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STEM CELL BIOLOGY

Advancements in reprogramming strategies for the generation of induced pluripotent stem cells

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Abstract Direct reprogramming of somatic cells into induced pluripotent stem (iPS) cells has emerged as an invaluable method for generating patient-specific stem cells of any lineage without the use of embryonic materials. Following the first reported generation of iPS cells from murine fibroblasts using retroviral transduction of a defined set of transcription factors, various new strategies have been developed to improve and refine the reprogramming technology. Recent developments provide optimism that the generation of safe iPS cells without any genomic modification could be derived in the near future for the use in clinical settings. This review summarizes current and evolving strategies in the generation of iPS cells, including types of somatic cells for reprogramming, variations of reprogramming genes, reprogramming methods, and how the advancement iPS cells technology can lead to the future success of reproductive medicine.

Keywords Induced pluripotent stem cell · Reprogramming strategies

Capsule Current and evolving reprogramming technologies that are critical for the generation of iPS cells with the benefits and drawbacks of each technology highlighted.

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Introduction

The pursuit for patient-specific and disease-specific therapy has been spear-heading technological breakthroughs in stem cell research area. Research on embryonic stem (ES) cells which have the ability to multiply indefinitely while maintaining pluripotency and capabilities to differentiate into three germ layers has started since 1980s [1, 2]. Embryonic stem cells have the potential to treat multiple genetic diseases but immune rejections and ethical issues surrounding the use of human embryos as ES cell source have been hampering the research progress [3–5].

Cell-differentiation and specialization were thought to be unidirectional and reprogramming was rarely observed [6]. However, nuclear-transfer studies have shown that adult cells can be reprogrammed into embryonic state through nuclear content transfer into viable oocytes or fusing somatic cells with ES cells [7, 8]. However, these techniques still require the use of embryos. An important

milestone in stem cell research was achieved by Yamanaka and colleagues [3] in 2006 when they successfully induced adult somatic cells into pluripotent cells. These cells termed as induced pluripotent stem (iPS) cells overcomes the issues of immune rejection as cells can be derived from patient's own cells and there would not be ethical issues regarding the use of human embryos. Induced pluripotent stem cells have also been found to resemble ES cells in many ways including the cell morphology, expression of surface markers, telomerase and gene expression as well as the capability to form embryoid bodies [3, 9, 10].

Yamanaka and colleagues [3] tested genes involved in maintenance of pluripotency, long-term maintenance of ES cell phenotype and high proliferation of ES cells in culture. They managed to reprogram murine fibroblast into iPS cells using 4 transcription factors. Although this is a ground-breaking research, as with other initial researches there are still a number of problems related to the reprogramming method used by the group. As stated by the authors, the iPS cell derivation was of very low frequency. A study done by Mali et al. [11] found the reprogramming efficiency of human iPS cells from fibroblasts was approximately 10^{-4}. The use of viral vectors has led to multiple viral integrations in iPS cell genomes. Viral integration could cause the gene to be unstable and therefore may lead to mutagenesis [6, 12]. The silencing of the transduced exogenous gene could be incomplete, which could disrupt the subsequent differentiation of iPS cells [5]. The presence of c-Myc as one of the transcription factors involved in reprogramming has been linked to carcinogenesis and reactivation of this gene will have cancer-causing potential in iPS cells [6, 9, 11].

From the time iPS cells' reprogramming was published, many other groups have been successfully reprogramming adult cells from various sources using various reprogramming strategies. In this review paper, we will be examining the different sources of somatic cells that have been investigated by various groups to generate iPS cells. We will also be focusing on the different reprogramming strategies that have been studied to overcome the problems that are currently preventing iPS cells from being applied in clinical settings. The potentials of iPS cell technology in reproductive medicine, cell-based therapies and biomedical research are too big to ignore and proper reprogramming strategy is essential to create patient- and disease-specific stem cells that can be used for regenerative medicine.

Sources of somatic cells

Genetically identical mouse clones have been produced using SCNT technology from a variety of differentiated cell types [13, 14] or from embryonic cells (ES) by tetraploid blastocyst complementation [15, 16]. Importantly, the reprogramming of the somatic cells using SCNT [17] or by nuclear fusion with ES cells [7, 8] signifies that the unfertilized eggs and ES cells contain factors that can confer pluripotency to somatic cells. These factors were used to successfully generate iPS cells from fibroblasts of both mouse [3] and human [9, 18]. Fibroblasts were a viable candidate for the first reprogramming effort as they have been shown to be amenable to reprogramming by nuclear transfer in mouse [14] and cell fusion in both mouse and human [7, 8]. In addition, fibroblasts are easily derived by a simple technique [19] and they are often used as "feeder cells" in ES cell media for optimal cell growth, making them compatible with the ES cell culture conditions.

Due to the successful reprogramming of the fibroblasts, a range of other cell types have been analyzed for their capacity to be reprogrammed. Other cell types that have been successfully reprogrammed are stomach cells [20], liver cells [20, 21], neural progenitor cells [22, 23], lymphocytes [24], B-cells [5], keratinocytes [25], human blood [26, 27], human cord blood [28, 29], human amniotic cells [30], human peripheral blood [31, 32], human platelets [33], human astrocytes [34], and human adipose tissues [35, 36]. What has been identified throughout the process of reprogramming of various cells is that the type of cells influences the reprogramming capacity. This could be due to the ability of the gene delivery vector to transfer the reprogramming factors efficiently into the given cell type. For instance, in a study by Stadtfeld et al. [37], lower titer of adenovirus was required to reprogram mouse liver cells than that of fibroblasts.

As such, several parameters that need to be considered for cell selection are: (i) the ease at which reprogramming factors can be introduced. For instance, quiescent cells are refractory to retroviral gene delivery, (ii) the convenience of derivation of the cell type. Neural progenitor cells which can be reprogrammed with a single factor are not easily accessible without performing highly invasive procedures making them poor candidates for clinical setting; and, (iii) the age and source of the cells. Cells harboring genetic defects can undermine the therapeutic potential of the iPS cells. Hence, although many cell types have been reprogrammed to date, fibroblasts would still remain as the choice for basic research as they are easily attainable and susceptible to a large number of gene delivery vectors.

Reprogramming genes

Direct reprogramming was initially performed through retroviral transduction of 24 candidate genes into mouse fibroblasts. This pool of 24 genes was ultimately narrowed down to four transcription factors, *Oct4*, *Sox2*, *c-Myc*, and *Klf4* [3] that were sufficient to mediate reprogramming. Yamanaka's approach of genetically induced reprogram-

ming itself was not revolutionary, and the four reprogramming factors identified were already known to contribute to cell proliferation and maintenance of pluripotency [38–42]. For example, *Oct4* is known as a transcription factor required to form the inner cell mass in early embryos and to maintain pluripotency in ES cells [39]. In ES cells, *Oct4* has been shown to co-operate with *Sox2* to regulate various other pluripotency regulatory genes. Kruppel-like factor 4 (*Klf4*), is required for the establishment of left-right asymmetry in the early embryo. *c-Myc* participates in the maintenance of ES pluripotency via the LIF-STAT3 pathway [41] and may induce global histone acetylation [43], thus allowing *Oct4* and *Sox2* to bind to their specific target loci.

Some variations of the above-mentioned factors can be used to successfully reprogram cells. In mouse fibroblasts, *Sox1* and *Sox3* can replace *Sox2*, although it affects the reprogramming efficiency; *L-Myc* and *N-Myc* can replace *c-Myc*, and *Klf4* can be substituted with *Klf2* [44, 45]. Partially different set of factors (*OCT4*, *SOX2*, *NANOG* and *LIN28*) has been reported to be sufficient to reprogram human fibroblasts [18]. For human foreskin fibroblasts, a combination of six transcription factors (*OCT4*, *NANOG*, *SOX2*, *LIN28*, *C-MYC* and *KLF4*) has been shown to significantly increase the reprogramming efficiency [46]. Interestingly, Scholer and his colleagues [22] have demonstrated that *OCT4* alone was able to convert human neural stem cells into iPS cells, termed as "one factor (1F) iPS". Another new discovery has shown that although *Oct4* was essential, it can be replaced with a nuclear receptor gene, *Nr5a2,* in the derivation of iPS cells from mouse somatic cells [47].

Other methods to reduce the number of reprogramming factors have taken advantage of endogenously expressed reprogramming factors, thereby precluding the need for ectopic expression of these factors. For instance, *c-Myc* can be excluded for reprogramming of mouse and human fibroblasts as these cells express *c-Myc* and *Klf4* [45]. Mouse neural progenitor cells have been reprogrammed using only *Oct4/Klf4* or *Oct4/c-Myc* since the cells express *Sox2* and *c-Myc* at higher levels compared to ES cells [22]. Unfortunately, these two studies have also shown that the exclusion of the factors could affect the efficiency of reprogramming.

Although different reprogramming protocols have been reported, the delivery of the original four transcription factors remains as the most commonly method used to date. However, one must realize that these factors are oncogenes and they can lead to the formation of tumours in chimeras and in offsprings derived from these iPS cells [4]. Therefore, future generation of iPS cells must avoid the use of these oncogenes to render iPS cells suitable for clinical application.

Methods for reprogramming

Gene delivery is an exceptionally important aspect of research into iPS cells reprogramming, particular in the efficiency and the safety of the gene delivery system. The experiences from the past 15 years of using myriads of gene delivery systems in clinical gene therapy have facilitated the progress of iPS cells research. Clearly now, the iPS cells researchers have the choice of using either viral or non-viral method of gene delivery (Table 1 & Fig. 1). Lately, a new method of reprogramming without the use of genetic materials has been shown to be applicable for the production of the iPS cells.

Gene delivery methods

Viral delivery system

The initial generations of iPS cells employed retrovirus to shuttle the four transcription factors into mouse and human fibroblasts [3, 9]. The Moloney Murine Leukemia Virus (MMLV)-based retroviral vectors used in these studies are known to undergo silencing in the ES cells [48], which provides an advantage for the initial attempts as the temporal requirement of factor expression was undefined. Later generations of iPS cells were produced using lentiviruses. This was driven by several factors. First, unlike retroviruses, lentiviruses infect both dividing and non-dividing cells, hence could vastly improve the rates of cells transduction. Second, recombinant lentiviruses can be pseudotyped with alternative envelope proteins to broaden the viruses tropism. The commonly used Vesicular Stomatitis Virus glycoprotein (VSVg) is highly effective in a wide variety of cells. Fourth, constitutive or inducible expression of the transcription factors can be attained, depending on the type of promoter used. Fifth, the production of lentiviruses is not overly complicated. Therefore, very efficient, stable, reproducible gene expression could be achieved and a wide variety of cells could be transduced with the use of lentiviruses. However, a number of limitations and drawbacks inherent to the standard retroviral and lentiviral reprogramming technology persist, which will hinder the practical use of the iPS cells in the clinical settings. Although retroviruses and lentiviruses are very efficient at delivering genes, the use of these viral systems has been criticized for their permanent integration into the host genome. This phenomenon can by itself cause reactivation of the silenced exogenous *Klf4* and *c-Myc* and give rise to tumors upon differentiation of the iPS cells [49, 50]. The use of multiple individual viral vectors to deliver each transcription factor (*Oct4*, *Sox2*, *c-Myc*, and *Klf4*) to generate reprogramming can result in a high number of genomic integrations. The presence of multiple

Table 1 The advantages and disadvantages of different reprogramming methods for induction of pluripotent stem cells

Methods	Type	Subtypes	Advantages or disadvantages
Gene delivery methods	Viral delivery system	Lentivirus	Advantages
			- Infect both dividing and non-dividing cells
			- Recombinant can be pseudotyped to broaden tropism
			- Highly efficient
			- Constitutive and inducible expression can be attained
			Disadvantage
			- Integration into host genome
		Retrovirus	Advantages
			- Undergo silencing in ES cells
			- Highly efficient
			Disadvantage
			- Integration into host genome
		Adenovirus	Advantage
			- Non-integrating vector
			Disadvantages
			- Poor gene transfer
			- Control of gene expression level difficult
	Non-viral system	Episomal plasmids	Advantages
			- Non-integrating vector
			- Able to replicate autonomously giving rise to prolonged expression
			Disadvantage
			- Low reprogramming efficiency
	Excision strategies	Cre-*lox*P recombination	Advantages
			- Gene expression profile closer to hES cells compared to human iPS cells
			- Efficient excision of integrated transgenes
			Disadvantages
			- Genomic instability and genome rearrangements
			- *lox*P site remain integrated in genome
		piggyBac transposon	Advantages
			- Self-excision
			- Precise and efficient excision
			Disadvantage
			- pBt gene may remain active post-transposition
Non-DNA methods	Protein-mediated	Poly-arginine peptide tags	Advantages
			- Reprogrammed cells from embryonic fibroblast cells were indistinguishable from the classic embryonic stem cells
			Disadvantages
			- Not clear is can be used for adult cells
			- Short half-life

Enhancing DNA reprogramming efficiencies	Manipulation of cell-culture conditions	Oxygen level manipulation
		Advantages
		- No genetic manipulation
		- No protein carriers needed
		Disadvantage
		- Short-term induction
	miRNAs	miR-294, miR-302
		Advantages
		- Able to replace c-Myc
		- Increased efficiency
		Disadvantages
		- Not specific
		- Target point may be too upstream in the cell signalling pathway
		- Expression not easily controlled post-induction
	Small molecules and soluble factors	Chromatin modification modifiers
		Advantages
		- Modulate chromatin modification
		- Target cell-signalling pathways
		Disadvantage
		- Non-specific and broad action
		- May cause dysregulation of gene expression

proviral integrations across the genome alters the genomic construction and may consequently disturb the function of cellular genes [3]. In addition, the random integration nature of the lentiviral vector may lead to inappropriate insertion of the transgene near *proto-oncogene*, thus increasing the cancer formation risks [51].

Doxycycline-inducible lentiviruses have provided a more attractive approach as they permit temporal control over factor expression [37, 52]. Throughout the use of the inducible lentiviruses, researchers have shown that expression of the factors is necessary until the late stage of iPS [53]. With the use of the inducible lentiviral system, Maherali et al. [54] have generated "secondary" iPS cells. These "secondary" iPS cells were produced by the reactivation of the integrated transgenes in the differentiated cells derived from the primary iPS cells, upon re-induction. Interestingly, the reprogramming efficiency for the secondary iPS cells is more than 100-fold higher than that found in the primary iPS cells.

The endeavors to make iPS cells more therapeutically applicable have led to the pursuit of non-integrating viruses approaches. A key potential technology is the development of integrase-defective lentiviruses that have a reduced propensity for integration into the host genome [55]. This is achieved by mutating the integrase coding sequence in the viral genome. Though previously thought to be transcriptionally inactive, they are in fact as efficient as their integrating counterparts for effective transduction [56].

Adenovirus, which is a non-integrating vector and remains as an epichromosomal form in cells, offers a flexible platform. However, adenovirus is generally rather poor at gene transfer, probably due to the limited availability of primary receptors and/or co-receptors required for cell binding and internalization in vitro [57]. It is also very difficult to control the level of gene expression in infected cells, particularly if constitutive promoter is being used. In addition, repeated infections are required for certain cell types and the reprogramming kinetics are delayed [58].

Non-viral delivery system

The work on the adenovirus delivery system has shown that the iPS cells could be generated without the use of integrating virus and supports the claim that transgenes integration into the genome is not required for in vitro reprogramming [58]. The retroviral system, which shows silencing of all four reprogramming genes, indicates that the iPS cells are fully reprogrammed and no longer depend on exogenous expression of the transgenes [59]. The success of the non-integrating vector with transient gene expression to generate iPS cells has provided an opportunity to potentially develop a non-viral delivery strategy, which is safe, cost-effective, easier to manufacture and manipulate.

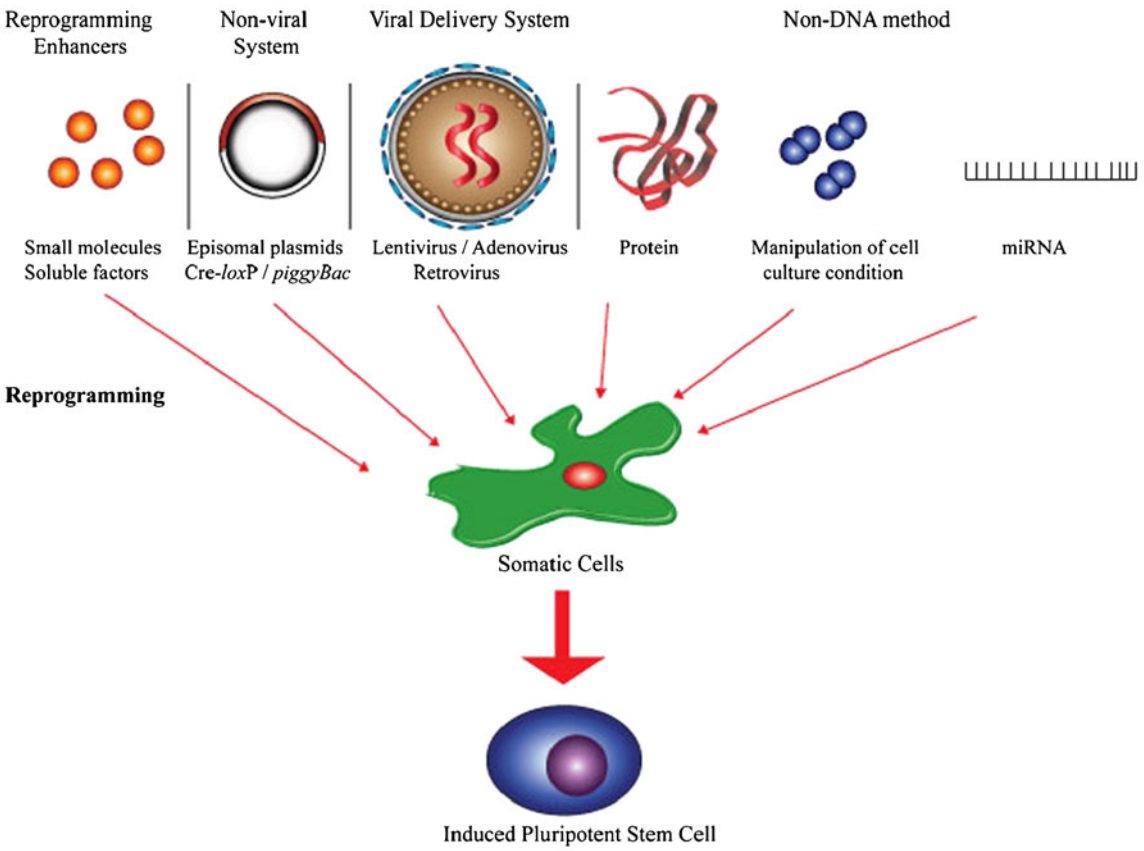

Fig. 1 The current reprogramming strategies used to induce pluripotent stem cells from adult somatic cells. The first method introduced was the viral delivery system involving the use of adenovirus, retrovirus and lentivirus. But since then, non-viral methods have been explored. For example, episomal plasmids for gene delivery and cre-loxP and piggyBac transposon are used as the excision strategy. Protein-tagging, cell culture manipulations and miRNAs are non-DNA modification methods

Plasmid transfection The non-viral system usually employs plasmids carrying the reprogramming genes encapsulated by lipid or cationic polymers and subsequently transfected into the cells to be reprogrammed. Plasmids are episomally maintained and usually exhibit short duration of gene expression. Okita et al. [60], described the first successful attempt in generating iPS cells from mouse embryonic fibroblasts by serial and transient expression of 2 plasmids, one expressing *c-Myc* and a second construct expressing *Oct4*, *Klf4* and *Sox2* (a polycistronic plasmid unit), using Lipofectamine as the transfection agent. Although plasmid incorporation into the host genome was seen using the serial transfection method, no integration was detected using the transient transfection protocol [60]. This study took advantage of the fact that stable integration efficiency during plasmid transfection is as low as 1–0.01%. Gonzales et al. [61] simplified the protocol by the delivery of "all-in-one" single plasmid containing all the four reprogramming factors, using nucleofection transfection technology. Nucleofection is a proprietary technology provided by Amaxa [Cologne, Germany] based on electroporation technique that combines specific cell-type solutions with specific electrical parameters generated by the Nucleofector™ device to deliver DNA directly to the nucleus, which may consequently lead to enhanced gene expression [62]. Unlike the serial transfection procedure by Okita et al. [60], the iPS cells generated using nucleofection did not show evidence of transgene insertion in their genome.

In contrast to the regular plasmid vectors, episomal plasmid vectors are able to replicate themselves autonomously as extrachromosomal element. As such, episomal plasmid vectors exhibit prolonged expression of the reprogramming genes in target cells. This type of plasmid can be stably maintained in transfected cells via drug selection. The removal of drug selection results in gradual loss of the vector during multiple rounds of cell division, at a rate of 5% per cell cycle [63]; thus, cells devoid of plasmids can be easily isolated. Derived from the Epstein-Barr virus, oriP/EBNA1 episomal plasmid vectors have been used for reprogramming of human somatic cells [64]. In the study, three combinations of oriP/EBNA1, all of which includes *OCT4, SOX2, NANOG, LIN28, c-MYC, KLF4* and *SV40LT* (SV40 large T antigen), successfully generated the first human iPS cells, which had not

undergone genomic manipulation. Nevertheless, the reprogramming efficiency of this approach was extremely low (3–6 colonies per 10^6 input cells).

Excision strategies The removal of exogenous reprogramming factors from genomic integration sites can be achieved by two strategies, Cre-l*oxP* recombination and *piggyBac* transposition. In the Cre-l*oxP* strategy, a *loxP* site was positioned in the 3′ LTR of lentivirus vectors that contain a Dox-inducible minimal CMV promoter to drive the expression of the reprogramming factors [65]. During proviral replication, the *loxP* was duplicated into the 5′ LTR, resulting in genomic integration of transgene flanked by two *loxP* sites. Subsequent transient expression of Cre enabled the excision of the floxed reprogramming factors. Interestingly, the iPS cells generated using this strategy displayed a gene expression profile closer to that of hES cells compared to human iPS cells. Unfortunately, Cre-mediated excisions of transgene can lead to genomic instability and genome rearrangements. To overcome these potential drawbacks, a single polycistronic vector, containing the four reprogramming factors connected with 2A peptide linkers, was developed [66, 67]. The multiproteins vector does not need multiple integrations in genome, therefore minimizing overall genome modification [68]. Cre expression resulted in the efficient excision of the integrated transgenes. However, a *lox*P site and vector DNA external to the *lox*P sites still remain integrated in the genome, retaining the possibility of interrupting promoters, coding sequences and regulatory elements.

On the other hand, the *piggy*Bac (PB) transposon is capable of excising itself without leaving any remnants of exogenous DNA in the cell genome [69, 70]. Recently, iPS cells have been generated from fibroblasts using a PB method to deliver a polycistron construct carrying the reprogrammed genes linked with a 2A peptide linkers which were positioned between PB 5′ and 3′ terminal repeats [71]. With the expression of transposase in the programmed cell lines, traceless elimination and precise excision of the integrated reprogramming genes were observed [66, 72, 73]. Apart from the precise and efficient excision, the PB reprogramming method demonstrates comparable induction efficiencies to the retroviral methods [73]. In addition, unlike retroviral gene delivery method, piggyBac method does not need specialized biohazard containment facilities, rather, a conventional plasmid working area and normal transfection protocols are sufficient.

Non-DNA methods

Although a number of strategies have been developed to minimize the genomic integration, which may subsequently reduce the potential risks, all the methods developed to date still involve the use of genetic materials, which may still pose unexpected genetic modifications. Two possible ways to avoid the introduction of exogenous genetic materials to target cells are; (i) delivery of the reprogramming proteins directly into the cells, and (ii) manipulation of cell culture condition parameters.

Protein-mediated

Zhou et al. [74] reported the first successful generation of protein-induced pluripotent stem cells (piPS cells). In the study, purified recombinant reprogramming factor proteins (OCT4, SOX2, KLF4 and C-MYC) were fused to poly-arginine peptide tags to allow passage of the proteins through the plasma membrane upon addition to the culture medium. After four rounds of protein transduction, and subsequent culture for 30–35 days in the presence of histone deacytelase inhibitor, valproic acid (VPA), three iPS cells colonies from initial 5×10^4 mouse embryonic fibroblast (MEF) cells were obtained. The piPS cells were found to be morphologically similar to the classic mouse embryonic stem (mES) cells, by forming compact domed small colonies. Using bisulfite genomic sequencing analyses, it was discovered that the *Oct4* and *Nanog* promoters were methylated in both piPS cells and mES cells. Global gene expression analysis identified that the piPS cells were similar to mES cells. In addition, the piPS cells were able to differentiate into various cell types, such as neurons, cardiomyocytes, pancreatic and hepatic cells. These results show that the piPS cells are morphologically, molecularly and functionally similar to the classic mES cells [74]. Nevertheless, albeit the successful reprogramming of the target cells in the study, it is not clear if protein transduction can be used routinely for adult cells, which have proven more difficult to be reprogrammed compared to embryonic cells [75]. In addition, the requirement for multiple protein transductions due to the short half-life of the recombinant proteins may hamper the reprogramming process [76].

Manipulation of cell-culture conditions

Interestingly, Page et al. [77] has reported successful reprogramming of primary adult human fibroblasts by manipulating cell culture conditions alone. In the study, the stem cell genes *OCT4, SOX2* and *NANOG* were shown not to be completely dormant in untreated skins cells, as was initially presumed. The mRNAs of those genes were detectable at basal level and were not being translated. These existing, yet dormant genes were activated by lowering the amount of atmospheric oxygen the cells were exposed to, and by adding fibroblast growth factor 2

(FGF2) (for maintaining the pluripotency of embryonic stem cells) to the culture medium. Furthermore, once the reprogramming factors were activated and began expressing proteins, it was found that these proteins migrated back into the nucleus of the skin cells, precisely as it would occur in iPS cells. Although the results showed that it was possible to manipulate endogenous expression of stem cell genes in somatic cells without genetic manipulation, this short-term induction may not be sufficient for generating genuine pluripotency.

micro RNAs

The crucial roles of microRNAs (miRNA) in the control of pluripotent stem cells were clearly established by the discovery that ES cells lacking mature miRNAs exhibit defects in proliferation and differentiation [78, 79]. Recently, it has been discovered that miRNAs play an important part in the gene networks controlled by the pluripotency factors *Sox2*, *Oct4* and *Nanog*. These factors not only bind to the promoters of most miRNAs that are expressed in mouse ES cells, which include the subset of the miR-290 and miR-302 clusters, but also regulate the expression of these miRNAs [80, 81].

The effects of miRNA expression to promote somatic cell reprogramming were first investigated by Judson et al. [82]. A subset of miR-290 cluster, known as the embryonic stem cell cycle (ESCC) regulating miRNA, was introduced in mouse embryonic fibroblasts together with retrovirus expressing *Sox2*, *Oct4* and *Klf4*. Among the different miRNAs tested, miR-294 exhibited the highest effect on reprogramming with increased efficiency of iPS cells generation from 0.01–0.05% to 0.1–0.3%. However, miR-294 did not show any effect when introduced with *Sox2*, *Oct4*, *Klf4* and *c-Myc*. This implies that the miRNA can replace c-Myc in promoting the differentiation of somatic cells into iPS cells.

The mouse miR-291/294/295 homologous human counterpart, miR-302, was also found to be predominantly expressed in hES and iPS cells, but not in differentiated cells [83, 84]. Therefore, miR-302 could also be applied to enhance the reprogramming efficiency of somatic cells to iPS cells. With induced miR-302 expression beyond 1.3-fold of the concentration in hES cells, Lin et al. [85] reprogrammed human hair follicle (hHF) cells to iPS cells. The expression of the miR-302 significantly decreased the epigenetic regulators AOF2, DNMT1 and MECP1/2, leading to global genomic DNA methylation and histone modifications. The reprogrammed miR-iPS cells were reversible and dependent on AOF2-DNMT1 suppression. This result shows that epigenetic reprogramming of the genomic methylation patterns was necessary for the efficient reprogramming of the hHF cells to iPS cells.

Enhancing DNA reprogramming efficiencies

Several small molecules and soluble factors have been used to enhance reprogramming efficiencies. These small molecules aid reprogramming by modulating chromatin modifications (such as DNA methyltransferase inhibitors, histone deacytelase inhibitors and histone methyltransferase inhibitor)[86], or by targeting cell-signaling pathways (MEK inhibition, CDK1/cyclin B inhibition, TGF-β inhibitor)[87, 88]. Most of the chromatin modification modifiers have been employed together with retroviral vectors, which are subjected to silencing by chromatin modification. An important caution to the use of these epigenetic modifiers is that their broad and non-specific effects may elicit an overall dysregulation of gene expression. For example, the use of 5-azacytidine has been shown to cause tumour in mice with global alteration of DNA methylation level [89].

iPS cells and reproductive technology

The ongoing researches and events on iPS cells have demonstrated a desirable model, which could be translated to treat infertility in the coming days. The derivation of germ cells from iPS cells is more difficult compared to autologous stem cells. Any errors occurring during germ cell differentiation will cause birth defects or germ cell tumours. There have not been any reports of human germ cell generation using iPS cells to date, although there have been some groups researching into this area.

ES cells have been shown to be able to differentiate into gametes and primordial germ cells. However, due to the ethical issues involved in the manipulation of ES cells, reprogramming of terminally-differentiated somatic cells or adult stem cells are extensively explored. Park et al. [90] have derived primordial germ cells (PGCs) from the inner cell mass of blastocysts and human fibroblasts using Oct-4, c-Myc, SOX2 and Klf4. However, they found that the induced PGCs from human iPS cell lines were not able to initiate imprint erasure at their selected imprinted genes. This may indicate an epigenetic reprogramming problem, which hampered the process of successful reprogramming of induced PGCs from terminally-differentiated somatic cells. More work is currently being carried out to identify the solution.

Other groups have found that amniotic fluid cells or amniocytes gave higher reprogramming efficiencies compared to other terminally-differentiated somatic cells. Anchan et al. [91] and Galende et al.[92] have demonstrated that cells found in the amniotic fluid generate iPSCs at a higher rate of approximately 200% increase and at 5–6 days compared to about 10 days or more than 2 weeks from keratinocytes or mouse embryonic fibroblasts. Anchan

et al. [91] used only two reprogramming factors (Klf4 and c-Myc) for pluripotency induction compared to the usual quartet cocktail. The amniocytes were shown to be able to function as both the source of iPSC and also a better feeder layer material for reprogramming purposes. They have suggested that amniotic fluid cells could be stored just like cord blood samples in the cord blood bank for future autologous therapeutic purposes.

Gong et al. [93] has published a method using cell to cell interactions without any genetic modification to induce stem cells from ovarian stromal cells. They cocultured ovarian cells with a fibroblast monolayer using DMEM as the culture media with β-mercaptoethanol, nonessential amino acids and mouse leukaemia inhibitory factor. However they have not confirmed whether the adult ovary-derived colony-forming cells (OCC) were derived from ESC-like cells from the ovarian stromal tissue or non-germline ovarian stromal cells. The OCC was shown to differentiate both in vitro and in vivo but they did not confirm the pluripotency of the cells.

Conclusion

Somatic cells reprogramming into a pluripotent state was originally achieved by the infection of retrovirus carrying four reprogramming factors (*Oct4*, *Sox2*, *c-Myc*, and *Klf4*) into mouse fibroblasts [3]. Within 5 years of the initial reprogramming demonstration, amazing progress has been achieved in the reprogramming strategies; such as the alternative choice of target cell type to be reprogrammed, variations of the reprogramming factors and their delivery methods. A strong requirement for a simple, robust, standardized and reproducible reprogramming strategies to generate genuine iPS cell colonies is the driving factor behind the creative process in method design. While new strategies are continually tested and unveiled, the foundation of iPS cells technology ultimately lies on the safety profile of the iPS cells for clinical use. The increasing trend in the stem cell research community for the use of integrating viruses suggests that clinical applications in the future will eventually meet regulatory issues. Permanent genetic modifications caused by multiple viral insertions, in addition to the use of oncogenes as the reprogramming factors, are the reasons for the concern. This recalls serious consequences in retrovirus-mediated gene therapy for severe combined immunodeficiency disease [94]. As such, it is tempting to envision future reprogramming techniques to employ a non-integrating vector carrying minimal oncogenic reprogramming factors with high gene delivery efficiency on target cells that are easily attainable. A perfectly designed reprogramming strategy, which leaves no trace of the reprogramming process itself, or with total absence of genetic modification, is highly desired. Recent advancements in the iPS cells technology described above have shown increasing potential to facilitate future reproductive technology. Treatment of infertility using the patient's own cells will eliminate chances of histo-incompatibility, disease-transference risks and even zoonotic risks. However, several challenges needed to be overcome first before this method could be tested clinically.

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