REVIEWS

Methods for making induced pluripotent stem cells: reprogramming à la carte

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Abstract | Pluripotent stem-cell lines can be obtained through the reprogramming of somatic cells from different tissues and species by ectopic expression of defined factors. In theory, these cells — known as induced pluripotent stem cells (iPSCs) — are suitable for various purposes, including disease modelling, autologous cell therapy, drug or toxicity screening and basic research. Recent methodological improvements are increasing the ease and efficiency of reprogramming, and reducing the genomic modifications required to complete the process. However, depending on the downstream applications, certain technologies have advantages over others. Here, we provide a comprehensive overview of the existing reprogramming approaches with the aim of providing readers with a better understanding of the reprogramming process and a basis for selecting the most suitable method for basic or clinical applications.

Inner cell mass
(ICM). In mammals, a cluster of pluripotent cells found inside the blastocyst that give rise to all the cells of the body of the embryo proper. Embryonic stem cells, which are derived from ICM cells, are the closest *in vitro* counterpart of ICM cells.

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Self-renewal and pluripotency are defining properties of embryonic stem cells (ESCs). They refer, respectively, to the ability to proliferate indefinitely without commitment *in vitro* and to the capacity to differentiate into cell lineages belonging to the three embryonic germ layers[1–3]. ESCs are derived from the inner cell mass (ICM) of pre-implantation embryos[1,2], but alternative approaches — such as nuclear transfer, cell fusion or direct reprogramming (reviewed in REF. 4) — are now available that allow the generation of pluripotent stem-cell lines directly from differentiated adult somatic tissue. These have widened the range of applications in which these cells can be used[5–7]. Among these methodologies, direct reprogramming through the ectopic expression of defined transcription factors[8] — in this Review referred to simply as 'reprogramming' — represents a simple way to obtain pluripotent stem-cell lines from almost any somatic tissue and mammalian species. The use of such cells also circumvents the ethical issues associated with human ESCs.

Reprogramming entails the in *trans* expression in a somatic cell of a set of core pluripotency-related transcription factors (in most cases OCT4 (also known as POU5F1), SOX2, KLF4 and MYC (also known as c-MYC) (OSKM)). When successful, tightly compacted colonies appear on the culture dish; these colonies resemble ESCs morphologically, molecularly and phenotypically[9–12]. These induced pluripotent stem cells (iPSCs) are relevant to a range of applications, including: autologous cell therapy; the modelling of monogenic and multigenic diseases; the study of complex genetic traits and allelic variation; and as substrates for drug, toxicity, differentiation and therapeutic screens. To serve these various purposes, a multitude of protocols for iPSC generation have been developed in recent years. They use, for example, different mouse[13–16] and human donor populations[17–19], or vary the number, identity and delivery mode of the reprogramming factors[20–22].

iPSCs represent a widely available, non-controversial and practically infinite source of pluripotent cells. Unlike human ESCs, their usage is not restricted, so most laboratories can now develop research programmes using human pluripotent stem-cell lines. However, one needs to choose a strategy to obtain iPSCs that is suited to the research aims. The simplest approach is to obtain an existing line from another laboratory, but there are also now many options available for generating them in-house. The scope of this Review is to provide an overview of these methodologies and the way they influence the ease, efficiency or kinetics of reprogramming, as well as their expected effects on the genome, epigenome and transcriptome of the pluripotent lines generated. We will see that, as with choosing the appropriate menu for a specific diet, different reprogramming strategies are appropriate

Table 1 | **Considering reprogramming in the light of downstream applications**

Application	Species of choice	Donor cell type	Reprogramming cocktail	Delivery mode	Recommendations
Study reprogramming mechanism	Mouse	Cells from chimeric mice from iPSCs obtained using an inducible system (secondary iPSC setup)	OSK/OSKM* as reference, any additional factor possible	Inducible lentivirus	To understand reprogramming, compare as many factors and cell types as possible
Study pluripotency/ differentiation	Mouse/ human	MEFs/fibroblasts	OSK/OSKM; OSNL‡	Retrovirus; RNA?	To understand pluripotency and improve differentiation protocols, reliable and reproducible reprogramming methods are best. Non-integrative methods may reduce genetic heterogeneity among cell lines
Disease modelling and drug screening	Human/ pig	Reprogrammable cells easily available from patients or cell repository	OSK/OSKM; OSNL	Retrovirus; RNA?	The starting cell population may be limited, so efficient methods are needed to generate models. Safety is not a crucial issue but avoiding integration would reduce genetic heterogeneity among cell lines
Cell therapy	Human	Reprogrammable cells easily available from patients, cell repository or HLA-matched iPSCs obtained from cord blood	Need to avoid potent oncogenes or inhibitors of tumour supressors	Non-integrative	When generating cells for transplantation into patients, safety is the major issue. Non-integrative methods still need to be compared in terms of iPSC quality and differentiation, as well as efficiency

Depending on the specific purpose of reprogramming, a series of choices have to be made when considering how to generate induced pluripotent stem cells (iPSCs). HLA, human leukocyte antigen; MEFs, mouse embryonic fibroblasts. *The OSK and OSKM combinations were developed by Shinya Yamanaka. OSK describes the combination of the transcription factors *OCT4*, *SOX2* and *KLF4*, and OSKM describes the combination of *OCT4*, *SOX2*, *KLF4* and *MYC*. ‡The OSNL combination was developed by James Thomson. OSNL describes the combination of the *OCT4*, *SOX2*, *NANOG* and *LIN28* transcription factors, also known as the 'Thomson factors'.

for different studies, with the correct approach depending on the priorities of the specific application for which the cells are to be used (TABLE 1).

Because the iPSC field has been extremely prolific during the past few years, it is beyond the scope of this Review to give an exhaustive list of all existing approaches. Supplementary information S1–S4 (tables) provide more details, and we provide a comprehensive database of reprogramming experiments in human and mouse cells at http://intranet.cmrb.eu/reprogramming/home.html.

On the variability of reprogramming

Although direct reprogramming is conceptually and technically simple, it is an extremely slow and inefficient process influenced by several variables that affect its efficiency, reproducibility and the quality of the resulting iPSCs. Before choosing a reprogramming approach it is therefore important to identify these variables. Depending on the application, the appropriate protocol will not only have to take into account the efficiency but also the reproducibility or the quality of the reprogrammed cells. Although this is conceptually straightforward, there is as yet no clear consensus on how to properly measure reprogramming efficiencies (BOX 1), reproducibility or iPSC quality (BOX 2), making it difficult to properly evaluate these parameters[23–25]. Despite this caveat, some guidelines can be extracted from the literature, thereby allowing an estimate of the effect of these variables on reprogramming

The donor cell type

Reprogramming requires the delivery of certain factors into a specific cell type and their adequate expression under defined culture conditions for a period of time, which varies depending on the cell type, species and delivery method. Depending on the donor cell type, reprogramming is achieved with different efficiencies and kinetics. For example, 8–12 days are required to reprogramme mouse embryonic fibroblasts (MEFs) using retroviruses, whereas the same process takes 20–25 days for human foreskin fibroblasts (HFFs). So far, fibroblasts remain the most popular donor cell type, and were used in more than 80% of all reprogramming experiments published. As a result, several studies have analysed the reprogramming capacity of alternative cell types that are of particular interest owing to their ease of reprogramming (FIG. 1), availability or therapeutic relevance. Compared with fibroblasts, human primary keratinocytes transduced with OSKM reprogramme 100 times more efficiently and twofold faster. Moreover, these cells can be obtained simply by culturing a plucked hair[17]. Alternatively, cord blood CD133$^+$ cells require only OCT4 and SOX2 to generate iPSCs. In theory, their availability through cell banks could offer a logistic advantage over the use of other adult somatic cell types for the purpose of creating iPSC banks covering a consistent range of haplotypes[18].

The increase in reprogramming efficiency and/or decrease in factor requirement of specific donor populations are attributed to high endogenous levels of certain reprogramming factors — which obviates their expression in *trans* — and/or intrinsic epigenetic states that are more amenable to reprogramming. The first hypothesis is supported by the fact that neural progenitor cells, which express SOX2 endogenously, reprogramme in the absence of exogenous SOX2 (REFS 15,16) or with OCT4 alone[26].

The differentiation status of the starting cell type also affects reprogramming efficiency. For example, haematopoietic stem and progenitor cells generate 300 times more iPSC colonies than do terminally differentiated B and T cells[27]. The differences in reprogramming among cell types are not restricted only to the efficiency, but can also affect the quality of the iPSCs. For instance, iPSCs

OCT4
(Also known as POU5F1). A POU homeodomain transcription factor that has a crucial role in early embryonic development and is necessary for the maintenance of embryonic stem cell pluripotency.

SOX2
Transcription factor of the SRY-related HMG-box family involved in the regulation of embryonic development and in the determination of cell fate. SOX2 is required to maintain self-renewal of undifferentiated embryonic and neural stem cells.

KLF4
A member of the Krüppel-like family of zinc finger transcription factors that is involved in cell proliferation, differentiation and survival. KLF4 has both transcriptional activation and repression domains.

MYC
(Also known as c-MYC). *MYC* is among the most frequently dysregulated oncogenes in human cancer. This transcription factor controls the expression of hundreds of target genes, many of which are also oncogenes or tumour suppressors, and have roles in cell proliferation and the cell cycle.

Box 1 | **Efficiency of reprogramming**

At first glance, a simple way to evaluate different reprogramming protocols is to compare their efficiency. Two measures help in defining this concept: the ratio between the number of donor cells receiving the full set of reprogramming factors and the number of reprogrammed colonies generated; and the kinetics of induced pluripotent stem cell (iPSC) generation. Although these two variables might seem straightforward to determine, in practice it may be difficult to assess them, not only for technical reasons but also because of a lack of consensus in the community on how to measure them. For example, the efficiency of infection or transfection is usually estimated indirectly (the constructs usually lack a reporter gene) or the number of infections or transfections and the timing of plating of the cells varies between groups.

In addition, the effect of different reprogramming factors on cell proliferation is usually not evaluated and the time to complete the process is not equally estimated because the criteria for assessing successful reprogramming vary — for example, the simple appearance of embryonic stem cell-like, alkaline phosphatase (AP)-positive clones or colonies in which the endogenous expression of the transcription factors *OCT4* or *NANOG* is properly upregulated. This last aspect is particularly important because clear differences appear in the success of line establishment depending on the criterion used to identify reprogrammed clones. For instance, a higher number of 'false positives' is found among AP-positive clones than among *NANOG*-positive clones.

The difficulty in comparing reprogramming efficiencies is well illustrated when retroviral delivery of the transcription factors *OCT4*, *SOX2*, *KLF4* and *MYC* (OSKM) and OSK are performed in parallel in mouse embryonic fibroblasts and compared. Using OSKM, a larger number of colonies appears on the plate and does so earlier than when using OSK. However, unlike when OSK is used, many of these colonies contain partially reprogrammed cells that have not properly upregulated the expression of endogenous pluripotency genes such as *NANOG*, *OCT4* or *SOX2*. Depending on the criteria used to assess reprogramming (colony appearance, AP staining or *NANOG* expression), efficiency could be inaccurately estimated.

derived from mouse tail-tip fibroblasts have a higher tendency to form teratomas than do those derived from MEFs or hepatocytes[28]. The choice of cell type is therefore an important aspect to consider before starting any experiment. It will usually depend on cell availability and will affect the requirement for ectopic factors, the efficiency and kinetics of reprogramming, and the quality of the resulting iPSCs.

The reprogramming cocktail

Pluripotency. After choosing a starting cell type, one needs to select a cocktail of reprogramming factors (FIG. 1) and, if required, facilitating compounds. Many of the factors that induce reprogramming are genes that are normally expressed early during development and are involved in the maintenance of the pluripotent potential of a subset of cells that will constitute the ICM of the pre-implantation embryo and, later, the embryo proper. This is the case for OCT4, SOX2 and NANOG, which are core pluripotency transcription factors. When NANOG is expressed along with OSKM in mouse B cells, the time until colony appearance is reduced by half compared with that taken by OSKM alone[29]. When UTF1, another pluripotency transcription factor, is expressed with OSKM in human primary fibroblasts, more colonies with high levels of alkaline phosphatase are generated[30]. Similarly, when compared with OSK alone, the overall number of iPSC colonies is increased tenfold when the transcription factor SALL4 (which has been associated with pluripotency) is co-expressed in human fibroblasts[31]. The ectopic expression of these factors may allow the establishment of an embryonic-like transcriptional cascade that is sustained and stabilized by the reactivation of the endogenous core pluripotency network.

Cell proliferation. Other factors, such as MYC and KLF4, directly or indirectly affect cell proliferation. Telomerase reverse transcriptase (TERT) and the SV40 large T antigen (SV40LT), two proteins that have positive effects on proliferation, increase the appearance of ESC-like colonies when combined with OSKM[32]. The influence of cell-cycle regulators on reprogramming has also been highlighted using chemical compounds. Specific inhibition of the mitogen-activated protein kinase kinase (also known as MEK) signalling using a compound (PD0325901) increases the number of fully reprogrammed colonies obtained from neural precursor cells infected with *Oct4* and *Klf4* (REF. 33). MicroRNAs (miRNAs) are also known to influence pluripotency and reprogramming[34], and some miRNAs from the miR-290 cluster — called the ESC-specific cell-cycle regulating (ESCC) miRNAs — contribute to the unique cell cycle of ESCs[35]. The introduction of OSK plus miR-291-3p, miR-294 or miR-295 into *Oct4–GFP* reporter MEFs increases the number of GFP^+ colonies compared with OSK alone. miR-294 has the most marked effect, increasing the efficiency of reprogramming tenfold. These ESCC miRNAs are believed to be downstream effectors of MYC and show clear potential to enhance the production of mouse iPSCs[36]. Finally, some factors inhibit reprogramming barriers, such as senescence and apoptosis, and allow an increase in both the speed and efficiency of reprogramming; for example, in mouse cells, inhibition of p53 or members of its pathway using short hairpin RNAs or knockout alleles has this effect[30,37–41].

Epigenetics. Chromatin remodelling is a rate-limiting step during iPSC generation[42], and chemical compounds that alter DNA methylation or chromatin modifications improve reprogramming in various cell types. Treatment with the DNA methyltransferase inhibitor 5′-azacytidine or histone deacetylase (HDAC) inhibitors (such as hydroxamic acid (SAHA), trichostatin A (TSA) and valproic acid (VPA)) improves reprogramming in MEFs[42]. By combining the glycogen synthase kinase 3 inhibitor CHIR99021 with tranylcypromine (Parnate) — an inhibitor of lysine-specific demethylase 1 — human primary keratinocytes reprogramme with only *OCT4* and *KLF4* (REF. 43). Moreover, VPA enables the induction of pluripotency in neonatal HFFs and dermal fibroblasts with *OCT4* and *SOX2* alone[44]. During embryonic development, the G9a histone methyltransferase mediates the epigenetic repression of *Oct4* (REF. 45), which might explain why an inhibitor of G9a (BIX-01294) allows reprogramming of MEFs with only OCT4 and KLF4 (REF. 46). Butyrate also affects histone H3 acetylation and promoter DNA demethylation, and alters the expression of endogenous pluripotency-associated genes, including developmental pluripotency associated 2 (*DPPA2*)[47]. Vitamin C also significantly improves the reprogramming of MEFs and adult mammary gland fibroblasts, in part by alleviating cell senescence[48] and inducing DNA demethylation[49].

OSKM
Combination of the OCT4, SOX2, KLF4 and MYC transcription factors, also known as the 'Yamanaka factors'. This was the first combination that was reported to reprogramme somatic cells into a pluripotent state.

Cord blood
The fraction of blood remaining in the placenta and the umbilical cord after childbirth. Cord blood is a rich source of haematopoietic stem cells, which have been used extensively for transplantation in the treatment of diseases such as leukaemia and other cancers.

CD133$^+$ cells
Cells expressing the CD133 antigen, a 97 kDa glycoprotein composed of five transmembrane domains. This cell-surface marker is expressed by immature haematopoietic stem/progenitor cells but not their mature counterparts.

Box 2 | **Induced pluripotent stem cell quality**

To correctly evaluate a reprogramming strategy, one should rely on concrete and reproducible criteria to define the quality of the induced pluripotent stem cell (iPSC) lines that it can generate. These criteria relate, in part, to the resemblance of the iPSCs to embryonic stem cells (ESCs). The functional and molecular similarities between ESCs and iPSCs are well documented. Comparative analysis of genetically matched mouse ESCs and iPSCs reveals that, in terms of transcriptional and methylation profiles, iPSCs and ESCs are almost identical[109]. In most cases, however, iPSC lines are not genetically matched (particularly those generated from human samples) and have been reprogrammed and cultured in different conditions, which result in transcriptionally, epigenetically and phenotypically heterogeneous lines[110].

Moreover, although genomic integrity during and after reprogramming has still not been clearly assessed, it represents an important aspect in evaluating the final quality of an iPSC. When using viral or non-viral integrative vectors, the lines generated are unavoidably heterogeneous because each of them contains a specific set of randomly distributed transgene insertions[62], obscuring comparative genomic analysis and limiting their use in therapeutic set-ups.

Reprogramming also involves the overexpression of potent oncogenes — including the transcription factor *MYC* — which have a direct effect on genome stability. Furthermore, the observation that impairing important tumour suppressor pathways and DNA replication checkpoints improves iPSC production suggests that reprogramming exerts a selective pressure on cells in the initial donor population that are genetically or epigenetically impaired in these pathways.

The methods currently available need to be compared according to these criteria to allow for a proper evaluation of their effect on iPSC quality and the requirements linked to these cells' downstream use.

NANOG
A homeobox transcription factor expressed in undifferentiated cells, including fetal gonads (ovary and testis), inner cell mass and embryonic stem cells. NANOG expression in the inner cell mass prevents this from differentiating into extra-embryonic endoderm and trophectoderm.

Alkaline phosphatase
A hydrolase enzyme responsible for dephosphorylating molecules such as nucleotides, proteins and alkaloids under alkaline conditions. It is often used as marker of pluripotency.

p53
A tumour suppressor that responds to diverse cellular stresses by regulating genes involved in cell-cycle arrest, apoptosis, senescence, DNA repair and changes in metabolism. Downregulation of p53 improves reprogramming efficiency.

Moloney murine leukaemia virus
(MMLV). A retrovirus composed of an ssRNA genome replicating through a DNA intermediate that integrates into the host genome. MMLV infects only actively dividing cells.

The culture conditions

After deciding on the combination of factors that are best suited to a specific cell type, one needs to consider the conditions in which the cells will undergo reprogramming. For example, culture conditions, supportive cells and medium composition are all parameters that have been shown to modulate reprogramming efficiencies. Reprogramming under hypoxic conditions of 5% O_2 (similar to those found in some stem-cell niches, such as the bone marrow), instead of the atmospheric 21% O_2, increases the reprogramming efficiency of mouse and human cells by 40- and fourfold, respectively. When combined with VPA, the efficiency increases to 200-fold in mouse cells[50]. Supportive feeder cells secrete growth factors that are required for ESC survival and/or proliferation and inhibition of ESC spontaneous differentiation[51]. Marson *et al.*[52] have shown that adding medium conditioned by cells expressing WNT3a promotes the generation of iPSCs in the absence of MYC. Moreover, by testing different culture conditions, Okada *et al.*[53] found that serum-free medium (KK20) allows iPSCs to be obtained at an earlier time point.

Therefore, the questions that need to be considered before a specific reprogramming method is selected are: which cell type, which factors and which culture conditions should be used? Although multiple combinations are possible, in most cases the reprogramming of MEFs and neonatal human dermal fibroblasts is accomplished using OSKM and ESC culture conditions. Sometimes, *MYC* is eliminated or substituted by other factors because of the oncogenic risk associated with this gene. Additional factors or compounds that could improve the quality of the reprogrammed cells are sometimes added to the OSKM set, although their use should be properly assessed in each situation. Below, we describe the reprogramming methods currently available. We hope that the overview of considerations and options presented above will help readers to understand why some methods are better suited to some applications and which hurdles the protocol modifications try to overcome (FIG. 1; TABLE 1). Reprogramming methods can be divided into two classes, those involving the integration of exogenous genetic material and those involving no genetic modification of the donor cells.

Integrative delivery systems

Viral delivery systems. The delivery of the OSKM transcription factors into mouse or human fibroblasts was originally achieved using Moloney murine leukaemia virus (MMLV)-derived retroviruses (FIG. 2) such as pMXs[54–56], pLib[12] or pMSCV[17,57]. These vectors have cloning capacities of around 8 kb, allow delivery of genes into the genome of dividing cells and are usually silenced in immature cells such as ESCs[58,59]. Silencing is important because only an iPSC that has upregulated the endogenous pluripotency gene network and downregulated the expression of the transgenes can really be considered to be fully reprogrammed[60]. The vector, in which the reprogramming cDNA is cloned, provides a viral packaging signal, as well as transcription and processing elements. On transfection into a packaging cell line that expresses a specific viral envelope protein (which determines the range of cell types that can be infected), high titre, replication-defective viruses are produced; they can infect donor cells with efficiencies of up to 90%. The efficiency of iPSC generation using MMLV-derived retroviruses expressing each gene in the OSKM set separately is ~0.1% in mouse embryonic fibroblasts and ~0.01% in human fibroblasts.

Lentiviral delivery vectors (FIG. 2) have also been successfully used to express different sets of reprogramming factors in somatic cells[22,61]. They are generally derived from HIV, exhibit slightly higher cloning capacities (8–10 kb) and usually have higher infection efficiency than MMLV-based retroviruses. Moreover, they allow infection of both dividing and non-dividing cells. The efficiency of reprogramming using lentiviral vectors is comparable to that with MMLV-derived retroviruses. Compared with MMLV-derived vectors, lentiviruses are less effectively repressed in pluripotent stem cells[62]; this can complicate the identification of bona fide iPSC clones[60]. Although this issue could not be addressed using constitutive lentiviral vectors[22,61], Tet-inducible reprogramming lentiviruses allow expression of the reprogramming factors in a controllable manner. Although their preparation is slightly more complicated and time-consuming than that for MMLV-derived retroviruses, the main advantage they present is their availability as inducible systems.

Although they are efficient and reproducible, reprogramming using viruses entails the production of potentially harmful viral particles that express potent oncogenes such as *MYC*. iPSC lines generated using these vectors carry randomly distributed viral transgene insertions[63], which could disrupt the expression of tumour suppressor genes if there are insertions in the open reading

frames or alter the expression of oncogenes if inserted nearby. Moreover, they unavoidably generate heterogeneous iPSC lines, which could complicate comparative analysis. Even if properly silenced, viral transgenes can eventually be reactivated during differentiation or during the life of iPSC-derived or transplanted animals, leading to tumours[11]. These tumours result either from basal expression levels of the *MYC* transgenes or other oncogene-related factors (if present in the reprogramming set) or tissue-specific reactivation of these transgenes owing to promoter- or enhancer-trapping events. The use of Cre-deletable[64] or inducible lentiviruses has solved some of these problems[65], but viral systems still lack the safety required for therapeutic applications.

Transfection of linear DNA. If aiming to avoid the use of viral vectors, standard DNA transfection using liposomes or electroporation is a good alternative (FIG. 3). Compared with viruses, however, transduction efficiency is much lower; substantially fewer donor cells receive the full set of reprogramming factors. A crucial improvement has been the design of polycistronic vectors that allow the expression of several cDNAs from the same promoter. These constructs include self-deleting 2A peptide sequences (~20 amino acids long) from the foot-and-mouth disease virus (FMDV) or other picornaviruses[66,67]. When cloned in between different cDNAs, 2A peptide sequences allow ribosomes to continue translating the downstream cistron after releasing the first protein with its carboxyl terminus fused to 2A. This results in the expression of almost stochiometric amounts of each protein encoded by the polycistron. Such a system has been successfully tested in ESCs[68].

Using a linearized 2A-peptide-based polycistronic vector flanked by *loxP* sites, Kaji and colleagues successfully reprogrammed mouse fibroblasts. Approximately 10% of the lines they generated showed single insertions of the construct, indicating that single-copy polycistronic OSKM expression cassettes are sufficient to achieve direct reprogramming. By transiently expressing the Cre recombinase, they then induced recombination between the *loxP* sites to delete the reprogramming construct[69]. Such a system is appealing for its simplicity; however, owing to the low percentage of cells transfected with the reprogramming construct and the inherent low efficiency of reprogramming, it requires a large number of donor cells, which may be difficult to obtain for certain cell types. Moreover, obtaining transgene-deleted iPSCs is not necessarily straightforward because many of the colonies with the deletion start to differentiate; this indicates that many clones obtained using this system represent reprogramming intermediates[69]. The main advantage of this approach is the possibility of deleting the reprogramming cDNAs in iPSCs, which would improve their differentiation potential and, perhaps more importantly, avoid the reactivation or constitutive expression of the reprogramming factors, thus, in theory, reducing their oncogenic potential.

The observation that polycistronic vectors allow reprogramming of somatic cells through a single insertion also encouraged some researchers to include them

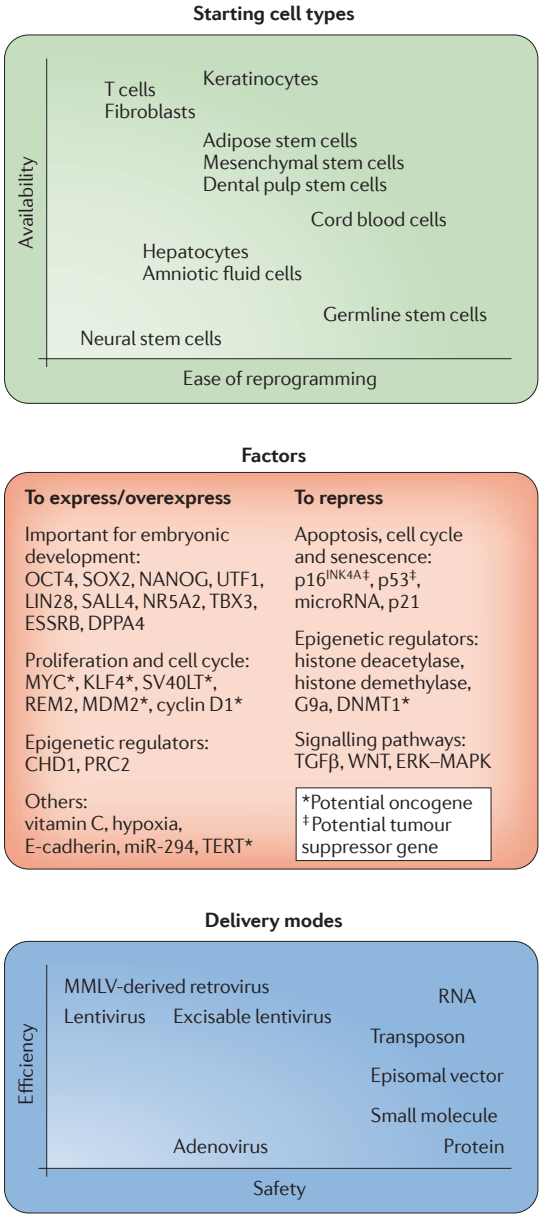


Figure 1 | **The reprogramming menu.** Any reprogramming experiment is determined by a number of preliminary choices regarding the donor cell type to reprogramme, the factors to use and the mode of their delivery. These choices depend not only on the availability of the cells but also on the purpose that the reprogrammed cells will serve. This figure shows how cell type, reprogramming factors and delivery method might each be evaluated and chosen. Information on the factors that are not commented on directly in the main text can be found using this online database: http://intranet.cmrb.eu/reprogramming/home.html. CHD1, chromodomain-helicase-DNA-binding protein 1; DNMT1, DNA methyltransferase 1; DPPA4, developmental pluripotency associated 4; E-cadherin, epithelial cadherin; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MMLV, Moloney murine leukaemia virus; PRC2, Polycomb repressive complex 2; SV40LT, SV40 large T antigen; TERT, telomerase reverse transcriptase; TGFβ, transforming growth factor-β.

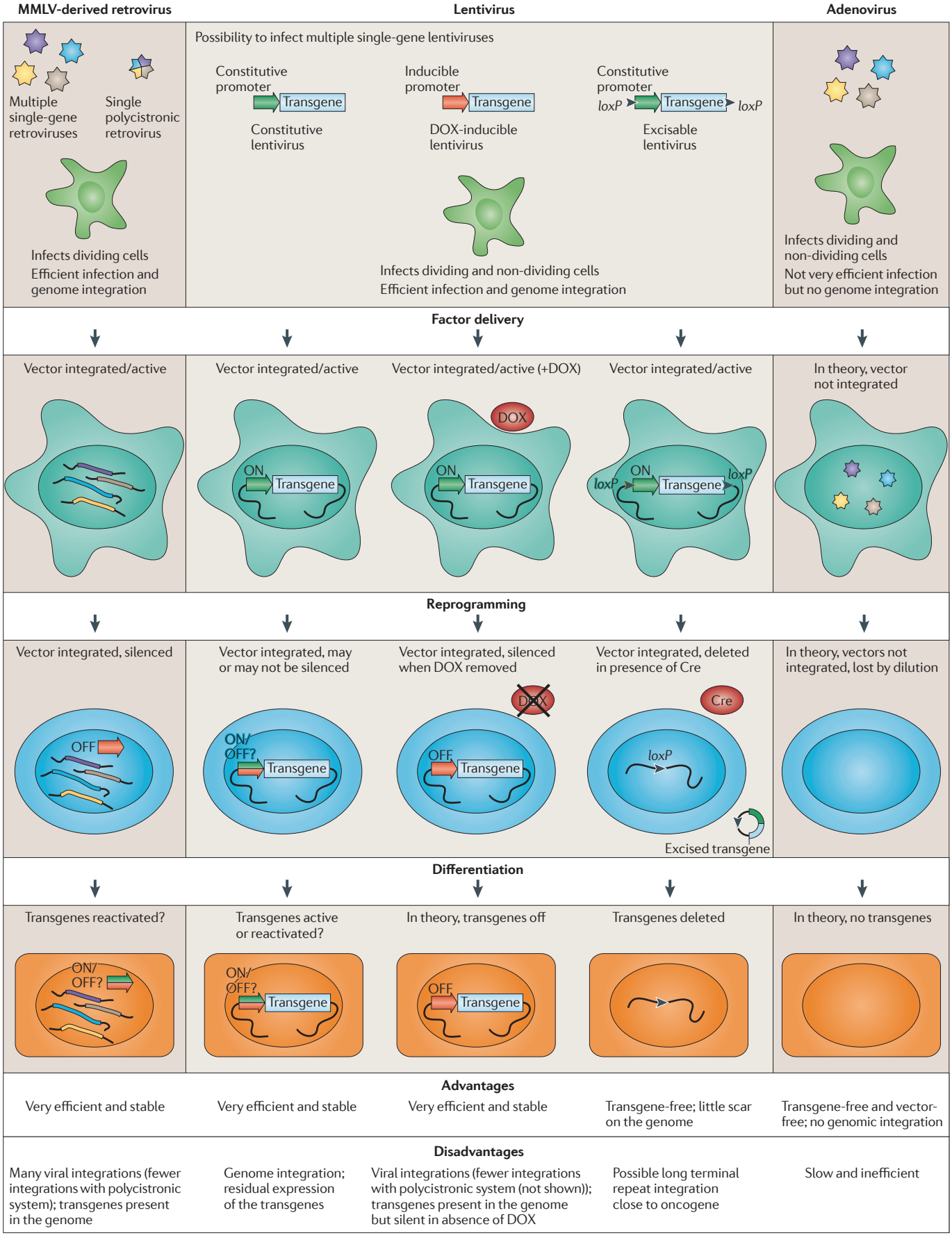
Transfection
Delivery of nucleic acids (plasmid DNA, linear DNA or RNA) into cells by a non-viral method. Common transfection methods include calcium phosphate treatment, electroporation, nucelofection and the use of cationic lipid vehicles.

Lentiviruses
A genus of retroviruses with long incubation periods that cause chronic, progressive and usually fatal diseases, such as HIV in humans. They are the only retroviruses that are able to replicate in non-dividing cells.

Tet-inducible
An inducible promoter system based on the tetracycline operon, which is present in a variety of vectors. In Tet-OFF vectors, gene expression is turned on when tetracycline or doxycycline is removed from the culture medium, whereas Tet-ON systems are induced only when doxycycline is added.

Cre
Cre is a 38-kDa type I topoisomerase protein from bacteriophage P1 that catalyses site-specific intramolecular (excision or inversion) and intermolecular (integration) recombination between *loxP* sites. The *loxP* site consists of two 13 bp inverted repeats separated by an 8 bp asymmetric spacer region.

Polycistronic
A transcription unit made up of several open reading frames, resulting in the translation of separate proteins. Internal ribosome entry site or 2A-peptide sequences allow such multigene expression constructs to be engineered.



◀ Figure 2 | **Viral delivery methods.** A flow diagram summarizing the main viral delivery methods, with their advantages and caveats shown below. For each of the methods, the design of the vector is shown at the top, followed by the status of the cell after initial delivery of the vector. The blue cells show the status of the vector in reprogrammed cells (induced pluripotent stem cells). The bottom level (orange cells) shows what might happen to the transgenes after the pluripotent cell is differentiated. The methods are described in more detail in the text. DOX, doxycyclin; MMLV, Moloney murine leukaemia virus.

in integrative MMLV-derived retroviral vectors[70] and lentiviral vectors[71,72] (FIG. 2), substantially reducing the number of genomic insertions compared with single-factor-expressing viruses. By including *loxP* sites, such vectors represent an easy way to induce transgene-free iPSCs from various donor sources with higher transduction efficiencies than naked DNA[73]. These vectors eliminate the oncogenic risk related to transgene reactivation and have a positive effect on the differentiation potential of the resulting iPSCs[73].

piggyBac transposon. To enhance the stable integration of non-viral constructs, Kaji and others moved to vectors based on the *piggyBac* (*PB*) transposon[74] (FIG. 3). The PB transposase is active in mouse[75] and human ESCs[76], and mediates a higher genome integration efficiency than random integration of linearized plasmids. The reprogramming system includes the PB transposase that mediates gene transfer and a transposon containing the sequence of interest flanked by the 5′ and 3′ terminal repeats required for transposition[74,77,78]. The *PB* system is usually composed of a donor plasmid containing the transposon, co-transfected with a helper plasmid expressing the transposase[74–76,79]. Cre-excisable linear transgenes leave a genomic scar, including the *loxP* site, after Cre deletion, whereas *PB*s are, in theory, precisely deleted without modifying the sequence of the integration site upon remobilization by the transposase. Using *PB*-based reprogramming vectors, a number of groups have induced mouse and human pluripotent stem cells from fibroblasts and subsequently deleted the transgenes[80,81], thus leading to theoretically genetically unmodified iPSCs. Among integrative methodologies, this approach is the only one that guarantees a precise deletion of the transgenes, although alterations are sometimes observed in the insertion sites, which therefore need to be sequence-verified[32].

In Cre or FLP recombination, recombination in *cis* between the target sites is highly favoured compared with recombination in *trans*, which leads to a unidirectional reaction and loss by dilution of the circular deleted fragment; by contrast, the PB transposase promotes deletion and integration at similar efficiencies, allowing the transposon to 'jump' from site to site until the transposase is expressed. As a result, the expression window of the PB transposase needs to be tightly controlled because long exposure times lead to several rounds of excision-integration, increasing the risk of non-conservative deletion. To reduce such risk and facilitate the isolation of cells without the transposon, it is highly recommended that negative selection genes, such as thymidine kinase (*Tk*), are included in the transposon.

Integrative delivery systems can enable efficient generation of iPSCs with single transgene insertions, which can be deleted after reprogramming. Deletion lowers the risks of insertional mutagenesis or oncogenesis (by precluding *MYC* reactivation) and improving the differentiation capacity of iPSCs (preventing basal expression of the core pluripotency reprogramming transcription factors *OCT4*, *SOX2* or *NANOG*). Although these are major improvements in terms of the safety and quality of iPSCs, their possible effects during the reprogramming process in terms of genomic stability or possible aberrant epigenetic remodelling still need to be evaluated.

Non-integrative approaches

Non-integrative approaches address a major limitation of iPSCs: the permanent genetic modification resulting from the integration of classic retroviral or lentiviral vectors, or the genomic scars left behind by deletion of Cre-deletable viral vectors, naked DNA transgenes or non-conservative transposon remobilization. The different approaches that are currently available can be subdivided into four main categories: integration-defective viral delivery (FIG. 2), episomal delivery, RNA delivery and protein delivery (FIG. 3). Although the kinetics of reprogramming vary between different starting cell types and species, the generation of stable iPSCs usually requires several weeks to complete. Depending on the starting population, some of the non-integrative approaches are difficult to apply owing to poor infection or transfection efficiencies, poor cell survival, long reprogramming kinetics or other limitations. These considerations underline one of the major drawbacks of these methodologies: they are usually inefficient and poorly reproducible, which is the principal reason why no consensus has yet been reached in the community regarding a method of choice.

Integration-defective viral delivery. One of the first attempts to generate integration-free iPSCs was reported by Stadtfeld *et al.*[82], who used a replication-defective adenoviral vector, pHIHG-Ad2. The authors cloned the OSKM set as single factors into pHIHG-Ad2 and were able to generate transgene-free iPSCs after infection of mouse hepatocytes with adenoviral particles. However, for mouse fetal liver and adult fibroblasts, the authors were able to obtain transgene-free iPSCs only when the vectors carrying *Sox2*, *Klf4* and *Myc* were complemented in *trans* by a stably integrated inducible *Oct4* transgene[82], owing to low infection efficiencies or the transcriptional status of these donor cells. The authors also identified several tetraploid iPSC clones derived from mouse fetal liver cells and mouse adult hepatocytes, probably reflecting the level of endogenous polyploidy of the liver[83,84]. Using similar vectors, Zhou *et al.* generated diploid transgene-free iPSC lines from human fetal fibroblasts[85]. The efficiency of iPSC generation using this system in the mouse ranges between 0.0001% and 0.0018%, which is approximately three orders of magnitude lower than that for retroviruses.

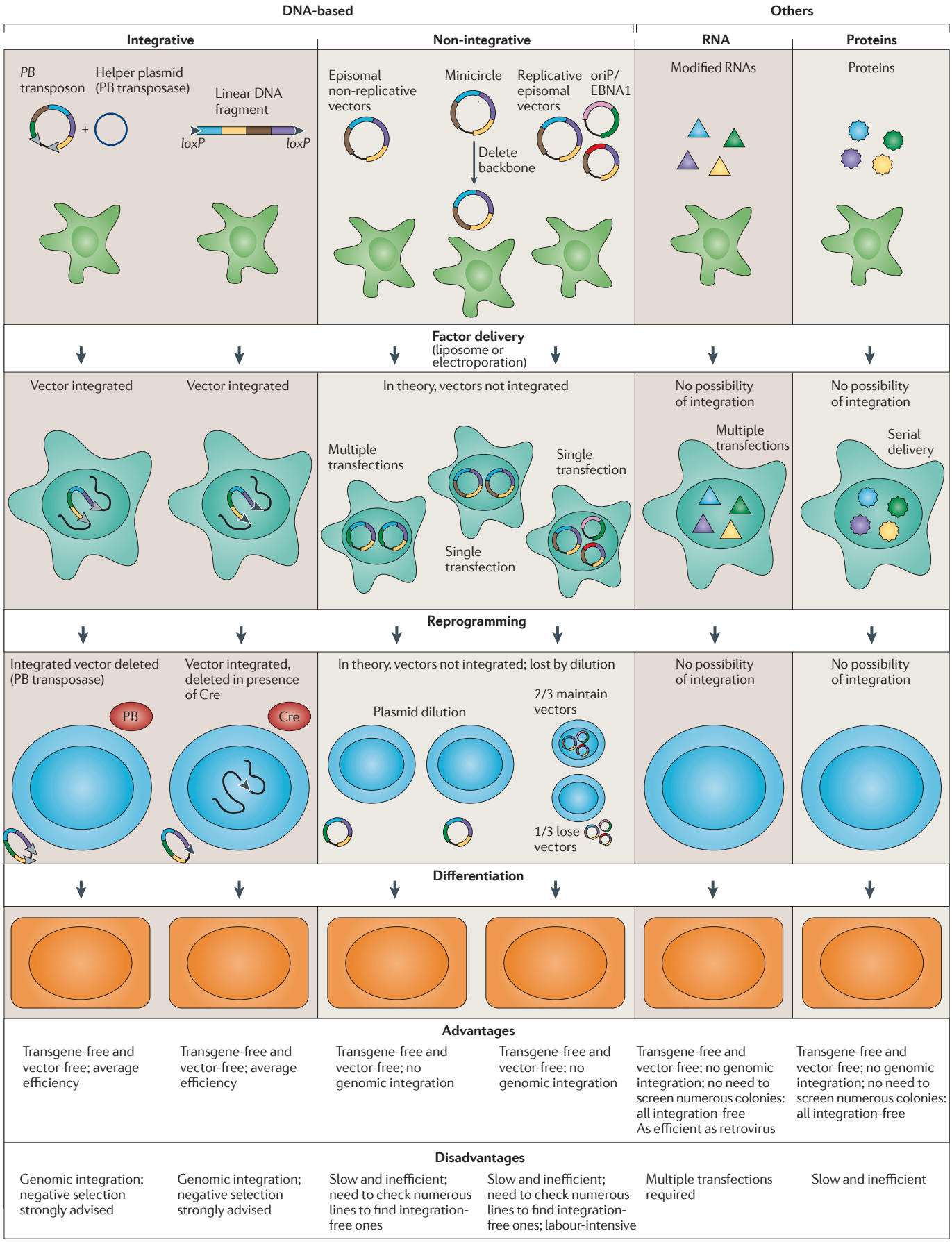
Human fibroblasts and terminally differentiated circulating T cells have also been successfully reprogrammed using F-deficient Sendai viral vectors[86,87]. These

piggyBac
(*PB*). A TTAA-specific transposon, originally described in the order Lepidoptera. This mobile genetic element stably transfers exogenous DNA into a variety of cells. The *PB* system is composed of a donor plasmid, co-transfected with a helper plasmid expressing the transposase. Once integrated, *PB*s can be precisely deleted upon remobilization by the transposase.

FLP
The FLP recombination system, derived from the 2μ plasmid of *Saccharomyces cerevisiae*, mediates site-specific intramolecular (excision or inversion) and intermolecular (integration) recombination between *FRT* sites. The *FRT* site consists of two 13 bp inverted repeats separated by an 8-bp asymmetric spacer.

Adenoviral vector
A vector based on adenoviruses, which are medium-sized viruses with a double-stranded linear DNA genome. Recombinant adenoviral vectors allow transient, high-level expression of exogenous genes without integrating into the host genome.

Sendai viral vector
A vector based on a negative sense, ssRNA paramyxovirus. F-deficient Sendai viral vectors replicate in the form of negative-sense ssRNA in the cytoplasm of infected cells, allowing the transfer of foreign genetic material.



◀ Figure 3 | **Non-viral delivery methods.** A flow diagram summarizing the main non-viral delivery methods, with their advantages and caveats shown below. DNA-based delivery methods include those that do or do not involve integration into the genome. For each of the methods, the design of the vector is shown at the top, followed by the status of the cell after initial delivery of the vector. The coloured bars represent the transgenes. The blue cells show the status of the vector in reprogrammed cells (induced pluripotent stem cells). The bottom level (orange cells) shows cells after differentiation — in each case the cells should be transgene-free. The methods are described in more detail in the text. The use of small molecules that accelerate or replace the action of reprogramming factors is not included in this figure; these could be added to any delivery method described in this figure or in FIG. 2. PB, piggyBac; oriP/EBNA1, oriP/Epstein–Barr nuclear antigen-1-based episomal vector.

vectors efficiently transfer foreign genes into a wide range of host cells[88] and replicate in the form of negative-sense ssRNA in the cytoplasm of infected cells[89,90]. Using Sendai viral vectors expressing OSKM, Fusaki *et al.*[86] generated iPSC lines and were able to isolate a few clones that showed no presence of viral RNA. Although an appealing method, the viral RNA replicase of these vectors is extremely sensitive to transgene sequence content. Furthermore, because these viral vectors replicate constitutively, they are difficult to eliminate from host cells, making it challenging to properly isolate transgene-free clones, even at high passage numbers[86]. High passage numbers also increase the probability of generating aneuploid iPSC lines owing to longer exposures to *Myc*.

Transient episomal delivery. As an alternative to integration-defective viruses, some authors have developed reprogramming strategies based on direct delivery of non-replicating[91–93] or replicating episomal vectors[94]. These methods are appealing because they are relatively simple to implement with a standard laboratory set-up and molecular biology experience, avoiding the time-consuming and labour-intensive production of viral particles.

By serial transfection of two plasmids expressing, respectively, OSK and *Myc*[91,95], or a single plasmid expressing the full OSKM set as a polycistron[92], iPSCs that showed no sign of plasmid integration were obtained from MEFs. Using such methods, only a low percentage (for example, 33%[91] and 8%[92]) of the iPSC lines generated are free of plasmid integration (FIG. 3). There are several possible reasons for this, including: low transfection efficiencies for large plasmids (5–10 kb) that result in few cells receiving the appropriate dose of plasmid over the full reprogramming period; premature dilution of the vectors in actively proliferating cells; or the active silencing of prokaryotic sequences contained in the backbone of these vectors in mammalian cells, leading to downregulation of the reprogramming factors[96]. These reasons probably explain this method's failure to produce iPSCs from HFFs or keratinocytes, because these cell types require sustained expression of OSKM for a longer duration than do MEFs to reach pluripotency (J.C.I.B. and F.G., unpublished data).

To circumvent the need for serial transfection and to solve the problem of episome dilution through cell division, Yu and colleagues used oriP/Epstein–Barr nuclear antigen-1-based episomal vectors (oriP/EBNA1)[94] (FIG. 3). oriP/EBNA1 vectors are maintained through cell division and under selection conditions as stable extra-chromosomal replicons that require only a *cis*-acting oriP element[97], a trans-acting *EBNA1* gene and a positive selection gene[98]. These vectors can be transduced into donor cells using standard transfection procedures and can be removed by culturing the cells in the absence of drug selection. By co-transfecting three oriP/EBNA1 vectors expressing respectively, *OCT4–SOX2–NANOG–KLF4*, *OCT4–SOX2–SV40LT–KLF4* and *MYC–LIN28*, and in the absence of any drug selection, Yu and colleagues successfully generated iPSC colonies from HFFs. Analysis of the derived subclones revealed that one-third of them were devoid of plasmid DNA. The reprogramming efficiency of human fibroblasts using oriP/EBNA1 vectors is, however, extremely low (3 to 6 colonies per million cells nucleofected), which may reflect the low transfection efficiency of such large plasmids (more than 12 kb), their gradual loss through cell division in the absence of drug selection or active silencing through DNA methylation, resulting in low levels of expression of the reprogramming factors. A major concern about this system is the use of the SV40LT antigen as one of the reprogramming factors. Because this potent viral oncoprotein is able to inactivate both the p53 and the retinoblastoma pathways, the result could be the generation of iPSC lines with higher tumorigenic potential. This aspect still needs to be properly addressed.

In order to decrease the size of the reprogramming episomes and delete potentially methylatable prokaryotic backbone sequences, minicircle vectors represent an interesting solution that allows the expression of the reprogramming factors as non-integrating, non-replicating episomes[93]. These vectors are supercoiled DNA molecules that lack a bacterial origin of replication and antibiotic resistance gene because their backbone is removed by PhiC31-mediated intramolecular recombination before purification[99,100]. Compared with plasmids, minicircle vectors show higher transfection efficiencies (their size is usually reduced by at least by 3 kb, the average size of the backbones usually found in episomal vectors) and longer ectopic expression of the transgenes due to lower activation of exogenous DNA-silencing mechanisms[99,100]. By cloning a 2A-peptide-based polycistronic cassette including *OCT4*, *SOX2*, *LIN28* and *NANOG* (OSLN), plus a GFP reporter gene in a single minicircle vector, Jia and colleagues[93] reprogrammed human adipose stem cells in 14–16 days with an average efficiency of ~0.005%. Subsequent Southern blot analysis suggested that none of these iPSC lines carried integration of the minicircle vectors, although more sensitive assays for integration, such as PCR, were not performed[93].

RNA delivery. In order to completely eliminate plasmid or viral vectors, Warren *et al.*[101] developed a system that achieves the efficient conversion of different human somatic donor cells into iPSCs using direct delivery of synthetic mRNAs (FIG. 3). The efficiency reached with this approach is much higher than that achieved with other non-integrative systems, with 2% of neonatal fibroblasts being converted into iPSCs in just 17 days. This system

Episome
An extrachromosomal DNA element that can replicate within a cell independently of the chromosome. Commonly used episomal vectors (also referred to as plasmids) contain an origin of replication and an antibiotic resistance cassette, allowing propagation in bacteria.

LIN28
Human LIN28 is a cytoplasmic RNA-binding protein containing an amino-terminal cold-shock domain and two carboxy-terminal CCHC zinc finger domains. It is expressed in various undifferentiated embryonic cell types, as well as adult cardiac and skeletal muscle cells. The expression of LIN28 is regulated by microRNAs.

PhiC31
The PhiC31 (ΦC31) integrase from bacteriophage PhiC31 is a serine-type site-specific recombinase that mediates the recombination between the heterotypic target sites *attB* and *attP*. Unlike Cre or FLP, this system allows irreversible deletion, inversion or integration between its target sites.

requires modification of *in vitro* transcribed RNAs in order for them to escape the endogenous antiviral cell defence response to ssRNA. Phosphatase treatment, incorporation of modified ribonucleoside bases substituting 5-methylcytidine for cytidine and pseudouridine for uridine, combined with the addition of a recombinant version of B18R protein in the medium, allowed for high, dose-dependent levels of protein expression with high cell viability. By delivering synthetic RNAs encoding OSKM and *Lin28*, reprogramming was achieved by serial transfection of different donor populations using a cationic vehicle[101]. Although this system is extremely appealing for its simplicity and efficiency, the high gene dosages of the reprogramming factors resulting from direct mRNA delivery may represent an oncogeneic risk owing to higher expression levels of *Myc* affecting genomic stability. A similar delivery method that avoids this potent oncogene would represent an improvement, although a direct assessment of the mutation load of iPSCs generated using this approach will be required, as for any of the approaches described here.

Protein delivery. Another way to avoid the introduction of exogenous genetic material into donor cells is to deliver the reprogramming factors as proteins (FIG. 3). Several studies have demonstrated that proteins can be delivered directly into cells *in vitro* and *in vivo* when fused with peptides mediating their transduction, such as HIV transactivator of transcription (Tat) and poly-arginine[102–104]. Using this approach, Zhou *et al.* generated recombinant OSKM proteins fused with a poly-arginine transduction domain. They expressed these engineered proteins in *Escherichia coli* in inclusion bodies; the proteins were then solubilized, refolded and further purified. By serial transduction of *Oct4–GFP* reporter MEFs with OSKM or OSK recombinant proteins, the authors obtained GFP^+ colonies if the HDAC inhibitor VPA was also added to the media[105]. Similarly, Kim and co-workers fused each of the OSKM factors with a myc tag and a tract composed of nine arginines. They generated four stable HEK293 cell lines expressing each of the four human recombinant reprogramming factors and applied the extracts of these cells to human neonatal fibroblasts for 8 hours per week, for a total of 6 weeks. They were able to obtain iPSC colonies after dissociation and replating on MEF feeder cells[106]. Although promising, protein-based strategies show extremely slow kinetics and poor efficiencies. Moreover, the recombinant proteins used in these approaches are usually difficult to reproducibly purify in the required amounts, making them difficult to use routinely in the laboratory.

Strategic choices and future directions

At present in the iPSC field, it is still difficult to choose a reprogramming strategy that is fitting for all purposes. This can be illustrated by considering two different research programmes: the first focused on deciphering the mechanisms of reprogramming, the second intended to generate clinically relevant iPSCs. In the first situation, the reprogramming approach needs to be robust and efficient; a delivery method and a combination of factors that can achieve this is required, regardless of the presence of genomic modifications. For example, using integrative inducible lentiviruses and subsequently deriving secondary iPSCs will meet these requirements. By contrast, the second case requires a non-integrative or semi-integrative approach in order to avoid or control genomic modifications. Multiple methods have been proposed, but all are inefficient. An RNA-based approach published recently[101] seems promising owing to the high efficiency achieved. In any case, the choice of starting-cell population will depend on the availability of the cell type, the ease with which it is reprogrammed and the efficiency it yields. Importantly, the use of 'safe' approaches does not necessarily prevent variability in the expression levels of lineage-specification genes or the occurrence of aberrant epigenetic remodelling, which may limit downstream applications of iPSC technology.

The improvement in reprogramming efficiency and/or kinetics that can be achieved with small molecules makes them an attractive avenue for further research, although they must be treated with caution because some can be tumorigenic (for recent reviews, see REFS 107,108). Small molecules represent a powerful alternative or support for reprogramming because they can target different cellular pathways that control cell fate, state and function, but their specificity is sometimes difficult to assess. Their progressive introduction in reprogramming protocols and/or implementation of large-scale screens will probably be key to identifying new pathways that might allow the replacement of current reprogramming factors or that might have a positive effect on iPSC generation.

Since the first published demonstration that fibroblasts can be reprogrammed by retroviral delivery of just four factors (OSKM), a substantial number of alternative approaches have been developed to induce pluripotency in somatic cells. To properly assess the improvement that each of the methods provides and to give a more precise idea of their real contribution to reprogramming, it will be crucial to test them using commonly accepted standards. In addition to the use of oncogenes in reprogramming cocktails and the issue of viral integration, reprogramming itself may have an effect on a cell's genome, especially given that the process takes many weeks and is rather inefficient. The low efficiency and slow kinetics might subject cells to detrimental alterations, such as the accumulation and/or selection of subtle genetic and epigenetic abnormalities before or during reprogramming, which could favour the activation of growth pathways and the inhibition of tumour suppressor pathways (F.G. and J.C.I.B., unpublished data). A crucial challenge in the iPSC field will be to properly determine how these various methodologies affect the quality of iPSCs in terms of transcriptional signatures, epigenetic status, genomic integrity, stability, differentiation and tumour potential. Whole-genome sequencing platforms will probably play an important part in the future in assessing the integrity of the genome of iPSCs and will certainly improve our understanding of the mechanism by which reprogramming occurs in a specific cell type.

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Competing interests statement
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