Minireview

Induced pluripotent stem cells free of exogenous reprogramming factors

Ramzey Abujarour and Sheng Ding

Address: Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.

Correspondence: Sheng Ding. Email: sding@scripps.edu

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Abstract

The development of novel approaches for reprogramming mouse and human somatic cells has enabled the generation of induced pluripotent stem cells that are free of exogenous genes.

The epigenome of differentiated somatic cells can be reprogrammed to a pluripotent state by nuclear transfer into enucleated oocytes or by fusion with pluripotent cells such as embryonic stem cells (ESCs) [1]. More recently, it has been shown that overexpression of defined transcription factors via transduction of viral vectors can reprogram mouse and human somatic cells to induced pluripotent stem cells (iPSCs) [2-4]. This new approach greatly simplifies the generation of pluripotent cells, bypassing many technical and ethical hurdles, and brings closer the possibility of using patient-specific cells in cell-based therapy. However, the use of viruses to deliver the reprogramming factors entails permanent genetic alterations that render the cells inappropriate for many *in vitro* and *in vivo* applications. Several approaches have recently been devised to generate iPSCs free of the exogenous reprogramming factor genes, including the use of non-integrating approaches for transgene delivery [5,6]. Four papers published this year describe a variety of novel approaches. Soldner *et al.* [7] have used the Cre/*loxP* recombination system to produce human iPSCs free of exogenous reprogramming genes. Woltjen *et al.* [8] and Kaji *et al.* [9] demonstrate that the *piggyBac* (PB) transposon system can be used both to introduce reprogramming genes and induce pluripotency and then to remove the transgenes from established iPSC lines. Finally, Yu *et al.* [10] describe the successful use of another type of non-integrating vector to obtain iPSCs free of vector and transgenes.

Towards reprogramming without a trace

ESCs are derived from the inner cell mass of mammalian embryos, and are characterized by their capacity to self-renew indefinitely in culture and their potential to differentiate into all cell types of the body. During development, cells become more restricted in their ability to generate other cell types and somatic cells do not normally revert to an earlier, more primitive developmental stage. Nonetheless, the developmental memory of a somatic cell can be erased, and the cell can be induced to revert to a pluripotent stage by forced expression of a combination of transcription factors that usually includes Oct4, Sox2, Klf4 and c-Myc [2,3]. The same approach has been used to reprogram human adult fibroblasts and keratinocytes into iPSCs [3,11]. Reprogramming adult human cells from patients with complex diseases such as Parkinson's disease and Alzheimer's disease holds great promise of providing invaluable disease models, as well as platforms for drug screening. Along the road, iPSC-derived specialized cells could also serve in transplantation therapy.

As a proof of concept, disease-specific iPSCs have been generated [12-14], and have been used to model disease [14] as well as to ease the symptoms of sickle-cell anemia in animal models after gene correction and proper differentiation [15]. However, current methods for generating iPSCs are unsuitable for therapeutic applications. Most methods rely on the use of retroviruses or lentiviruses to permanently

integrate the reprogramming factor genes into the genome of the target cell. Although the reprogramming factors are often silenced after complete reprogramming, the iPSCs maintain significant residual transgene expression and could display transgene reactivation. This could have an impact on their differentiation into specialized cells and, more importantly, increase the risks of tumorigenesis [16]. The added risk of insertional mutagenesis highlights the need for the development of safer non-integrating vehicles to deliver the reprogramming factors.

Early attempts to generate iPSCs without viral integration included the repeated transient transfection of plasmid-based vectors into mouse embryonic fibroblasts [5], and the use of adenoviruses in mouse liver cells [6]. However, in both cases, the reprogramming efficiency was extremely low and the kinetics was too slow, and no iPSCs have been generated from human cells using such methods. Soldner *et al*. [7] have now successfully exploited the Cre/*loxP*-recombination system to efficiently reprogram fibroblasts into iPSCs from five patients with idiopathic Parkinson's disease using excisable lentiviruses. Transgenes were removed by transfecting the iPSCs with Cre-recombinase and applying selection to remove untransfected cells or isolating transfected cells by cell sorting. Out of 180 clones isolated, 16 had lost the integrated transgenes (approximately 9% excision efficiency), and maintained a pluripotent state for more than 15 passages.

When these iPSCs were induced to differentiate under neuronal differentiation protocols, dopaminergic neurons were derived regardless of the age of the donor, thus highlighting the feasibility of using iPSC-derived cells in transplantation therapy for Parkinson's disease in the future [7]. Interestingly, Soldner *et al*. found, by genome-wide gene-expression analysis, that the factor-free iPSCs are more similar to human ESCs than they are to the parental iPSCs carrying the transgenes. These observations suggest that residual transgene expression could have an effect on the molecular characteristics of reprogrammed cells. The complete removal of vector and transgene sequences from established iPSCs is therefore essential if iPSCs and ESCs are to be accurately compared.

An alternative method for removing the reprogramming factors from iPSCs relies on the PB transposition system, in which a transiently expressed transposase catalyzes the excision of transgenes flanked by inverted terminal repeats [8,9]. The PB system is effective in mouse and human cells, and performs efficient and precise excision without leaving a footprint behind [17,18]. Woltjen *et al*. [8] generated iPSCs by transfecting mouse embryonic fibroblasts with a plasmid expressing the PB transposase and a vector containing Oct4, Sox2, Klf4, and c-Myc open reading frames linked with 2A peptide sequences and flanked by the required inverted terminal repeats. The polycistronic sequence served to reduce the required number of excisions. The efficiency and kinetics of iPSC generation using this method were similar to those observed using other viral vector approaches. Out of 48 iPSC lines established, two contained only a single copy of the polycistronic sequence, indicating that the reprogramming factors are sufficient in single copy for reprogramming. The transient expression of PB transposase in the two single-copy cell lines and subsequent subcloning resulted in the removal of the linked reprogramming factor DNA at an efficiency greater than 2%; within these subclones, the majority examined (10 out of 11) had reverted to the wild-type sequence. The factor-free iPSCs were fully reprogrammed as determined by their contribution to chimera development and tetraploid embryo complementation. Woltjen *et al*. [8] were also successful in generating iPSCs from human embryonic fibroblasts using the PB transposon system, although no removal of transgene sequences was shown. In an accompanying paper, Kaji *et al*. [9] combined a Cre/*loxP*-based method with a non-viral 2A system and found that expression of the exogenous transgenes could no longer be detected in stably reprogrammed iPSCs derived from mouse embryonic fibroblasts, suggesting that they had been eliminated.

The removal of reprogramming transgenes using Cre/*loxP*-recombination or the PB transposition system provides a practical approach for the generation of factor-free human iPSCs, but requires additional tedious steps that might hinder widespread applications of iPSCs for various applications. In addition, in the case of the recombinase-based approach, residual vector sequences are left behind, increasing the risk of insertional mutagenesis. Yu *et al*. [10] have made an effort to simplify the derivation of transgene-free human iPSCs by exploiting an *oriP*/EBNA1 (Epstein-Barr nuclear antigen-1)-based episomal vector. Plasmids containing *oriP* maintain stable extrachromosomal replication in 1% of transfected cells when the viral protein EBNA1 is provided, being lost at a rate of 3 to 5% per cell generation after removal of selection [19]. Yu *et al*. [10] transfected human fibroblasts once with a combination of episomal vectors expressing two to three reprogramming factors from IRES2-linked open reading frames.

Initial attempts to generate iPSCs by delivering IRES2-linked open reading frames for the human reprogramming factors OCT4, SOX2, KLF4, c-MYC, NANOG and LIN28 via multiple *oriP*/EBNA1 vectors failed as a result of substantial cell death, possibly due to the high level of c-MYC expression. However, when the SV40 large T (*SV40LT*) gene was included in the mixture to counteract the possible side effects of c-MYC, the authors managed to derive iPSCs from human foreskin fibroblasts in two independent experiments [10]. PCR analysis of iPSC clones revealed persistence of the episomal vectors over a prolonged period of time, perhaps a requirement for successful reprogramming, but no integration in the genome was observed. Subcloning of iPSC lines (at passage 9 and 10) to select for spontaneous loss of episomes led to the isolation

of episome-free subclones (more than one-third of all subclones derived). The iPSC subclones were fully reprogrammed and had normal karyotypes.

This approach is promising as there is no integration of transgenes into the genome, and the exogenous DNA can be removed by gradual loss of the episomes during extended culture without drug selection, and without the need for further genetic manipulation. However, the protocol as it stands now requires the delivery into somatic cells of a large number of genes (six reprogramming factors in addition to *SV40LT* and *EBNA1*) in multiple vectors (two to three), with low reprogramming efficiency (around 0.001%). In addition, the fact that episomal loss is spontaneous and not directed will require subcloing of iPSC lines and prolonged culture. Improved efficiency and a simpler approach are therefore needed for this method to be more widely applied.

Improvement still needed

The first iPSCs derived from murine somatic cells were reported three years ago, followed by similar studies in human cells a year later. Despite great advances, much still needs to be clarified before iPSCs can be fully utilized in basic research and clinical applications. Reprogramming of somatic cells by forced expression of defined factors is clearly different from reprogramming through somatic cell nuclear transfer or fusion with pluripotent cells. The efficiency and kinetics of reprogramming are not the same, with reprogramming by defined factors being a random process that requires many progressive nonspecific epigenetic remodeling events to occur over a prolonged period of time (usually 2 weeks for mouse cells, and 3-4 weeks for human cells).

Whether reprogramming by defined factors can be further optimized will depend on a better understanding of the mechanisms involved, and improvements in the methods for generating transgene-free iPSCs such as those discussed here [7-10]. It might be possible to combine multiple approaches to achieve this, such as combining episomal vectors for gene delivery and small molecules to improve reprogramming efficiency and shorten the time required. The latter will be essential, as prolonged protocols and extended culture times and subcloning might introduce genetic or epigenetic abnormalities that would render the iPSCs unsuitable for clinical application. What will be even more desirable is to avoid genetic material and manipulation altogether. Indeed, the number of reprogramming factors required to generate iPSCs has been reduced [20,21], and neural stem cells have recently been reprogrammed by the forced expression of Oct4 only [22]. In the future, it may be possible by treatment with small molecules, direct introduction of proteins, or a combination of both to generate iPSCs without any genetic manipulation.

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