TIMELINE

A decade of transcription factor-mediated reprogramming to pluripotency

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Abstract | The past 10 years have seen great advances in our ability to manipulate cell fate, including the induction of pluripotency *in vitro* to generate induced pluripotent stem cells (iPSCs). This process proved to be remarkably simple from a technical perspective, only needing the host cell and a defined cocktail of transcription factors, with four factors — octamer-binding protein 3/4 (OCT3/4), SOX2, Krüppel-like factor 4 (KLF4) and MYC (collectively referred to as OSKM) — initially used. The mechanisms underlying transcription factor-mediated reprogramming are still poorly understood; however, several mechanistic insights have recently been obtained. Recent years have also brought significant progress in increasing the efficiency of this technique, making it more amenable to applications in the fields of regenerative medicine, disease modelling and drug discovery.

In the late 19th century, August Weismann described a genetic theory known as the Weismann barrier[1]. He postulated that because inheritance is only mediated by germ cells (that is, eggs and sperm), unnecessary genetic codes must be deleted or terminally inactivated in somatic cells that are committed to a specific state. As a result of this theory, in the mid-20th century, Conrad Waddington developed a model that depicts normal embryonic development as a ball rolling downhill to its final differentiated state[2] (FIG. 1). Similar to Weismann's theory, this so-called Waddington's landscape model proposed that the destiny of lineage-committed cells was permanent. Shortly thereafter, however, in 1962, Sir John Gurdon reported the first example of cellular reprogramming through somatic cell nuclear transfer (SCNT)[3,4] (FIGS 1,2). During SCNT, the nucleus of a somatic cell is transferred to an enucleated egg, which then starts to divide, generating an embryo that is genetically identical to the donor of the somatic cell. This demonstrated that the nuclei of somatic cells maintain all genetic information, and that they can be reprogrammed to an embryonic, pluripotent state (that is, rejuvenated) by experimental manipulation. Gurdon's discovery was followed by limited progress for 40 years in understanding nuclear reprogramming (FIG. 2). During this time, however, Sir Martin Evans, Matthew Kaufman and Gail Martin established pre-implantation embryo-derived self-renewable cell lines, which Martin named embryonic stem cells (ES cells). These lines have an inherited developmental capacity to generate all cell types in our body, a property known as pluripotency, and they changed the world of developmental biology[5,6]. Finally, in the late 20th century, several groups reported cloned animals, such as sheep and mice, which were developed using SCNT, thereby expanding the applications of SCNT beyond frogs[7,8].

Parallel studies revealed that the profile of gene expression in somatic cells can be changed through fusion with other cell types, thus causing reprogramming of these cells. This was first documented in 1983, with experiments showing that silenced muscle-specific genes in human amniocytes are activated after cell fusion with mouse muscle cells (to generate so-called heterokaryons)[9]. In the same year, it was demonstrated that inactivated X chromosomes in female somatic cells, such as thymocytes or bone marrow cells, could be reactivated by fusion with teratocarcinoma-derived cells in which both X chromosomes were active[10]. Others reported that by cell fusion with pluripotent cells such as ES cells, somatic cells (for example, fibroblasts and T lymphocytes) could be epigenetically reprogrammed to express pluripotency-associated genes that are predominantly expressed in pluripotent cells *in vitro* and/or *in vivo*, including octamer-binding protein 3/4 (OCT3/4; also known as POU5F1)[11,12] (FIG. 1). This suggested that pluripotent stem cells (PSCs) have the potential to reprogramme somatic cells toward pluripotency. Thus, these landmark reports not only presented the prospect of somatic cell rejuvenation, but also suggested the existence of one or more reprogramming factors that can 'erase' the 'memories' of somatic cells.

Further evidence of the existence of reprogramming factors came from studies showing direct fate conversion of mammalian cells by introducing a single transcription factor[13]. cDNA subtraction to identify novel myoblast-specific genes led to the discovery of three genes that were expressed predominantly in proliferative myoblasts. The ectopic expression of one of them, myoblast determination protein (MYOD), alone was sufficient to transform mouse fibroblasts to myoblasts, which expressed myoblast marker genes such as myosin (FIG. 1). Years later, it was demonstrated that the ectopic expression of erythroid transcription factor GATA-binding protein 1 (GATA1) could convert myeloblasts to megakaryocyte and erythrocyte precursors[14] (FIG. 1). In addition, CCAAT/enhancer-binding protein-α (CEBPα) or CEBPβ could convert B lymphocytes to macrophages[15] (FIG. 1). This process of converting somatic cells into a different somatic lineage is known as transdifferentiation.

Transdifferentiation has also been achieved in *Drosophila melanogaster*. For instance, a mutation in the homeotic gene *Antennapedia* was found to be responsible

for converting an antenna into a leg[16–21], and the targeted expression of a gene homologous to the mammalian paired box 6 (*PAX6*) gene induced ectopic eye structure formation in mutant *D. melanogaster* lacking eyes[22]. These studies provided strong evidence that transcription factors, especially the master regulators of cell identity, can modify cell fate.

The confluence of all of these works led to the seminal discovery of induced PSCs (iPSCs) (FIG. 2), which are pluripotent cells that are derived from somatic cells, and that functionally bear a striking resemblance to ES cells. The discovery of iPSCs has also paved the way for direct cell fate conversion (also known as transdifferentiation) experiments, in which the expression of sets of tissue-specific transcription factors can induce a different fate, across germ layers and by bypassing the pluripotent state, opening new doors in the field of cellular reprogramming. iPSCs are promising for many applications, including drug discovery, disease modelling and regenerative medicine[23] (FIG. 2).

In this Timeline article, we first introduce how reprogramming factors were discovered and how they led to the first report of iPSCs 10 years ago. We also describe other factors that contribute to transcription factor-mediated reprogramming and outline the current knowledge of such mechanisms. Finally, we briefly discuss future perspectives on transcription factor-mediated reprogramming.

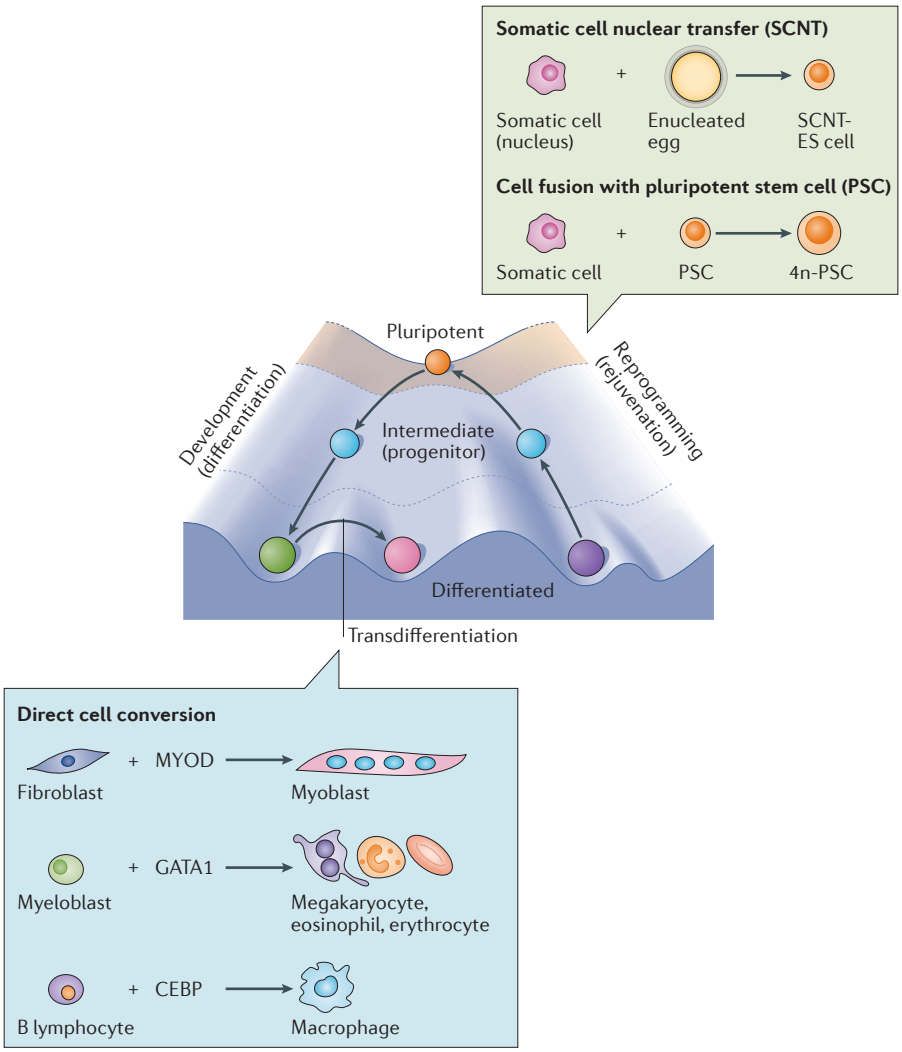


Figure 1 | **Early studies of cell fate plasticity.** Initially, it was believed that acquisition of cell fate can occur unidirectionally, from an immature (pluripotent) to a mature (differentiated) state, and this idea has been depicted as a ball rolling down from the top of Waddington's 'mountain' to the bottom of a 'valley'. However, a series of landmark experiments showed that cell fate is flexible and reversible. It is now known that cells can, in fact, transition from a differentiated to a pluripotent state (depicted as climbing up Waddington's hill) in the course of rejuvenation or reprogramming. The first experimental indications of this cellular plasticity were provided by approaches involving the transfer of somatic nuclei into an enucleated egg or fusion of a somatic cell with a pluripotent stem cell, which have shown that epigenetic memories of the somatic genome can be erased and that cells can be rejuvenated to pluripotency. It has also been demonstrated that ectopic expression of tissue-specific transcription factors can convert a differentiated cell to a cell of another lineage, a process known as transdifferentiation (direct cell conversion) and depicted as moving from one valley to another valley across the ridge of Waddington's landscape. CEBP, CCAAT/enhancer-binding protein; ES cell, embryonic stem cell; MYOD, myoblast determination protein. The image depicting Waddington's landscape is reprinted from The Strategy of the Genes. A Discussion of Some Aspects of Theoretical Biology, Waddington, C. H. (George Allen & Unwin, 1957). Reproduced by permission of Taylor & Francis Books UK.

Induction of pluripotency

Previous works, as described above, suggested the existence of reprogramming factors that can change cellular fate. If this is the case, then what are these reprogramming factors? In this section, we describe the seminal work that led us to the discovery of these factors and their use for the generation of the first iPSCs.

Identifying candidate reprogramming factors. According to previous experiments, genes that contribute to the identities of fertilized eggs or ES cells were thought to be, in large part, responsible for the reconstitution of specific transcriptional networks during reprogramming. Thus, in 2000, we began analysing mouse ES cells (as ES cells are much easier to use and are robustly expandable in comparison to fertilized eggs) to identify genes underlying their characteristics, such as pluripotency and infinite proliferation. This led to the identification of ES cell-specific genes, referred to as ES cell-associated transcripts (ECATs). We then analysed the functions of these ECATs by various experiments, including the generation of knockout ES cells and knockout mice.

Using this approach, we discovered that NANOG homeobox (*Nanog*) is an ECAT and demonstrated that this gene is crucial for the maintenance of pluripotency in both ES cells and early embryos[24,25]. In addition, we found that the overexpression of NANOG allowed mouse ES cells to self-renew, even without the presence of leukaemia inhibitory factor (LIF), which is an essential cytokine for the maintenance of mouse cell pluripotency[26] in serum-containing medium. LIF was already known to stimulate the signal transducer and activator of transcription 3 (STAT3) pathway,

and until then it was considered to be a necessary medium supplement to prevent ES cell differentiation in culture[27,28]. NANOG overexpression enabled us to compare the gene expression of ES cells cultured with or without LIF[24,26], revealing that Krüppel-like factor 4 (KLF4) is a downstream target of LIF–STAT3 signalling in mouse ES cells. Similarly to NANOG, KLF4 overexpression induced LIF-independent self-renewal of mouse ES cells, suggesting that KLF4 is another component of the pluripotency network and is induced by the LIF–STAT3 pathway[29]. This indicated that STAT3 and KLF4 may be candidate reprogramming factors. At the same time, other research groups were reporting the identification of molecules that are specific for mouse ES cells and/or necessary for the maintenance of their characteristics. These molecules included MYC (a well-known proto-oncogene that promotes cellular proliferation and survival)[30], β-catenin (a regulator of the WNT signalling pathway)[31] and T-cell leukaemia/lymphoma protein 1 (TCL1; an activator of the PI3K pathway), as well as a dominant-negative form of growth factor receptor-bound protein 2 (GRB2)[32–34], which inhibits the activity of GRB2 (an adaptor molecule that is important for the activity of the RAS–MAPK pathway). These molecules were subsequently added to our list of candidate reprogramming factors (Supplementary information S1 (table)). Furthermore, it was known at that time that OCT3/4 and SOX2 function as core transcription factors of pluripotency networks by regulating the expression of pluripotency-associated genes[35–37]. Thus, we predicted that they could be strong candidates as reprogramming factors and included them in our list.

The first report of mouse iPSCs. Our next step was to deliver the genes that had been identified as potential reprogramming factors (BOX 1) into mouse embryonic fibroblasts (MEFs) and investigate whether they indeed induced pluripotency. For this, we used a retroviral transduction system developed by Toshio Kitamura of the University of Tokyo, because of its high efficiency in gene delivery[38].

We prepared plates that contained MEFs in which β-galactosidase was placed under the F-box only protein 15 promoter (*Fbxo15*–β-geo) to test the 24 candidates (see BOX 1 for details). We introduced each candidate individually and also used a mixture of all 24 candidates (Supplementary information S1 (table)). None of the factors alone supported cell survival in the presence of antibiotic; however, surprisingly, 22 colonies were formed in the wells that were transduced with all 24 genes. These colonies resembled those of ES cells and not the MEFs from which they originated. At first, we doubted our results, and only after several repeated experiments were we finally convinced that this 24-gene pool indeed contained our sought-after reprogramming factors.

Next, we decided to narrow down the pool of 24 candidates to a minimal set of reprogramming factors. To simplify this challenge, we first removed single factors from the 24-gene list, thereby testing 24 combinations of 23 factors each. We reasoned that if we obtained ES cell-like colonies by the transduction of 23 factors, we could dismiss the omitted candidate gene. This strategy worked beyond our initial expectations, allowing us to narrow down the list to ten genes in the first round of experiments. In the next round, we found that the elimination of four genes, encoding OCT3/4, SOX2, KLF4 and MYC (hereafter referred to as OSKM factors), reproducibly inhibited colony formation. Finally, we tested the ability of the combination of these four genes to induce pluripotency, thereby confirming that they comprise the minimal set of bona fide reprogramming factors[39]. The cells generated with the use of OSKM factors had ES cell-like properties, and we named them iPSCs. Although these first-generation iPSCs still displayed somewhat incomplete pluripotency (BOX 2), they represent the first examples of the reprogramming of somatic cells towards pluripotency using defined factors.

Human iPSCs. After successfully generating mouse iPSCs, we began a similar study involving human iPSCs. In addition to the OSKM factors, we tested the influence of additional factors, including Simian virus 40 large T antigen (SV40LT) and human telomerase reverse transcriptase (hTERT), which can immortalize human diploid cells. After initial difficulties in optimizing the methods for human cells owing to poor transduction efficiency, we demonstrated that the OSKM factors were also sufficient to generate human iPSCs[40]. A group led by James Thomson at Wisconsin University reported the development of human iPSCs using four alternative factors at approximately the same time[41]. Two of the factors, OCT3/4 and SOX2, were common, whereas two others, NANOG and LIN28, were not. Both sets of reprogramming factors are now used globally. Interestingly, combinations of these two sets have a synergistic effect on the generation of human iPSCs[42,43].

Reprogramming enhancers

Although OSKM expression reprogrammes somatic cells to generate iPSCs, the efficiency of reprogramming is quite low. Therefore, many researchers have attempted to discover new molecules, referred to here as 'reprogramming enhancers', which facilitate reprogramming and increase its efficiency.

Pluripotency-associated genes. Further studies of reprogramming factors revealed that the role of SKM, but not OCT3/4, is redundant[44]. SOX2 can be replaced with SOX1 and SOX3, whereas KLF4 can be substituted by KLF2 or KLF5 (REF. 45), which also have redundant roles in the self-renewal of mouse ES cells[45]. Moreover, all MYC family members can enhance the efficiency of reprogramming. Of note, it was previously demonstrated that the oncogenic transformation activity of L-MYC (encoded by the *MYCL1* gene) was much lower than that of other members of the MYC family, such as MYC and N-MYC, thus allowing the generation of safer human iPSCs for clinical applications[46].

Apart from using different combinations of the minimal pluripotency factors, the expression of other pluripotency-associated genes, which are highly expressed in ES cells, can enhance the reprogramming efficiency or even replace some of the reprogramming factors. For example, transduction of T-box transcription factor TBX3 in mouse, undifferentiated embryonic cell transcription factor 1 (UTF1) in human or Sal-like protein 4 (SALL4) in human, together with OSKM, facilitated reprogramming efficiency[47–49]. Oestrogen-related receptor-β (ESRRβ) in mouse and NANOG in human can replace KLF4 (REFS 50,51), whereas nuclear receptor subfamily 5 group A member 2 (NR5A2) and TCL1A can replace OCT3/4 (REFS 51,52). A set of microRNAs (miRNAs) including miR-291-3p, miR-294 and miR-295 effectively promote iPSC generation by OSK[47–49,53]. Moreover, RNA-binding proteins including LIN28 and E3 ubiquitin-protein ligase TRIM71 facilitate reprogramming towards pluripotency[41,43,54]. The activity of several of these reprogramming enhancers can be predicted on the basis of the knowledge of pluripotency networks (that is, defined sets of factors that are essential for the

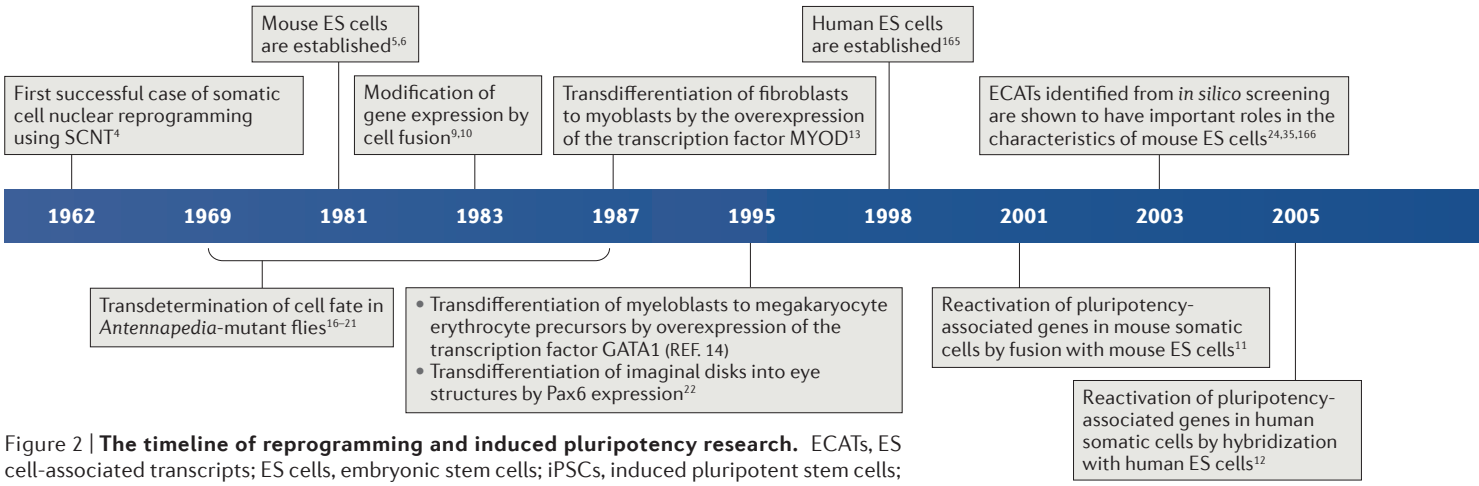


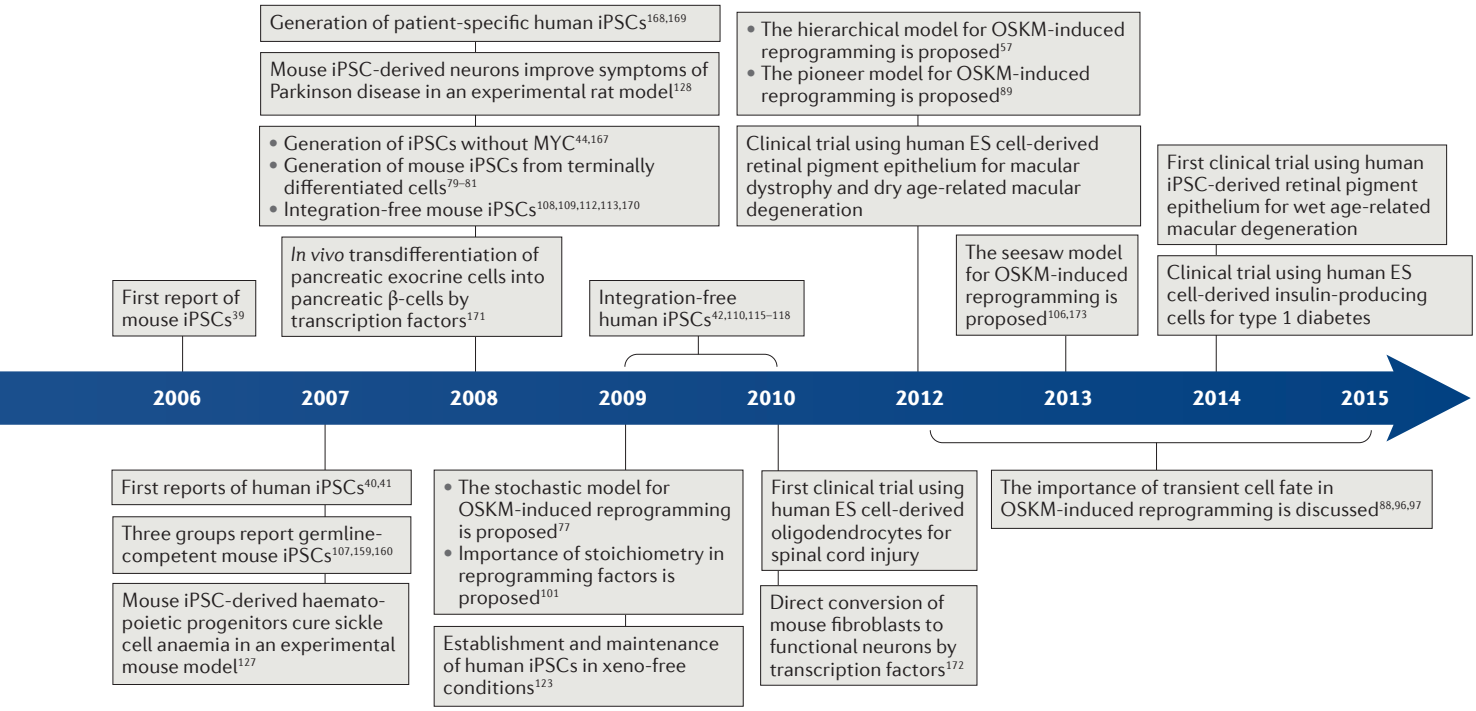
Figure 2 | **The timeline of reprogramming and induced pluripotency research.** ECATs, ES cell-associated transcripts; ES cells, embryonic stem cells; iPSCs, induced pluripotent stem cells; OSKM, octamer-binding protein 3/4 (OCT3/4), SOX2, Krüppel-like factor 4 (KLF4) and MYC; Pax6, paired box 6; SCNT, somatic cell nuclear transfer.

maintenance of pluripotency, and the relationships between these factors). For example, the gene encoding ESRRβ is a direct target of NANOG (and can rescue the disruption of the pluripotency network that results from NANOG deficiency)[55], whereas KLF4 can promote the expression of ESRRβ via transcription factor CP2-like protein 1 (TFCP2L1)[56]. Thus, at least some reprogramming factors can be substituted by their downstream targets, although in this case, efficiency of reprogramming is rather low[57]. Single-cell gene expression analyses for intermediate reprogrammed cells revealed that a combination of ESRRβ, SALL4, LIN28 and NANOG (rather than OSKM) was sufficient for reprogramming mouse fibroblasts to iPSCs, albeit with low efficiency[57]. Many of these genes are likely to be part of the reprogramming network induced by OSKM. Thus, studying the downstream targets of OSKM may help to understand the molecular mechanisms of reprogramming toward pluripotency and increase the efficiency of OSKM-mediated reprogramming.

Cell cycle-regulating genes. On the path towards becoming iPSCs, somatic cells should not only achieve pluripotency, they should also acquire the capacity to proliferate indefinitely, which is important for the self-renewal of PSCs. Indeed, one of the original reprogramming factors, MYC, is a well-known oncogene that can promote cell proliferation. It was easy to hypothesize that other cell cycle regulators might therefore also affect the transition to a pluripotent state. The first major finding indicating that this is indeed the case was the discovery that the tumour suppressor p53 has an inhibitory effect on iPSC generation[58–63]. Several studies have shown that the inactivation of p53 drastically enhances the efficiency of iPSC generation in both mice and humans, concluding that p53 was associated with DNA damage and cellular senescence during reprogramming[58–63]. Additionally, cell cycle-dependent kinase inhibitors such as CIP1, INK4A and ARF have been shown to block iPSC generation[59,61,63]. Conversely, the forced expression of cell cycle-enhancers GTP-binding protein REM2 or cyclin D1, together with OSKM, improves the reprogramming efficiency (similar to the expression of the MYC family genes)[43,64]. Thus, reprogramming enhancers may remove obstacles on the path toward pluripotency.

Epigenetic modifiers. Cellular reprogramming is associated with epigenetic changes, and thus epigenetic modifiers have been studied with respect to their involvement in reprogramming (reviewed in REF. 65). Enzymes that regulate the post-translational modifications of histones (such as their acetylation or methylation), which largely affect transcription, are one example of such epigenetic modifiers[66–73]. These enzymes can affect (by upregulation or downregulation) the expression of pluripotency-associated, as well as somatic, genes and thereby drive cell fate in a particular direction (more towards either pluripotency or differentiation). For example, pluripotency is promoted if expression of pluripotency genes is enhanced and/or expression of somatic genes is suppressed, and vice versa. Accordingly, modifying the expression of these enzymes — WD repeat-containing protein 5 (WDR5)[69] and histone-Lys *N*-methyltransferases EZH2 (REF. 70), SETDB1 (REF. 73), SUV39H1 (REF. 73), and DOT1L[73], to list a few examples — by either overexpression or downregulation has been shown to negatively or positively influence the efficiency of reprogramming. This effect depends on the nature of the deposited epigenetic histone marks and thus the specific roles in transcriptional regulation that are served by these factors. It is also notable that molecules that are used as enhancers of pluripotency and that contribute to epigenetic changes are not necessarily epigenetic modifiers themselves. One notable example is vitamin C, which has been shown to enhance reprogramming efficiency by promoting the activity of histone demethylases JHDM1A and JHDM1B (also known as KDM2A and KDM2B)[72].

In addition to the enzymes that are involved in histone modifications, histone



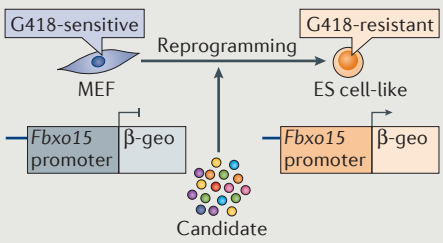
composition — in particular, histone variants T_H2A and T_H2B, which are highly expressed in oocytes — can affect the reprogramming efficiency of mouse fibroblasts to iPSCs[74]. Similarly, removal of histone H1 from chromatin (through protein Arg deiminase type 4 (PADI4)-mediated citrullination), was shown to affect chromatin condensation, resulting in reprogramming progression in mice[73]. Finally, reprogramming efficiency can also be influenced by the methylation status of the DNA itself. As long as DNA methylation is dispensable for reprogramming (as exemplified by unperturbed reprogramming upon knockout of DNA methyltransferases)[75], correct DNA demethylation seems to be a necessary step on the path to pluripotency and has been found to be important in the early stage of reprogramming (rather than in the acquisition of pluripotency itself)[76].

Mechanisms of reprogramming

Even 10 years after its initial discovery, the mechanisms of OSKM-mediated reprogramming are still not fully understood. However, some mechanistic insights have been obtained and will be briefly discussed here (for more details, refer to REF. 174 for a review).

Elite model. An 'elite' model was proposed to explain the low efficiency of transcription factor-mediated reprogramming towards pluripotency, during which only a small number of cells are successfully reprogrammed[77] (FIG. 3a). This model posits that only a rare population of somatic progenitor and/or stem cells — the 'elites' — which are present in the somatic cell population, can be reprogrammed to iPSCs, whereas the differentiated cells within the population are resistant to OSKM-mediated programming. Indeed, the somatic cell population is generally heterogeneous and contains stem cells[78]. However, this model has been ruled out by lineage-tracing studies and clonal analyses, which have shown that iPSCs can be generated from terminally differentiated cells such as T and B lymphocytes, pancreatic β-cells and albumin-expressing hepatocytes[79–81].

Box 1 | **Identifying and screening for pluripotency factors**

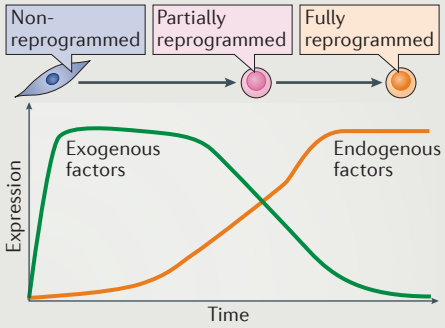


One of the most important details in the generation of induced pluripotent stem cells (iPSCs) was the identification of the key pluripotency factors. To establish the list of candidate genes (see Supplementary information S1 (table)), we first used *in silico* subtraction to identify cDNAs that are specifically enriched in embryonic stem cells (ES cells) in comparison to somatic cells. Using this sequence information, we obtained full-length sequences of cDNAs encoding novel genes that were designated ES cell-associated transcripts (ECATs)[24].

Yoshimi Tokuzawa, a former graduate student in the Yamanaka laboratory, was a key person in the discovery of iPSCs. She generated knockout mice and ES cells with the disruption of one of the ECATs — F-box only protein 15 (*Fbxo15*) — by using the promoter trap strategy[35]. In this case, β-galactosidase (β-geo) disrupted the gene, and its expression could be used as a reporter of *Fbxo15* promoter activity, as cells expressing β-geo would be resistant to geneticin (G418).

As FBXO15-null mutant mice developed normally and were fertile, we could readily isolate FBXO15-null mouse embryonic fibroblasts (MEFs). As expected, with no expression of FBXO15 in somatic cells, MEFs were not resistant to G418 treatment, but FBXO15–β-geo ES cells could survive even in an exceptionally high concentration of G418, suggesting that the endogenous *Fbxo15* promoter was strongly and tightly regulated (see the figure). We then used these FBXO15–β-geo MEFs as a system to screen for reprogramming factors. This system was based on transducing various combinations of candidate genes and evaluating the ability of MEFs to survive G418 treatment (see the figure).

Box 2 | **OSKM-induced reprogramming often results in partially reprogrammed cells**



In OSKM (octamer-binding protein 3/4 (OCT3/4), SOX2, Krüppel-like factor 4 (KLF4) and MYC)-induced reprogramming, the expression of exogenous OSKM should be continued for a sufficient amount of time but then silenced at approximately the time point of cell fate commitment to the embryonic stem cell (ES cell)-like state in the late stage of reprogramming, to generate fully reprogrammed induced pluripotent stem cells (iPSCs; see the figure)[93]. These fully reprogrammed iPSCs show complete characteristics of ES cells, including supporting the full-term development of tetraploid blastocyst-complemented embryos[144–146]. Cells that continue to express exogenous reprogramming factors do exhibit some ES cell-like characteristics, including similar morphologies, robust proliferation, activation of pluripotency-associated genes, teratoma-forming activity and chimeric contribution, but these characteristics are inferior to those of ES cells[39]. In addition, their epigenetic statuses retain traces of somatic cell characteristics[86]. Therefore, these cells are only partially reprogrammed and can subsequently undergo defective differentiation[39]. This represents a major hurdle to practical applications of iPSCs, but certain approaches can already be used to increase the efficacy of reprogramming. For instance, experiments have shown that full reprogramming is promoted by a longer culturing time[93]. A major improvement was also provided by the introduction of reporter systems driven by endogenous *Oct3/4* or *Nanog* promoters, rather than F-box only protein 15 (*Fbxo15*) (BOX 1), to select for reprogrammed cells. As the expression of OCT3/4 and NANOG appears after that of FBXO15 (REFS 107,159,160), this method can be used to select more mature or higher-quality mouse iPSCs. Additionally, it has been demonstrated for human iPSCs that the reprogramming efficacy can be further enhanced by the co-expression of reprogramming enhancers, together with the reprogramming factors themselves[58,59,61–63,161,162]. Furthermore, it has been clearly shown that the culture environment is important for successful reprogramming, and modification of the culture medium can increase the reprogramming efficiency. For instance the LIF–STAT3 (leukaemia inhibitor factor–signal transducer and activator of transcription 3) pathway was shown to be an active player in induced mouse pluripotency[163]. Treatment with inhibitors of MEK (for example, PD0325901) and glycogen synthase kinase 3 (for example, CHIR99021) — which are termed 2i — allows mouse ES cells to undergo LIF-independent self-renewal and increases the homogeneity of the cell population, thus facilitating the efficiency of fully reprogrammed mouse iPSC generation[134,164]. These pathways synergistically affect both the induction and the maintenance of mouse pluripotency[29].

Altogether, it is now well established that fully differentiated cells can also initiate reprogramming, and that the low efficiency of OSKM-mediated reprogramming stems from the fact that the majority of cells never complete the process, and only a small number become iPSCs (FIG. 3a).

Stochastic and deterministic reprogramming. Successful reprogramming depends on the occurrence of many events, sequentially or in parallel. As previously described, some events are important early during reprogramming, including the suppression of somatic cell genes, the mesenchymal-to-epithelial transition[82,83] and changes from oxidative phosphorylation to a glycolysis-based metabolism[84,85]. At the same time, DNA damage, oncogene-induced senescence and apoptosis block reprogramming progression[58–63]. During later stages of reprogramming, late pluripotency-associated genes are activated, tissue-specific transcription factor-encoding and developmental genes are repressed, and some of their promoter regions acquire bivalent trimethylation of histone 3 Lys4 (H3K4) and H3K27 (REFS 86–88). All of these events must occur to complete reprogramming toward iPSCs.

Assuming that OSKM could, in principle, lead to the reprogramming of all transduced somatic cells (FIG. 3a), reprogramming could occur either in a deterministic or a stochastic manner (FIG. 3b). If reprogramming occurred in a deterministic manner, iPSCs should appear at a fixed, predictable time, as reprogramming events in all transduced cells would be synchronized. The stochastic model predicts that reprogramming events may or may not be achieved. Therefore, according to the stochastic mechanism, iPSCs appear at different, random times, and it is not possible to predict whether the progeny of somatic cells becomes an iPSC or not[77].

The low probability of completing reprogramming events, combined with the necessity to overcome roadblocks, results in low efficiency of reprogramming and in stochastic acquisition of iPSC fate mediated by OSKM factors (FIG. 3a).

Reprogramming is a two-step process. How exactly the ectopic expression of OSKM induces the transition to a pluripotent state remains an area of intensive investigation. Studies have indicated that OSKM function as 'pioneer factors', binding to chromatin regions that are not accessible to other factors and leading to the remodelling of chromatin regions, thus activating or repressing gene expression[89].

In the early phase of reprogramming, OSKM occupy many genomic loci, including loci that they do not bind to in ES cells[89]. In particular, MYC binds to genomic loci with methylated H3K4, which is a mark of open chromatin. These loci include the enhancers and promoters of genes that determine the somatic identity of the cell, and this leads to the silencing of somatic genes[89,90] (FIG. 3c). Simultaneously, OSKM bind at enhancers and then at promoters of early pluripotency-associated genes, inducing their expression[89]. This process seems to be stochastic[57] and is largely inefficient, owing to the presence of repressive methyl histone marks, which cover many genes that are involved in pluripotency induction and are responsible for closed chromatin conformations[89]. This obstacle can be alleviated with the use of histone deacetylase inhibitors[66,67,91], which allow opening of the chromatin. Notably, however, the expression of late pluripotency genes takes place in a 'second wave' (REFS 88,92,93) and seems to be a much more predictable event that occurs hierarchically[57]. OSKM can access the loci of late pluripotency genes in the late phase, but not the early phase, of reprogramming[89] (FIG. 3c). Thus, it seems that the initial, stochastic early phase of reprogramming leads to the generation of partially reprogrammed cells, which can then enter a second, more deterministic and hierarchical late phase. In line with this two-step reprogramming model, it was demonstrated that OSKM could be replaced with their downstream targets, including LIN28, SALL4, ESRRβ and NANOG, for mouse iPSC generation[57]. This complex, stepwise nature of OSKM-mediated reprogramming may contribute to its low efficiency.

Reprogramming as a reversal of development. As mentioned above, reprogramming efficiency induced by transcription factors is quite low, and the population of OSKM-transduced cells consists mainly of non-reprogrammed cells, making the analysis of reprogramming very difficult. However, it is now possible to purify these rare cells undergoing reprogramming with the use of specific surface markers (such as stage-specific embryonic antigen 1 (SSEA1) for mouse and TRA-1-60 for human)[43,88,94,95]. Analysis of such purified cells revealed that, upon reprogramming, cells transiently expressed mesendodermal genes, which in early embryos mark the region where the epiblast (the embryonic tissue that gives rise to the whole embryo proper) starts differentiating[96]. Therefore, such transient mesendodermal gene expression is a common event during the developmental transition of pluripotent cells to differentiated cells *in vivo*, and during reprogramming of differentiated cells to the pluripotent state *in vitro*. Also, in the late stages of human cell reprogramming, transient activation of some genes associated with pre-implanted embryos, such as developmental pluripotency-associated 3 (*DPPA3*), DNA methyltransferase 3-like (*DNMT3L*) and *miR371*, was observed[97]. Additionally, human endogenous retroviruses are transiently hyperactivated during reprogramming of human cells[98], similar to what has been observed in mesendodermal cells derived from human PSCs[99], as well as human pre-implantation embryos[100]. Taken together, these observations suggest that cellular reprogramming to pluripotency follows, at least in part, a reversal of the path of early embryonic development.

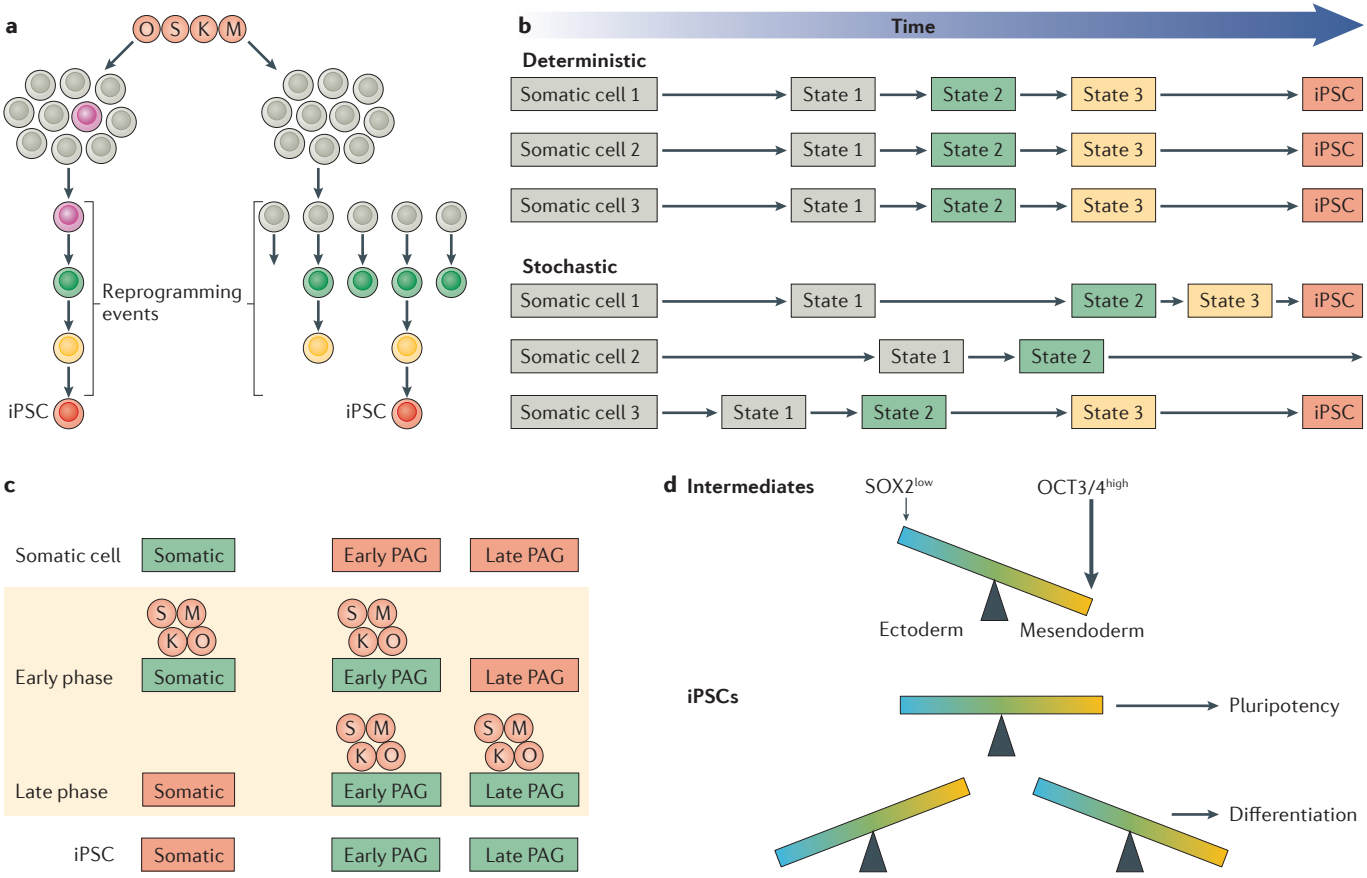


Figure 3 | **Mechanistic insights into transcription factor-mediated reprogramming towards pluripotency. a** | The 'elite' model of reprogramming suggested that only a small number of cells (with stem or progenitor cell characteristics, shown in magenta) present within a population have the potential to be induced by pluripotency factors, and that only these cells can contribute to the generation of induced pluripotent stem cells (iPSCs; left). It is now known that fully differentiated cells can also undergo reprogramming. In fact, many cells within the population initiate the process; however, owing to the fact that reprogramming is complex and comprises many events, the majority of cells never complete the process, and only a small number become iPSCs (right). **b** | Reprogramming can potentially occur either in a deterministic (top) or a stochastic (bottom) fashion. In the deterministic process, all cells follow exactly the same path towards pluripotency, and thus cells are reprogrammed with a fixed latency. In the stochastic reprogramming process, transitions between states vary within the cell population, and thus cells reach the iPSC state with different latencies. **c** | Reprogramming occurs in two phases: early and late. In somatic cells, somatic genes are activated (shown in green), but pluripotency-associated genes (PAG) are silenced (shown in red). In the early phase of reprogramming, OSKM factors (octamer-binding protein 3/4 (OCT3/4), SOX2, Krüppel-like factor 4 (KLF4) and MYC) bind to enhancers and promoters of somatic genes and early PAGs but cannot access late PAG loci. Only in the late phase of reprogramming can OSKM access late PAGs and initiate their expression. In iPSCs, exogenous OSKM factors are no longer expressed, and both early and late PAGs, but not somatic genes, are fully activated. **d** | Stoichiometry of factors is important for reprogramming. In the early part of the reprogramming process, OCT3/4 levels are high and SOX2 levels are low, and this induces transient mesendodermal features in intermediates, which are important for the further progression of reprogramming (top). For stable pluripotency, levels of cell fate-determining factors should be balanced (bottom).

Levels and stoichiometry of factors. Reprogramming differentiated cells to iPSCs is a slow process. Continuous OSKM expression is required for iPSC generation, until the stages of intermediate reprogrammed cells with unstable fates have been passed. Disruption of the expression of OSKM transgenes before this commitment (which occurs ~8–12 days after OSKM transduction in mouse cells) results in the failure of cells to become iPSCs, even if they already express SSEA1, which is a representative marker of pluripotency and reprogramming in mice[92,93].

Another factor that influences the success rate of reprogramming is the appropriate stoichiometry of OSKM expression[101–103]. For example, fibroblasts derived from mice carrying doxycycline-inducible OSKM expression cassettes that allow homogenous expression with favourable levels for iPSC generation can be reprogrammed with high efficiency (although not at 100%)[104]. Increased KLF4 expression is also effective for reprogramming[105]. Unfavourable OSKM expression seems to withdraw the intermediate cells from the reprogramming process, and, as a consequence, these cells are unable to ever become iPSCs[43,88]. Moreover, it has been reported that the levels of both OCT3/4 and SOX2 are important for reprogramming efficiency, with high OCT3/4 expression and low SOX2 expression leading to the highest efficiency[101,102].

In the early phase of reprogramming towards pluripotency, OCT3/4 activates the expression of mesendodermal genes and suppresses that of ectodermal genes. Conversely, SOX2 promotes ectodermal gene expression and lowers mesendodermal gene expression (FIG. 3d). In addition, this OCT3/4^{high}, SOX2^{low} stoichiometry is probably important not only in the early phase of reprogramming, but also in the late phase, when the respective endogenous genes are expressed, as it has been shown that endogenous OCT3/4 levels are high in this phase, whereas SOX2 levels remain low[57,96]. This expression pattern (OCT3/4^{high}, SOX2^{low}) elicits the mesendodermal features, which, as discussed above, are observed in cells undergoing reprogramming and seem to be important for the establishment and maturation of intermediate cell states[88,96] (FIG. 3d). Collectively, this indicates that the timing and levels of expression, as well as the stoichiometry of pluripotency factors, determine the route of reprogramming. This may allow cells to reach intermediate states, thereby positively contributing to the efficiency of iPSC generation.

However, it has also been shown that expression levels of factors with counteracting functions (such as OCT3/4 and SOX2, or ectoderm- and mesoderm-specific genes) need to be balanced to facilitate cells reaching fully reprogrammed and pluripotent states[94]. These observations led to the 'seesaw model' of cellular reprogramming[106] (FIG. 3d). According to this model, the imbalance of the linage-specifying factors would lead to an undesirable fate and failure to reprogramme somatic cells to iPSCs.

Rapid evolution of iPSC technology

From their discovery, there has been much anticipation regarding the potential clinical applications of iPSCs. However, first-generation mouse iPSCs show several unfavourable features, which restrict their usability. Most notably, they can have incomplete pluripotency (BOX 2). Recently, significant progress has been made in improving the quality of iPSCs and increasing the efficiency of the process of generating fully reprogrammed cells.

Another crucial issue with first-generation iPSCs was the use of retroviral vectors to deliver the reprogramming factors. These vectors integrate into the genome of host cells, potentially causing disruption or aberrant activation of neighbouring genes, and pose a risk of reactivation of the reprogramming factors themselves. Indeed, the reactivation of MYC, one of the OSKM factors, sporadically induced tumour formation in iPSC-derived chimeric mice[107]. A major milestone in solving this issue was the introduction of efficient, integration-free methods for cell reprogramming. Alternative induction methods have been developed that involve the transient expression of reprogramming factors, including adenoviruses[108], plasmids[42,109–111], transposons[112–114], Sendai viruses[115,116], synthetic mRNAs[117] and recombinant proteins[118]. Currently, episomal vectors, Sendai viruses and synthetic mRNAs are widely used for generating integration-free iPSCs. More recently, a report showed that a set of chemical compounds is sufficient to reprogramme mouse fibroblasts to iPSCs[119]. This new generation protocol may allow us to achieve more controllable reprogramming than that induced by transcription factors. Additionally, since 2009, xeno-free conditions have been developed to overcome the problems associated with traditional culture methods and to eliminate undefined animal components[120–126]. Currently, combinations of chemically defined media and recombinant matrix proteins, such as vitronectin or laminin, are widely used for the generation and maintenance of human iPSCs[120,124,125].

These advancements have enabled the use of iPSCs for therapeutic purposes[158]. The proof of concept was provided by a sickle cell anaemia mouse model treated with iPSC-derived haematopoietic progenitors[127]. Similarly, neural cells derived from mouse iPSCs have been used successfully to cure Parkinson disease and spinal cord injury in experimental mouse models and found to be safe[128,129]. A safe and effective engraftment of human iPSC-derived neural stem and progenitor cells for the treatment of spinal cord injury has even been confirmed in non-human primates[130].

Conclusions and future perspectives

During the 10 years since its birth, iPSC technology has already shown potential for clinical applications and disease modelling[23,158]. This potential is further strengthened by combining iPSC technology with genome engineering[131], which allows the correction of mutations in patient-derived iPSCs, as well as modification of reporter lines to facilitate differentiation towards specific cell types. The finding that it is possible to modulate cell identity has also introduced the possibility of direct reprogramming, in which cell fate conversion is achieved by the overexpression of multiple transcription factors without reverting to a pluripotent state.

Nevertheless, some important issues regarding the generation of iPSCs remain unsolved. In pre-implantation embryos, PSCs are referred to as 'naive', and they become 'primed' during post-implantation development. Mouse ES cells and iPSCs have homogeneous characteristics, resembling cells of the early epiblasts of mouse pre-implantation embryos, and are considered to be 'naive' (REF. 132). Features of naive pluripotency[133] have been extensively studied[134], and recently many efforts have been made to obtain human naive pluripotent cells[135–141]. Current human ES cells and iPSCs, however, resemble the late epiblast cells of post-implantation embryos, which are 'primed' (REF. 132). Primed pluripotent cells show heterogeneity in the cell population and have distinct differentiation potentials[142,143]. This diversity makes the standardization of human PSCs difficult.

Moreover, although the pluripotency of fully reprogrammed mouse iPSCs seems to be indistinguishable from that of mouse ES cells[144–146], this remains controversial for human iPSCs, which seem to show differences in gene expression and DNA methylation patterns compared to human ES cells (reviewed in REF. 147). The diversity of genetic backgrounds of iPSCs (or donors), as well as the finding that there are differences between clones of human ES cells, has made the analyses difficult[148], and further analyses will be required to better characterize the pluripotent state of human iPSCs. The recent development of human SCNT technology offers a possible way to overcome these issues[149–151], as it enables the direct comparison of ES cells, SCNT-ES cells and iPSCs with the same genetic background[152–154]. Another important limitation is that reprogramming is a rare and stochastic event that often leads to partially reprogrammed iPSCs, which can subsequently undergo defective differentiation[155,156]. Hence, iPSCs must be carefully evaluated for their pluripotency before clinical use.

Despite its caveats, cellular reprogramming to pluripotency is a powerful technology that allows the generation of hundreds of iPSC clones from a single donor. The establishment of a quality control procedure that enables the identification of safe, fully reprogrammed iPSC clones will significantly expand the clinical applications of iPSCs. Clinical trials using human ES cell-derived cells have already begun under approval by the US Food and Drug Administration[157,158]. More recently, in Japan, the first clinical trial for wet age-related macular degeneration using iPSC-derived retinal pigment epithelium has begun, with an aim of assessing the safety and effectiveness of iPSC-based therapies in humans. We now hope that further trials of iPSC-based therapies, accompanied by basic research focused on gaining more detailed, molecular insights into the process of cellular reprogramming itself, will result in unveiling the full potential of iPSCs in the next decade.

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Competing interests statement

The authors declare competing interests: see Web version for details.

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