stem cell research&therapy

REVIEW

Experimental approaches for the generation of induced pluripotent stem cells

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Abstract

Derivation of autologous induced pluripotent stem cells (iPSCs) through direct reprogramming of easily accessible somatic cells holds the potential to transform the field of regenerative medicine. Since Takahashi and Yamanaka's groundbreaking study describing the generation of iPSCs by retroviral-mediated delivery of defined transcription factors, substantial progress has been made to improve both the efficiency and safety of the method. These advances have provided new insights into the molecular mechanisms of reprogramming and promise to accelerate the clinical translation of iPSC technology. Here, we summarize current reprogramming methodologies with a focus on the production of transgene-free or genetically unmanipulated iPSCs and highlight important technical details that ultimately may influence the biological properties of pluripotent stem cells.

Introduction

The potential use of embryonic stem cells (ESCs) for cell replacement therapies is limited by ethical concerns and the technical hurdles associated with their isolation from human embryos. In addition, as the genetic identity of the donor egg from which the ESCs are derived most likely will differ from that of potential recipients, patients who receive ESC-derived cells or tissues may face the same complications that result from organ transplantation (for example, immunorejection, graft-versus-host disease, and need for immunosuppression). To circumvent these obstacles, considerable effort has been invested in attempting to derive ESC-like cells by reprogramming somatic cells to an embryonic state. Although exciting results have been achieved by means of somatic cell nuclear transfer, cell fusion, and culture-induced reprogramming [1], these procedures are technically demanding and inefficient and therefore unlikely to become a common approach for producing patient-specific pluripotent cells. In 2006, a major breakthrough was reported in Japan by Takahashi and Yamanaka, who described the generation of induced pluripotent stem cells (iPSCs) from mouse fibroblasts via overexpression of defined transcription factors [2]. Shortly afterwards, the original experimental protocol was replicated and optimized by several laboratories, confirming that iPSCs share the gene expression profile, epigenetic modifications, and proliferation rates as well as the pluripotency of ESCs [3]. Importantly, iPSCs were subsequently derived from adult human fibroblasts, providing a feasible way of producing tailor-made pluripotent cells with clinical translational potential [4-7].

The fact that iPSCs can be derived from easily accessible somatic cells, such as skin fibroblasts or keratinocytes [8], has opened up new horizons in the field of regenerative medicine [9]. Jaenisch and colleagues [10,11] showed – as proof of principle of the therapeutic potential of iPSCs – the rescue of sickle cell disease and Parkinson disease in mouse models after transplantation of iPSC-derived hematopoietic stem cells and dopaminergic neurons, respectively. Similarly, correction of a mouse model of hemophilia A was recently demonstrated by intra-liver injection of endothelial cells and endothelial progenitor cells differentiated from iPSCs [12]. On the basis of the unlimited capacity to be propagated *in vitro*, iPSCs are good targets for genetic manipulation by gene therapy or gene correction by homologous recombination. Ideally, iPSC-based therapies in the future will rely on the isolation of skin fibroblasts or keratinocytes, their reprogramming into iPSCs, and the correction of the genetic defect followed by differentiation into the desired cell type and transplantation. Although this technology holds the potential to revolutionize drug discovery and regenerative medicine, important technical issues associated with the derivation of iPSCs still hinder its clinical translation. In this review, we outline current reprogramming approaches developed to improve the safety and efficiency of the method and highlight its critical

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aspects that may have an impact on the quality of the iPSCs.

Delivery of reprogramming factors by integrating viral vectors

Several experimental strategies have been developed to derive iPSCs from differentiated somatic cells (summarized in Figure 1). Direct reprogramming was originally achieved by retroviral transduction of transcription factors. Retroviruses are highly efficient gene-transfer vehicles because they provide prolonged expression of the transgene after genomic integration and have low immunogenicity. Most replication-defective retroviral vectors derive from the Moloney murine leukemia virus by replacing the viral structural genes (*gag*, *pol*, and *env*) with the gene of interest while retaining the *cis*-acting sequences. These include the 5′ and 3′ long terminal repeats (LTRs), a packaging signal, and elements involved in reverse transcription and chromosomal integration. To produce recombinant retroviruses, the plasmid is introduced into a packaging cell line that provides the viral proteins in *trans*. Using four individual retroviruses encoding the transcription factors Oct4, Klf4, Sox2, and c-Myc, Takahashi and Yamanaka [2] were able to obtain pluripotent cells by selecting for Fbx15 expression in ESC culture conditions. Fbx15, though an ESC marker, is not essential for the maintenance of the pluripotent state, and this, together with the timing of drug selection, may explain why the first iPSC lines failed to generate adult chimeric mice and exhibited a global gene-expression profile that was similar but not identical to that of ESCs. Further studies showed that delayed selection for the expression of key regulators of pluripotency such as Oct4 or Nanog yielded germline-competent iPSCs [13-15]. Importantly, fully reprogrammed clones could also be derived from genetically unmodified cells by means of colony morphology-based selection [16], thus enabling many laboratories to use the technique without requiring specific cell lines. Shortly after the original report by Takahashi and Yamanaka, their group [5] and teams led by James Thomson [6] in Wisconsin and George Daley [4] in Boston were able to produce iPSCs from human fibroblasts by using a similar experimental design. In all cases, the resulting human iPSCs (hiPSCs) were remarkably similar to human ESCs in terms of morphology, surface marker expression, methylation status in the promoter regions of pluripotency-associated genes, *in vitro* differentiation, and teratoma formation. After these first studies, retroviruses were used to reprogram somatic cells from patients with a variety of diseases, including amyotrophic lateral sclerosis, Parkinson disease, type 1 diabetes mellitus, Huntington disease, and Down syndrome [17,18], providing an unprecedented opportunity for disease modeling and drug screening.

Delivery of reprogramming factors via lentiviral vectors was first reported by the Thomson lab [6] and later was successfully employed to generate hiPSCs from various cell types, including skin fibroblasts, keratinocytes [19], and adipose stem cells [20]. Lentiviruses are a subclass of retroviruses capable of transducing a wide range of both dividing and non-dividing cells. Lentiviral insertion sites are often found anywhere within the transcriptional unit, whereas gamma-retroviruses tend to integrate near transcriptional start sites [21]. Another major difference between retroviral and lentiviral vectors is the degree of silencing to which they are subject in pluripotent cells. For retroviruses, silencing is almost complete and provides a way to identify fully reprogrammed clones [22] but also decreases the efficiency of the process. Lentiviruses seem to escape silencing to varying degrees, depending in part on the species and the promoter sequence. Moreover, position effects are often associated with variegated expression of the transgene. Even with doxicycline-inducible systems, low levels of transgene expression have been found to affect the transcriptome of iPSCs [23]. This adverse effect is more pronounced with constitutive promoters like human EF1α (elongation factor-1 alpha), whose activity in the context of reprogramming results in continued transgene expression that severely impairs differentiation both *in vivo* and *in vitro* [24]. The lack of silencing of lentiviral vectors increases the efficiency of iPSC generation, but transgene excision may be required to generate fully functional pluripotent cells [24].

Excisable vectors for the production of transgene-free induced pluripotent stem cells

Despite the fact that viral vectors have proven to be effective tools for reprogramming, the resulting iPSC clones usually display several proviral integrations, ultimately increasing the risk of insertional mutagenesis. In addition, spontaneous transgene reactivation may occur and lead to tumor formation [13]. Furthermore, we have shown that residual transgene expression affects the developmental potential of iPSCs [24]. These findings have encouraged researchers to look for alternative gene-delivery methods to generate transgene-free iPSCs that are suitable for basic research and clinical applications.

A feasible way to decrease the risk of insertional mutagenesis is to combine the reprogramming factors into a single polycistronic vector by inserting a 'self-cleaving' 2A peptide or an internal ribosome entry site (IRES) sequence between two consecutive open reading frames. With this approach, expression of the four factors can be accomplished in virtually every transduced cell, with a consequent increase in the reprogramming efficiency. A number of studies have recently demonstrated the advantage of using polycistronic vectors for iPSC

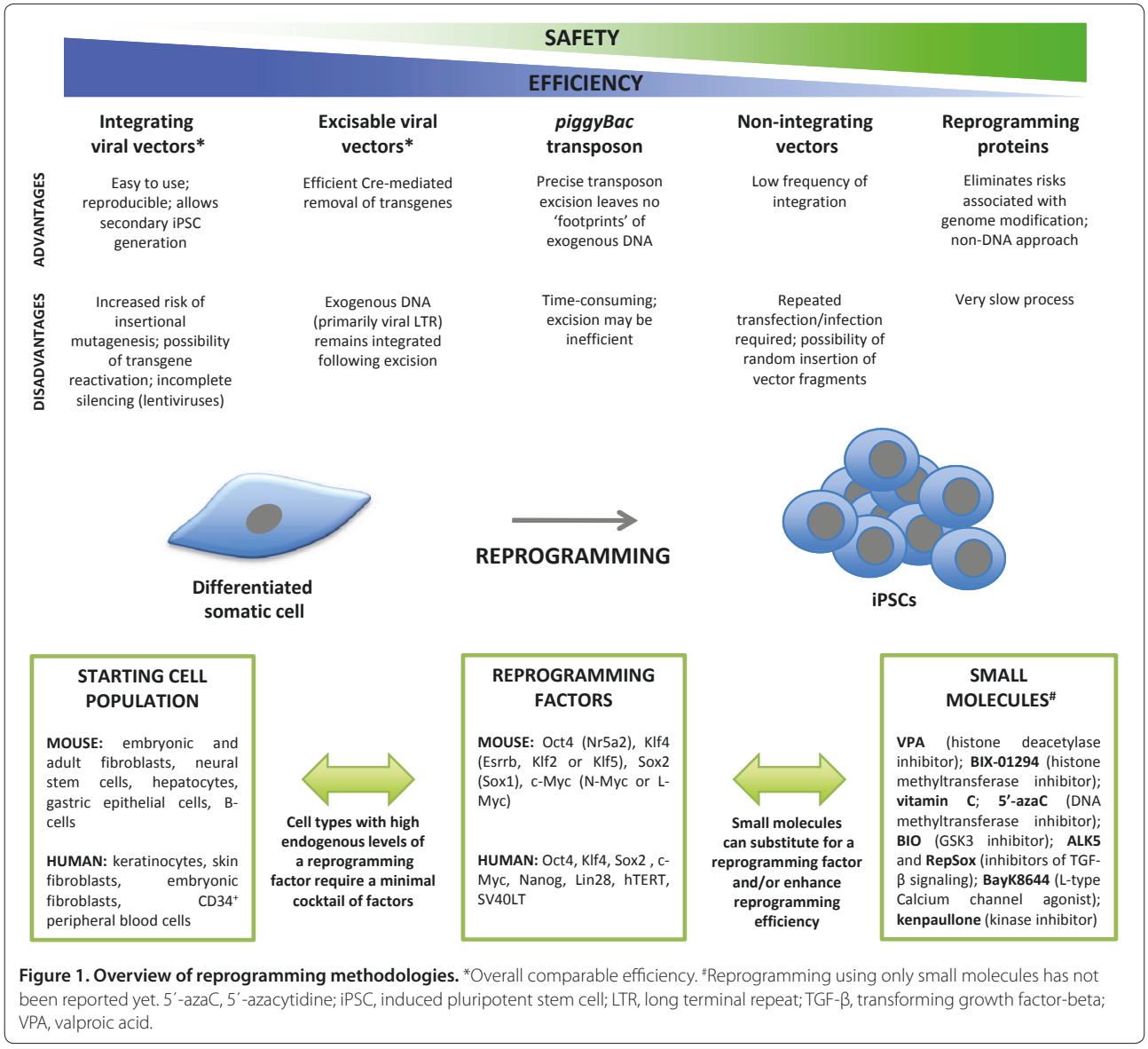

Figure 1. Overview of reprogramming methodologies. *Overall comparable efficiency. #Reprogramming using only small molecules has not been reported yet. 5′-azaC, 5′-azacytidine; iPSC, induced pluripotent stem cell; LTR, long terminal repeat; TGF-β, transforming growth factor-beta; VPA, valproic acid.

generation. Our group showed efficient conversion of adult mouse fibroblasts to iPSCs (0.5% to 1% of transduced cells) by using either a constitutive or an inducible version of the Stem Cell Cassette (STEMCCA) polycistronic lentiviral vector [25]. In this vector, a combination of 2A peptides with an IRES sequence allowed the appropriate expression of the four reprogramming factors. Similarly, the Jaenisch group [26] developed a single polycistronic Dox-inducible lentiviral vector encoding the four factors separated by three different 2A peptides and demonstrated successful reprogramming of mouse fibroblasts and neonatal human foreskin keratinocytes with 0.0001% and 0.00001% efficiencies, respectively. Notably, these studies demonstrated that somatic cells carrying a single proviral copy of the polycistronic cassette can be reprogrammed to pluripotency, substantially reducing genomic modification of the target cell.

The possibility of deriving iPSCs with single proviral integrations has stimulated the development of methodologies aimed at removing the transgenes by Cre/loxP technology. To this end, a loxP site is inserted into the 3′ LTR region of the lentiviral vector. During the normal reverse transcription cycle of the virus before integration, the loxP sequence is duplicated into the 5′ LTR region, creating a loxP-flanked or 'floxed' version of the vector that integrates into the host chromosome. After reprogramming, transgene-free iPSCs can be obtained by treatment with Cre recombinase and selection of clones that have undergone excision. Soldner and colleagues [23] performed excision of multiple integrated lentiviral vectors in hiPSCs by transfecting the cells with a plasmid

co-expressing Cre and the *puro* gene followed by selection of resistant clones. Similarly, a Cre-expressing plasmid or adenovirus was employed to remove three or four copies of a polycistronic vector from the genome of mouse iPSCs [27]. Moving the technology a step forward, we accomplished highly efficient removal of a single copy of the 'floxed' STEMCCA vector in mouse iPSCs through adenoviral-mediated expression of Cre in the absence of selection [24]. Likewise, efficient excision of a single integrated human STEMCCA vector has been achieved by transfection of a Cre-IRES-Puro plasmid to obtain hiPSCs free of exogenous transgenes (Somers A *et al.*, in press). These approaches, however, do not completely eliminate the theoretical risk of insertional mutagenesis since approximately 200 base pairs of exogenous DNA remain in the host genome after excision. The fact that the remaining sequence is an inactive viral LTR, together with the possibility of sequencing the integration site, may improve the applicability of these approaches in the clinical arena.

As an alternative to integrating viral vectors, the *piggyBac* (PB) transposon/transposase system has been recently adapted for iPSC generation. PB transposons are characterized by a high transposition activity in mammalian cells and a precise self-excision from genomic loci in mouse ESCs [28]. Woltjen and colleagues [29] engineered a PB transposon-based multiprotein expression vector to generate iPSCs carrying a single genomic integration. Remarkably, as a result of the transient expression of PB transposase, 90% of the clones showed no traces of exogenous DNA after excision. Yusa and colleagues [30] further optimized the protocol by including a negative selection cassette to facilitate the identification of integration-free iPSCs. This method is significantly less efficient than lentiviral-mediated reprogramming [31] but is substantially safer. Nevertheless, its potential use in a clinical setting will require a thorough examination of the iPSC clones to exclude the presence of transposon-induced genomic rearrangements [32].

Non-integrating vectors and direct delivery of reprogramming proteins

IPSCs result from the overexpression of transcription factors over several days during which the endogenous factors are activated and maintain the pluripotency gene network [33,34]. In principle, transient expression of the reprogramming factors from non-integrating vectors could provide the level and duration of expression that are required to induce pluripotency. A number of studies have described the successful derivation of mouse iPSCs by using plasmids [35,36] and adenoviral vectors [37], providing proof of principle that proviral insertions are not necessary for iPSC generation. Most recently, expression of reprogramming factors from a non-viral minicircle vector proved capable of converting human adipose stem cells to pluripotency [38]. Likewise, Fusaki and colleagues [39] used RNA Sendai virus-based vectors to achieve reprogramming of human somatic cells and were able to derive transgene-free hiPSCs by antibody-mediated negative selection. Alternatively, Thomson and colleagues [40] employed an oriP/EBNA1 episomal vector to reprogram human fibroblasts. Derived from the Epstein-Barr virus, these plasmids replicate extrachromosomally but are gradually lost from cells upon removal of drug selection. The strategy of these authors entailed the introduction of a polycistronic vector encoding the genes *Oct4*, *Sox2*, *Nanog*, *Lin28*, *c-Myc*, *Klf4*, and *SV40LT* and selection of stable episomes that provided persistent gene expression to achieve reprogramming followed by the identification of vector-free subclones after several passages in the absence of selection. As with the other non-integrating delivery systems, the reprogramming rates were very low (approximately 0.0005%). To date, it remains unclear whether these approaches could be translated to the clinic given the low efficiencies reported. In addition, the possibility that vector pieces have integrated into the host genome is difficult to rule out by polymerase chain reaction analysis and this matter may require whole-genome sequencing [9].

One possible way to induce pluripotency in somatic cells while avoiding the risks of genomic modifications is through direct delivery of reprogramming proteins. Such a strategy has been reported by different groups. To enable cellular uptake, the reprogramming factors were tagged to a poly-arginine protein transduction domain and produced in *Escherichia coli*. As a result of repeated incubation of mouse fibroblasts with the purified tagged proteins and the histone deacetylase inhibitor valproic acid (VPA), a few iPSC colonies emerged on days 30 to 35 [41]. Kim and colleagues [42] recently extended these findings to produce hiPSCs by means of a slightly different approach, which employed whole-protein extracts from HEK293 cells stably expressing the tagged reprogramming proteins. Finally, a recent report demonstrates that a single transfer of ESC-derived proteins is able to induce pluripotency in adult mouse fibroblasts [43]. Although the generation of these so-called protein hiPSCs (p-hiPSCs) is quite inefficient (about 0.001% of input cells) and takes about 8 weeks, the study is proof of concept that human somatic cells can be reprogrammed by direct protein delivery. Importantly, p-hiPSCs were produced in the absence of chemicals such as VPA, which may induce mutations [9].

It is widely accepted that the choice of the delivery method will impact the reprogramming efficiency, which is defined as the number of formed colonies divided either by the number of cells seeded or, more accurately, by the number of cells that were effectively transduced/transfected with the reprogramming factors [44].

Interestingly, even at a high multiplicity of infection, the number of colonies that emerge in a typical experiment using retro/lentiviruses is low, and most studies have reported reprogramming rates of 0.001% to 1%. In fact, the evidence indicates that in addition to the delivery method, these values will be subject to other sources of variation that include the transcription factors and target cell type employed, the age of the donor, the passage number of the cells (inversely correlated with efficiency), and whether the specific protocol includes splitting of cells after infection/transfection. These variables hinder proper comparisons across the studies, even when similar delivery methods are used.

Transcription factor cocktails, donor cells, and chemical additives: the various ways to create an induced pluripotent stem cell

In their seminal work, Takahashi and Yamanaka [2] screened a library of 24 transcription factors for their ability to induce pluripotency in somatic cells and ultimately selected four (Oct4, Klf4, Sox2, and c-Myc), which are commonly referred to as the 'Yamanaka factors'. Exogenous expression of these genes was found to be effective also in reprogramming human somatic cells, as reported one year later [5]. Concurrently, the Thomson group [6] demonstrated hiPSC derivation by using a slightly different set of transcription factors, namely Oct4, Sox2, Nanog, and Lin28. At present, Oct4 is the only factor that cannot be replaced by other Oct family members [45], and this is consistent with its critical role in maintaining pluripotency [46]. A recent report, however, indicates that Oct4-independent reprogramming is possible through overexpression of the orphan nuclear receptor Nr5a2 [47], which operates in part by activating endogenous Nanog and Oct4. Interestingly, another orphan nuclear receptor, Esrrb, is capable of replacing Klf4 [48]. Moreover, p53 siRNA (short interfering RNA) and UTF1 were found to significantly increase the efficiency of iPSCs generated with the Yamanaka factors [49]. Not surprisingly, few factors are required to impart pluripotency in cell types that already express high endogenous levels of a reprogramming factor. For example, fibroblasts can be reprogrammed without c-Myc [45] and overexpression of only Oct4 converts neural stem cells into iPSCs [50], albeit at very low rates. Together, these studies provide new insights into the molecular basis of nuclear reprogramming and indicate that different sets of exogenously introduced transcription factors can jump-start the endogenous pluripotency gene network. Whether the choice of the reprogramming cocktail contributes to the variability observed across iPSC lines deserves further investigation.

Nuclear reprogramming to pluripotency is a gradual process that involves the conversion of the epigenetic state of a differentiated cell into that of an ESC-like cell [1]. To date, iPSCs have been produced from several types of somatic cells, including embryonic and adult fibroblasts [2], keratinocytes [8], neural stem cells [51], hepatocytes and gastric epithelial cells [52], B lymphocytes [53], and peripheral blood cells [54]. However, as each mammalian cell type is characterized by a distinct epigenome, some of them appear to be more amenable to reprogramming. Thus, mouse embryonic fibroblasts reprogram more rapidly and efficiently than adult fibroblasts [55], and reprogramming is faster in mouse stomach and liver cells compared with fibroblasts [52]. Similarly, reprogramming appears to be at least 100-fold more efficient in human keratinocytes than in fibroblasts [8], although this has not been reproduced by others yet [19]. The use of 'reprogrammable' mouse strains could provide further insight into the reprogrammability of different specialized cell types [56]. In fact, a secondary system for the inducible expression of the reprogramming factors was recently employed to analyze the impact of the differentiation status of the cell on reprogramming. By examining the reprogrammability of hematopoietic cells at different stages of development, Eminli and colleagues [57] demonstrated that immature blood cells reprogram more efficiently than their differentiated progeny. These analyses may assist in the selection of suitable targets for the efficient generation of iPSCs.

Alternatively, small molecules that enhance reprogramming efficiency or even substitute for a reprogramming factor have been identified. Some of these compounds induce epigenetic changes by inhibiting the activity of chromatin remodeling factors. In the presence of the DNA methyltransferase inhibitor 5′-azacytidine (5′-azaC) or the histone deacetylase inhibitor VPA, the efficiency of reprogramming can be enhanced by approximately 10-fold and 100-fold, respectively [58]. Furthermore, VPA is able to compensate for c-Myc and Klf4 during conversion of human fibroblasts to iPSCs [59]. Likewise, the G9a histone methyltransferase inhibitor BIX-01294, together with BayK8644, an L-type calcium channel agonist, allows reprogramming of mouse embryonic fibroblasts transduced with Oct4 and Klf4 only [60]. Among the compounds that target signal transduction pathways, inhibitors of transforming growth factor-beta signaling have been shown to increase reprogramming efficiency and substitute for Sox2 and c-Myc [61,62]. Also, the kinase inhibitor kenpaullone was found to replace Klf4 [63], although the underlying mechanism is unknown at present. Finally, a recent report demonstrates that vitamin C improves the generation of mouse iPSCs and hiPSCs [64], in part by alleviating cell senescence through p53 repression and probably by acting as an agonist of the hypoxia-inducible factor pathway. These results are in agreement with the recent finding that

hypoxic conditions also improve iPSC production [65]. Of note, many of these molecules have pleiotropic effects that could result in transient or permanent epigenetic or genetic alterations, hindering the use of chemically induced iPSCs for therapeutic purposes.

Conclusions

Reprogramming with defined transcription factors provides a simple way of producing customized pluripotent stem cells with enormous therapeutic potential. Although viral transduction of the Yamanaka factors remains the most common strategy for producing iPSCs, significant progress has been made in improving the efficiency and safety of the technique. Excisable vectors may prove suitable for most applications, and methods that rely on non-integrative vectors or protein delivery might become routine once their efficiency is enhanced. The use of high-throughput screening technologies to identify small molecules that modulate the expression and/or activity of regulators of pluripotency in somatic cells could potentially allow reprogramming by purely chemical means. Whether the choice of the donor cell and reprogramming method eventually have an effect on the ability of iPSCs to differentiate into functional cell types will require additional investigation. Given the rapid pace of the field, further optimization of the protocols coupled with a thorough analysis of the iPSC lines generated will facilitate the clinical translation of this technology.

Abbreviations

ESC, embryonic stem cell; hiPSC, human induced pluripotent stem cell; iPSC, induced pluripotent stem cell; IRES, internal ribosome entry site; LTR, long terminal repeat; PB, *piggyBac*; p-hiPSC, protein human induced pluripotent stem cell; STEMCCA, Stem Cell Cassette; VPA, valproic acid.

Competing interests

Boston University (Boston, MA, USA) has filed a STEMCCA vector patent, which is licensed to Millipore Corporation (Billerica, MA, USA). However according to Boston University competing interests policies the authors do not have a competing interest.

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