes could cause mutations, disrupt endogenous gene regulation or become inappropriately reactivated in terminally differentiated cells. These caveats not only create heterogeneity among iPSC clones, thus complicating mechanistic studies and disease modeling; but also increase the risk of tumor formation in vivo, making iPSCs unsafe for clinical applications. To overcome these issues, researchers have devised diverse solutions to generate iPSCs that are free of integrated transgenes. By providing a brief overview of the existing methods of generating transgene-free iPSCs (Tg-free iPSCs), we hope to facilitate the process of evaluating and choosing the right approach for a specific research goal.

Generation of transgene-free iPSCs using viral vectors

The choice of integrative viral vectors as delivery vehicles for reprogramming factors is not accidental. Viral vectors have two main merits: (1) ease of delivery to a wide range of cell types; and (2) stable and high levels of reprogramming factor expression, both of which are critical for reprogramming and are exactly what transgene-free methods need to emulate. One of the ideas to rid iPSCs of transgenes is to excise the proviruses following the completion of reprogramming using the cre-lox system. Because of their large cargo capacity, lentivectors can accommodate all reprogramming factors as a polycistron, which helps to reduce the number of viral insertions, thus facilitating excision. Excisable lentiviral vectors have been used by different groups to generate Tg-free iPSCs [1, 2]. This approach matches the efficiency of the original retroviral method and is straightforward to adopt, though there are several drawbacks. The excision step is cumbersome and exposes iPSCs to additional

culture manipulations. Moreover, it requires the introduction of additional genetic materials (cre expression vectors), which carries the risk of altering the genome. Another concern is the possibility of inducing chromosomal translocation when Cre-mediated recombinations happen between two proviruses, although screening for clones with a single copy of the lentivector can reduce this risk [2]. Last but not least, a small piece of the vector is left in the integration site even after excision, which necessitates additional characterizations to rule out the possibility of insertional mutagenesis.

To circumvent the integration step altogether, many groups have resorted to non-integrating viruses as delivery vehicles for reprogramming factors. The Hochedlinger group and the Freed group generated integration-free iPSCs from mouse hepatocytes and human fibroblasts, respectively, using replication-incompetent adenoviral vectors [3, 4]. The reprogramming by adenoviral vectors is highly inefficient (two orders of magnitude lower than that of retroviral vectors) and restricted to permissive cell types. The low efficiency was attributed to the failure to sustain factor expression due to a gradual loss of viral vectors in dividing cells. Although the authors did not find any evidence of vector integration after extensive characterizations, a high frequency of tetraploidy was found in one study [3].

Sandai virus (SeV), another non-integrating virus, was used to reprogram human cells with greater success [5, 6]. SeV is an RNA virus that does not go through a DNA phase, therefore eliminating the risk of modifying the host genome. In contrast to adenoviral vectors, a non-transmissible SeV vector can sustain high-level factor expression, because they are replication competent. Another unique feature of the SeV vector is that it allows the expression of transgenes to be dialed up/down by simply cloning them into different positions. Indeed, by tweaking the stoichiometry of the four factors, the authors achieved vastly different reprogramming efficiencies, the best being ~10 times higher than that of retroviral vectors. Using replicating viruses is a double-edged sword when it comes to removing them. Since the viral genome is detected in iPS cells after 20 passages, SeV-infected cells have to be actively removed by an antibody-mediated negative selection. Recently, two mutant variants of the Sandai viral vector have been developed to address this issue [6, 7].

Generation of transgene-free iPSCs using plasmid DNA-based vectors

Making high titer viruses is labor intensive, requires special knowledge in virology, and demands strict biosafety protocols. Furthermore, any virally derived iPSCs, even those purged of viral genome, will not likely be accepted in the clinic without further scrutiny for issues such as immunogenicity. To generate non-viral Tg-free iPSCs, many groups have explored the use of DNA-based expression vectors including plasmid, minicircle vector, piggyBac transposon and episomal plasmid. Generally speaking, transient transfection of DNA vectors is less efficient than viral transduction. To improve the probability of reprogramming, it is beneficial to use a polycistronic expression cassette, which ensures that every transfected cell gets the full complement of factors. Using polycistronic plasmid vectors, two groups successfully generated of Tg-free iPSC from MEFs, albeit at a much lower efficiency [8, 9]. Both studies used repeated transfection regiments to maintain the expression level of reprogramming factors during the initial critical period of reprogramming. However, applying the same strategy to human fibroblasts was less successful [10, 11], possibly due to the fact that human fibroblasts are notoriously hard to transfect with large DNA constructs and require prolonged expression of reprogramming factors [12].

Minicircle vectors are circular expression cassettes produced from regular plasmids by removing the bacterial backbone through intramolecular recombination. Compared to their parental plasmids, minicircles are smaller in size and less prone to silencing, both of which make them attractive for reprogramming human somatic cells. A group in Stanford generated Tg -free iPSCs from human adipose stem cells using a minicircle vector expressing Oct4, Sox2, Nanog and Lin28 [13]. Despite the improvements over plasmid vectors, minicircle vectors are still far less efficient in reprogramming compared to retroviral vectors. In addition, the protocol is complicated, requiring a large number of starting cells, a fluorescence-activated cell sorting step and multiple transfections of minicircles. It is also unclear whether minicircles will be able to reprogram adult human fi-

broblasts [14].

Since the major issue with transient transfections is the rapid loss of reprogramming factors in dividing cells, several methods have been developed to overcome this. PiggyBac transposon is an invertebrate transposable element that is highly active in mammalian cells [15]. Upon transient transposase expression, piggyBac transposons can mediate stable integration of the reprogramming cassette into the genome, thus maintaining a high level of expression of the reprogramming factors. More importantly, piggyBac transposons can be excised by transiently expressing the transposase in iPSCs without introducing any mutation to the integration site. This strategy has been used by three groups to reprogram mouse cells with efficiencies that rival that of the retroviral method [16-18]. In two cases, the majority of the iPSC lines revert to a wild type genome after piggyBac transposon excision. However, the excision step is inefficient and shares the same drawbacks as discussed for excisable lentiviral vectors. To facilitate the screening for excision events, one group inserted a negative selection maker in the piggyBac transposon [16]. Alternatively, the transposase may be delivered in mRNA form, which could increase transfection efficiency and reduce the risk of transposase vector integration. To date, two papers reported the reprogramming of human embryonic fibroblasts using the piggyBac transposon system, but both stopped short of excising the transposons [17, 18].

An alternative way to sustain stable expression of reprogramming factors is by means of the non-integrating oriP/EBNA1-based episomal vectors (referred to as episomal vectors hereafter). The cis element-oriP and the trans factor encoded by the EBNA1 gene together control the replication and partition of the episomes within each cell cycle. Because episomal vectors are self-sufficient, they can function in a variety of cell types via a single transfection. Another attractive feature of episomal vectors is that the removal of episomal transgenes happens automatically, and at a pace that perfectly matches the needs of the different stages of reprogramming. A small portion of the episomes is lost per cell cycle due to errors in plasmid replication and partition [19]. This process happens slowly in the initial phase of reprogramming because somatic cells have longer cell cycles. This is beneficial for maintaining high levels of transgene expression for a longer period. By the time fully reprogrammed iPSCs emerge, transgene expressions are no longer needed. Accordingly, episomal vectors are purged faster in rapidly proliferating iPSCs. The Thomson group first reported the generation of Tg-free iPSCs from human foreskin fibroblasts using a combination of 7 factors (OCT4, SOS2, KLF4, c-MYC, NANOG, LIN28 and SV40 large T antigen). After testing an extensive list of combinations and arrangements of the factors, the authors found three conditions that gave rise to *bona fide* iPSCs. As expected, all iPSC clones tested lost the episomal vector after expansion [20]. The reprogramming efficiency in the original report is still low (~0.0005% or 100 fold lower than that of retroviral method). Soon after, two papers (including one from the same group) reported that the reprogramming efficiency of episomal vectors can be 100 times more efficient in bone marrow and cord blood mononuclear cells (MNCs) compared with that of fibroblasts [21, 22]. Moreover, Tg-free iPSCs were also derived from adult peripheral blood MNCs, offering a promising method for obtaining high-quality patient-specific iPSCs from one of the most accessible human tissues [21]. Recently, an enhancement of two orders of magnitude in reprogramming efficiency was also achieved in human fibroblasts by using novel reprogramming enhancers and an optimized episomal vector design [23]. Because episomal vectors are simple to prepare (same as plasmid DNA prep), require only a single transfection, and are proven effective in reprogramming multiple cell types, they will likely to see wide acceptance in the future.

Generation of transgene-free iPSCs using DNA-free methods

All DNA-based reprogramming methods share the common risk of permanent genome modification. Even though this risk can be reduced by screening for iPSC clones that are free of vector sequences using qPCR and Southern blot, micro insertions/deletions remain difficult to detect. However, when reprogramming factors are delivered as mRNAs or proteins, such risk can be completely eliminated.

Instead of being produced from transgenes, reprogramming proteins can be directly delivered into the cells. One of the methods for mak-

ing reprogramming proteins permeable to the cell membrane is fusing them with cell-penetrating peptides such as poly-arginine. Cell-penetrating reprogramming proteins have been shown to localize correctly in the nucleus [24, 25]. However, they are only stable for a short time and therefore require repeated applications in order to support reprogramming. Cell-penetrating reprogramming proteins either purified from E. coli or in cell extracts from HEK293 cells have been used to reprogram mouse and human cells, respectively [24, 25]. Alternatively, reprogramming proteins can be delivered by streptolysin O-mediated reversible permeabilization. One group reprogrammed adult mouse fibroblasts by a single introduction of wild type ES extract by reversible permeabilization [26]. All reports of protein-based reprogramming suffer from slow kinetics and low efficiencies (2 months, 0.001% in the case of human cells). In addition, it is technically challenging to produce large quantities of pure and biochemically active reprogramming proteins. Similarly, the use of undefined cell extracts may have unpredictable effects on iPSCs and is unfavorable for mechanistic studies or clinical applications.

Synthetic mRNAs have been shown to promote fast and efficient reprogramming in several human cell types (We would like to refer the readers to our recent commentary for a detailed discussion on this method) [27, 28]. However, the protocol is technically challenging and has not been independently reproduced.

MicroRNAs (miRNAs) are important regulators of development and are thought to play an important role in maintaining the pluripotent phenotype. Several papers have described the enhancing effects of certain miRNAs on reprogramming when they are used in conjunction with viral transgenes [29-33]. Remarkably, five miRNAs (mir-302a-d and mir-367) expressed from a lentiviral vector are sufficient to reprogram human cells without any exogenous transcription factors [34]. Later, another group generated Tg-free iPSCs in mouse and human by transfections of mature miRNAs (different combination, i.e. mir-200c, mir-302s and mir-369s family) [35]. Because miRNAs are easy to synthesize and easy to transfect due to their small size, they represent an attractive option for labs that do not have experience in DNA or viral vectors technologies. Although the reported efficiency in human cells is relatively low, it could be improved through the discovery of better miRNA combinations, more stable synthetic miRNAs and improved delivery methods.

The enthusiasm over iPSCs has fueled the explosion of alternative methodologies for the generation of Tg-free iPSCs. Given the pace of the iPSC field, the integration-based reprogramming methods will probably be replaced by transgene-free methods in the near future. While the past research has been primarily focused on increasing reprogramming efficiency and eliminating transgenes, works has been started to evaluate the quality of Tg-free iPSCs [2, 36]. In order to identify the best quality iPSCs for future clinical applications, it is especially important to map the genetic and epigenetic abnormalities in Tg-free iPSCs derived by different methods.

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