Review

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Regenerative Medicine

Current reprogramming systems in regenerative medicine: from somatic cells to induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) paved the way for research fields including cell therapy, drug screening, disease modeling and the mechanism of embryonic development. Although iPSC technology has been improved by various delivery systems, direct transduction and small molecule regulation, low reprogramming efficiency and genomic modification steps still inhibit its clinical use. Improvements in current vectors and the exploration of novel vectors are required to balance efficiency and genomic modification for reprogramming. Herein, we set out a comprehensive analysis of current reprogramming systems for the generation of iPSCs from somatic cells. By clarifying advantages and disadvantages of the current reprogramming systems, we are striding toward an effective route to generate clinical grade iPSCs.

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Induced pluripotent stem cells (iPSCs) paved the way for research fields including cell therapy, drug screening, disease modeling and the mechanism of embryonic development. Previously, nuclear transfer or the fusion with embryonic stem cells (ESCs) in somatic cells was fraught with technical, ethical, immune and logistical barriers [1]. Cell extracts from embryonic carcinoma cells or ESCs, which mediated nuclear reprogramming, constituted an attractive alternative to cell fusion or nuclear transfer. Notably, they upregulated ESC genes and downregulated somatic cell markers and epigenetically modified histones [2]. Thus far, these extracts have not successfully reprogrammed somatic cells into iPSCs with full differentiation potential.

Excluding embryonic materials has been deemed as the obligatory approach to obtaining available iPSCs. By the retroviral transduction of 24 candidate genes and subsequent narrowing down to four transcription factors (TFs), namely Oct4, Sox2, Klf4c, and c-Myc (OSKM), Takahashi and Yamanaka [3] made a breakthrough in 2006, converting mouse fibroblasts to iPSCs. These reprogrammed cells complied with the major aspects of pluripotency (Figure 1), including morphology, proliferation, pluripotent marker expression, self-renewal, multilineage potency and germ-line transmissibility, which were similar to ESCs [4]. Although the viral transduction of OSKM remains the most common strategy to provide a fast way to produce iPSCs with numerous therapeutic implications, poor reprogramming efficiency is still a key concern [5].

To improve the reprogramming efficacy, alternative factors and newer methods should be rigorously tested to ensure quality of the resultant iPSCs. For successful generation of iPSCs from mouse fibroblasts, Sox1 and Sox3 are perfect substitutes for Sox2; furthermore, L-Myc and N-Myc can replace c-Myc [4,6]. In addition, somatic cell reprogramming was originally achieved by gene delivery systems

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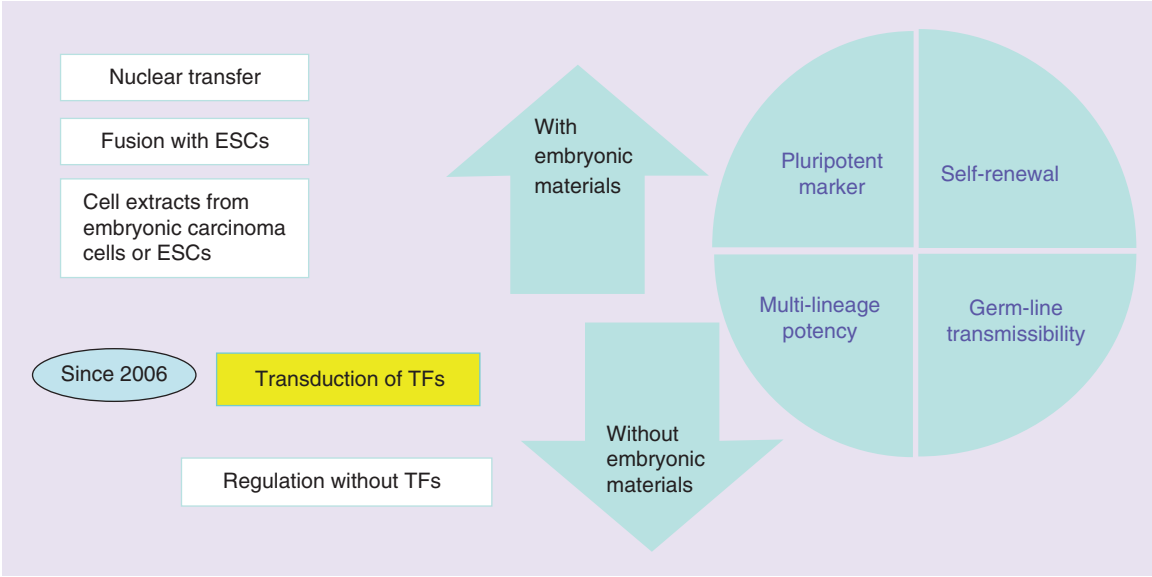

Figure 1. Reprogramming with or without embryonic materials and the characteristics of the resulting cells are demonstrated.
ESC: Embryonic stem cell; TF: Transcription factor.

via integrating viruses, but this resulted in integration into the host genome and caused random mutations within target cells [7]. Since the complete elimination of transgene integrations has been the major goal for delivery systems, several nonviral delivery systems for introducing TFs to somatic cells have been developed, with the aim of enhancing reprogramming efficacy and reducing abnormal chromosomes. In this review article, we set out a comprehensive analysis of reprogramming systems for the generation of iPSCs from somatic cells as a guide to the application of current generation systems (Figure 2). By analyzing advantages and disadvantages of the current reprogramming systems, we are striding toward an effective route to generate clinical grade iPSCs (Table 1).

The reprogramming kinetics & efficiencies

To better evaluate the reprogramming systems, investigators are seeking for various methods and markers to determine the efficiency in the reprogramming process. Epithelial characteristics and activation of some ESC markers are acquired in somatic cells after initiation of reprogramming through MET transition, which is deemed as a critical but nonessential step for reprogramming. Later, pluripotency-related genes are activated, and markers of AP, SSEA1, NANOG and the surface marker TRA-1-60 gradually turn to be expressed in the different reprogramming stages [8–11]. Cell surface markers of CD44 and ICAM1 can be used to indicate the gradual reprogramming process including mesenchymal state, epidermal state, early pluripotent state and late pluripotent state [12]. The ratio between the number of original cells receiving the set of TFs and the number of genuine iPSC colonies and the kinetics of reprogramming are important for the successful reprogramming, while they are hard to be measured. Besides, the donor cell type and defined culture conditions will undoubtedly influence the reprogramming efficiencies and kinetics. Compared with fibroblasts, human primary keratinocytes can be reprogrammed 100-times more efficiently than MEFs [13]. And the intrinsic epigenetic states in specific donor cells contribute to the higher efficiency, fewer TFs and the quality of the resulting iPSCs [14]. For example, neural stem cells with endogenous expression of Sox2 can be reprogrammed in the absence of Sox2 or with Oct4 alone [15,16]. Concurrently, an increase in proliferation rate and a decrease in cell size are molecularly accompanied with the sequential transition [17]. Telomerase reverse transcriptase and the SV40 large T antigen, which have positive effects on proliferation, can also increase the quantity of resulting iPSCs [18]. Small molecules and miRNA which are able to regulate the cell cycle may take effect to increase the number of fully reprogrammed colonies [19,20]. Intriguingly, hypoxic conditions [21], growth factors secreted by feeder cells [22] and additions in culture medium [23] can absolutely improve the reprogramming efficiency. The kinetics are regulated by multiple factors, consequently there is no golden standard for accurate evaluation about the reprogramming for various reprogramming conditions.

Viral vector approaches for reprogramming

During the reprogramming process, induction silencing occurs gradually but viral genes are expressed constitutively. Despite the possibility of making safe

iPSCs, nonintegrating viruses display a rather low gene transfer capacity and thus repeated infections are often required for many cell types. Consequently, retroviruses [24] and lentiviruses [25] are still the widely applicable delivery systems.

Retrovirus

As the most common choice in studies, retroviruses from replication-defective vectors can infect their target cells and deliver their viral payload but avoid cell lysis and death by inhibiting the lytic pathway. The infectivity of retroviruses is limited to dividing cells, thus the cell type for reprogramming is under restrictions. Retrovirus-mediated iPSCs stained positive for alkaline phosphatase, showed renewed expression of pluripotency genes, exhibited ultrastructural features including massive glycogen granules in the cytoplasm [26] and formed teratomas *in vivo* [27]. Recently a polycistronic cassette encoding four TFs separated by 2A peptides was tested in a retrovirus under an LTR or EF1α promoter, and the efficiency was much higher (up to 0.6%) than any other vectors [28]. In brief, the insertional mutagenesis, residual expression and reactivation of TFs, as well as titer loss during viral concentration and storage inhibited the infection of species and cell types resulting in reprogramming limitations.

Lentivirus

HIV1-based VSV-G-pseudotyped lentiviruses, as a subclass of retroviruses, are efficient and easy to transduce nondividing cells. However, the unpredictable integration will disturb the internal genes and bring about the activation of oncogenes. In the pluripotent state, their poor silence will make their constitutive versions less suitable for reprogramming attempts [29]. Adult mouse fibroblasts can be efficiently converted to iPSCs by using the Stem Cell Cassette (STEMCCA) polycistronic lentiviral vector [30]. Similarly, a single polycistronic Dox-inducible lentiviral vector was developed and successfully reprogrammed somatic cells with relative higher efficiency [31]. What is more, only a single proviral copy with high fidelity was required in the reprogramming process [30,31]. Cells reprogrammed with the stemgent human TF lentivirus set [32] began to show iPSCs morphology four days posttransduction. As it uses a type of retrovirus, this technique is limited by the same fundamental drawbacks. Moreover, its inefficient packaging cell lines also contribute to VSV-G toxicity.

Adenovirus

Adeno-associated virus (AAV) delivery systems lack pathogenicity, are capable of infecting dividing and

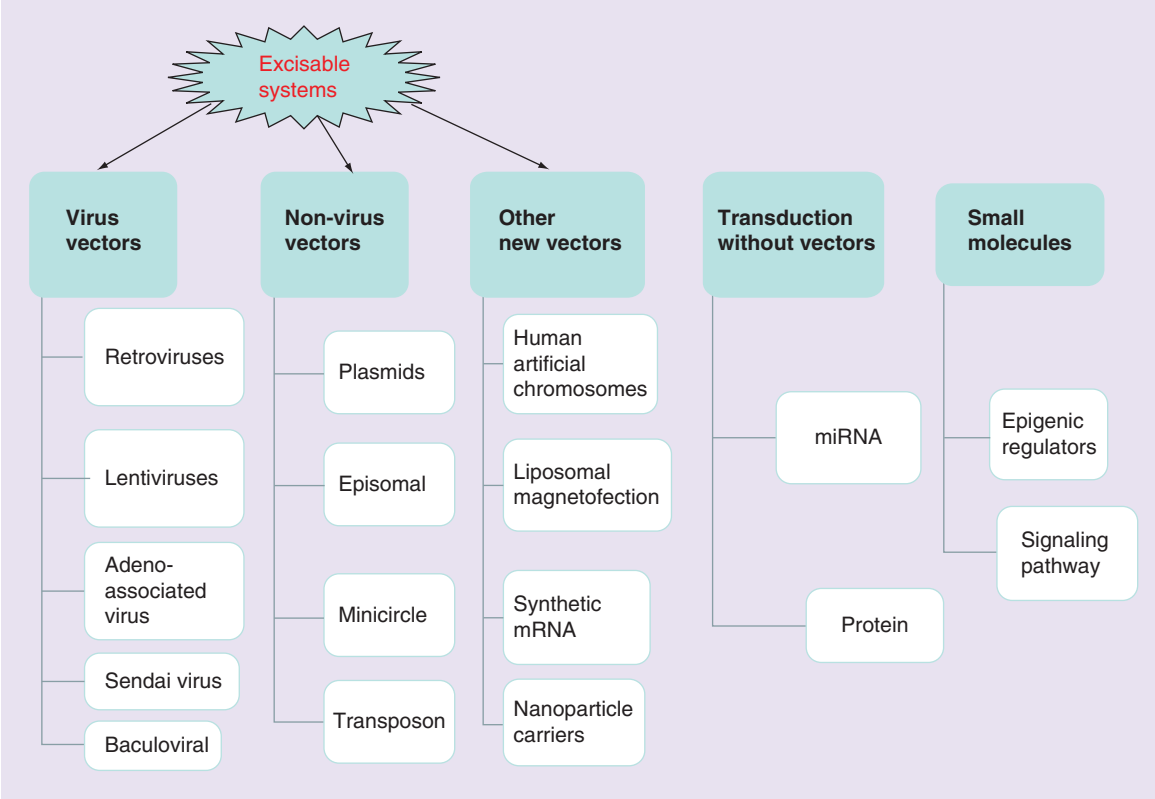

Figure 2. A comprehensive survey of reprogramming systems for the generation of induced pluripotent stem cells from somatic cells as a guide to the application of current generation systems.

Table 1. Advantages and disadvantages of the current reprogramming systems.

Classification	Vectors	Advantages	Disadvantages
Virus	Retrovirus	High efficiency, avoid cell lysis and death	Limited cell type, insertional mutagenesis, residual expression of TFs, titer loss during viral concentration
	Lentivirus	High efficiency, broadened tropism, ease of handling, availability of inducible systems	Unpredictable integration site, poor silence, insertional mutagenesis, residual expression of TFs, titer loss during viral concentration, inefficient packaging cell lines
	Adenovirus	Lack pathogenicity, broadened tropism, site-specific integration	Low efficiency
	Sendai virus	High efficiency, without integration into the host genome, broadened tropism	Sustained cytoplasmic replication of viral vectors
	Baculoviral	High efficiency, broadened tropism, site-specific integration, without appreciable cytotoxicity, flexibility in transgene exchange, low genome toxicity	Perturb the transcription of 12 genes involved in the Toll-like receptor signaling pathway
Nonviral approaches	Standard plasmids	Without integration into the host genome, without chromosomal abnormalities	Low efficiency, additional TFs requirement, result in cell death, transiently express transgenes, low efficiency, insufficient excision of integrated vectors
	Episomal plasmids	Simplest disintegration approach, reasonable efficiency, broadened tropism	Low efficiency, repetitive induction, insufficient excision of integrated vectors
	Minicircle plasmids	Nonintegrating, inexpensive, low immunogenicity	Low efficiency, occasional integration, additional TFs requirement, multiple transfections, insufficient excision of integrated vectors
	Transposons	Higher efficiency than plasmids, broadened tropism	Mutations in the genome, low efficiency, repetitive induction, insufficient excision of integrated vectors
Other burgeoning delivery systems	miRNA	Small, without genome integration, readily synthesized, function longer than the coding RNAs,	Low efficiency, transient action, multiple transfections
	Artificial chromosome system	Episomal transmission, transfer of multiple large transgenes long-term stable maintenance of single copy episomes, without integration into the host chromosomes, can be transferred from one cell to another	Low efficiency, limited by technical difficulties of gene loading into the HAC, ill-defined structures
	Synthetic messenger RNA	High efficiency, without transgene integration, rapid kinetics, obviation of a clean-up phase to purge the vector.	Relatively laborious
	Liposomal magnetofection	How efficiency, stable, integration-free, under the least toxic conditions	The distribution of aggregate complexes over the cell surface may be ununiform
Direct protein transduction		Without the genetic modification, simpler and faster	Low efficiency, require repetitive induction, and/or produce insufficient excision of integrated vectors
Small molecules		High efficiency, improve the efficiency of TF-mediated reprogramming, reduce the transcriptional factors	Unable to recapitulate the series of TFs and generate iPSCs with full pluripotency and differentiation potency

HAC: Human artificial chromosome; iPSC: Induced pluripotent stem cell; TF: Trancription factor.

nondividing cells and can stably integrate into the host cell genome at a specific site, which distinguishes them from lentivirus-based approaches. Adenoviruses can infect all cell types with the exception of some lymphoid cells, and their gene expression is not consistently and sufficiently long enough within this system. Transgene-free human iPSCs can be generated through the site-specific integration and excision of transgenes combined with the LoxP/Cre system. AAV serotypes 2 and 6 were superior to other serotypes in their transduction efficiency, and this is correlated with the abundance of their respective receptors [33]. Even so, the reprogramming efficiency of these two serotypes is very low both in mouse and human cells [34,35]. As an alternative for standard adenovirus, introducing artificial DNA double-strand breaks is unnecessary in the reprogramming process by helperdependent adenoviral vector (HDAdV), and 7–81% of colonies were gene-targeted for complete iPSCs generation [36,37]. In consequence, considerable works are obligatory for optimized transgene expression and higher efficiencies in the reprogramming process.

Sendai virus

The Sendai virus vector, a negative-strand RNA virus in the paramyxovirdae family, is nonpathogenic to humans. It will replicate in the cytoplasm of target cells but does not go through a DNA phase [38]. It is gradually depleted from the iPSCs cytoplasm after several passages, efficiently generating transgene-free iPSCs starting with different cell types as well as in feeder-free conditions [39,40]. During the division of iPSCs, although viral vectors were slowly diluted, the sustained replication of viral vectors had to be cleared [41]. In a cost-effective manner, this vector efficiently demonstrates constant reprogramming results [42]. Then temperature-sensitive mutations, which can accelerate future clinical application of iPSCs by less invasive methods, were introduced for the complete removal of viral constructs at nonpermissive temperatures [43].

Baculoviral

In addition to mentioned viral vectors, baculoviral (BV) can transduce various mammalian cells without considerable cytotoxicity [44,45]. This virus delivers genes with high efficiency in human ESCs and delivers genes in almost all medaka ESCs [46]. After three successive transductions of mouse embryonic fibroblasts (MEFs) with BacMam particles, iPSCs colonies were generated and the efficacy was shown to be increased to 64–98% [47]. Although BV may trigger innate responses in mammalian cells [33,34], the transduction of MSCs activates only slight and transient responses in the Toll-like receptor 3 pathway [48], and no well-known cytokines and sensors or their downstream signaling mediators were altered by this way [49]. Recently, BV transduction successfully reprogrammed human fibroblasts by site-specific integration into the AAVS1 locus [50]. Attributing to the high integration efficiency, flexible transgene exchange and low genome toxicity, BV-transcription activator-like effector nuclease system may offer great potential for precise genetic manipulation in iPSCs generation [51,52]. To promote the technology far away, BV as transgenic vector of radionuclide reporter gene imaging technology bring up to monitor stem cell transplantation therapy [53].

Nonviral approaches to reprogramming

To avoid interference with the host genome during the reprogramming process, safer methods must be developed. Several nonviral vectors including plasmids [54], episomal vectors [55], minicircles [56] and transposons [57] have been described for iPSC reprogramming. However, these nonviral approaches are inefficient, require repetitive integration, and produce deficient excision of vectors.

Standard plasmids

Plasmids are nonvirus vectors that do not integrate into the genome of iPSCs and produce chromosomal abnormalities [58] but are characterized by low reprogramming efficiency [59]. Their occasional genomic integration requires additional TFs and results in cell death when nucleofection occurs. Most regular plasmid vectors lack the ability to replicate themselves in mammalian cells, leading to gradual cell division, and even then, they only transiently express transgenes. Established iPSCs are morphologically similar to ESCs, and express pluripotent markers of ESCs at comparable levels [60]. To ensure efficient and controlled generation, reprogramming plasmids have been equipped with a particular bacteriophage site and a specific expression vector to enhance integration into the genome [61].

Episomal plasmids

The Epstein–Barr nuclear antigen-1-based episomal system, a simplest disintegration approach, indicates appreciable efficiency while only requires one transfection with Maxiprep DNA. The vectors have been extensively used to generate footprint-free iPSCs, replicate themselves autonomously as extrachromosomal elements in both dividing and nondividing cells, persist throughout reprogramming and subsequently diminish in iPSCs [62]. As current protocols of generating integration-free human iPSCs from keratinocytes are generally inefficient, the simple transfection of episomal vectors was able to achieve a reprogramming efficiency of approximately 0.14% on average [63]. The delivery of episomal vectors into cells

may be a problem for primary somatic cells, which may be solved by using the adenovirus episomal vector hybrid system [64], a system utilizing Cre-mediated site-specific recombination to excise an episomal vector from a target recombinant adenovirus genome. In summary, episomal vectors are superior to conventional plasmid vectors because of the increased duration of reprogramming factor expression in target cells.

Minicircle plasmids

Minicircle plasmids are nonintegrating and inexpensive delivery vectors of low immunogenicity, but protocols for their use are inefficient, result in occasional integration, and require additional factors and multiple transfections. Minicircles are special episomal DNA vectors devoid of any bacterial plasmid backbone [65] and are significantly smaller than standard plasmids. The repeated transfection of minicircle DNA vectors into somatic cells and abundant cell sources are amenable to efficient reprogramming into transgene-free iPSCs [65]. In comparison with standard plasmids, minicircle DNA benefits from higher efficiency and longer ectopic expression but accompanied with lower activation of exogenous silencing [56], which enhances its transfection efficiency and the survival rate of target cells. Although the minicircle theoretically should not integrate into the target cells, there is still a relatively small chance of integration. Intriguingly, the minicircle-based generation of iPSCs is compatible with the production of chicken chimeras [66].

Transposons

Transposons are able to move from one locus to another within the chromosome and cause mutations or genomic rearrangements within the genome. First discovered by Barbara McClintock in 1950 [67], they can be grouped into two classes: class I copy themselves after being firstly transcribed to RNA, then being reversely transcribed to DNA, finally they are inserted at another position into the genome; class II move directly from one locus to another but excise themselves from the original location and insert themselves into a new locus. Transposases are normally located at each end of the transposon, can act on almost any DNA sequence which is flanked by the terminal repeat sequences [68] and mediate a higher genome integration efficiency than plasmids [69]. Genetic screens conducted on this transposase have since resulted in a hyperactive variant capable of efficient transposition in vertebrates and mammalian cells [69], enabling novel methods of genetic engineering in animal models, a variety of human cell types and gene therapy trials [70]. Furthermore, following stable genomic integration, the reexpression of the transposase can result in transposon excision [71]. The piggyBac (PB) transposon belongs to the class II mobile genetic elements and requires only the inverted terminal repeats (ITR) and active transposase to catalyze insertion and/or excision [69,70]. The unique characteristics of PB transposons including efficient genomic integration, unlimited cargo capacity, robust gene expression, and even seamless excision [72] make this system one of the best choices for generating 'genetically clean' iPSCs. The use of PB in a plasmid containing both a transposase and transposon greatly increased the probability of transposase integration, but using a transposon and transposase from separate vectors circumvented this. In addition, the delivery of PB plasmid vectors into cells is dependent on transfection reagents, and the insertion sites in each cell are uncontrolled. The Sleeping Beauty (SB) transposon system was reconstructed from fragments belonging to the Tc1/mariner superfamily and resembles an ancestral transposon [73]. The SB transposon does not exhibit an integration bias towards particular genetic elements, thereby reducing the risk of insertional mutagenesis. Furthermore, unlike the alternative transposon PB, SB has no SB-like elements within the human genome, which minimizes the possibility of mobilizing endogenous transposon elements [57]. The SB transposon-reprogrammed iPSCs showed long-term proliferation *in vitro* over 40 passages and expressed typical surface markers of ESCs [74]. Together with its simple and inexpensive production, SB-mediated gene transfer can be used to generate mouse iPSCs from different genetic backgrounds [75].

Excisable systems

Cre–loxP system

Cre-deletable systems have made it possible for the removal of the integrated transgenes from the genome [64]. During the normal viral reverse transcription cycle before integration, the loxP sequence is duplicated into the 5′ LTR region to create a loxP-flanked version, and then integrates into the targeted genome. The deletion of the loxP-flanked transgene cassette requires the introduction of Cre recombinase activity, which has been accomplished with Cre-encoding plasmids [76], lentiviral Cre constructs [64] and adenoviral Cre constructs [77]. By contrast, the delivery of Cre mRNA [78] to obtain transgene-free iPSCs involves the daily transfection of mRNA for a week to perform excision, so this mRNA-mediated progress is more inefficient, laborious and less appealing. Then, transgene-free iPSCs can be obtained by treatment with Cre recombinase and selection of excised iPSC clones. Both excised and non-excised iPSCs expressed pluripotency markers and were able to differentiate *in vitro*, and non-excised cells can form germ-line competent chimeras *in vivo* [64]. More recently, by a single application

of TAT–Cre recombinant protein for 5 h, the process of obtaining transgene-free iPSCs with minimal technical complexity was accelerated [79]. Cre recombinase resulted in multiple transgene excisions, potentially leading to genome rearrangement and genomic instability. The efficient and reliable induction of Cre recombinase activity in loxP-modified iPSCs and subsequent selection of cleaned clones represents a roadblock for the widespread use of Cre-deletable systems [79].

Exercisable site-specific integration

Although the nonintegrating methods are rapidly becoming a standard approach, methods based on the site-specific integration of reprogramming factor genes hold the potential for the efficient generation of genetically amenable iPSCs suitable for future gene therapy applications. As a class of artificial restriction enzymes, transcription activator-like effector nucleases (TALENs), can be efficiently delivered by the type III secretion system [80], and significantly promote homologous recombination over 1000-fold. A recent study using plasmid transfection of human primary cells has demonstrated the generation of iPSCs by zinc finger nuclease (ZFN)-mediated targeted insertion of TF genes into the CCR5 locus, though a relatively low reprogramming efficiency was reported [81]. It appears that the low cotransfection efficiency of ZFN and a large donor DNA carrying TF genes represents a major obstacle for the reprogramming of human primary cells by ZFN technology. The expression of TFs can be efficiently accomplished in almost every transduced cell when combining with a single polycistronic vector by inserting a 'self-cleaving' 2A peptide or an internal ribosome entry site sequence between two consecutive open reading frames [31].

Other burgeoning delivery systems

miRNA transduction

miRNAs, which are very small, can be readily synthesized and delivered into cells. After that, they remain stable for several days and function longer than the coding RNAs but without risk of genome integration. Specific miRNA mimics or miRNA inhibitors promote the reprogramming of somatic cells into iPSCs [82]. The overexpression of miR-302a, miR-302b and miR-200c can improve the reprogramming efficiency but reduce the handling time and tumorigenicity efficiently [83]. In an episomal system, ESC-specific miRNAs (miR-302/367 cluster) increased the iPSC colony-forming efficiency in fibroblasts and epithelial cells [84]. Another ESC-specific miRNA (miR-294) can replace exogenous c-Myc in the reprogramming of MEFs towards iPSCs and improve the reprogramming efficiency without c-Myc. miR-302b, which shares the same seed sequence as miR-294, can also improve the reprogramming efficiency [20]. Based on the regulation of the miRNA processing, Lin28 can replace Klf4 and c-Myc and improve the reprogramming efficiency in combination with Nanog [85]. Miyoshi *et al.* generated mouse and human iPSCs by direct delivery of ESC-specific miRNAs without any vector-based gene transfer [86]. However, attributing to the transient action, multiple transfections are required for complete reprogramming with miRNAs.

Artificial chromosome system

Human artificial chromosomes (HACs), which can be transferred from one cell to another, are used for episomal transmission and the transfer of multiple large transgenes. The functional centromere of HACs enables long-term stable maintenance of single copy episomes but without integration into the targeted genome. Despite these obvious advantages over viral vectors, the use of HACs for reprogramming was limited by technical difficulties of transgene insertion and the undefined structures [87,88]. Recently, by the use of 21HAC vector, MEFs was successfully reprogrammed to iPSCs [89]. Global gene expression patterns demonstrated that the HAC-based iPSCs are relatively uniform at a level comparable to retrovirus-based iPSCs [89]. Next, the cells which spontaneously lost the HAC were isolated and, consequently, HAC-free iPSCs were established [89]. HAC1 carried four TF partially reprogrammed MEFs, but HAC2 carried four TFs and a p53-knockdown cassette efficiently reprogrammed MEFs [89]. Satellite-DNA-based artificial chromosomes (SATACs) have already passed the obstacles, including large-scale purification, transfer into various cells and embryos, germ-line transmission and generation of transgenic animals [90]. The reprogramming of MEFs was efficiently induced by HACs with engineered OSKM factors carrying an N-terminal flag-tag and a C-terminal polyarginine tail [91].

Synthetic messenger RNA

The use of synthetic mRNA to generate iPSCs is extremely attractive for regenerative medicine, which benefits from the avoidance of common drawbacks in DNA-mediated or virus-mediated reprogramming strategies. Exogenous DNA must be delivered into the cytoplasm and placed into the chromosome for successful reprogramming. In contrast, exogenous mRNA only needs to be transmitted through the cell cytoplasm and leaves the integration out undoubtedly. In contrast to retrovirus-derived iPSCs, synthetic mRNA-derived iPSCs do not differ significantly from the parental fibroblasts. Thus synthetic mRNA gradually became an important alternative to DNA-based integration for cell reprogramming. Furthermore, hepatic differentiation studies indicated that mRNA-based iPSCs can

differentiate into hepatoblasts efficiently [92]. Synthetic mRNA-based integration-free techniques successfully generated iPSCs from adipose tissues of a patient under feeder-free conditions and put forward iPSCs as a potential personalized regenerative medicine [93]. This system can reprogram enormous cell types to pluripotency with high efficiency and direct the resulted iPSCs into terminally differentiated cells [94]. Notable advantages of the mRNA approach include high efficiency, rapid kinetics, and obviation of a clean-up phase to purge the vector. Still, this method is relatively laborious, but when reprogramming without feeders, there is reduced labor and material costs [95].

Liposomal magnetofection

Liposomal magnetofection (LMF) is based on the use of superparamagnetic particles and cationic lipids and shows better transfection efficiency than other nonviral delivery systems; however, the ununiform distribution of aggregate complexes on the cell surface should be eliminated. Under a dynamic gradient magnetic field, the transfection was less cytotoxic and the efficiency was greater by almost 21 and 42% in comparison with LMF and lipofection, respectively [89]. LMF based-iPSCs are able to present similar characteristics to ESCs, including cellular morphology, surface marker expression, embryoid body formation, teratoma formation, direct differentiation into terminal cells, and chimeric mouse production [96]. Park *et al.* produced a stable and integration-free iPSC line by a single LMF procedure and a half-dose of plasmid, while the *in vitro* and *in vivo* pluripotency were similar to other cell lines. Thus, LMF may represent an outstanding technique for the generation of virus-free iPSC lines and could lead to enhanced stem cell therapy [96].

Synthetic carriers

There is growing applications in nanoparticle and synthetic carriers as reprogramming systems for generation of iPSCs. For instance, after retinoic acid (RA) was efficiently incorporated into poly(N-isopropylacrylamide)-co-acrylamide nanoparticles, this nanoparticle could be a potentially powerful carrier for effective RA delivery to direct human iPSC fate to the neuronal lineage [97]. Tavernier *et al.* generated mouse iPSCs from MEFs using a different cationic lipid carrier fused with OSKM mRNAs [98].

Direct protein transduction

Protein delivery, without genetic modification, provides a substantially simpler and faster approach than the currently progressive genetic reprogramming systems, but the efficiency is too low to be practical for research and clinical applications. Protein transduction of TFs tagged with polyarginine has generated mouse iPSCs in the presence of valproic acid (VPA) [99] and generated human iPSCs without VPA [100]. By fusing in frame to a glutathione-S-transferase tag and to the transactivator transcription-nuclear localization signal polypeptide, recombinant OKSM proteins successfully generated stable iPSCs [101]. After optimizing cationic bolaamphiphile-protein complex ratio to 7:1 and incubating for 3 hours, the reprogrammed human fibroblasts were shown to exhibit the characteristics of ESCs, including the expression of pluripotent genes, teratoma formation, and differentiation into various terminal cells [102].

Although protein transduction is able to convert the immature fetal cells, adult somatic cells are difficult to be reprogrammed [18]. More recently, Human umbilical cord blood neural stem cells have been successfully reprogrammed with HEK293cell extracts containing three TFs recombinant proteins in combination with additional small molecules under low oxygen condition [103]. What's more, after the cell penetrating TAT domain from HIV1 to be conjugated with cationic liposomes or combination with VPA, the transduction efficiency was increased [104,105]. In the absence of any chemical treatment, the system may allow the translation of iPSC technology into the clinical applications [94,100]. However, to successfully reprogram somatic cells to pluripotent state, purification of sufficient desired proteins is necessary.

Small molecules

There is growing evidence indicating that small molecules may revolutionize the iPSC field by replacing current delivery systems and extremely enhancing reprogramming efficiency. They are particularly useful for partially reprogrammed cells and cells resistant to reprogramming. A majority of these chemicals are inhibitors of epigenetic regulators and inhibitors of signaling pathways.

Epigenetic regulators

To potentially reprogram somatic cells by sole chemical supplements, high-throughput screening technologies can be used to identify detailed small molecules for modulating the expression and regulating pluripotency. Huangfu *et al.* [106] demonstrated that the treatment of MEFs with a histone deacetylase inhibitor (VPA) could improve the reprogramming efficiency in OSKM- and OSK-infected MEFs by 100-fold and 50-fold, respectively. Although overexpression of Mbd3/NuRD does not have any positive or negative effect on iPSC induction efficiency, combined with Nanog overexpression improves both reprogramming kinetics and efficiency [107]. However, another recent study reported that Mbd3/NuRD is required for effi-

cient iPSC generation from neural stem cells, pre-iPSCs and epiblast-derived stem cells [108]. In the context of iPSC reprogramming, combined overexpression of the histone variants TH2A and TH2B, the efficiency of iPSC generation was improved and further enhanced by additional overexpression of the phosphorylation-mimic form of nucleoplasmin through the induction of an open chromatin structure [109]. More recently, Li *et al.* [110] demonstrated that under the transduction with lentiviral vectors expressing only Oct4, treatment with small molecules is sufficient to generate functional iPSCs. Intriguingly, the combination of VPA, CHIR99021, 616452, tranylcypromine, forskolin and dznep can reprogram MEF into iPSCs with 2i media [111]. What is more, under the condition without any transgene, mouse iPSCs were efficiently generated only with a combination of seven small molecule compounds [9].

Signal pathways

In the same manner, specific signaling modulators are also sufficient to generate functional fibroblast-derived iPSCs [112]. The combination of TGF-β, WNT and FGF pathways resulted in regulating pluripotency in different species [113]. With the combination of TGF-β receptor inhibitor, MEK inhibitor and thiazovivin, the reprogramming efficiency was improved for more than 200-fold [114]. Recent studies demonstrated that the Ink4/Arf and p53–p21 pathways serve as a barrier to iPSC generation [115–117]. Thus, it will be worthwhile to test whether the combination of transient p53 inhibition and delivery of reprogramming factors via nonintegrating vectors could generate genetic-modification-free human iPSCs with a higher reprogramming efficiency. Furthermore, the signaling pathway regulators can also eliminate the requirement for transduction with certain reprogramming factors. In the absence of exogenous c-Myc, Wnt3a-conditioned medium can also help to reprogram somatic cells with high efficiency [23]. In addition, the MEK and TGF-β pathways without delivery of exogenous transcription factors efficiently generated iPSCs [118]. Above all, small molecules are the most promising resources for successful reprogramming of high-quality clinical-grade iPSCs with a minimum of genomic operation. However, it is currently unknown whether small molecules alone can recapitulate the series of TFs and generate iPSCs with full pluripotency and differentiation potency.

Epigenetic barriers & reprogramming process

The epigenetic status may be altered during reprogramming process [119], chromatin remodeling complexes and certain histone variants play important roles in the acquisition and subsequent maintenance of the permissive pluripotent chromatin state [120]. Reconfiguration of chromatin structure including DNA methylation, histone modifications and nucleosome remodeling come out after initiation of reprogramming. Repres-

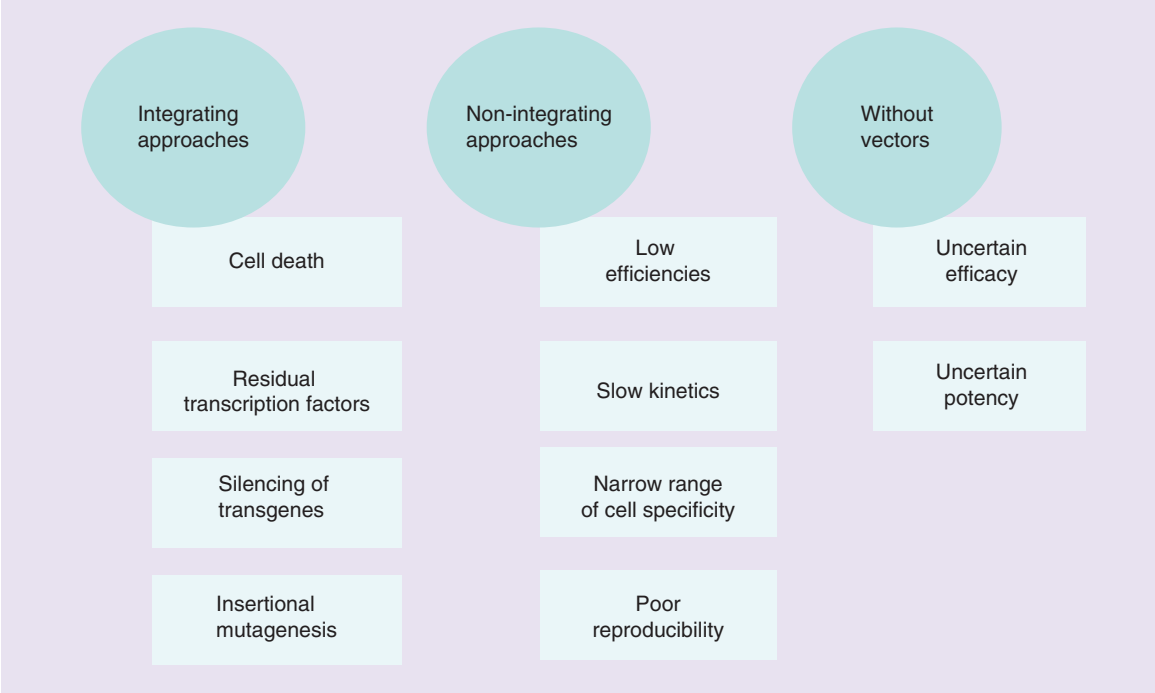

Figure 3. The obstacles exist in systems with or without vectors for induced pluripotent stem cells generation.

sive chromatin comprises a major mechanistic barrier in reprogramming process [121,122], and global DNA demethylation is a conserved and required feature of reprogramming [123]. Most histone variants incorporated into chromatin in a replication-independent manner and may contribute to the epigenetic barrier during reprogramming [124]. Additionally, both repressive H3K9me2/3 histone methylation and the presence of 5mC may act as a barrier to the reprogramming process [125,126]. In the initial phase of reprogramming, rapid genome-wide changes of H3K4me2 distribution are one of the earliest events [17], then dramatic changes at promoter and enhancer regions of more than a thousand genes were observed. On the other hand, NANOG overexpression and inhibition of DNA methylation synergistically enhance the final phase of reprogramming [127]. Clearly, reprogramming process was accompanied by silencing of somatic cell genes, resetting of pluripotency, and altered epigenetic status. In the contrast, evidence appeared to show that some epigenetic alteration is not obligatory for successful reprogramming. Although somatic methylome is altered after initiation of reprogramming, *de novo* deposition of methylation is not a requirement [128]. Depletion of Tet1 and Tet2 may result in significantly reduced efficiency of iPSC colony formation [129], while they are clarified to be only necessary for somatic cells to undergo the MET during iPSC reprogramming [129].

To clarify the detailed transition during the reprogramming process, comparative analysis of genetically matched mouse ESCs and iPSCs was performed and revealed identical transcriptional and methylation profiles [130], while in other cases, iPSCs are not identical but with transcriptional, epigenetic and phenotypical heterogeneous lines when compared with ESCs [131]. The differences between ESCs and iPSCs may be due to the preexist mutations in original cells or long time culture or the reprogramming technology [132]. With integrative vectors, the reprogrammed cells tend to be heterogeneous by transgene insertions [133]. By a whole exome sequencing of human foreskin fibroblasts and their derived iPSCs, the aberrations can be attributed to *in vitro* passaging for 7%, preexist mutations in the parental fibroblasts for 19%, and the remaining 74% of the mutations were acquired during cellular reprogramming. Another report suggested that the mutation intensity during reprogramming is nine fold higher than the background mutation rate in culture [134]. What is more, genomic copy number variation rates were negatively associated with the dosages of TFs, and high-performance engineered factors may result in less genomic copy number variation rates than the classic TFs at the same dosage [135]. The genomic integrity of the partially purified reprogramming protein-based mouse iPSCs was compared with mouse iPSCs developed from viral-based strategies, and they were able to maintain genomic integrity better than current viral reprogramming methods [136]. While some researchers demonstrated that most of the genetic variation in iPSC clones is not caused by reprogramming *per se*, but is rather a consequence of the mutational history derived from individual cells [137]. In consist with this, one case demonstrated that genome stability can persist throughout reprogramming, and it is possible to generate iPSCs without gene mutations with current reprogramming methods [138]. Taken together, it is obligatory to cross epigenetic barriers in somatic cells for successful reprogramming, but various delivery systems will lead to epigenetic alterations in resulting pluripotent cells.

Conclusion & future perspective

Somatic cell reprogramming was originally achieved by gene-delivery systems via integrating viruses. To apply these systems to clinical usage, obstacles including resulting cell death, remnant expression of transgenes, immunogenicity and insertional mutagenesis should be stepped over for generation of virus-free and transgene-free iPSCs. One major goal of reprogramming research is to eliminate or reduce transgene integrations since the advent of iPSC technology. There is no gold standard for an iPSC reprogramming strategy because these nonintegrating approaches exhibit limitations such as low reprogramming efficiencies, slow reprogramming kinetics, a narrow range of cell specificity, and poor reproducibility [79]. Thus, gene-delivery reprogramming approaches remain major strategies for generation of iPSCs for basic research. Excisable vectors are applicable for most virus-based systems; once the efficiency of disintegration vectors, miRNA mimics, direct protein transduction and small molecules is enhanced, the alternative routes may be the most promising approaches to avoid genome alterations. The obstacles to overcome in all of the systems for iPSC generation are summarized in Figure 3. In addition to the mentioned delivery systems, these values are also subject to the donors' age, the different combination of reprogramming factors, the somatic cell types and the passage number of target cells. Meanwhile, reprogramming systems absolutely overcome the epigenetic barriers and may lead to epigenetic abbreviations. Considering the mentioned factors for successful and available reprogramming, the further optimization of the reprogramming protocols, accompanied with a thorough analysis of the generated iPSCs, will facilitate the clinical applications of the iPSC technology and produce desired terminal cells for regenerative medicine.

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Executive summary

Various reprogramming systems & induced pluripotent stem cell generation

- Induced pluripotent stem cell (iPSC) technology has been improved by various reprogramming systems.
- Low reprogramming efficiency and genomic modification steps still inhibit clinical use of iPSCs.
- One major goal of reprogramming research is to eliminate or reduce transgene integrations since the advent of iPSC technology.

Epigenetic status & iPSC generation

- It is obligatory to cross epigenetic barriers in somatic cells for successful reprogramming.
- The epigenetic status may be altered during reprogramming process.

Conclusion & future perspective

- Advantages and disadvantages of the current reprogramming systems may help scientists to generate clinical grade iPSCs.
- Improvements in current vectors and the exploration of novel vectors are required to be investigated thoroughly.

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