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Introduction

The evolving biology of cell reprogramming

Ian Wilmut*, Gareth Sullivan and Ian Chambers

*MRC Centre for Regenerative Medicine, University of Edinburgh, Chancellor's Building,
49 Little France Crescent, Edinburgh, EH16 4SB, Scotland, UK*

Modern stem cell biology has achieved a transformation that was thought by many to be every bit as unattainable as the ancient alchemists' dream of transforming base metals into gold. Exciting opportunities arise from the process known as 'cellular reprogramming' in which cells can be reliably changed from one tissue type to another. This is enabling novel approaches to more deeply investigate the fundamental basis of cell identity. In addition, new opportunities have also been created to study (perhaps even to treat) human genetic and degenerative diseases. Specific cell types that are affected in inherited disease can now be generated from easily accessible cells from the patient and compared with equivalent cells from healthy donors. The differences in cellular phenotype between the two may then be identified, and assays developed to establish therapies that prevent the development or progression of disease symptoms. Cellular reprogramming also has the potential to create new cells to replace those whose death or dysfunction causes disease symptoms. For patients suffering from inherited cases of degenerative diseases like Parkinson's disease or amyotrophic lateral sclerosis (also known as motor neuron disease), the future realization of such cell-based therapies would truly be worth its weight in gold. However, before this enormous potential can become a reality, several significant biological and technical challenges must be overcome. Furthermore, to maintain the credibility of the scientific community with the general public, it is important that hope-inspiring advances are not over-hyped. The papers in this issue of the *Philosophical Transactions of the Royal Society B*: *Biological Sciences* cover many areas relevant to this topic. In this *Introduction*, we provide an overall context in which to consider these individual papers.

Keywords: stem cells; regenerative medicine; pluripotency; reprogramming; disease modelling;
transcription factors

1. INTRODUCTION
During the past 50 years, a number of key experiments have shown that cell fate is much less rigidly fixed than previously understood. The experimental approaches that were used in these studies included somatic cell nuclear transfer (SCNT) [1,2], cell fusion [3], introduction of selected transcription factors [4] or of extracts [5]. This stream of research culminated six years ago when it was established that after the introduction of key transcription factors into somatic cells, a small proportion acquired the characteristics of an embryonic stem (ES) cell [6]. These cells are known as induced pluripotent stem or iPS cells. The term 'reprogramming' has been used to describe the above experiments in a number of different contexts and this has become confusing. In this review, 'factor-directed reprogramming' will indicate that transcription factors have been introduced into cells to induce them to revert to an earlier stage of development. Cells turned back to an earlier stage of development in this way are said to be 'reprogrammed'

while the process is described as 'reprogramming'. By contrast, the use of this approach to change the fate of the cell to another lineage will be termed 'directed fate conversion'. This is in contrast to 'directed differentiation' when treatment induces cells to convert to their natural progeny.

The discovery that factor-directed reprogramming could convert somatic cells to pluripotent cells, which are potentially capable of forming any tissue in the body, had a seismic effect on stem cell biology and its potential applications. The establishment of these procedures depended upon some knowledge of the molecular mechanisms that specify and maintain pluripotent cell identity to suggest candidate transcription factors that might bring about the desired change. The initial protocols have been adapted and refined and although factor-directed reprogramming to pluripotency is now routine, it remains an inefficient, slow process. A deeper understanding of the mechanisms that maintain pluripotency and of those involved in the process of this change can be expected to continue to lead to improvements in the procedures.

Our understanding of the abilities and limits of factor-directed reprogramming exemplified by induced pluripotency has evolved at a mind-boggling pace. Since that seminal discovery, new experiments have

seen factor-directed reprogramming of many different cell types, as well as the direct fate conversion of fibroblasts to other non-pluripotent cell types, including neurons and cardiomyocytes. Moreover, the application of these procedures to the study of human disease has resulted in significant insights into disease ontology brought about through the production of patient-specific induced pluripotent cells. The papers in this issue of the *Philosophical Transactions of the Royal Society B: Biological Sciences* cover many areas relevant to this fascinating new research. In this *Introduction*, we provide an overall context in which to consider these individual papers.

2. FACTOR-DIRECTED REPROGRAMMING TO PLURIPOTENCY

Pluripotency is the ability of a cell to differentiate into cell types representative of each of the primary embryonic germ layers. A new research era was launched when Takahashi & Yamanaka [6] demonstrated that, following enforced expression of four transcription factors normally expressed in pluripotent cells (*Oct4*, *Sox2*, *Klf4* and *c-Myc*), a small proportion of differentiated cells can be induced to adopt a phenotype similar to ES cells; these are called iPS cells. This initial experiment with mouse foetal fibroblasts was quickly extended to many different adult mouse cell types— (liver and stomach [7]; B-lymphocytes [8]; adult neural cells [9]). Three independent groups exemplified this in human cells [10–12]. These or very similar methods are now used routinely in many different laboratories around the world. Since the initial reports, a number of other genes that contribute to this reprogramming process have been identified. In some cases, these are able to replace one of the four factors identified by Yamanaka (often referred to as the Yamanaka factors), while in others they complement the standard protocol. This topic is reviewed by Okita & Yamanaka [13] in this issue.

Oct4, *Sox2*, *Klf4* and *c-Myc*, were originally introduced using retroviral vectors [6]. While the procedure is very repeatable, it has several limitations. Namely, it is slow, requiring three to five weeks or more for colonies of apparently pluripotent cells to be available for passage, and only a small proportion of treated cells become pluripotent [14]. More importantly, the viral integrants in the pluripotent cells carry the risk of causing harmful mutations. In addition, *c-Myc* is an oncogene and caused tumours in 20 per cent of animals when iPS cells were introduced into chimeras [15]. Ideally, the retroviral vectors in iPS cells are silenced allowing the endogenous regulatory mechanisms to function and control the balance between pluripotency and differentiation. However, as a practical matter, it is important to demonstrate that this retroviral gene silencing has occurred and is maintained after differentiation; otherwise, the transgenes may interfere with the normal physiological function of the cells.

Empirical attempts to overcome these limitations are described in table 1 and are further discussed by Okita & Yamanaka [13]. The PiggyBac vector has been used to introduce all four proteins in a single

transcript [19]. This modified transposon has the advantage that, in principle, it can be removed from iPS cells at the end of the treatment without any mutation. A number of non-integrating vectors have been used, including Sendai virus [31,39]. This RNA virus carries no risk of altering the host genome, infects a great variety of different cell types, can accommodate the four gene-coding sequences and can be completely removed from the iPS cells. Advantage has also been taken of the fact that some cell types express one or more of the Yamanaka factors. In this way, iPS cells were obtained from murine neural stem cells, which express *Sox2* and *c-Myc*, by introducing *Oct4* alone [40]. It was also notable that the conversion of neural stem cells was quicker than that of fibroblasts, demonstrating that the nature of the somatic cell influenced the efficiency of iPS cell production [9,41].

In part, the inefficiency of Yamanaka's procedure is owing to sub-optimal expression of the transgenes [42]. This was demonstrated by comparing iPS cell production efficiencies between a conventional procedure and one in which somatic cells derived from iPS cells were reprogrammed for a second time. This is known as 'secondary reprogramming'. In both cases, doxycycline was used to turn-on the expression of the four vectors, but while the location and number of viral inserts were variable in primary somatic cells, the expression of the viral inserts in the secondary line was uniform and known to be effective [42]. The efficiency of iPS cell production was between 25- and 50-fold greater in secondary fibroblasts than after primary infection. This seems to indicate that in some cases an inadequate number of copies of the genes insert or that random integration may take place at inappropriate sites and suggests that other approaches such as exposure to small molecules, proteins or mRNA could be more efficient and/or more reproducible. Introduction of modified mRNA encoding the four Yamanaka genes, but with OCT4 at higher levels, was more efficient and quicker than the standard use of retroviral vectors [38]. Modified ribonucleoside bases incorporated into the synthesized mRNA were designed to reduce the innate anti-viral immune response and increase cell viability and enabled them to direct reprogramming by daily transfection. Further studies are required to confirm that this procedure is repeatable and effective in different cell types.

Effective selection of small molecules to manipulate complex process such as factor-directed reprogramming is a major challenge, but significant progress has been made as discussed by Efe & Ding [43] in this issue. 'Pluripotin' is a compound that maintains mouse ES cells in a pluripotent state through many passages in the absence of feeders, serum or cytokine. This effect was later shown through action on ERK1 (extracellular-signal-regulated kinase 1) and RasGAP (Ras GTPase activating protein); the authors comment that the use of small molecules established more stable and uniform populations of cells. With regard to factor-directed reprogramming, the combined inhibition of the ERK1 pathway and glycogen synthase kinase 3 (GSK3) has been shown to promote factor-induced reprogramming to a basal 'ground state' [41].

Table 1. Summary of strategies to generate induced pluripotent stem cells, including species that have successfully been reprogrammed.

method	mode	pros	cons	species reprogrammed	references
retroviral	integrative	virus efficiently silenced/efficient and tried and tested	modifies genome, potential reactivation	mouse/human/rat	[6,11,16]
lentiviral	integrative	virus efficiently silenced/efficient and tried and tested	modifies genome, potential reactivation	mouse/human/pig/rat/sheep	[12,14,17,18]
piggyBac	integrative + excision	leaves no footprint	excision inefficient and screening laborious; possibility of mobilization of endogenous transposons	mouse/human/horse	[19–23]
lentivirus + Cre	integrative + excision	efficient and the vector can be removed	leaves footprint of vector	mouse/human	[19,24,25]
plasmid + Cre	integrative + excision		leaves footprint of vector	mouse	[19]
plasmid	integrative		modifies genome; requires repeated transfection	human	[26–28]
episomal vectors	non-integrative vector		possible integration of DNA	human	[29]
adenoviral	non-integrative vector		possible integration of DNA	mouse	[30]
Sendai	non-integrative vector			human	[31]
protein (Arginine tag)	protein-based no nucleic acids	no genetic modification	very inefficient; requires repeated treatments	mouse/human	[32,33]
small molecules + virus		increase efficiency	integrative	mouse/human	detailed list in review [34]
small molecules + episome	non-integrative vector	increase efficiency	possible integration of DNA	human	[35]
mini-circles	non-integrative vector		possible integration of DNA	human	[36,37]
modified RNA	RNA-based, no vectors (non-integrative)	no genetic modification	requires repeated treatments; nucleic acid possible retro-transposition	human	[38]

Additional compounds that have global effects upon chromatin organization may facilitate the effect of introduced proteins. Compounds that are effective in some circumstances include valproic acid and butyrate (inhibitors of histone deacetylase), RG108 (a DNA methyltransferase inhibitor) and parnate (an H3K4 histone demethylase inhibitor). Other compounds are able to replace specific factors (see Efe & Deng [43]). Most strikingly, a combination of three inhibitors increased the efficiency of reprogramming of human fibroblasts by the four Yamanaka factors by 200-fold [44]. It is a particular challenge to replace all of the transcription factors with small molecules, but human fibroblasts have been reprogrammed effectively by *Oct4* and a cocktail of chemicals [45].

3. DIRECTED FATE CONVERSION TO A DIFFERENT SOMATIC CELL TYPE

A landmark experiment in the molecular basis of cell identity was performed over 25 years ago, when gene-transfer experiments showed that ectopic expression of the transcription factor MYOD was sufficient to convert fibroblasts to muscle fibres [4]. Inspired by these experiments and by the new methods for the production of pluripotent stem cells, the possibility of inducing fibroblasts to acquire the characteristics of neurons [46] or cardiomyocytes has been investigated [47]. Both groups selected transcription factors that are specifically expressed in the target tissues, have known roles in differentiation to the target tissue or are known to promote epigenetic changes. Initially, 19 [46] or 14 [47] transcription factors were examined for their ability to activate tissue-specific reporters as the basis for their selection protocol. The same reductionist strategy successfully deployed by Takahashi & Yamanaka was then used in which transcription factors were removed from the pool individually to define the minimum effective group.

Together, *Brn2*, *Myt1l*, *Zic1*, *Olig2* and *Ascl1* were able to induce the change to a neural phenotype. Immature neurons were seen three days after the treatment of mouse embryonic fibroblasts (MEFs) and by day 5 neuronal cells with long branching processes were present. Although tail tip fibroblasts did not exhibit comparable neuronal cells before day 12, final efficiency of conversion was comparable, being 1–8% with both MEFs and tail tip fibroblasts. Further analysis demonstrated that *Ascl1* was sufficient to induce formation of immature neurons, but that inclusion of *Brn2* and *Myt1* generated mature neurons with efficiencies of up to approximately 20 per cent. Neurons from both donor cells types were able to form synapses and express markers of cortical identity. A low proportion of induced neuronal cells expressed markers of GABAergic neurons. Further research is required to identify protocols that lead to the formation of other neural cell types.

The reporter gene was activated in over 20 per cent of cardiac fibroblasts when *Gata4*, *Mef2c*, *Mesp1* and *Tbx5* were introduced [47]. Subsequent analyses established that *Mesp1* was not essential. Detailed analyses of the chromatin of the cells showed that fate change induced by the pool of three genes had altered their epigenetic status to be very similar to that of cardiomyocytes.

Interestingly, monitoring gene expression following this fate change established that during this process none of the reprogrammed cells passed through a progenitor state. The same pool of three genes was able to direct a fate change of tail tip fibroblasts, although there were some differences in the nature of the cardiomyocytes that were induced. A small proportion of the cardiomyocytes derived from cardiac fibroblasts (1%) showed the electrophysiological and beating characteristics of cardiomyocytes.

A different approach to direct fate change of somatic cells depended upon the observation that during derivation of iPS cells, colonies were present with a round morphology that resembled that of haematopoietic cells [48]. In subsequent analysis, forced expression of *OCT4* alone made it possible to isolate CD45 haematopoietic progenitors from primary human fibroblast cultures. The subsequent fate of these cells depended upon the culture environment; under appropriate conditions, multipotent haematopoietic progenitors able to form cells of the myeloid, erythroid and megakaryocytic lineages were obtained without first becoming iPS cells.

4. MECHANISMS THAT UNDERPIN PLURIPOTENCY

Since pluripotent stem cells were first cultured in 1967 [49], much has been learned about the mechanisms that maintain pluripotency [50,51]. Early lessons led to the direct isolation of ES cells from mouse embryos [52,53]. The subsequent isolation of human ES cells [54] highlighted notable differences between mouse and human ES cells in morphology and in growth factor requirements. The more recent establishment of pluripotent cell lines from the post-implantation mouse epiblast (known as epiblast stem cells or EpiSCs) that resemble human ES cells in growth factor requirements and gene expression has raised the possibility that, although derived from pre-implantation embryos, human ES cells may be most closely related to the post-implantation human epiblast. The recent establishment of EpiSCs from pre-implantation mouse embryos cultured in human ES cell culture conditions adds weight to this proposition [55,56].

Ordinarily, mouse ES cells require the combined actions of leukaemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) to remain in a pluripotent state [57]. However, LIF and BMP can be dispensed with provided the cells are treated with inhibitors of particular signalling pathways [58]. Inhibition of the mitogen-activated protein kinase (ERK1/2) pathway as well as inhibition of GSK3 is able to maintain pluripotency. These results indicate that shielding mouse ES cells from pro-differentiative signals enables the stabilization of the pluripotent phenotype and has been interpreted to indicate that cells cultured in this way are in a basal 'ground state' in which they can propagate indefinitely provided they are insulated from signalling pathways. However, the possibility remains that undetected autocrine signals may be required to maintain the cells in an undifferentiated state.

As well as signalling requirements, particular transcription factors have important roles in maintaining

pluripotency. Three of the most critical factors are *Oct4*, *Sox2* and *Nanog*. Expression of Oct4 is required for the initial specification of pluripotent cells *in vivo* [59]. Maintenance of the pluripotent state depends on keeping *Oct4* expression between upper and lower limits; loss of expression leads to trophectoderm differentiation [59,60] while a higher level induces differentiation to mesoderm and endoderm [60]. *Sox2* is required for epiblast maintenance [61] and acts upon many of the same target genes as *Oct4* by binding to DNA sequences adjacent to the *Oct4* binding site [62]. The level of expression of *Nanog* determines self-renewal in mouse ES cells as overexpression renders the cells independent of cytokine [63]), while reduced expression causes a reduction in self-renewal efficiency [64]. *Nanog* is also required for pluripotent cell specification both during development and in the final stages of reprogramming to 'ground state' pluripotency [65]. This requirement is discussed in the paper by Theunissen & Silva [66] in this issue. *Nanog* is also needed to allow the completion of primordial germ cell development [64]. This dual requirement is interesting in view of the two waves of epigenetic change that occur at the blastocyst stage and during primordial germ cell development. The confluence and overlap in mechanisms occurring during these processes is the subject of the paper from Hajkova [67] in this issue.

Much of the present understanding of the mechanisms that regulate pluripotency or initiate differentiation has been gleaned by manipulating and observing a very small number of genes. In contrast, new studies that monitor changes in thousands of genes and use bioinformatics tools to identify interactions between genes are revealing large networks of interactions [62,68]. Genome-wide chromatin immunoprecipitation (ChIP) has been used extensively to identify transcriptional networks. Oct4, Sox2 and Nanog have feedback effects upon one another and they co-bind to promoters of many genes [69,70]. Moreover, many genomic sites to which Oct4, Sox2 and Nanog are co-localized also bind additional pluripotency transcription factors, suggesting that extensive combinatorial regulation of transcription underpins stable pluripotent cell identity [71,72]. Downstream genes include members of signalling networks, chromatin-remodelling proteins and micro-RNAs that are hypothesized to maintain pluripotency while also inhibiting differentiation.

While both mouse and human ES cells have transcriptional networks based around *Oct4* (*OCT4*), *Sox2* (*SOX2*) and *Nanog* (*NANOG*), differences between the species emphasize the idea that human ES cells are more like mouse EpiSCs than mouse ES cells [73]. In addition, protein interaction analyses are revealing associations between critical protein components in the network [74–77]. While all the above studies are important for generating hypotheses, many of the measurements have the limitation that they are snapshots of a fixed moment and thus operate as population averages, ignoring potentially functional variation within apparently homogeneous cell populations.

ES cells in culture show a marked variability in the expression of some key regulatory genes, including pluripotency genes such as *Nanog*, and *Sox2*, as well as lineage-affiliated genes such as *T-brachyury* and *Hex* as discussed by Osorno & Chambers [78] in this issue. Cells that express low levels of Nanog can revert to a high Nanog-expressing state, but are also more prone to differentiate. This has led to the hypothesis that heterogeneity allows differentiation to proceed while retaining a pluripotent population [64]. This idea is pursued in relation to the transition of ES cells to EpiSCs by Osorno & Chambers.

In this issue, Huang [79] discusses such heterogeneity using mathematical representations of cell identities in terms of the quantitative state of gene expression in a cell at a given time. While many possible combinations of individual gene expression states appear combinatorially possible, only a subset of these exists in reality. Furthermore, cells expressing similar levels of pluripotency genes may have reached that position from different starting points and be on different trajectories [79].

5. CHANGES DURING INDUCED PLURIPOTENT STEM CELL DERIVATION

In these circumstances, it is perhaps not surprising that there is considerable variation between cells in response to factor-induced reprogramming. Among fibroblasts selected initially as all having a high level of expression of *Thy1* as a marker of fibroblast phenotype, roughly half the cells had lost *Thy1* after five days of treatment, but only a small proportion of these cells become fully reprogrammed iPS cells [80]. By contrast, extended induction of transgene expression for several weeks suggests that most, if not all, cells have the ability to become iPS cells [81]. This difference between the initial reaction and the final response to prolonged treatment has been interpreted to indicate stochastic variation between cells in the precise exposure to reprogramming factors or in the ability of the cells to respond to those factors. Sequential detailed analyses of cells during the process of reprogramming can identify the population that is being successfully reprogrammed and so provide information on how to improve the procedure.

During secondary reprogramming of fibroblasts, expression of mesenchymal genes was reduced by 5–40-fold in four days along with the induction of genes concerned with proliferation. Variation in gene expression was observed in lines established after successful reprogramming, but in general they had become very similar to ES cells. Partially reprogrammed cells derived from foetal fibroblasts or B-lymphocytes showed incomplete inhibition of different lineage-specific genes and DNA hypermethylation of loci associated with pluripotency [82]. These observations led to the hypotheses that partial reprogramming might reflect inadequate inhibition of transcription factors associated with the somatic cell phenotype and failure to fully demethylate lineage-specific genes. Indeed, the efficiency of reprogramming was increased using RNAi to reduce the level of key transcription factors and of small molecules to promote demethylation [82].

Direct analysis of chromatin changes in secondary MEFs during the first days after induction of transgene expression is possible by careful monitoring of cell

division in secondary somatic cells that carry inducible vectors [83]. The predominant initial change was to silence somatic genes rather than activate pluripotency genes and this effect increased gradually with additional cell divisions. Rapid, genome-wide changes in the permissive histone modification, H3K4me2, were observed at large numbers of loci, including large numbers of genes related to pluripotency or regulation of development. Interestingly, these changes often preceded the change in gene expression and occurred in cells that had not yet divided. These results reveal an unexpected chromatin-remodelling effect of the reprogramming factors that precedes transcriptional activation of ES cell-specific genes.

High-resolution, time-lapse imaging technology has also been used to identify retrospectively those cells from which iPS cell colonies were derived following the treatment of secondary somatic cells carrying doxycycline inducible vectors described above [84]. Strikingly, all iPS cell colonies arose from small, fast-dividing cells that first became apparent soon after induction. These cells had a cell cycle length of 12.2 ± 2.8 h after the first division, which was strikingly shorter than that of published figures for embryonic fibroblasts (18–22 h), but very similar to that of murine ES cells (11–16 h). The fact that all of the iPS cells were derived from the small cells emphasizes the importance of this early change and argues in favour of there being a single important step at this stage. It will be interesting to see how this can be resolved with the model which is often accepted of an unpredictable, inefficient progress through several independent steps—the stochastic model of iPS cell production.

6. MOLECULAR MECHANISMS OF REPROGRAMMING IN OTHER MODEL SYSTEMS

In addition to direct observations during iPS cell production, four other model systems have provided insight into molecular mechanisms of cellular de-differentiation. These are SCNT, exposure of mammalian somatic nuclei to amphibian oocyte cytoplasm, analysis of epigenetic changes during the development of germ cells and cell fusion. It is noteworthy that studies in all four situations describe rapid changes in chromatin structure including DNA methylation, but the mechanisms remain to be fully understood.

Following the transfer of nuclei into enucleated oocytes, direct observation by immunohistochemistry demonstrated major changes in DNA methylation and histone modifications within hours of nuclear transfer [85]. Early experiments demonstrated dramatic reductions in the expression of genes associated with the somatic donor cells by the blastocyst stage [86,87]. However, recent work has shown that even at this stage of development, abnormalities of gene expression were present in most of the 87 individually analysed murine blastocysts [88]. In this study, nuclei from Sertoli cells, cumulus cells and ES cells were compared and characteristic perturbations to gene expression were apparent in the three groups of embryos. Repression of gene expression was by far the more common perturbation with expression of seven genes depressed in embryos derived from all three different donor cell types—*Asz1*, *Magea5*, *Magea3*, *Xlr3a*, *Xlr5c*, *Hemt1*

and *Tktl1*. These genes may be suitable early markers of successful reprogramming after nuclear transfer as their normal expression in cloned embryos was associated with the most nearly normal overall pattern of gene expression.

An alternative system for analysis of reprogramming events after nuclear transfer is to follow the changes in the nuclei of mammalian somatic cells injected into *Xenopus* oocytes [89,90]. In the *Xenopus* system, large numbers of nuclei can be studied, with a majority of them being within the germinal vesicle [91]. Transplanted nuclei begin transcribing pluripotency genes within 24–72 h, depending upon the stage of development from which the cell was obtained. ChIP assays revealed an increase in histone H3 lysine 4 dimethylation (H3K4me2) in the regulatory and coding regions of pluripotency genes in reprogrammed nuclei. Interestingly, the extent of histone H3 lysine 4 trimethylation (H3K4me3) varied and was concluded to be a function of the timing of reprogramming. H3K4me3 was not at all seen on genes such as *β-globin* that are not transcribed, but was present on pluripotency genes in some nuclei [89].

There is some evidence that axolotl oocyte cytoplasm has a greater ability than that of *Xenopus* to remodel mammalian chromatin [92]. There were dramatic changes in gene expression within 6 h of treatment in breast cancer cells permeabilized before immersion in extract, including the expression of some genes that were silenced in the cell line. This expression occurred without DNA replication, and was associated with demethylation of DNA and removal of repressive histone marks at the promoters of tumour suppressor genes. This remodelling activity was found only in prophase oocytes and not in extracts from ovulated eggs. Furthermore, a similar activity was present in only limited amounts in mouse ES cell extracts [92,93]. These studies in amphibians provide opportunities for identifying additional factors that might be used in factor-directed reprogramming protocols.

In addition to DNA demethylation during the formation of pronuclei referred to above, similar changes occur during the development of primordial germ cells and this, too, is amenable to manipulation and analysis. At both of these stages, components of the base excision DNA repair (BER) pathway accumulate at the time of DNA demethylation and molecular inhibitors of the BER pathway prevent DNA demethylation in germ cells [93], suggesting that this pathway may have a role in demethylation. Changes in DNA methylation are associated with the loss of repressive histone modifications, such as H3K9me3 and H3K7me3 and it has been proposed that these marked changes in chromatin structure may reflect disassembly and rebuilding of chromatin, rather than the action of several histone modification enzymes [67].

When human somatic cells are fused to murine ES cells, in some cases, gene expression of the human nucleus is modified to closely resemble that of human ES cells. Indeed, expression of key pluripotency genes is initiated within 24 h [94]. Use of this approach to analyse mechanisms of reprogramming is discussed by Piccolo and colleagues [95] in this issue. Conversely, expression of tissue-specific genes is significantly reduced within 48 h. All of these

changes occur before fusion of nuclei in these cells, which are known as heterokaryons. If the nuclei are removed from ES cells before fusion of the cytoplast to the human somatic cell, the degree of nuclear re-modelling is greatly reduced [96]. Following fusion of human B cells to mouse ES cells, approximately 15 per cent of the heterokaryons express SSEA4 and other cell surface markers of pluripotency. This approach can be used to select those cells that express the pluripotency genes *OCT4*, *CRIPTO* and *NANOG* [94,97]. It is then possible to search for the key molecular mechanisms that bring about remodelling [98]. Oct4 and the remodelling complexes PRC1/PRC2 are all essential for reprogramming to pluri-potency. Oct4 protein from the mouse ES cell migrates into the human nucleus over the period of 6–34 h during which the volume of the nucleus swells by approximately 2.5-fold, although these changes are not themselves predictive of successful reprogramming. Other changes that were associated with successful remodelling include an increase in the level of phos-phorylated histone H3 at serine 10 (H3S10ph) and the re-distribution of heterochromatin protein 1 (HP1α) in human nuclei. Inhibition of phosphorylation of H3S10 dramatically reduced H3S10ph levels in human B cell nuclei, and prevented HP1α redistribution, but did not abolish successful reprogramming. This result suggests that although changes in H3S10ph and HP1α characterize reprogrammed cells, neither is essential for successful reprogramming to pluripotency and it con-trasts with the observations of changes in these proteins in the nuclei incubated in *Xenopus* oocytes [89].

Together, these different observations reveal that reprogramming as achieved by the four Yamanaka fac-tors is slow and particularly inefficient in comparison with the changes in these other model systems. It is per-haps unsurprising that exposure of a somatic nucleus to the complex mileu of an oocyte cytoplasm or a pluripo-tent cell would result in a more rapid change than can be achieved by merely adding four transcription factors. It is possible that differences in the concentration of the active agents in relation to the quantity of chromo-somes are contributory. Alternatively, reprogramming by transcription factors may appear more passive compared to exposure to extracts because following expression, transcription factors must accumulate and be formed into effective multi-protein complexes in order to function. In addition, the more rapid repro-gramming may reflect the action of other factors that are present in oocyte or ES cell cytoplasm that have not yet been identified.

A recent search for such factors identified components of the ATP-dependent Brahma (or Brahma-related-gene 1)-associated factor (BAF) chromatin-remodelling complex as being able to significantly increase the repro-gramming efficiency of fibroblasts by the four Yamanaka factors. These observations are interesting given the role of an ES cell form of BAF in maintaining pluripotency [99–101] and the detection of Brg1 and Baf155 in Oct4 protein complexes [76]. This may suggest that BAF acts by facilitating chromatin binding of Oct4 to promoters during factor-directed reprogramming. It remains to be seen if this effect can be confirmed and extended by the identification of other factors.

7. THE ACCURACY OF FACTOR-DIRECTED REPROGRAMMING
While it was immediately obvious that profound changes had been induced in iPS cells, it is always important to assess the accuracy of reprogramming by a number of different measures. These may include confirming the presence of key markers of cell iden-tity, genome-wide measures of gene expression and epigenetic status, or confirmation of appropriate phys-iological function. A brief survey of the literature revealed differences in gene expression between pluri-potent stem cell populations [102]. In some cases, the abnormal pattern of gene expression suggested a memory of the original phenotype presumably because of a limitation of the reprogramming procedure [103,104], but interestingly, iPS cell populations with abnormal gene expression could be coaxed to a more ES cell-like pattern either by prolonged culture or treat-ment with small molecules. It has been suggested that advantage might be taken of this memory by using donor cells of the tissue type that one ultimately wishes to produce from the iPS cells. In that way, the cells produced from the iPS cells might more closely resemble endogenous cells of the same type as the donor cells. While superficially attractive, this approach assumes that the epigenetic memory of the donor phe-notype is the only failure of reprogramming, whereas a procedure that fails to reprogramme accurately may well have other limitations.

Unexpected differences between human ES cell lines from different laboratories were revealed by simi-lar analyses [105]. In many cases, iPS cell lines resembled the ES cell lines from the same laboratory suggesting that a significant proportion of the variation was induced by the procedures that were used for the derivation and maintenance of the cell lines. Together, these observations suggest that there is a need for refine-ment not only of the procedures for reprogramming cells, but also for ES cell derivation and maintenance. Attainment of these advances will be assisted by a greater understanding of the mechanisms that underpin pluripotency.

8. USING INDUCED PLURIPOTENT STEM CELLS TO MODEL HUMAN DISEASE
The ability to produce pluripotent cells from a patient's somatic cells has led to the establishment of iPS cells from many disease conditions including Par-kinson's disease and amyotrophic lateral sclerosis (ALS), also known as motor neuron disease, as dis-cussed by Unternaehrer & Daley [106] in this issue. These disease-specific iPS cells are being used in an unprecedented way to discover the molecular basis of the inherited disease and to establish assays to identify small molecules that prevent or delay the development of disease symptoms. The progress and the likely challenges can be illustrated in relation to familial dys-autonomia (FD), ALS, muscle wasting diseases and diabetes.

As discussed in detail by Lee & Studer [107] in this issue, the study of FD provides an excellent proof of the concept that it is possible to use iPS cells and their derivatives to obtain important insight into the molecular

mechanisms that cause inherited diseases, particularly where there is little prior knowledge. FD is a rare autosomal recessive disease associated with deficiencies of the autonomic nervous system and dysfunction of small-fibre sensory neurons. It is caused by single-point mutation in intron 20 of the I-κ-B kinase complex-associated protein (*IKBKAP*) gene, which leads to exon 20 skipping and reduced levels of full-length IKAP protein (Lee & Studer). *IKBKAP* is part of the transcription elongation complex and it is widely expressed, raising the interesting question of why a defect in a gene with such wide expression should have such an exquisitely cell-specific phenotype. There are no mouse models of the disease.

A number of iPS cell lines were derived from patients with the disease and their unaffected relatives with ES cell lines also used as control [108]. IKBKAP expression was determined in tissues from all three germ layers: neural crest precursors, neural rosette cells, haematopoietic cells, endothelial cells and endodermal precursors. The lower absolute level of normal IKBKAP in all cell types of the patient-derived cells mimicked the differences *in vivo* [108]. Microarray analysis of gene expression in neural crest precursors revealed decreased expression of several genes concerned with peripheral neurogenesis. In a wound-healing assay, FD-derived neural crest precursors migrated less efficiently than control cells. Finally, it was demonstrated that the plant hormone kinetin was able to decrease the quantity of mutant protein and partially restore the level of normal protein, but long-term treatment was required to restore generation of autonomic neurons. As the mutation has its effect during development and very early life, this raises the practical challenge that full prevention of the disease symptoms may require treatment during foetal life. In this project, the cells studied were all precursor populations and the authors emphasize the need to extend these studies to the full range of terminally differentiated populations as methods for their derivation become available.

ALS is a relentless progressive family of different, but related, conditions. The common characteristic is the death of both upper and lower motor neurons in the brain stem and the spinal cord. During development of the disease, the motor neurons accumulate inclusions that typically contain ubiquitinated proteins, including TAR DNA-binding protein (TDP43). Approximately 5 per cent of cases are inherited, but the cause of the vast majority of cases is unknown. Among inherited cases, approximately 20 per cent are associated with mutations in superoxide dismutase (SOD1) of which more than 150 have been identified. Causative mutations have been identified in several other genes that are all involved in protein processing [109]. Table 2 describes the known mutations. At present there are no effective treatments for ALS.

Research with mice carrying mutant human *SOD1* transgenes showed that the disease arises from the presence of a corrupted protein rather than a loss of function. In a more detailed analysis, removal of the transgene specifically from motor neurons or glia showed that onset of the disease is associated with the mutant gene in the motor neurons, but that fatal progression of the disease reflected the presence of the transgene in the glia [145].

In the first laboratory based studies of the disease, motor neurons and glia were brought together in co-culture [146–148]. In these cases, the cells were derivatives of ES cells or of primary cells, and not iPS cells. The motor neurons were derived from human ES cells in all studies and they were cultured with glia that carried a transgene which directs the expression of a mutant human *SOD1* allele. In one case, the glia were from transgenic mice [147], while in the other, the cells were primary human astrocytes transduced with the mutant human gene [148]. Both groups observed death of motor neurons under these co-culture conditions, thus mimicking events *in vivo*. Inclusion of an antioxidant reduced the incidence of cell death suggesting that therapies of this kind may be beneficial and should be investigated further [148].

While these results demonstrate that cell culture can be used to model some aspects of degeneration in ALS, a note of caution is necessary because the transgene expressed the mutant protein at a higher than native level, and in one study expression of the mutant protein by the native promoter did not cause the death of neurons in the culture assay that was used [147]. This highlights the limitations of iPS cells in this specific context. Ongoing projects in several laboratories examine the role of other candidate genes in the aetiology of ALS. It is now known that aberrant accumulation of TDP43 occurs in over 90 per cent of cases of ALS, while mutations in this gene account for only 2 per cent of inherited cases. It remains to be seen whether novel treatments developed on the basis of a new understanding of the causative mechanisms in inherited cases will also be effective in sporadic cases.

In the paper by Melton [149] in this issue, it has been proposed that the emerging methods will make it possible to investigate even more complex diseases such as type 1 diabetes. In most cases, type 1 diabetes is an autoimmune disease in which immune cells attack and deplete the pancreatic beta cells that normally monitor and maintain blood glucose levels. While this process is well-documented, the initial trigger that sets off this chain of events is unknown. In order to study the molecular basis of the disease, pancreatic beta cells, thymic epithelia and haematopoietic stem cells from diabetic patients must be produced and the relationship between these different cells must be analysed within immune-deficient mice.

iPS cells offer exciting new possibilities for understanding the complex processes that underlie the initiation and pathology of muscle diseases, as discussed by Shadrach & Wagers [150] in this issue. Muscle tissue is unusual in that it is possible to obtain the specialized muscle-forming stem cells known as satellite cells in a biopsy. Research using these cells has established that loss of satellite cell function can lead to the failure of muscle to recover, particularly in cases of progressive muscle disease such as Duchenne muscular dystrophy. Recent progress in the isolation of muscle satellite cells and elucidation of the cellular and molecular mediators controlling their activity indicate that these cells

Table 2. Mutations linked with familial ALS (fALS). ALS, amyotrophic lateral sclerosis; FTD, frontotemporal lobe dementia; AD, autosomal dominant; AR, autosomal recessive.

familial ALS designation	location of mutation	inheritance	no. indentified mutations	chromosomal locus	onset	references
ALS1	*SOD1*	AD	+120	21q	adult	[110]
ALS2	*ALSIN*	AR	10	2q33	juvenile	[111–114]
ALS3	not established	AD	not established	18q21	adult	[115]
ALS4	*SETX*	AD	3	9q34	juvenile	[116,117]
ALS5	not established	AR	not established	15q15	adult	[118]
ALS6	*FUS*	AD/AR	16	16q21	adult	[119–121]
ALS7	not established	AD	not established	20p13	adult	[122]
ALS8	*VAPB*	AD	1	20q13.3	adult	[123,124]
ALS9	*angiogenin/VEGF*	AD	6	14q11	adult	[125–128]
ALS10	*TDP43/TARDP*	AD	30	1q36.2	adult	[109,129–135]
ALS-FTD	*MAPT* (Tau)	AD	+50	17q21.1	adult	[136–139]
FTD3	*CHMP2B*	AD	+6	3	adult	[140]
ALS	*DCTN1*	AD	3	2p13	adult	[141,142]
ALS	*OPTN*	AD	>3	10	adult	[143]
ALS	*VCP*	AD	>1	9p13.3	adult	[144]

represent promising therapeutic targets. Such satellite cell-based therapies may involve either direct cell replacement or development of drugs that enhance endogenous muscle repair mechanisms. Ultimately, the ability to derive transplantable muscle stem cells from patient-specific iPS cells would make it possible to create gene-corrected cells that would be genetically matched to individual patients.

The study of FD and the recent demonstration of long Q-T syndrome in cardiomyocytes derived from iPS cells [151] indicate that it is possible to use iPS cells and their derivatives to characterize the molecular mechanisms that cause some inherited diseases. It may be important that the disease phenotype of both of these conditions is apparent at or soon after birth and reflects the influence of dominant mutations. By contrast, more common degenerative diseases such as Parkinson's and Alzheimer's diseases usually develop over many years and involve effects of several genes and environmental influences.

There is no absolute requirement for knowledge of the causative mutation, provided that it is very likely that the disease was inherited. iPS cells may make it possible to search for genes that modify the effect of mutations. These might be identified by contrasting iPS cells from patients within the same family who carry the same mutation, but who do not exhibit disease symptoms as the lack of effect of the mutation may reflect the influence of genes that are able to ameliorate the harmful effect of the mutation. An understanding of the means by which this occurs may suggest new approaches to therapy.

The use of reprogrammed cells should be complemented by other approaches to study inherited diseases, including the use of gene targeting to either introduce candidate mutations into ES cell lines or to correct a mutation to confirm its role in causing a disease phenotype. There is also an opportunity to request permission to derive ES cells from embryos that are being discarded because they were identified by pre-implantation genetic diagnosis as carrying a mutation that causes a serious inherited disease.

Together, these opportunities hold out the long-term promise of providing the first effective treatments for at least some degenerative diseases.

9. PRACTICAL CONSIDERATIONS FOR DEPLOYMENT OF REGENERATIVE STRATEGIES

In the longer term, it may be possible to replace the dead or non-functioning cells that cause disease symptoms with healthy, laboratory produced cells. However, a great number of biological and technical challenges will have to be overcome before this becomes possible. At present, clinical trials are in progress in which human ES cell derivatives are being considered for spinal cord repair [152] and immortalized foetal neurons are being developed for the treatment of stroke [153].

One biological challenge is the immune rejection that may follow transplantation of cells from an unrelated embryo or individual. The ability to produce pluripotent cell lines from an adult by reprogramming creates the possibility of providing patient-specific cell lines. However, the effort and cost involved would probably be prohibitive, and alternative strategies are suggested by Taylor *et al.* [154] in this issue. It has been proposed that banks of appropriately selected cell lines could provide matched cell lines for a majority of the population. Similar conclusions were drawn from analyses in Britain and Japan [155,156] and suggest that banks of selected iPS cells from blood group O donors with conserved homozygous human leucocyte antigen (HLA) haplotypes would be the most effective way of providing cells for treatment of a majority of the population. Specifically, it is estimated that around 10 lines that are blood group O and homozygous for HLA haplotypes would be expected to provide a perfect HLA-A, -B and -DR match for around 38 per cent of recipients and be beneficial for 67 per cent of recipients taken at random [156]. A total of around 150 lines would provide beneficial lines for essentially the entire British population. Racial differences could then be accommodated by exchanging lines between different countries. It should be noted that the use of such

partially matched cells would require chronic immune suppression and thus while valuable for off-the-shelf therapies at scale, these are not optimal cells. Before cells can be used for therapy, there is also a need to resolve a number of other biological issues, none of which are trivial. These include establishing methods for the large-scale production of clinical grade cells at specific stages of differentiation, storing and shipping those cells, and transplanting them to the appropriate location within the body.

In addition to these biological challenges, novel approaches are required for funding both the particularly expensive pre-clinical phases of development and the treatments themselves. As has been emphasized by Prescott [157] in this issue, regenerative treatments by cell therapy are more expensive than conventional pharmacological treatments, but, therapeutic benefits may last longer and therefore be more cost-effective in the long term.

10. WHAT ARE THE LIMITS TO THE OPPORTUNITIES TO MODIFY CELL FATE?

Our understanding of the stability of cell phenotype has been transformed in a totally unexpected manner during recent decades. The examples of cellular reprogramming tell us that cell fate is plainly far less rigidly determined than was previously believed. However, the limitations to our opportunities to change cell fate are still not clear. Is it possible, for instance, to produce any cell type from any donor cell type, given the introduction of the necessary regulatory molecules and an appropriate culture environment?

Cell reprogramming has already created extraordinary opportunities for research. In time, reprogramming studies may offer new drug treatments that prevent degenerative changes or induce stem or progenitor cells to differentiate into the affected lineage in a specific condition. Cell transplantation might also become available for some degenerative diseases. The pace and scope of these advances are clearly unpredictable. In all likelihood, cell therapy will not be possible for all degenerative and genetic diseases for reasons that we cannot yet foresee. Furthermore, the development of treatments will almost definitely happen over a period of decades, and not merely years. Despite these notes of caution, we can be optimistic that the pace of advance will not slacken and that scientists can hope to deliver the therapeutic gold that patients so urgently seek.

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