INDUCED PLURIPOTENT STEM CELLS A BRIEF REVIEW

Dr Stuart P. Atkinson
Dr Lyle Armstrong

ABSTRACT

Induced pluripotent stem cell generation is regarded as one of the most important discoveries in cell biology of recent times. The demonstration of the reversion of somatic cells to cells which resemble embryonic stem cells (ESCs) by the viral transduction of 4 transcription factors has started an explosion of research into induced pluripotent stem cells (iPSC). iPSC have many intriguing possibilities, such as the generation of patient specific stem cells for cell replacement therapy, the study of diseases and the testing of novel drugs to treat such diseases. However, many hurdles need to be overcome before they are routinely used as true representations of ESC. Herein, iPSC studies to date are discussed alongside their potential advantages and disadvantages.

THE BEGINNINGS OF A REVOLUTION

The field of embryonic stem cell (ESC) research was shaken in 2006 by a paper from the lab of Shinya Yamanaka which demonstrated the generation of induced pluripotent stem cells (iPSCs). This publication, which entails the reprogramming of a mouse somatic cell genome to that resembling a pluripotent cell (1) has been heralded as one of the most important discoveries in cell biology and in the future may provide a limitless source of patient specific stem cells. In this seminal paper, the retroviral transduction of just four genes (Oct4, Sox2, Klf4 and c-Myc) partially reprogrammed mouse embryonic fibroblasts (MEFs) and adult tail tip fibroblasts (TTFs) to show characteristics reminiscent of mouse embryonic stem cells (mESCs). Initial studies utilised retrovirus-mediated gene transfer for their perceived ability to undergo silencing in the pluripotent state after genomic integration (2, 3). The iPSCs generated in this study were similar to mESC in terms of morphology, growth rate, gene expression and the epigenetic status, but were nevertheless obviously different to mESC. Further analysis showed that iPSCs were able to differentiate into cell types representative of all three primordial germ layers in teratoma assays and formed embryoid bodies, two key tests of pluripotency. However, these cells could not contribute to chimaeric animals when injected into mouse blastocysts, (perhaps a more robust test of pluripotency) and did not fully silence transgene expression.

Yamanaka's lab addressed some of the problems from this paper by using a slightly altered strategy (4). Previously, an antibiotic resistance gene was knocked into the Fbx15 locus, to allow for selection upon attainment of pluripotency (1). This study instead utilised the gene Nanog, which is more closely associated with the pluripotent nature of ESCs than Fbx15 (4). MEFs using this strategy gave rise to stable clones with similar gene expression and epigenetic status to ESCs, while they effectively silenced transgene expression. Importantly these iPSC showed contribution to chimaeric animals with germline transmission, another key test for the pluripotency of iPSC. However, it was also reported that up to 20% of chimaeric mice generated showed the formation of tumours attributed to the reactivation of the c-Myc transgene.

Soon, multiple studies began to show that iPSC technology was readily transferable and repeatable, so allowing an explosion of research interest in this field. Maherali et al and Wernig et al, both reported the generation of iPSC by retrovirally mediated transduction of Oct4, Sox2, Klf4 and c-Myc into MEFs and TTFs using Nanog or Oct4 selection, with reprogramming taking three weeks at an efficiency of 0.05-0.1% (5, 6), similar to Yamanaka's studies (1, 4). Both papers analysed the epigenetic status of their iPSC in some detail, and reported similarities between iPSC and mESC with regard to both histone modifications and DNA methylation. These iPSCs also showed contribution to chimaeric animals and germline transmission, with Wernig et al reporting the generation of embryo's derived entirely from iPSC following injection into tetraploid blastocysts (perhaps the best assay for pluripotency and developmental potential). However, while mid- and late-generation embryos were generated, no live births were reported, perhaps suggesting, that by the most rigorous tests, these cells were not equivalent to mESC.

HUMAN iPSC

The next major breakthrough came with the generation of human iPSC (7, 8). Yamanaka's group extended their earlier work and showed that adult dermal fibroblasts could be reprogrammed by retroviral transduction of OCT4, SOX2, KLF4 and C-MYC with good efficiency, but only after the cells where transduced with a mouse retrovirus receptor (7). Cells with hESC-like morphology were picked and expanded and showed all the hallmarks of pluripotency, including teratoma formation, appropriate gene expression and the ability to differentiate into multiple tissues. James Thomson's laboratory found that a slightly different set of factors, OCT4, SOX2,

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NANOG and LIN28, were sufficient to allow iPSC generation from foetal and adult fibroblasts (8) via lentiviral, rather than retroviral transduction, allowing the transduction of non-dividing cells (9), which was not previously permitted by the use of retroviruses (10). iPSC generated showed all the signs of a pluripotent cell type and their data further suggests that Lin28 was not required, but aided an increased frequency of reprogramming events. These two studies using similar but distinct, methods underscores the validity of iPSC technology and removes questions of reproducibility while also showing redundancy in which genes can affect the reprogramming process.

Closely following this came the generation of iPSC from another lab using a similar protocol (11). Addition of a ROCK-inhibitor, which can aid hESC growth when dissociated to single cells (12), enhanced the formation of iPSC from hESC-derived embryonic fibroblasts and importantly iPSC were also generated in the absence of C-MYC or KLF4, although at a lower efficiency. Although these initial strategies were useful for foetal cells, adult somatic human cells required the addition of hTERT and SV40 Large T antigen, two more potentially oncogenic factors, to allow for iPSC generation (11). Other studies have also reported that 6 factors are required for reprogramming of human fibroblasts (13, 14). Lentiviral expression of OCT4, SOX2, KLF4 and C-MYC in combination with NANOG and LIN28 were used to generate iPSCs from human neonatal foreskin fibroblasts and compared to four factor reprogramming (OCT4, SOX2, NANOG and LIN28) iPSC generation was quicker and more efficient (13). Another study found that SV40 Large T antigen, in addition to either of the four factor combinations of reprogramming factors, could increase efficiency by 23-70-fold in foetal fibroblasts and immortalised adult fibroblasts, using non-integrating lentivirus' (NILs) in order to minimise viral integration (14). This study also reported that SV40 Large T antigen could synergise with Oct4/Sox2/Nanog, Oct4/Sox2, or Oct4 alone to generate iPSCs, but only some clones were shown to be able to differentiate into cell types representative of all three primordial germ layers in teratoma formation assays. The use of Large T indicates that tumour suppressor pathways may need to be downregulated in order for the reprogramming process to take place at a respectable efficiency. A tumour suppressor response may be induced due to replicative stress on the cells, which induced a DNA-damage response (15, 16). This study also reported the generation of iPSC from fibroblasts homozygous for the sickle cell anaemia mutation, showing the capability of generating human models of disease in hESC-like cells. This is an important aspect of iPSC generation, with the generation of disease-specific iPSC perhaps as important as patient-specific iPSC and will be discussed further in this review.

iPSC generation from human neonatal foreskin fibroblasts was reported with four factors, although repeated retroviral transduction was required (17). Within this study, it was noted that partially reprogrammed clones still expressed the ectopic genes while the fully reprogrammed clones appeared to have silenced the exogenous genes more efficiently.

iPSCs Generation in More than Just Fibroblasts

It is important to establish that various cells types can be used for iPSC generation from a therapeutic aspect and also to assess the potential for a defined terminally differentiated cell type to be an amenable target of iPSC generation. It is still not fully understood if all cells can be targets for iPSC generation, although studies have shown that not just fibroblasts can be reprogrammed. Mouse β-pancreatic cells (18), and adult mouse liver and stomach cells (19) were shown to be relevant targets for iPSC generation. All cell types were deemed pluripotent and iPSC contributed to cells of all three germ layers in chimaeric embryos, although only one animal survived to birth. Interestingly iPSC from liver and stomach were less tumourigenic than MEF-derived iPSC, although perinatal death was higher and, interestingly, when c-Myc was removed only a 20-40% reduction in efficiency was observed, compared to a 90% drop in MEF reprogramming.

Another study reported the reprogramming of a terminally differentiated cell type but required the interruption of the cellular state before iPSC could be generated (20). Terminally differentiated mature B-cells from an iPSC-derived chimaeric mouse could only be reprogrammed after cellular "sensitisation" by expression of the myeloid transcription factor C/EBPα. C/EBPα can disrupt the function of Pax5, a transcription factor understood to play a major role in mature B-cell development and function, and hence suggests that not all terminally differentiated cells could be reprogrammed using the same factors.

iPSC technology was utilised in a novel reprogramming study in which transdifferentiation of an adult cell type to another adult cell type, without the requirement to revert these cells to an ESC-like state using transcription factors specific to that cell lineage was demonstrated (21). Specifically, they converted pancreatic exocrine cells to cells that closely resemble pancreatic β-cells by retroviral transduction of 3 genes; Ngn3, Pdx1 and Mafa, which are all involved in β-cell development. As β-cells are the insulin secreting cells destroyed in diabetes, this study is of obvious therapeutic interest.

An elevated efficiency and speed of reprogramming of human keratinocytes in comparison to fibroblasts was also observed (22). The advantage of using keratinocytes was further demonstrated by the generation of iPSC from cells isolated from single plucked human hairs, thus bypassing the requirement for skin biopsies when requiring fibroblasts.

REVEALING THE REPROGRAMMING PROCESS - MOLECULAR MECHANISMS AND SECONDARY iPSC GENERATION

The process of reprogramming somatic cell genomes towards a pluripotent-like state seems now to be a reachable goal, but little is actually known about how this is achieved at the molecular level. Understanding the roles of each of the reprogramming factors is of importance to understand the process and the relatively low efficiency of reprogramming and extended lengths of time required suggests that reprogramming is a gradual stochastic event rather than a set of specific events, which also requires to be understood.

Oct4, Nanog and Sox2 had been previously associated with the pluripotent state (23), with the use of such factors in iPSC generation thought to "kick-start" pluripotency-associated transcriptional circuits in non-pluripotent target cells. c-Myc induces the expression of genes involved in proliferation and self-renewal (24) and can also mediate global histone acetylation(25) perhaps mediating chromatin decondensation to allow the functions of DNA-binding transcription factors 26). Klf4 may function to downregulate p53 transcription (26), which has been shown to regulate Nanog expression (27) and also inhibits ES differentiation (28). Lin28 is involved in RNA processing (29), and modulates the expression of Let7 micro-

RNA's (miRNAs) in stem cells to maintain pluripotency in mESC (30). The importance of miRNA in the maintenance of pluripotency is just becoming uncovered (31, 32).

Studies intent on understanding the molecular mechanisms behind iPSC generation have begun to make use of "secondary systems" in which iPSC are generated by lentiviral transduction of doxycycline (dox) -inducible transgenes and are then injected into blastocysts to form chimaeric mice from which fibroblasts, or other cell types, carrying the dox-inducible transgenes can be isolated (33-39). Dox-induction of these cells allows for reprogramming of a homogenous cell type, allows the induction process to be controlled to more fully understand the process and importantly, this also allows for self-selection of reprogrammed cells after dox-removal, as only cells with activated endogenous pluripotency genes will grow effectively. SSEA1 and alkaline phosphatase (AP) activity have been shown to be early markers of the reprogramming process, while endogenous Oct4 and Nanog expression and telomerase activity were only found in fully reprogrammed cells (33). Longer transgene expression was linked to enhanced reprogramming, but continued transgene expression ablated the differentiation capacity of iPSC. In a similar study, secondary iPSC generation was reported to be 20-50 fold more efficient that the "primary" system and multiple cell types could be used, suggesting that iPSC can be generated from cells with different developmental origins and epigenetic states, using this system (34). Again, longer transgene expression led to enhanced iPSC generation and, interestingly a DNA methyltransferase inhibitor, **5-Aza-deoxycytidine (5-Aza)** was shown to boost the reprogramming process while a histone deacetylase inhibitor, Trichostatin A (TSA), did not. This suggests that DNA methylation is more important to the reprogramming process, although the effects of modulating histone methylation were not studied. The time scale of reprogramming of the secondary cells (9-13 days) was noted to be similar to that of directly reprogrammed cells (10-14 days) suggesting that direct viral integration is not an essential part of the reprogramming process, and further suggests that reprogramming is driven by stochastic epigenetic events requiring a minimum time of transgene expression.

Integrative genomic analysis of reprogramming of mouse fibroblast and B-lymphocytes using this secondary system was reported next (35). An immediate response of transgene induction was the decrease of mesenchymal related genes and an increase in proliferation associated, stress-induced and anti-proliferative genes. This study also reported that the vast majority of transduced cells remain in an intermediate reprogramming state, suggesting that stochastic events are required for cells to become completely reprogrammed. Such partially reprogrammed clones were studied in order to understand the barriers to the reprogramming process and showed incomplete activation of pluripotency related genes, incomplete deactivation of genes related to the original cell phenotype and incomplete epigenetic reprogramming. Interestingly, it was also reported that inhibition of DNMT1 led to the full reprogramming of these stable partially reprogrammed cells, indicating that reprogramming of the epigenotype of the cell is vitally important in iPSC generation.

A more detailed analysis of reprogramming at different stages was undertaken in mouse cells with transcription factor binding, gene expression and histone methylation examined at different stages of reprogramming (37). c-Myc was observed to be very important to early reprogramming, especially for the silencing of fibroblasts-specific genes, contrary to its usually perceived function as a transcriptional activator. Further, it is proposed that the lack of Oct4, Sox2 and Klf4 co-binding in partially reprogrammed cells contributes to the lack of full reprogramming. This lack of binding was suggested to be due to the lack of expression of Nanog, which allows the targeting of reprogramming factors.

Human cells were also studied by generation of secondary system, by generating iPSC from fibroblasts and/or keratinocytes using dox-inducible lentviruses and then differentiating them to fibroblasts, for further dox-mediated reprogramming studies (38, 39). Secondary human fibroblasts were reprogrammed with an efficiency of 1-3% upon dox-induction. Induction times of 6-8 days were required at the minimum to produce iPSC, but prolonged dox-induction (20-25 days) gave highly homogenous pluripotent iPSCs with good efficiency.

Overall, these papers suggests that timing of transgene expression/silencing is critical to the reprogramming process, but also that reprogramming is, in part, a defined set of molecular events, although stochastic events are likely also to be required for fully reprogrammed iPSCs to be generated.

CHANGING THE REPROGRAMMING MIXTURE

Changing the Factors

It has already been demonstrated that there is some redundancy in factor requirement for reprogramming with the reports of two differing sets of four reprogramming factors working to a similar degree (7, 8). This suggests that other factors may also be able to influence the reprogramming process, and indeed, the removal of some factors, such as the oncogene c-Myc, would be beneficial. Analysis of gene expression in mESC has demonstrated that N-Myc, which has a less oncogenic nature (40), was expressed at a higher level than c-Myc and could substitute for c-Myc in iPSC generation (41). Other studies have also shown that c-Myc is non-essential for the human (42) and mouse (42, 43) reprogramming process with the tumourigenic potential of mouse iPSC generated being much lower, with all chimaeras generated surviving past 100 days. Similarly, it has been found that Sox3, 15 and 18 could substitute for Sox2, Klf1, 2 and 5 could substitute for Klf4 and both N-Myc and L-Myc could substitute for c-Myc in iPSC generation from MEFs (42). Use of the "substitutes" Sox1 and 3, Klf2 and N- and L-Myc led to iPSC generation from MEFs that gave rise to teratomas upon injection into nude mice.

Further additional reprogramming factors beyond the six "classic" factors have been reported. Addition of viruses carrying UTF1 and siRNA against p53 highly enhance the generation of fully pluripotent iPSC and could substitute for C-MYC (44). UTF1 is linked with chromatin mediated transcriptional repression (45) and so may function to aid differentiation, but is also **reported to highly expressed in ESCs and downregulated upon differentiation (46). Enhanced generation of iPSC after p53 siRNA expression again suggests that perhaps the downregulation of tumour-suppressor pathways are required as previously discussed with the use of SV40 Large T antigen**. iPSCs were also reported to be derived from MEFs with the addition of the nuclear receptor Esrrb, in the place of c-Myc and Klf4 (47) and were shown to be fully pluripotent, formed chimaeric animals and contributed to the germline. **Esrrb has been previously reported to be important in pluripotency**

and self-renewal of ES cells (48, 49) and it's capability to substitute for Klf4 and c-Myc was reported to be partly due to Essrb's ability to upregulate many key ESC-associated genes and a high degree of similarity in their transcriptional regulatory circuits.

A novel paper in the field of iPSC generation next showed the possibilities of reprogramming by transduction of miRNA (50). **miRNA are single-stranded RNA molecules of 21-23 nucleotides in length, which are partially complementary to one or more messenger RNA (mRNA) molecules and are involved in regulation of gene expression. Multiple miRNAs are expressed as cluster of genes in a single cistronic sequence which is later cleaved to give the mature miRNA**. A miR302-cluster transgene was retrovirally transduced into several tumour cell lines and cells with ES-like morphology, marker expression and rate of cell division were readily produced. All iPSC clones formed teratomas and also formed EBs and differentiation led to the production of neuronal cells. It is noted that MECP2 and the p66 component of MECP1 are targets of miR302 and analysis of the Oct4 promoter did show demethylation.

Changing the Cell to Change the Factors

Other cell types have been studied for their potential use in iPSC generation to minimise transgene requirements. Many cell typed in mouse and human, may already express reprogramming factors at a high enough level to remove them from the factors used in viral transduction. Multiple recent studies have shown that mouse neural cell types can be reprogrammed by lower numbers of transgenes, due to their endogenous expression of Sox2 and c-Myc (51-58). Mouse neural progenitor cells (mNPCs) were reprogrammed to iPSC with only two factors (Oct4 and Klf4) at a low efficiency (51) and use of an inhibitor of the K9-specific histone methyltransferase inhibitor (BIX-01294) improved the efficiency to that of the 4 factors, functionally replacing c-Myc and Sox2 (59). Mouse adult neural stem cells (mNSCs) (52) were used to generate iPSC without Sox2 and without c-Myc or Klf4, but with much delayed kinetics. Further studies showed that the use of two factors (Oct4/Klf4 or Oct4/c-Myc) allowed generation of iPSC which were shown to be pluripotent in nature with no reported tumourigenesis in chimaeric and F1 mice. Changes in growth conditions to minimise differentiation stimuli (use of serum free medium with MEK/ERK and GSK3 inhibitors supplemented with LIF) allowed for the generation of iPSC with only Oct4 and Klf4 in mNSCs in another study (55). iPSC were also generated from mouse meningiocytes (56), a multipotent cell type which expresses high levels of Sox2 and are easy to extract (in animals). iPSC clones generated were highly homogenous and gave a high yield of chimaeric animals. Recent studies have been able to generate iPSC from mouse NSCs, by expressing Oct4 alone (57). These cells were shown to be pluripotent, formed teratomas and exhibited transmission through the germline showing that, in principle, Klf4, c-Myc and Sox2 transgene expression are not required for reprogramming.

Small Molecules and Soluble Factors: Towards Transgene Free iPSC?

The addition of small molecules into the reprogramming mix has allowed improvement of iPSC generation suggesting the possibility of transgene free reprogramming, simply by the addition of appropriate small molecules or soluble factors to the cell culture medium.

Alongside previous uses of the small molecule BIX-01294, a K9-specific histone methyltransferase inhibitor (51), retroviral-mediated generation of iPSC was shown to be greatly enhanced using MEFs by the addition of **5-Aza**, dexamethasone (synthetic glucocorticoid), and various histone deacetylase inhibitors (valproic acid (VPA), TSA and suberoylanilide hydroxamic acid (SAHA)) (60). MEFs have also been reprogrammed by Oct4 and Klf4 alone, with the aid of two small molecules which inhibited K9-specific histone methyltransferase activity (BIX-01294) and an L-channel calcium agonist **(BayK8644)** (58). Similarly, primary human neonatal fibroblasts have been reprogrammed with only OCT4 and SOX2 transduction followed by one month in ESC culture conditions, following an initial two weeks of treatment with the histone deacetylase inhibitor, VPA (61).

Naturally occurring signaling molecules, rather than artificial drugs, that can modulate the expression of ESC-associated transcription factors could be utilised as soluble factors that enhance reprogramming. For this reason, the WNT pathway was studied (62) and it was discovered that modulation of the WNT-pathway can affect iPSC generation (63). In an attempt to compensate for excluding c-Myc, reprogrammed cells were cultured in WNT3a-conditioned medium and by approx 3 weeks, homogenous ESC-like colonies of cells were observed which were deemed to be fully pluripotent.

IPSC- FROM BENCH TO BEDSIDE?

Models of Human Disease

Multiple studies have addressed the potential use of iPSC to treat disease utilising studies in mouse models and using human fibroblasts carrying mutations relevant to specific diseases. Mouse models of human sickle cell anaemia (SCA) (64), Parkinson's Disease (PD) (65) and Haemophilia A (66) have all been used to investigate possible therapeutic uses of iPSC technology. iPSC technology was combined with gene therapy to correct the mutant human SCA allele, with subsequent haematopoietic progenitor differentiation and transplantation effectively leading to the recovery of the mouse (64). In another study, iPSC generated from MEFs were differentiated to mNPCs, which were then transplanted into foetal mouse brains where they showed functional integration and differentiation into various neural cells (65). The same cells were then transplanted into adult rat brain after administration of 6-hydroxy dopamine (6-OH-DA) into the striatum, which specifically kills dopamine neurons, providing a useful model of Parkinson's disease. **Encouragingly, improved behaviour was observed 4 weeks after transplantation**. TTFs were used to generate iPSC without c-Myc and were differentiated towards an endothelial progenitor cell type and where then injected into the livers of mice carrying the mutation associated with Haemophilia A (65). Following a deliberate injury to the tail, mice with the mutation that did not receive the iPSC-derived progenitors died wheras those that did survived as well as control mice. However, it was noted that 16 of the 36 chimeras generated in one of these studies died within 8 months, with many showing tumours making it clear that the use of integrating viruses and oncogenes as reprogramming factors will hinder any therapeutic use of iPSC technology.

iPSC have also been generated from other model animals (67-69) such as the rhesus macaque (67) and rat (68, 69). These studies are useful as the rhesus macaque is the most relevant primate model for human disease, and also shows that reprogramming is conserved across species.

Disease and Patient Specific iPSC

iPSC technology has been immensely useful in the generation of "disease-specific" iPSC in humans (70-73). This offers the opportunity to recapitulate and study normal and "affected" tissue formation, enabling further studies into respective diseases and drug development or treatments.

Possible treatment of type 1-diabetes was studied by combined use of iPSC technology with directed differentiation of human iPSCs to reprogram human foreskin fibroblasts to iPSC with subsequent generation of islet-like clusters which were shown to partially recapitulate their normal in vivo function (70). Dermal fibroblasts from an 82 year old patient with amyotrophic lateral sclerosis (ALS), a neurodegenerative disease, were also used for iPSC generation (71). iPSC were differentiated into motor neurons and glial cells which still carried the mutation present in ALS, allowing for more in-depth studies of the molecular mechanisms of this disease. These studies underline the ability to generate human models of disease with iPSC technology and also that the technology can also be applied to "old" fibroblasts, proof of concept that iPSC technology can be utilised with any age of cell. Activation of telomerase and elongation of telomeres is likely to be an important part of establishing stable iPS cells, especially if using donor cells from aged patients. Telomerase activity and telomere extension has been observed in iPSC generated from MEFs and also from "old" dermal skin from aged mice, which had telomeres of similar length to iPSC generated from "young" donors (74). iPSC have also been generated from a patient with spinal muscular atrophy (72) in which disease-specific changes in cell survival and function were reported. Differentiation towards the cell type affected in SMA (motor neurons) was allowed at early time points, but was affected upon further culture of the cells where motor neuron production was slowed or motor neurons were degenerated. A further study also reported the generation of iPSC from a wide range of genetic diseases, with either Mendelian or complex inheritance (73).

Safe iPSC?

Initial iPSC studies utilised retroviruses for gene transfer into target cells, for their ability to be silenced (2, 3), but alongside their inability to infect non-dividing cells (10), it was noted that silencing was not maintained in iPSC (11, 71). Constitutive lentiviral use, in which transgene silencing is poor (8, 41, 75) was superseded by inducible lentiviral methods which hoped to attain full silencing of transgene expression upon attainment of the pluripotent state. However, the common problem with these vector types is the possibility of integrative mutations, or reactivation of transgenes, which has been shown to lead to tumourigenesis (4). Studies on viral integration seem to demonstrate that there are possible "hot-spots" for viral integration, but the majority seem to be random (19, 76-78).

Solutions to these problems have been the centre of intense recent research and multiple techniques have been found to enhance iPSC technology. The use of a polycistronic transcript, in which all transgenes are present in a single linear form, could theoretically reduce the number of integrations required for iPSC generation down to just one and has been utilised to generate iPSC from both MEFS and TTFs with good efficiency (0.5-1%) (79, 80). Most of the iPSC showed only one integration site and full transgene silencing. A further study in this area was able to show that human neonatal human foreskin fibroblasts could be reprogrammed similarly (81). The iPSC were generated with relatively low efficiency, although they did express common pluripotency markers, formed teratomas with cells representative of all three primordial germ layers and some differentiation potential was reported.

The use of adenoviral transgene transduction, which does not lead to transgene integration, was studied next (82). Hepatocytes, which are easily infected by adenovirus, were successfully reprogrammed with no integration, and iPSC generated showed endogenous expression of pluripotency-associated genes. The adeno-iPSC also formed teratomas in nude mice, while some chimerism and germ line transmission was observed. However, this was only observed in one cell type, the efficiency of reprogramming was extremely low (0.001-0.0001%) and 23% of the iPS lines were tetraploid. A similar study generated iPSC utilising a plasmid based approach (83), which did not require the use of a virus, although again this was only in one cell type and at very low efficiency.

Recent studies have solved problems associated with the techniques outlined above by the use of plasmid-based transduction of transgenes using the piggyBac (PB) transposition system and Cre-lox technology (84, 85) which allows for seamless integration then excision of transgenes (86). In the first study, stable iPSC are generated from MEFs and HEFs using dox-inducible transgenes using the piggyBac system (85) and seamless removal of the transgenes from iPSCs was observed following transient transposase expression. Further, pluripotency of the MEF-derived iPSCs was confirmed via a tetraploid embryo complementation assay. The second study utilised a single plasmid with a 2A-peptide-linked reprogramming cassette, flanked by loxP sites, to generate iPSCs in MEFs, with subsequent removal of the transgene by Cre expression (84). The single plasmid cassette was then combined with piggyBac technology to induce stable formation of iPSC from HEFs, although removal of the plasmid was not reported. These methods seem to be simple (use of plasmids and accessible transfection products), can be used in any cell type (not restricted by tendency to be infected by virus) and may allow for accurate transgene removal subsequent to iPSC generation. This technique, while still initially requiring transgene integration, does remove the possibility of transgene re-expression and associated tumourigenic consequences. This enhanced technique was then utilised in a therapeutic context, with the generation of disease-specific transgene-free human iPSCs (87). Fibroblasts from sporadic-PD sufferers were reprogrammed into iPSC, and subsequently, the transgenes were excised through expression of Cre-recombinase, moving the major therapeutic goals of iPSC technology significantly closer. Further, the paper also suggests that after the excision of the viral transgenes, the expression profile of these iPSC was more similar to that of hESCs than previously generated iPSC lines. This suggests a small amount of "leaky" expression from transgenes is still enough to perturb the gene expression profile of such iPSCs.

Selection Criteria for iPSC

If iPSC technology matures rapidly, it will be possible to generate large numbers of iPSC lines, but what criteria do we

use to select for therapeutically useful iPSC? Pluripotent ESC can be distinguished by morphological criteria, by surface-antigen detection, by loss of transgene expression and by analysis of gene expression, protein expression and studies of DNA methylation and histone modifications. Lineage specific differentiation, teratoma formation, germline transmission and tetraploid complementation assays are also routinely used to identify pluripotent cells. However, to do all these assays for each iPSC clones would be a great undertaking, or can not be undertaken, such as chimera formation and tetraploid complementation in the case of human iPSC.

This conundrum has been the topic of some debate and minimum criteria to test the pluripotent nature of iPSC have been suggested (88-90). These include, morphological attributes (including self-renewal), correct expression of pluripotency and lineage-associated genes, transgene independence and proof of functional differentiation. For human iPSCs, the main functional test is suggested to be the differentiation if iPSC into cell types representative of all three primordial germ layers in teratoma formation.

SO IS THIS THE END OF ES RESEARCH?

The short answer, of course, is no.

Research into ESCs has allowed us to understand some of the key genetic and epigenetic pathways which govern ESC pluripotency and self-renewal (91, 92), and without this data iPSC technology may not have been developed. Indeed, iPSC technology was partly inspired by the fact that somatic human cells could become reprogrammed by fusion with hESC so suggesting that factors present in hESC allows the reprogramming of a somatic cell genome (93). Further, there are still many questions which can be answered by more studies in ESC, as we still do not fully understand the pluripotent state of ESC, although gene expression (49, 91, 94, 95), protein interaction (96), epigenetic (92, 97-99) and miRNA analysis (31, 32) are surging forward. Only by such analysis and comparisons will we understand if iPSCs are true representations of ESCs. We also still do not have gold standard differentiation assays for many cell types and as functional analysis is mooted as being a possible test for the "worthiness" of iPSCs for therapy (88-90) it seems reasonable to suggest that further extended studies in ESCs are essential. Also, mentioned throughout the literature is the possible tumourigenic nature of iPSC. This is a major problem, as both the transgenes utilised and integrative viruses have been associated with tumourigenesis (100-107). Perhaps further studies in ESC biology, SCNT and associated techniques will allow us to discover new and improved ways to induce pluripotency in somatic cells without the risk of tumourigenesis,

Indeed, many top scientists, some highly involved in the iPSC field, have called for iPSCs to be studied alongside, rather than instead of, ESC biology and associated techniques, such as somatic cell nuclear transfer (SCNT) (108-112). Although, iPSC technology does not have the associated ethical problems that hESC derivation/biology and SCNT technologies (113, 114), there are many reasons why these associated technologies should progress side-by-side. Current disadvantages in iPSC technology may mean that proper therapeutic use may be decades away, in which time hESC could produce many important breakthroughs. hESCs will also be required as controls for analysis of iPSCs, further giving reason for continued research on them. Also, importantly, iPSCs will not tell us anything about early human development, which could be uncovered by the use of techniques, such as SCNT. Important processes, which are likely to be epigenetic in nature (115), that control early development may be totally by-passed, meaning many discoveries which may actually make iPSC a better, quicker and more therapeutically relevant technology may lie undiscovered.

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