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Mouse induced pluripotent stem cells

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ABSTRACT The recent discovery that it is possible to directly reprogramme somatic cells to an embryonic stem (ES) cell-like pluripotent state, by retroviral transduction of just four genes *(Oct3/4, Sox2, c-Myc* and *Klf4),* represents a major breakthrough in stem cell research. The reprogrammed cells, known as induced pluripotent stem (iPS) cells, possess many of the properties of ES cells, and represent one of the most promising sources of patient-specific cells for use in regenerative medicine. While the ultimate goal is the use of iPS cells in the treatment of human disease, much of the research to date has been carried out with murine cells, and improved mouse iPS cells have been shown to contribute to live chimeric mice that are germ-line competent. Very recently, it has been reported that iPS cells can be generated by three factors without c-Myc, and these cells give rise to chimeric mice with a reduced risk of tumour development.

KEY WORDS: *mouse, stem cell, induced, pluripotent, reprogrammed*

Introduction

In 2006, Yamanaka and co-workers surprised the scientific community when they reported that both mouse embryonic fibroblasts and tail tip fibroblasts could be reprogrammed to a pluripotent state similar to that observed in embryonic stem (ES) cells, by retroviral transduction of just four genes (Takahashi and Yamanaka, 2006). The discovery of these 'induced pluripotent stem (iPS) cells' was generally regarded as a major development in stem cell research and gave new insights into the pathways involved in the maintenance of pluripotency. Due to the complexity of genetic and epigenetic changes involved in cell differentiation (Surani *et al.,* 2007), it had been doubted if it would ever be possible to reprogramme somatic cells to pluripotency. With the first successful cloning experiments in mammals, it was verified that such reprogramming was, indeed possible (Wilmut *et al.,* 1997). However, the landmark discovery by Takahashi and Yamanaka less than a decade later signalled a development, which few expected so soon.

By definition, pluripotency is the ability of a cell to give rise to all cell types of an adult organism, without the self-organising capability to form the whole organism (Niwa, 2007). *In vivo*, pluripotency is observed in early embryos while *in vitro*, pluripotency may be maintained in ES cells. ES cells may be harvested from the inner cell mass (ICM) of blastocyst stage embryos. These cells, which were first isolated from mouse embryos, can proliferate indefinitely and possess the potential to develop in an unrestricted manner (Evans and Kaufman, 1981; Martin, 1981). In culture, the pluripotency of mouse ES cells must be maintained by addition of factors such as leukaemia inhibitory factor (LIF), which promote proliferation while preventing differentiation. Human ES cells lines have also been generated (Thomson *et al.,* 1998), and their potential as donor sources of specialised cells in cell transplantation therapies has been widely acknowledged (Yamanaka, 2007). However, some major concerns remain for ES cell transplantation. Tissue rejection due to the patients' immune response represents a real limitation of the use of ES cells for transplantation. Another concern is that in the process of isolating ES cells, human embryos are inevitably destroyed (Yamanaka, 2007). This has been a source of constant controversy since the development of the first human ES cell lines, and it has become an important ethical and political issue. These problems may, however, be overcome by reprogramming differentiated cells to an ES cell-like, pluripotent state. Such cells could be customised for individual patients and used in the treatment of disease.

In the last decade, a number of methods have been found to induce pluripotency artificially in somatic cells, including somatic

Abbreviations used in this paper: diMeH3K9, dimethylation of histone 3 lysine 9; ES, embryonic stem; Gcnf, germ cell nuclear factor; GFP, green fluorescent protein; ICM, inner cell mass; iPS, induced pluripotent stem; LIF, leukaemia inhibitory factor; Lrh1, liver receptor homologue 1; MEFs, mouse embryonic fibroblasts; Puro^r, puromycin resistance gene; SCNT, somatic cell nuclear transfer; SSEA1, stage-specific embryonic antigen 1; Sox2, SRY-type high mobility group box 2; TTFs, tail tip fibroblasts.

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cell nuclear transfer (SCNT) and cell fusion (Tada *et al.,* 2001; Cowan *et al.,* 2005; Hochedlinger and Jaenisch, 2006; Yang *et al.,* 2007; Egli *et al.,* 2007). Much of the research in this area has been carried out with mice, but the ultimate goal of stem-cell scientists remains the production of patient-specific pluripotent cells and their use in treatment of disease. Because both SCNT and cell fusion have posed technical and ethical problems as methods of reprogramming somatic cells, Takahashi and Yamanaka's method of reprogramming by defined factors has been hailed as the 'holy grail' of stem cell research. This method circumvents many of the problems associated with both SCNT and cell fusion and is regarded as the method with the best potential for producing patient-specific pluripotent stem cells for use in regenerative medicine. Consequently, this review will focus on this most recent method and what is known of the molecular mechanisms therein.

Production of induced pluripotent stem (iPS) cells

Since somatic cells can be reprogrammed by fusion with ES cells, it was reasoned by Takahashi and Yamanaka in their landmark experiment that ES cells contain factors that induce pluripotency, and these factors were also likely to be involved in the maintenance of pluripotency in ES cells. Based on this hypothesis, 24 different factors were selected, each of which were deemed to have a potential role in the induction of pluripotency (Takahashi and Yamanaka, 2006). These 24 factors were subdivided into three groups. The first group included transcription factors specifically expressed in ES cells such as Nanog, Oct-3/4, Sox2, UTF1, Sall4, Sox15 and Rex1. The second group included tumour and growth-related gene products which play key roles in ES cells such as c-Myc, Stat3, β-catenin, Grb2, Klf4, Eras and TCL1. The final group consisted of factors that are expressed specifically in ES cells, but whose functions have yet to be fully characterised. These include ESG1, ECAT1, Fbx15, ECAT8, DNMT3L, GDF3, ECAT15-1, Fthl17 and Stella. To determine which, if any of these factors induced pluripotency, an assay system was developed whereby induced expression of a pluripotency marker gene could be detected. The gene used was *Fbx15*, which is specifically expressed in ES cells, but is not required for development or for self-renewal of ES cells (Tokuzawa *et al.,* 2003). Using homologous recombination, a βgeo cassette (a fusion of the *β-galactosidase* and *neomycin resistance* genes) was placed under the control of the *Fbx15* promoter. Thus, upon the induction of pluripotency, the *Fbx15* promoter would drive transcription of the neomycin resistance gene (Fig. 1). It was expected that even a partial induction of pluripotency would result in somatic cells becoming resistant to G418 (Takahashi and Yamanaka, 2006). Each of the genes for the 24 candidate factors were introduced into Fbx15$^{\beta geo/\beta geo}$ mouse embryonic fibroblasts (MEFs) by retrovirus-mediated transfection and these cells were subsequently cultured on ES cell medium containing G418. While no drug-resistant colonies were observed following introduction of any single factor, a number of colonies resistant to G418 were observed following introduction of all 24 factors. Some of these clones had morphology and doubling times similar to ES cells and exhibited ES cell markers, as determined by RT-PCR. This suggested that a particular combination of some of the 24 factors caused the ES cell marker genes to be re-activated. These cells, which had been reprogrammed by defined factors, were designated induced pluripotent stem (iPS) cells. Subsequent, stepwise removal of individual factors from the pool of 24, identified 10 factors which, when removed individually, prevented the formation of G418-resistant colonies. When these 10 genes were introduced in combination into MEFs by retroviral transduction, more ES cell-like colonies were formed than with all 24 factors. Removal of individual factors from the 10-factor pool identified just four genes that when removed resulted in no colonies (*Klf4*, *Oct3/4*), greatly reduced colony numbers (*Sox2*), or colonies containing cells with non-ES cell-like morphology (*c-Myc*). Thus, the factors encoded by these genes played important roles in inducing pluripotency in MEFs. Combination of these four genes alone resulted in formation of G418-resistant colonies, comparable in number to those formed by the 10 factors. G418-resistant colonies could not be formed by any combination of two factors, while combination of three factors only resulted in colonies, which could not be maintained in culture (minus *Klf4* or *Oct3/4*), or had non-ES cell-like morphology (minus *Sox2* or *c-Myc*). Thus, it was possible to produce iPS cells by transduction of just four genes- *Oct3/4*, *Sox2*, *Klf4* and *c-Myc*. It was initially surprising that Nanog, which in addition to Oct3/4 and Sox2 is a core transcription factor in the maintenance of pluripotency (Boyer *et al.,* 2005), was found not to be required for iPS cell generation. However, more recent studies have clarified the role of Nanog. In mouse ES cells, Nanog suppresses cellular differentiation but is not required for ES cell self-renewal (Chambers *et al.,* 2007).

Using primers, which amplified transcripts of the endogenous genes only, RT-PCR experiments found that iPS cells express most ES cell marker genes. Notably however, endogenous levels of *Oct3/4* and *Sox2* remained relatively low

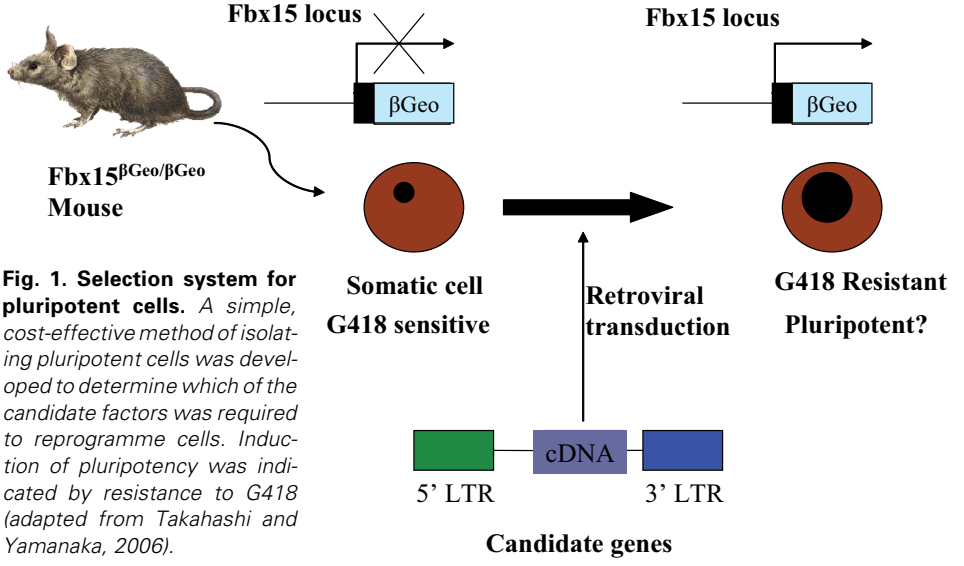

Fig. 1. Selection system for pluripotent cells. *A simple, cost-effective method of isolating pluripotent cells was developed to determine which of the candidate factors was required to reprogramme cells. Induction of pluripotency was indicated by resistance to G418 (adapted from Takahashi and Yamanaka, 2006).*

in the majority of clones. Using chromatin immunoprecipitation analysis, a number of epigenetic changes, which are associated with gene activation and are characteristic of ES cells, were observed in iPS cells. These changes included increased acetylation of histone H3, and a decrease in dimethylation of histone 3 lysine 9 (diMeH3K9) at the promoters of *Oct3/4* and *Nanog*. However, CpG islands in the promoter regions of these genes remained methylated in iPS cells, unlike the same regions in ES cells. DNA microarrays revealed that many of the genes up-regulated in iPS cells are expressed specifically in ES cells. However, a number of genes were found to be up-regulated to a greater extent in ES cells than iPS cells, including *Dnmt3a* and the *LIF receptor* gene, among others. Taken together, these results showed iPS cells to be similar to ES cells, but not identical.

To determine whether they were functionally pluripotent, iPS cells were injected into immune-deficient mice. Similar to ES cells, many of the iPS clones formed tumours called teratomas, which contain tissues originating from all three germ layers. Histological examination showed that teratomas formed by a number of iPS clones contained differentiated cells from all three germ layers, including neural tissues, cartilage and columnar epithelium. In addition, iPS cells were shown to form embryoid bodies in non-coated plastic dishes. By immunostaining for proteins specific to each germ layer it was found that these embryoid bodies contained differentiated cells originating from each germ layer. Following successful generation of iPS cells from MEFs, tail-tip fibroblasts (TTFs) were isolated from an Fbx15$^{\beta geo/\beta geo}$ mouse, which expressed green fluorescent protein (GFP), under the control of the constitutive CAG promoter. Following introduction of the four necessary transcription factors, a number of iPS cell colonies were established. When injected into nude mice, these iPS cells again contributed to all three germ layers in teratomas, indicating pluripotency. In addition, iPS clones expressing GFP, were introduced into blastocysts by microinjection. Chimeric mouse embryos, which constitutively expressed GFP in all three germ layers developed from these blastocysts. Thus, the pluripotency of iPS cells was further verified. However, no live chimeras were born, and chimeric embryos were observed only up to day E13.5, further highlighting the fact that Fbx15-selected iPS cells are significantly different from ES cells. Despite the clear differences observed between Fbx15-selected iPS cells and ES cells, the discovery of iPS cells was clearly of great scientific significance. Pluripotency had been induced in somatic cells, and a comprehensive examination of the known functions of each of the four factors was required to understand how they each contribute to the reprogramming process.

The Four Factors: *Oct3/4*, *Sox2*, *c-Myc* and *Klf4*

Oct3/4 is a transcription factor known to play a key role in the maintenance and self-renewal of pluripotent cells. It is specifically expressed in pluripotent cells, such as ES cells. ES cells cannot be established from Oct3/4 knockout embryos, while repression of Oct3/4 in ES cells results in differentiation into trophoblast-like cells (Niwa *et al.,* 2000), demonstrating the essential role played by Oct3/4 in the maintenance of pluripotency. Interestingly, Oct3/4 is also important in promoting differentiation, as its overexpression by as little as 50% results in differentiation of ES cells into mesoderm and endoderm (Niwa *et al.,* 2000). Together, these findings suggest that Oct3/4 expression levels require very tight regulation in ES cells. Oct3/4 has been shown to have a large number of target genes, many of which also possess regulatory elements for Sox2 and Nanog (Boyer *et al.,* 2005). Such genes are frequently up-regulated or down-regulated in ES cells, and encode proteins involved in ES cell signalling. Oct3/4 is known to co-operate with Sox2 to regulate a number of genes, including *Sox2* and *Oct3/4* in a positive feedback loop (Chew *et al.,* 2005) and *Nanog* (Kuroda *et al.,* 2005). Binding sites for both Oct3/4 and Sox2 have also been found in a number of other genes specifically expressed in ES cells including *Fbx15* (Tokuzawa *et al.,* 2003) and *Lefty1* (Nakatake *et al.,* 2006). In addition, two regulatory elements exist, which act as stem-cell-specific enhancers of the *Oct3/4* gene. Many regulators are recruited to these elements, and shifts in the balance between positive and negative regulators may give rise to variation in the levels of *Oct3/4* expression, in response to external stimuli (Niwa, 2007). Liver receptor homologue 1 (Lrh1) acts as a positive regulatory factor for *Oct3/4* (Gu *et al.,* 2005), while germ cell nuclear factor (Gcnf) acts as a repressor by recruiting Dnmt3 and promoting methylation of the *Oct3/4* promoter (Sato *et al.,* 2006).

Sox2 (SRY-type high mobility group box 2) is a transcription factor, which shares the HMG box DNA binding motif with numerous proteins (Yamanaka, 2007). Like Oct3/4, Sox2 plays an important role in the maintenance of pluripotency in ES cells. Down-regulation of Sox2 in mouse ES cell lines promotes differentiation into trophectoderm as well as other lineages, clearly demonstrating the importance of Sox2 in maintaining pluripotency (Ivanova *et al.,* 2006). As discussed above, genes with Sox2 regulatory elements frequently contain Oct3/4 and Nanog binding sites, and Sox2 acts in combination with Oct3/4 to regulate many genes in ES cells (Boyer *et al.,* 2005). By formation of a heterodimer, Sox2 and Oct3/4 regulate both *Sox2* and *Oct3/4* themselves (Chew *et al.,* 2005), as well as *Nanog* (Kuroda *et al.,* 2005) and *Fbx15* (Tokuzawa *et al.,* 2003) among others. In addition, Sox2 may also act in combination with transcription factors other than Oct3/4 to activate ES-cell associated genes. *Rex1* is an example of such a gene, the transcription of which is regulated by the combined action of Sox2 and Nanog (Shi *et al.,* 2006). Mouse ES cells engineered to overexpress *Sox2* express markers associated with a wide range of differentiated cell types (Kopp *et al.,* 2008). Therefore it seems that, like for Oct3/4, tight regulation of the level of Sox2 is required for self-renewal of ES cells. Given the essential role played by Sox2 in the maintenance of pluripotency, the recent discovery that deletion of *Sox2* in mouse ES cells can be rescued by the introduction of *Oct3/4* came as a surprise. This seems to suggest that maintenance of *Oct3/4* expression may be the major function of Sox2 (Masui *et al.,* 2007).

c-Myc is a basic helix-loop-helix transcription factor with well characterised functions in cell growth, differentiation and proliferation. It is also a proto-oncogene however, and plays a significant role in most human cancers. c-Myc accelerates the cell cycle by activating the transcription of cyclin-E, which promotes the transition from G1 to S-phase (Hooker and Hurlin, 2006). c-Myc is an important downstream target of two separate pathways, each of which are known to support the maintenance of pluripotency in ES cells. These pathways are the LIF (leukaemia inhibitory factor)/STAT3 signalling cascade (Cartwright *et al.,* 2005) and the Wnt signalling cascade (Sato *et al.,* 2004). LIF is required

for the culture of mouse ES cells, and the signalling cascade induced by LIF results in the activation and translocation to the nucleus of STAT3. Activation of *c-Myc* transcription is one of the major functions of active STAT3, and further, expression of a stable form of c-Myc promoted ES-cell self-renewal in the absence of LIF. In contrast, over-expression of a dominant negative form of *c-Myc* promotes differentiation of mouse ES cells (Cartwright *et al.,* 2005). Thus, c-Myc is strongly implicated in the maintenance of pluripotency. The Wnt signalling cascade promotes the self-renewal of both mouse and human ES cells, independently of LIF/STAT3 signalling, and is thought to act by inhibiting GSK3β (Sato *et al.,* 2004). In the absence of LIF, GSK3β phosphorylates c-Myc, which is then targeted for proteasomal degradation. In ES cells, Wnt signalling may inhibit GSK3β and thereby maintain an increased level of c-Myc in ES cells (Cartwright *et al.,* 2005). In addition to its roles in the maintenance of pluripotency, c-Myc possesses further functions, which may also be responsible for its importance in the induction of pluripotency. Firstly, there are as many as 25,000 Myc binding sites in the genome, and c-Myc may bind at numerous sites and recruit histone acetylase complexes (Knoepfler *et al.,* 2006). In addition, by promoting progression from G1 to S phase, it may counteract the anti-proliferative effect of Klf4 (Yamanaka, 2007).

Like c-Myc, the Krüppel-like zinc finger transcription factor Klf4 is targeted by active STAT3. Over-expression of *Klf4* inhibits differentiation of ES cells (Li *et al.,* 2005), and it co-operates with both Oct3/4 and Sox2 to activate the proximal promoters of a small number of target genes, including *Klf4* itself and *Lefty1* (Nakatake *et al.,* 2006). One interesting characteristic of Klf4 is that it can act both as a tumour-suppressor and an oncogene. It is associated with tumour suppression because it activates p21, which suppresses proliferation. However, Klf4 also down-regulates *p53*, thereby promoting cell proliferation (Rowland *et al.,* 2005). Thus, in the absence of stable p21, Klf4 switches from a tumour-suppressor to an oncogene. It is also noteworthy that p53 acts as a negative regulator of *Nanog*, which is a key factor involved in the maintenance of pluripotency (Lin *et al.,* 2005). Thus, by down-regulating *p53*, Klf4 may indirectly result in the up-regulation of *Nanog*. More recently, Klf4 was shown to be dispensable in the maintenance of the undifferentiated state of mouse ES cells and that Krüppel-like factors (Klfs) are required for self-renewal of ES cells (Jiang *et al.,* 2008). In addition it was demonstrated that Klfs and Nanog share many common target genes and that Klfs regulate *Nanog*, indicating some integration between Klf and Nanog transcriptional circuitry.

Induction of pluripotency by Four Factors: the model

Understanding the key functions of each of the four transcription factors has enabled experts in the field to suggest a model of how they act in combination to induce pluripotency (Fig. 2). ES cells are very similar to tumours in that they display a 'transformed' phenotype. This means that they are immortal, proliferate rapidly and form tumours when transplanted into immune-deficient mice (Yamanaka, 2007). Thus, it is not surprising that two tumour-associated factors, c-Myc and Klf4, are required for iPS induction. c-Myc promotes numerous aspects of transformation (Adhikary and Eilers, 2005), and as such, may be responsible for inducing immortality in iPS cells. However, c-Myc also induces p53-dependant apoptosis. A function of Klf4 in the induction of iPS cells may be to down-regulate *p53* and counteract this pro-apoptotic effect of c-Myc (Rowland *et al.,* 2005). As discussed above, however, Klf4 also activates *p21* and as a result suppresses cell proliferation. By suppressing *p21*, another function of c-Myc may be to counteract the anti-proliferative effect of Klf4. Therefore, a delicate balance between the expression levels of c-Myc and Klf4 might have to be achieved in order for transformation to occur. Apart from its role in transforming cells, c-Myc may also play a role in loosening chromatin structure. This is important, as pluripotent stem cells are known to have open chromatin structure (Meshorer *et al.,* 2006). c-Myc may modify chromatin by binding at numerous sites, and by recruiting histone acetylase complexes (Knoepfler *et al.,* 2006). By introducing *c-Myc* and *Klf4* alone, tumour cells and not iPS cells may be induced. With an open and accessible chromatin structure, loosened by c-Myc, Oct3/4 and Sox2 can gain access to their target genes and promote transcription of genes necessary for the induction of pluripotency. As described previously, Klf4 also acts in combination with Oct3/4 and Sox2 to activate genes. In addition, by down regulating *p53*, Klf4 allows the up-regulation of *Nanog*, which together with Oct3/4 and Sox2 forms a transcription factor network necessary for maintenance of pluripotency (Boyer *et al.,* 2005). While the underlying mechanism for the production of iPS cells is still not understood fully, current work using doxycycline (dox)-inducible lentiviral vectors has helped to decipher the chronology of pluripotency marker gene expression in mouse iPS cells. Using flow cytometry to analyse the timing of specific marker gene expression, it was shown that *alkaline phosphatase* was expressed first, followed then by *stage-specific embryonic antigen 1* (*SSEA1*) whereas endogenous *Oct4* and *Nanog* expression was only detected in fully reprogrammed cells (Brambrink *et al.,* 2008). In a similar study, downregulation of surface antigen *Thy1* expression was observed before *SSEA1* activation during the early phase of reprogramming and activation of endogenous *Sox2*, *Oct4*, telomerase and the silenced X chromosome happened later in the reprogramming process (Stadtfeld *et al.,* 2008a). The ability to identify cells at specific intermediate stages in the reprogramming process (by their specific gene marker expression) should prove useful in further deciphering the molecular basis of this process and improving the methodology for generating iPS cells.

Another important consideration is the cell type used for reprogramming. Mouse iPS cells have been produced from adult liver and stomach cells (Aoi *et al.,* 2008), pancreatic β cells (Stadtfeld *et al.,* 2008b) and, using dox-inducible lentiviral vectors, cells from several other somatic tissues (Wernig *et al.,* 2008a). However, reprogramming of mature B lymphocytes requires an additional factor (C/EBP-alpha) or inhibition of Pax5 (Hanna *et al.,* 2008).

Generation of iPS cells of improved quality

While the discovery of iPS cells by Takahashi and Yamanaka represented a major breakthrough in stem cell research, Fbx15-selected iPS cells were only partially reprogrammed. This was evident from the fact that iPS cells differed from ES cells in terms of global gene expression and epigenetic marks. Failure of iPS cells to give rise to live chimeric mice provided further evidence for these differences (Takahashi and Yamanaka, 2006). If iPS cells

were to ever have a use in regenerative medicine, their quality would have to be improved. In 2007, three groups individually generated improved iPS cells by using either *Nanog* (Maherali *et al.,* 2007; Okita *et al.,* 2007; Wernig *et al.,* 2007) or *Oct3/4* (Wernig *et al.,* 2007) as a selection marker. Both of these factors are more tightly associated with pluripotency than *Fbx15*. Drug selection was used by each group to isolate reprogrammed cells, with Yamanaka's group inserting a green fluorescent protein (GFP)-internal ribosome entry site (IRES)-*puromycin resistance* gene (Puro^r) cassette into the *Nanog* locus of ES cells (Okita *et al.,* 2007). Following blastocyst injection, chimeric mouse embryos were obtained that contained the Nanog-GFP-IRES-Puro^r construct. MEFs were taken from these embryos and transfected with the four known reprogramming factors. The development of puromycin-resistant cell colonies that expressed GFP indicated that these cells expressed the pluripotency gene of interest (*Nanog* or *Oct3/4*) and thus, had been reprogrammed (Okita *et al.,* 2007). Each group achieved a significant improvement in the quality of iPS cells. Nanog- and Oct3/4-iPS cells were subjected to rigorous tests, which demonstrated that these iPS cells were almost indistinguishable from ES cells. Following induction of pluripotency, the retroviral transgenes were silenced in Nanog-iPS cells, unlike in Fbx15-iPS cells, possibly due to the action of the *de novo* methyltransferase, Dnmt3a2 (Okita *et al.,* 2007). Additionally, Nanog-iPS cells expressed most ES cell marker genes including *Oct3/4*, *Sox2* and *Nanog* from endogenous loci at levels comparable to ES cells. The promoter regions of the *Nanog* and *Oct3/4* genes were fully de-methylated, contrasting with the same regions in Fbx15-iPS cells. In addition, the global patterns of gene expression and DNA methylation were almost identical in Nanog-iPS cells and ES cells (Maherali *et al.,* 2007; Okita *et al.,* 2007; Wernig *et al.,* 2007). A notable exception was the expression levels of *Rex1*, which was lower in Nanog-iPS cells than in ES cells (Okita *et al.,* 2007). Histone modification was also highly similar in the improved iPS cells and ES cells, with a bivalent pattern of histone trimethylation, characteristic of ES cells, being observed in Nanog- and Oct3/4-iPS cells (Maherali *et al.,* 2007; Wernig *et al.,* 2007). The observation that silenced X-chromosomes from female somatic cells were re-activated upon generation of Nanog-iPS cells was also important in demonstrating the improved quality of iPS cells using the new selection process. Upon differentiation of Nanog-iPS cells, the X-chromosome was randomly inactivated; further demonstrating the complete reprogramming that had been achieved (Maherali *et al.,* 2007). Nanog- and Oct3/4-iPS cells were also found to be functionally pluripotent. Like Fbx15-iPS cells, they contributed to all three germ layers in both teratomas and embryoid bodies. However, unlike Fbx15-iPS cells, Nanog- and Oct3/4-iPS cells were able to produce live chimeric mice when introduced into blastocysts (Maherali *et al.,* 2007; Okita *et al.,* 2007; Wernig *et al.,* 2007). Significantly, all three groups provided compelling evidence for germ-line competency of iPS cells, with Yamanaka's group successfully raising progeny from Nanog-iPS chimeric mice (Okita *et al.,* 2007).

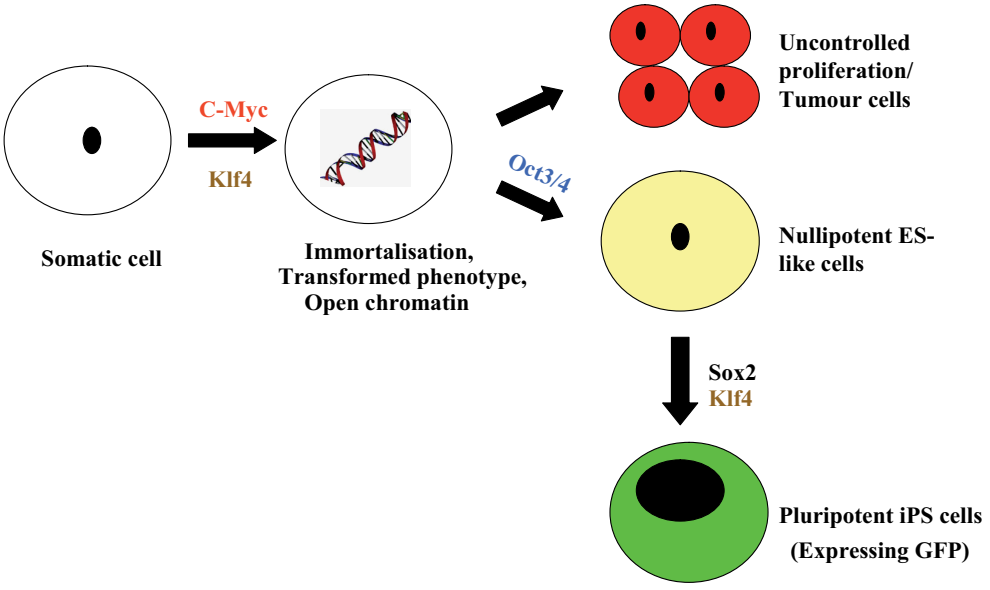

Fig. 2. Model for the generation of iPS cells by the retroviral transduction of four genes. *It is proposed that c-Myc induces cellular immortality and open chromatin structure and Klf4 is likely to suppress apoptosis and senescence. Oct3/4 probably changes cell fate from tumour cells to pluripotent cells and Sox2 is also necessary to establish pluripotency (adapted from Yamanaka, 2007).*

Further evidence for the complete reprogramming of Oct3/4- and Nanog- iPS cells was provided by each of the other two groups. Firstly, Jaenisch and colleagues performed the most rigorous test of pluripotency when they created 'all iPS embryos' by injecting Nanog-iPS cells into tetraploid blastocysts. These blastocysts have the developmental potential to form only placenta, and thus, any resulting embryos consist entirely of donor cells (Wernig *et al.,* 2007). Secondly, Hochedlinger's group used iPS cells to successfully reprogramme somatic cells by cell fusion (Maherali *et al.,* 2007). Taken together, the findings of each of these three groups provided incontrovertible evidence that Nanog- and Oct3/4-iPS cells are fully reprogrammed. Interestingly, Jaenisch's group were also able to derive iPS cells using these four factors from normal genetically unmodified somatic donor cells. The iPS cells were isolated based only on their morphology (Meissner *et al.,* 2007). This advance may be key in the application of iPS cell technology for human therapeutics.

Despite these developments, a number of issues remained with the technology before it could be used to generate patient-specific pluripotent stem cells. Firstly, it was unclear why iPS cells were induced with very low efficiency. Less than 1% of somatic cells expressing the four factors became iPS cells. While the reasons for this are uncertain, a number of possibilities have been suggested (Takahashi and Yamanaka, 2006; Okita *et al.,* 2007). It was suggested that tissue stem cells, which co-exist with fibroblasts in culture, might have been the origin of observed iPS cells. Another possibility is that expression levels of the four

factors might have to be confined within narrow ranges in order for pluripotency to be induced. Such appropriate expression may occur by chance, only in a small proportion of cells. Alternatively, expression of additional factors may be required to increase the efficiency of iPS cell generation. Secondly, because of the system of retroviral transduction required for induction of iPS cells, tumours developed in a high proportion of chimeric mice, and in mice derived after germline transmission, due to the reactivation of the c-Myc transgene. In order to be useful in a clinical context, it would be necessary to either develop a system of transiently delivering genes, or to remove the necessity for the c-Myc transgene completely.

Safer cell reprogramming

With the recent discovery that it is possible to generate iPS cells from human somatic cells (Takahashi *et al.,* 2007; Yu *et al.,* 2007; Lowry *et al.,* 2008; Park *et al.,* 2008; Mali *et al.,* 2008), the possibility that iPS cells could be used in patient-specific cell transplantation therapies moved one step closer to becoming a reality. However, the remaining presence of the *c-Myc* transgene in Yamanaka's protocol remained a barrier to any potential clinical application of iPS cells.

Two very recent studies have demonstrated that it is possible to produce mouse Nanog-iPS cells without the *c-Myc* transgene (Nakagawa *et al.,* 2008; Wernig *et al.,* 2008b). In these new papers, the remaining three reprogramming genes, namely *Oct3/4*, *Sox2* and *Klf4*, were retrovirally transduced into MEFs. While it had previously been found that no iPS cell colonies formed in the absence of exogenous *c-Myc* (Okita *et al.,* 2007), it was reasoned that this may have been due to the timing of drug selection. Thus, cells transfected with three genes only, were cultured for an extended period of time, before drug selection was applied. Both groups found that drug-resistant iPS cell colonies were indeed formed by the three factors devoid of *c-Myc* if drug selection was delayed sufficiently (Fig. 3). Yamanaka and co-workers found that reprogramming occurred in the absence of *c-Myc* if selection was delayed until 14 days after transduction (Nakagawa *et al.,* 2008), while Jaenisch and co-workers found that it was necessary to delay drug selection until 30 days after transduction (Wernig *et al.,* 2008b). Despite these differing results, both clearly demonstrated that the rate of reprogramming in the absence of *c-Myc* is significantly reduced when compared with iPS cells reprogrammed by four factors. Also, fewer iPS cell colonies were formed by three factors than four, while fewer background and non-iPS cell colonies were observed also (Nakagawa *et al.,* 2008). Together, these data suggest that iPS induction in the absence of *c-Myc* is less efficient but more specific than when *c-Myc* is present. Nanog-selected iPS cells generated without Myc expressed ES-cell marker genes at levels comparable to those in ES cells (Nakagawa *et al.,* 2008), and generated teratomas containing tissues from all three germ layers when injected into nude mice (Wernig *et al.,* 2008b). Importantly, they were also able to generate viable adult chimeric mice, indicating that the iPS cells generated without Myc were of high quality (Nakagawa *et al.,* 2008; Wernig *et al.,* 2008b). Contrasting with chimeras derived from iPS cells generated with Myc, those derived from Myc– iPS cells did not develop tumours within 100 days after birth (Fig. 3) (Nakagawa *et al.,* 2008). This reduced risk of tumour development is an important progression in iPS cell technology. Despite the finding that the *c-Myc* transgene is dispensable for reprogramming, it remained unclear whether endogenous Myc proteins were involved in iPS generation. Due to the decreased efficiency and delayed timing of reprogramming without *c-Myc*, it was clear that it does play a role. In the future, the discovery of small molecules, which can replace the role of *c-Myc*, would overcome both problems of tumourigenicity and reduced efficiency.

A comprehensive comparative genomic analysis of differentiated cells, stable partially reprogrammed cell lines, fully reprogrammed iPS cells and ES cells was undertaken in an effort to identify the reasons for the low efficiency of iPS cell production (Mikkelsen *et al.,* 2008). ES and iPS cells share similar gene expression patterns and epigenetic states whereas stable, partially reprogrammed cell lines show expression of some differentiation-associated genes and silencing (by DNA hypermethylation) of some pluripotency-associated genes. Interestingly, it was also

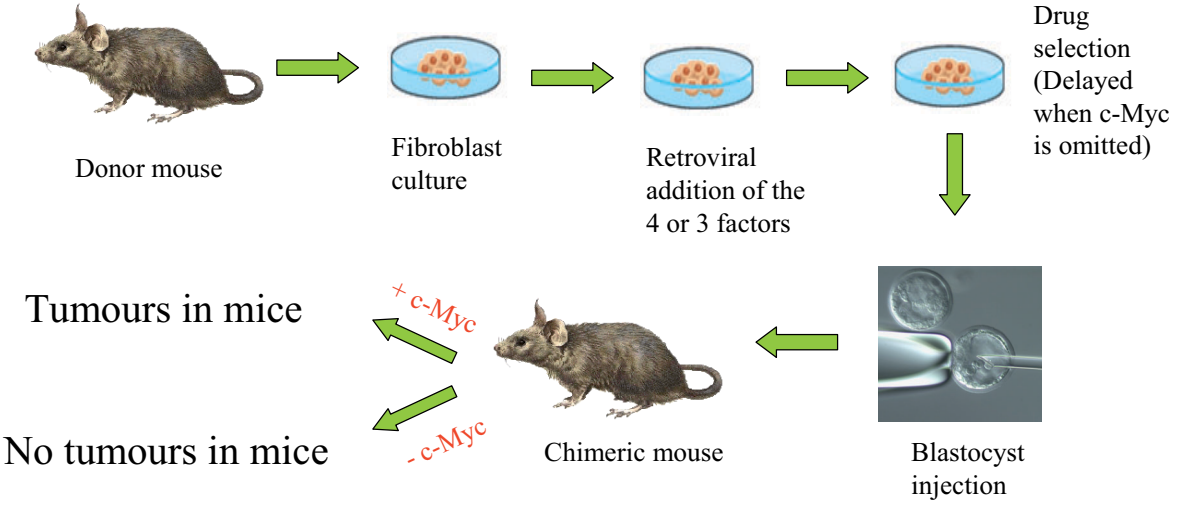

Fig. 3. Induction of induced pluripotent stem (iPS) cells and generation of chimeras. *Delaying the drug selection process allows the production of iPS cells without the requirement for c-Myc. Chimeric mice produced by blastocyst injection of these iPS cells show reduced tumour incidence (adapted from Pera and Hasegawa, 2008).*

demonstrated that low success rates in iPS cell generation can be ameliorated by transient RNA inhibition of transcription factors and treatment with DNA methyltransferase inhibitors, thereby suggesting that small molecule treatments can improve the efficiency of iPS cell production and the safety of iPS cells for clinical applications.

Another barrier to the application of iPS cells in human therapeutics is the risk posed by retroviral transduction of the three/four transcription factors. The difficulty of developing safe gene transfer methods is a challenge for both gene and cell therapy approaches. However, the potential of such therapies to treat human disease is so vast as to warrant extensive investigation. To date, the most efficient way to genetically modify cells is to introduce genes by retroviral integration thereby potentially causing insertional mutagenesis, proto-oncogene activation and tumourigenesis. For an extensive review of current attempts to improve the safety of retroviral integration, see Nienhuis *et al.,* 2006. Encouragingly Yamanaka's group have very recently demonstrated that retroviral integration into specific sites is not required for iPS cell generation (Aoi *et al.,* 2008). At this stage there is no proven safe option to generate iPS cells, without the risks associated with retroviral transfer. The use of RNAi and DNA modification enzyme inhibitors has been demonstrated to improve the efficiency and safety of iPS cell production (Mikkelsen *et al.,* 2008). It is hoped that a better understanding of the gene regulatory circuits and epigenetic modifications involved in cell reprogramming might point to improved small molecule treatments, an approach which is more likely to be applicable in the field of regenerative medicine.

Conclusions

Since the initial discovery of mouse iPS cells by Takahashi and Yamanaka in 2006, research in this area has advanced at an astonishing rate. In just over a year, the technology used to reprogramme mouse cells has been successfully extended to human cells, while some of the initial problems with mouse iPS cells, including tumourigenicity have been partly addressed. Nevertheless, extensive research is still required with mouse iPS cells before any potential therapeutic use of human iPS cells is realised. The cause of the low efficiency of iPS induction remains to be determined. In addition to gene activation by expression of transcription factors, epigenetic remodelling plays a key role in induction of cellular pluripotency. A greater understanding of this mechanism will be necessary to improve the efficiency of iPS cell generation. Moreover, retroviral transduction involves random integration into the genome and consequently poses a risk of mutagenesis. The future use of alternative gene delivery systems or small molecules, which can replace retroviral gene products, may circumvent this problem. Also, if iPS cells are to be used clinically, methods to direct differentiation and integrate them into tissues are still required. Despite this however, iPS cells represent one of the best hopes for producing patient-specific stem cells for cell-based therapies.

Note added in proof: Promising results demonstrating the therapeutic potential of iPS cells have been reported (Wernig *et al.,* 2008c). Mouse iPS cells were differentiated into mature, functional neuronal cells and improved the symptoms of a rat model of Parkinson's disease.

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