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A Review of the Methods for Human iPSC Derivation

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Abstract

The ability to reprogram somatic cells to induced pluripotent stem cells (iPSCs) offers an opportunity to generate pluripotent patient-specific cell lines that can help model human diseases. These iPSC lines could also be powerful tools for drug discovery and the development of cellular transplantation therapies. Many methods exist for generating iPSC lines but those best suited for use in studying human diseases and developing therapies must be of adequate efficiency to produce iPSCs from samples that may be of limited abundance, capable of reprogramming cells from both skin fibroblasts and blood, and footprint-free. Several reprogramming techniques meet these criteria and can be utilized to derive iPSCs in projects with both basic scientific and therapeutic goals. Combining these reprogramming methods with small molecule modulators of signaling pathways can lead to successful generation of iPSCs from even the most recalcitrant patient-derived somatic cells.

Keywords

Induced pluripotent stem cells; Reprogramming human somatic cells; Footprint-free iPSCs; Reprogramming with small molecules

1 Introduction

The discovery that somatic cells could be reprogrammed to a pluripotent state has profoundly altered the landscape in which stem cell research is conducted. Since the original demonstrations that mouse (1) and human (2–4) fibroblasts could be reprogrammed to become induced pluripotent stem cells (iPSCs) by viral overexpression of specific transcription factors, numerous methods have been developed to generate iPSCs. These methods have yielded increases in efficiency of reprogrammed cells and/or the generation of footprint-free iPSC lines that lack integration of any viral vector sequences into their genomes. This chapter will review the current state of iPSC reprogramming methods focusing on the reprogramming of human cells with occasional references to mouse cell reprogramming.

The original method of reprogramming murine fibroblasts by Yamanaka (1) utilized retroviral transduction of Oct4, Sox2, Klf4, and c-myc into mouse embryonic fibroblasts (MEFs) or tail-tip fibroblasts (TTF) derived from mice expressing β -galactosidase-neomycin fusion protein at the Fbx15 locus, which is specifically expressed in pluripotent stem cells and serves as an excellent marker for pluripotency. Drug selection with G418 after transduction of the four factors resulted in reprogramming of 0.02% of the MEFs or TTFs 14–21 days post-transduction. Wernig et al. (5) also succeeded in reprogramming MEFs by utilizing the same strategy with a drug selection marker knocked-in to the Nanog or Oct4

locus. This resulted in reprogramming in 0.05–0.08% of MEFs 20 days after transduction of the four Yamanaka reprogramming factors. Reprogramming of adult human dermal fibroblasts (HDFs) was first reported by the Yamanaka group to occur at an efficiency of ~0.02% at ~30 days after transducing the four reprogramming factors (2). A second group achieved reprogramming of ~0.1% fetal fibroblasts 14 days after infection of the four factors but was unable to reprogram adult fibroblasts with this reprogramming cocktail (4). Upon adding hTERT and SV 40 large T antigen to the reprogramming cocktail, ~0.25% of adult fibroblasts were reprogrammed. A lentiviral expression system was employed to deliver Oct4, Sox2, Nanog, and Lin28 to fetal MRC5 lung fibroblasts and newborn BJ-1 foreskin fibroblasts (3). iPSC colonies appeared 20 days post-transduction at an efficiency of 0.03–0.05% for fetal fibroblasts and 0.01% for newborn fibroblasts. After this initial discovery phase in the reprogramming field, modifications were made to redesign the reprogramming factor expression vectors and new modes of delivery were attempted to increase efficiency and minimize or remove vector sequences that were integrated into the reprogrammed iPSC genome.

2 Reprogramming Methods

2.1 Single Cassette Reprogramming Vectors with Cre-Lox Mediated Transgene Excision

As lentivirus, unlike retrovirus, can infect nondividing and proliferating cells it became the preferred delivery vehicle for expressing reprogramming factors in somatic cells. One of the concerns about reprogramming with four or more individual vectors was the suboptimal stoichiometry achieved using such a method (6). There were also worries about incorporation of the lentiviral vector sequences into the iPSC genome. The first concern was addressed with the development of a single cassette reprogramming vector in which each of the reprogramming factors was separated by a self-cleaving peptide signal (7–9). Some of these vectors were also engineered with loxP sites so that any integrated sequence could be excised by the overexpression of Cre-recombinase (8, 9). The first publication documenting Cre-mediated excision of integrated sequences from iPSCs was by episomal expression of a Cre-puromycin plasmid in iPSCs generated with four loxP containing lentiviral reprogramming vectors from patients with Parkinson's disease (10). After recombination and removal of transgene sequences the patients' iPSCs retained pluripotency and could be differentiated to dopaminergic neurons. It was later shown that adenoviral Cre was able to mediate excision of integrated transgene sequences in single cassette vectors (8, 9). A humanized version of one of these vectors with high reprogramming efficiency in mouse cells (0.5%) was constructed. The vector termed STEMCCA is now in wide use with reported reprogramming efficiencies of 0.1–1.5% (11).

2.2 Reprogramming by Nonintegrating Viruses

2.2.1 Adenovirus—Because adenovirus is a non-integrating virus it appears to be an excellent expression vehicle for generating iPSCs. However, the reprogramming efficiency of this method is only 0.001–0.0001% in mouse (12) and 0.0002% in human cells (13). For adenovirus to have any useful applications in reprogramming, considerable work has to be performed to optimize expression and increase reprogramming efficiencies.

2.2.2 Sendai Virus—Sendai virus has the advantageous property that it is an RNA virus that does not enter the nucleus and is therefore diluted out of cells ~10 passages after infection. A second desirable characteristic of Sendai virus is that it can produce large amounts of protein. When Sendai-based reprogramming vectors were made and used to generate iPSCs it was found that Sendai can reprogram neonatal and adult fibroblasts as well as blood cells (14–16). Cells are reprogrammed in ~25 days at an efficiency of 0.1% for blood cells and 1% for fibroblasts. Sendai is more difficult to work with than lentiviruses, but there are commercially available viral extracts for the Yamanaka factors that are ready to use. One disadvantage of Sendai-based reprogramming is that it takes ~10 passages for the virus to be completely lost from recently reprogrammed iPSCs and cells may need to be cultured at a higher temperature (39°C) to fully remove virus.

2.2.3 Protein—Expression of reprogramming factors as proteins would be an ideal method to generate footprint-free iPSCs. Unfortunately, it has been technically challenging to synthesize large amounts of bioactive proteins that can cross the plasma membrane. Two groups were able to make enough bioactive proteins in an *E. coli* expression system to reprogram 0.006% of mouse fibroblasts (17) and 0.001% of human fibroblasts (18). The low efficiency, technical challenges, and lack of published studies in non-fibroblast cell types suggest that much work needs to be done before protein reprogramming is a viable method.

2.3 Nonviral Reprogramming Methods

2.3.1 mRNA Transfection—The ability to express reprogramming factors as mRNA offers another method to make footprint-free iPSCs. Warren et al. were able to overcome several hurdles to transcribe mRNAs to efficiently express reprogramming factors (19). They were able to reprogram human fibroblasts at an efficiency of 1.4% within 20 days. By adding Lin28 to the Yamanaka reprogramming factor protocol, culturing at 5% O₂, and including valproic acid in the cell culture medium, the efficiency could be increased to 4.4%. Although reprogramming factor mRNAs are commercially available, this method suffers from the fact that it is labor intensive, requires addition of mRNA daily for 7 days, and has not been validated in cells other than fibroblasts.

2.3.2 miRNA Infection/Transfection—Several miRNA clusters are strongly expressed in ESCs. When synthetic mimics of the mature miR-302b and/or miR-372 plus the four lentiviral Yamanaka factors were added to MRC5 and BJ-1 fibroblasts there was a 10- to 15-fold increase in reprogramming efficiency in comparison with the four lentiviral factors alone (20). It was found that certain miRNAs could reprogram cells at high efficiency without the presence of the Yamanaka factors. Expression of the seed sequences for the miR302/367 sequence as lentivirus particles generated iPSCs in ~10% of BJ-1 fibroblasts 12–14 days after infection (21). Another miRNA reprogramming paper found that transfection of certain miRNAs could reprogram human cells. The mir-200c, mir-302s, and mir-369s were transfected into HDFs and adipose stromal cells four times over a 6-day period and reprogrammed 0.002% of the cells 20 days after the first transfection (22). There have been no published reports replicating results with any of the three variations of miRNA reprogramming so it is difficult to determine if this is a robust reprogramming method. If the efficiency of transfection of miRNAs could be improved and a canonical set of

reprogramming miRNAs were identified, this could be a promising tool for reprogramming iPSCs.

2.3.3 PiggyBac—PiggyBac is a mobile genetic element (transposon) that in the presence of a transposase can be integrated into chromosomal TTAA sites and subsequently excised from the genome footprint-free upon re-expression of the transposase. When cloned into a piggyBac vector and co-transfected into MEFs the Yamanaka factors can reprogram 0.02–0.05% of cells 14–25 days post-transfection (23, 24). The piggyBac vector could be cleanly excised from the iPSCs upon re-expression of the transposase. Human mesenchymal stem cells (MSCs) were reprogrammed at an efficiency of 0.02% using piggyBac with the addition of sodium butyrate; however, this was 50-fold less efficient than retroviral-mediated reprogramming of MSCs (25). PiggyBac appears to be a promising method for reprogramming mouse iPSCs but there is no published study showing that the vector can be excised from human iPSCs or that cell types other than MSCs can be adequately reprogrammed.

2.3.4 Minicircle Vectors—Minicircle vectors are minimal vectors containing only the eukaryotic promoter and cDNA(s) that will be expressed. A Lin28, GFP, Nanog, Sox2, and Oct4 minicircle vector expressed in human adipose stromal cells was able to reprogram 0.005% of the cells in ~28 days (26). The method was even less efficient at reprogramming neonatal fibroblasts and there are no published reports of successful reprogramming of other somatic cells. Therefore, more validation is necessary before this method can be widely used.

2.3.5 Episomal Plasmids—Transient expression of reprogramming factors as episomal plasmids would allow for the generation of footprint-free iPSCs. However, transient transfection with a standard plasmid vector does not result in expression for a long enough period of time to reprogram cells unless transfections are repeated daily and even then reprogramming efficiency is unacceptably low (27). OriP/EBNA-based plasmids are stably expressed for a longer period of time than standard plasmids, and therefore have been used to express reprogramming factors for the generation of iPSCs. A single transfection of three oriP/EBNA plasmids containing Oct4, Sox2, Nanog, Klf4; Oct4, Sox2, SV40 large T antigen; and c-myc and Lin28 into human foreskin fibroblasts resulted in 0.0003–0.0006% of cells being reprogrammed ~20 days post-transfection (28). Only one-third of the subclones from two of the original iPSC lines lost the episomal plasmid. Another study found similarly low levels of reprogramming in cord blood cells, although addition of thiazovivin enhanced the process tenfold. However, 0.035% of cord blood mononuclear cells were reprogrammed by day 12 (29). Unlike the study of Yu et al. (28), this study found that all iPSC lines lost the plasmid by passage 15. Co-expression of EBNA mRNA with the reprogramming vectors and reprogramming in defined E8 media under hypoxic conditions significantly improved reprogramming efficiency in fibroblasts to 0.006–0.1% with the variation attributable to the characteristics in the parental fibroblast lines that were being reprogrammed (30).

New oriP/EBNA vectors were constructed with the Yamanaka factors plus Lin28 in one cassette and another oriP/EBNA vector containing SV40 large T antigen (31). These vectors

were expressed in CD34+ cord blood, peripheral blood, and bone mononuclear cells in media supplemented with sodium butyrate, resulting in iPSC colonies in 14 days in 0.02, 0.009, and 0.005% of the cells, respectively. The transfected plasmids were lost by passage 12 with subsequent whole genome sequencing confirming loss of the plasmids in the bone marrow-derived iPSCs (32). Overall episomal reprogramming appears to be an effective strategy to generate footprint-free iPSCs with the only negative being an inability to reprogram fibroblasts at an acceptable efficiency without modifications to the way cells are cultured.

3 Oocyte Reprogramming as an Alternative Technology

The reprogramming of a somatic cell genome by transfer to enucleated oocytes has been shown to occur quickly and with nearly 100% efficiency in several species including mouse (33). However, ethical considerations and technical challenges have slowed progress of somatic cell nuclear transfer in humans. A recent report achieved positive results with reprogramming patient fibroblasts by transferring the fibroblast nucleus into an oocyte in which the nucleus was not removed (34). The resulting reprogrammed cells had expression profiles and epigenetic signatures that were very similar to pluripotent stem cell lines. Significant improvements still need to be made to technique as the reprogrammed cell types are not competent for therapeutic use because they are triploid. Future advances in this technology may further broaden the methods available to routinely reprogram somatic cells.

4 Enhancing Reprogramming Efficiency for Recalcitrant Cells

Even when using the same method there can be great variability in iPSC efficiency—especially in patient-specific disease lines. This variability is most likely attributable to the parental line that is to be reprogrammed, and could be due to disease-specific mutation(s) or an issue related to how the tissue source was collected, expanded, and stored long term. Various small molecules have been shown to enhance reprogramming efficiency (Table 1). Several known mechanisms enable these molecules to facilitate reprogramming including inhibition of histone deacetylation (25, 35), blockade of the TGF β and MEK signaling pathways (36, 37), enhancement of function of epigenetic modifiers (38), inhibition of the ROCK pathway (34), and induction of glycolysis (39). Amongst these small molecules the histone deacetylase inhibitors valproic acid and sodium butyrate are the most commonly used in reprogramming protocols. It should also be noted that culture of cells in 5% oxygen during the reprogramming process can also increase efficiency of iPSC derivation ~5-fold in mice and threefold in human cells (40). For samples that are particularly difficult to reprogram, the addition of a small molecule and culture in hypoxic conditions may yield enough improvements to generate iPSC clones (Table 2). The immense datasets from whole transcriptome studies of pluripotency networks are also likely to yield new transcription factors that can be added to the combinations that are currently in use or be used in new combinations to improve reprogramming efficiency (41–43).

One intriguing alternative to reprogramming difficult somatic cell lines may be to use embryonic stem cell-conditioned medium (ESCM) to induce expression of endogenous reprogramming factors. This strategy was used to reprogram rat limbal progenitors to iPSCs

at an efficiency of 0.002% without the exogenous expression of any reprogramming factors (44). The efficiency improved to 0.008% with the addition of valproic acid. The limbal progenitors have endogenous expression of Klf4, Sox2, and c-myc which was further upregulated by culture in ESCM with Oct4 also being induced after 10 days of ESCM culture. Such a strategy may not be effective in more differentiated cell types but the use of conditioned media along with small molecules may enhance the ability of exogenously expressed reprogramming factors to increase reprogramming efficiency, particularly in cells that are otherwise difficult to reprogram.

5 Choosing a Reprogramming Method

The primary factors to consider when deciding on a reprogramming method to produce iPSCs are what cell is being reprogrammed, the capacity of the reprogramming method to adequately reprogram this cell type, and whether the presence of integrated sequences in the iPSCs will hinder downstream application. How these factors are weighted will be dependent on the goals of the project. The reprogramming methods can be divided into six groups based upon efficiency, footprint, and number of different somatic cell types known to be reprogrammed by the method (Fig. 1). If there are no long-term translational goals for the iPSCs, a viral infection with the STEMCCA will suffice as this method works for many cell types and offers the option of excising the integrated sequences with Cre-recombinase at a later time point. Projects with translational aspirations should utilize a completely footprint-free method with consideration of whether fibroblasts or blood cells will be reprogrammed. Sendai virus works well with all cell types but requires ~10 passages for the generated iPSCs to be footprint-free. Reprogramming using the episomal method is excellent for blood cells but requires modification of standard culture conditions for fibroblasts. There are also differences in the amount of time it takes to lose the footprint between episomal and Sendai-based methods. There is no need to worry about the vestiges of a footprint remaining in iPSCs reprogrammed by mRNA, but the method is cumbersome and as of now only appears to work with fibroblasts. PiggyBac may be an attractive alternative but published studies in human cells are limited and excision of the piggyBac insertion has not been reported in human iPSCs. The remaining methods that have been discussed all either have severe limitations or have not been validated with the stringency required to express full confidence in their capabilities. Although there is no universal method that can handle every situation at least one of the methods described in this chapter should be able to cover nearly every need for a researcher attempting to produce iPSCs.

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Zero footprint Works for few cell types Good efficiency mRNA piggyBac	Zero footprint Works for many cell types Good efficiency Sendai virus Episomal	Large footprint Works for few cell types Good efficiency miRNA Viral	Large footprint Works for many cell types Good efficiency Viral
Zero footprint Works for few cell types Poor efficiency Minicircles Protein Adenovirus miRNA Transfection	Small footprint Works for many cell types Good efficiency Virus plus cre/lox		

Fig.1.

Classification of reprogramming methods by footprint, validation that multiple cell types can be reprogrammed, and efficiency. Further details are found in the text

Table 1

Compounds increasing iPSC reprogramming efficiency

Treatment	Process affected
Valproic acid	Histone deacetylase inhibition
Sodium butyrate	Histone deacetylase inhibition
PD0325901	MEK inhibition
A-83-01	TGF β -inhibition
SB43152	TGF β -inhibition
Vitamin C	Enhances epigenetic modifiers, promotes survival by antioxidant effects
Thiazovivin	ROCK inhibitor, promotes cell survival
PS48	PI3K/Akt activation, promotes glycolysis
5% Oxygen	Promotes glycolysis

Table 2

Increased efficiency of human iPSC generation with various treatment regimens

Treatment	Reprogramming method	Fold enhancement
Valproic acid	3-factor retroviral	>100 (33)
Sodium butyrate (NaB)	Episomal, piggyBac	15–50 (25)
SB431542 + PD0325901	4-factor retroviral	100 (34)
SB + PD + thiazovivin	4-factor retroviral	>200 (34)
A-83-01 + PD	4-factor retroviral	~90 (37)
PS48	4-factor retroviral	15 (37)
NaB + PS48	4-factor retroviral	~25 (37)
5% oxygen	4-factor retroviral	3 (38)