Review

Expert Opinion

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# New approaches for the generation of induced pluripotent stem cells

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***Introduction:*** The advent of induced pluripotent stem cell (iPSC) technology has opened up new vistas to generate patient-specific pluripotent stem cells from somatic cells. During the last 5 years, the iPSCs produced from a variety of somatic cell sources are found to be very similar, if not identical to embryonic stem cells. Invariably these cells are produced by viral transduction of four transcriptional factors that renders these cells unfit for therapeutic purposes.
***Areas covered:*** This review discusses current developments emphasising on new and improved methods of generating iPSCs, including minimal or no genetic modifications via excisable lentiviral and transposon vectors or through repeated application of transient plasmid, episomal and adenovirus vectors. Recent use of small molecules, synthetic mRNA and microRNAs is also reviewed.
***Expert opinion:*** iPSC technology is emerging as an unprecedented opportunity in biomedical research, disease modeling, drug discovery and regenerative medicine. However, to harness the full potential of this technology, a number of issues that need to be resolved pertaining to iPSC safety, stability, culture variability, their comparison with ES cells, the reprogramming mechanisms and better ways to direct a specific reprogramming process including lineage specifications.

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## 1. Introduction

Reprogramming of somatic cells has received much interest lately in regenerative medicine as it can offers a potential accessible source of cells that can be used to replace the defective or diseased cells in patients suffering with for example Alzheimer's, Parkinson's, Type 1 diabetes or cardiomyopathy that involves specific cellular pathology. The two most remarkable techniques used in reprogramming are, somatic cell nuclear transfer (SCNT) and induced pluripotent stem (iPS) cell generation. Both of the techniques can reprogram a differentiated cell back to an embryonic state [1,2]. Nuclear transfer experiments date back to the 1950s where frogs (*Rana pipiens*) were cloned by replacing nuclei of eggs with cells from the late blastula [3] and since then SCNT has been successfully reported in a number of mammalian species, including the birth of Dolly the sheep [4]. SCNT although remains the method of choice for generating isogenic pluripotent stem cells, this procedure is technically challenging and inefficient with low success rates and so far not successful in humans because of ethical constraints. The discovery of the induced pluripotent stem cell (iPSC) technique using defined transcription factors by Yamanaka invigorated this field and changed the paradigm and shifted the

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**Article highlights.**

- Over the past five years, the iPSC technology has significantly advanced. iPSCs have been generated from a broad range of somatic cell types [6-10] and species [11-15], differentiated into various seemingly functional cell types, and used to study basic biology and model human diseases. These studies have opened up new possibilities that iPSCs could be developed as unlimited and patient-matched cell sources for cell-based therapy or drug discovery and disease modeling. This review describes the recent progress in iPSCs research and development in improved techniques for generating these cells.
- The conventional iPSC technique that involves virus-mediated delivery of reprogramming factors, invariably leads to a permanent integration of oncogenes and potential genomic alterations.
- Recently purified recombinant reprogramming proteins fused with a poly-arginine cell-transduction domain were shown to be effective in generating iPSCs from fibroblasts.
- Modified RNA technology was also effective at redirecting stem cells to form other tissue types. Currently, scientists attempt to coax iPS cells to differentiate to clinically useful cell types by changing their external environment.
- However, some fundamental questions remain, such as why such cells show variations in pluripotency as determined by tetraploid complementation, which is the defining capability of a bona fide pluripotent stem cell line [16,17]. This also raises the question how to address the similar issue with human iPSCs, that cannot be subjected to a similar stringent test of pluripotency. Are human iPSCs an exact reproduction of human embryonic stem cells (ESCs), or have they inherited the incomplete pluripotency of their mouse iPSC counterparts? Due to the limitation of the use of tetraploid complementation test of pluripotency in human iPSCs, recently a number of studies have chosen to compare the gene expression profiles including epigenome, transcriptome, microRNA and genome-wide CpG methylation profiling of such cells with hESCs [18-23].
- Both iPSCs and ESCs are pluripotent – they can form any tissue in the body. Yet subtle differences exist as revealed recently, Su-Chun Zhang and his colleagues at the University of Wisconsin–Madison compared the ability of both types of pluripotent cell to form human neurons in a laboratory setting, and found that iPS cells did so with markedly lower efficiency than ES cells [41].
- It was demonstrated recently that reprogramming of somatic cells also resets genomic methylation, an epigenetic modification of DNA that influences gene expression, thus the resulting pluripotent stem cells might have different properties [42].
- Transgenic animal models have taught us a great deal about disease process in some of the diseases but question remains whether therapeutically significant insights will be gained from the study of animal models. In this context, iPSC cells derived from humans with inherited neurological disorders may provide invaluable tools for elucidating the mechanisms by which the disease-causing gene products kill neurons in the human cellular context and at endogenous levels of expression.
- Once disease-specific phenotypes are identified and translated into robust cell-based assays, the most consequential use of iPS cells derived from affected individuals will be the screening of candidate drugs by, for example, high-throughput platforms [56].
- As the mechanism of conventional iPS cell induction methods remains largely unknown, understanding this microRNA (miRNA)-mediated somatic cell reprogramming (SCR) mechanism may shed light on the improvement of iPS cell generation.
- There is no doubt that iPSC research will continue to offer new opportunities for stem cell research and regenerative medicine for years to come.

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debate away from SCNT program, particularly for humans with reduced ethical concerns [5]. Over the past five years, the iPSC technology has significantly advanced. iPSCs have been generated from a broad range of somatic cell types [6-10] and species [11-15], differentiated into various seemingly functional cell types and used to study basic biology and model human diseases. These studies have opened up new possibilities that iPSCs could be potentially developed as unlimited and patient-matched cell sources for cell-based therapy or drug discovery and disease modeling. This review brings out the recent progress in iPSCs research and development in improved techniques for generating these cells.

## 2. iPSCs pluripotent identity

The discovery that somatic cells can be reprogrammed to pluripotent stem cells (iPSCs) has opened up promising new avenues for regenerative medicine. However, some fundamental questions remain, such as why such cells show variations to pluripotency as determined by tetraploid complementation, which is the defining capability of a bona fide pluripotent stem cell line [16,17]. This also raises the question how to address the similar issue with human iPSCs, that cannot be subjected to a similar stringent test of pluripotency. Are human iPSCs an exact reproduction of human embryonic stem cells (ESCs), or have they inherited the incomplete pluripotency of their mouse iPSC counterparts? Due to the limitation of the use of tetraploid complementation test of pluripotency in human iPSCs, recently a number studies have chosen to compare the gene expression profiles including epigenome, transcriptome, microRNA and genome-wide CpG methylation profiling of such cells with hESCs [18-23]. These studies do indicate that although both iPSCs and hESCs cluster together as far as plurinet genes are concerned, there are also subtle differences between iPSCs and hESCs that have been attributed to a number of factors such as variation in data analyses, laboratory conditions and variations in protocols used for reprogramming. Further studies are



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required to identify specific genetic and epigenetic signatures that can be used to distinguish and classify iPSC lines, analyse their functionalities, including safety, self-renewal stability and differentiation potentials. This is particularly critical for evaluating human iPSCs for various applications, as there is a lack of authentic and stringent *in-vivo* pluripotency assays. Studies have shown that human (hESCs) are significantly different from mouse ESCs (mESCs) in terms of cell behaviours, gene expression, and signalling responses to several key developmental signaling pathways (e.g., TGF-β, bone morphogenic protein (BMP), leukemia inhibitory factor (LIF), fibroblast growth factor (FGF) and MAPK) in self renewal and differentiation, but correspond very closely to mouse epiblast stem cells (EpiSCs) that are derived from the postimplantation egg-cylinder-stage epiblasts of mouse. These observations support the notion that EpiSCs and hESCs (as well as human iPSCs generated with the conventional hESC culture conditions) are intrinsically similar, and suggest that mESCs and EpiSCs/hESCs represent two distinct pluripotent states: the mESC-like state representing the preimplantation inner cell mass (ICM) and the EpiSC-like state representing late epiblast cells, respectively. Therefore, creating mESC-like human pluripotent cells has attracted increasing interest. It was shown recently that a different type of human iPSCs that functionally behaves similarly to mESCs could be generated and maintained by combining the reprogramming approach and small-molecule modulators of TGF-β, MAPK and glycogen synthase kinase 3 (GSK3) [14]. Generation of such mESC-like human pluripotent cells would have significant values in both basic research and practical applications. These cells would be very useful to study the early human development that may not be easily pursued. Given the above considerations, generating reprogrammed cells to be identical to ESCs or with specific properties that distinguish those from ESCs would represent continued areas of interest for various applications.

## 3. Mechanism of reprogramming

Reprogramming needs to inactivate the somatic cell program and to activate the ES-cell-specific transcription programs of self renewal and pluripotency. One could speculate that the reprogramming factors contribute to both functions, as they can, in ES cells, be both repressive and activating. Thus, genes that encode somatic cell regulators could be repressed by the binding of the reprogramming factors, while self-renewal and pluripotency genes would be turned on [24]. Mechanism(s) that govern reprogramming in embryonic development, in SCNT and iPSC derivation process remain elusive. A number of studies have made efforts to identify the molecular factors involved in inducing or maintaining pluripotency. Examples include gene profiling and epigenetic mapping studies [25-33] conducted in either iPSCs or embryonic stem cells (ESCs). In addition, recent reports of the important roles of tumour suppressor genes p53, p16 and p19ARF shed some light on the iPSC induction process [34-38]. Another interesting advance came from a study that showed that activation-induced cytidine deaminase (AID) may be required for epigenetic reprogramming toward pluripotency in human cells [39]. However, the key molecular pathways/ processes through which iPSCs are derived from differentiated cells remain unknown. Recently Li *et al.*[40] have reported that caspases 3 and 8, two proteases previously associated with regulation of apoptotic cell death, play key roles in mediating the reprogramming of human fibroblasts into iPSCs. Both caspases are activated by Oct-4, the most important transcription factor in iPSC induction. The functional importance of the activation of the caspases was demonstrated by the fact that blocking caspase 8 led to a complete shutdown of the iPSC reprogramming process. Furthermore, they provided evidence that the retinoblastoma susceptibility (Rb) protein is a critical target of caspases 3 and 8 during iPSC nuclear reprogramming from human fibroblast cells. Recently we have also observed [2] that these caspases are also activated during reprogramming process involving non-genetic means. However, continuous activation of these caspases beyond reprogramming may be detrimental for the reprogrammed cells cultured *in vitro*. Some recent studies point to the possible pleiotropic effects of various ectopic transcription factors commonly used in reprogramming process through global perturbations of chromatin structure by histone acetylation that may lead to overcoming epigenetic barriers and DNA methylation – an important event in normal mammalian development [24].

## 4. Differentiation potentials of iPSCs

Both iPSCs and ESCs are pluripotent – they can form any tissue in the body. Yet subtle differences exist as revealed recently, Su-Chun Zhang and his colleagues at the University of Wisconsin–Madison compared the ability of both types of pluripotent cell to form human neurons in a laboratory setting, and found that iPS cells did so with markedly lower efficiency than ES cells [41]. As described above these differences may arise because of different conditions used for producing iPSCs including unique genetics of the somatic cells used. Therefore, a team led by Konrad Hochedlinger at Massachusetts General Hospital in Boston has now derived iPS and ES cells with identical DNA [16]. The iPS cells were less efficient than the ES cells at incorporating into chimeric mice – a standard test of pluripotency, or 'stemness'. The team added the stem cells into embryos from mice of a different colour; once each mouse matures, the colouring of its coat reveals how much the stem cells contributed to forming its tissue. When the scientists compared genome-wide expression patterns between the two cell types, they discovered that a small stretch of DNA on the long arm of chromosome 12 displayed significantly different gene activity. In this region, two genes and a slew of tiny regulatory sequences called microRNAs were consistently activated in the ES cells and silenced in

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the iPS cells, regardless of whether the reprogrammed cells came originally from skin, brain, blood or other tissue. Although the function of the key genes is unknown, this region is usually silenced in mouse sperm cells and activated in other types of cell, so reprogramming might somehow mimic the silencing process, the authors speculate. The discovery raises the possibility that human iPS cells carry similar silenced sequences that make them less effective than ES cells.

It was demonstrated recently that reprogramming of somatic cells also resets genomic methylation, an epigenetic modification of DNA that influences gene expression, thus the resulting pluripotent stem cells might have different properties [42]. It was observed that low-passage iPSCs derived by factor-based reprogramming of adult murine tissues harbour residual DNA methylation signatures characteristic of their somatic tissue of origin, which favours their differentiation along lineages related to the donor cell, while restricting alternative cell fates. Such an 'epigenetic memory' of the donor tissue could be reset by differentiation and serial reprogramming, or by treatment of iPSCs with chromatin-modifying drugs. In contrast, the differentiation and methylation of nuclear-transfer-derived pluripotent stem cells are more similar to classical embryonic stem cells than were iPSCs. The nuclear transfer is more effective at establishing the ground state of pluripotency than factor-based reprogramming, which can leave an epigenetic memory of the tissue of origin that may influence efforts at directed differentiation for applications in disease modelling or treatment.

## 5. iPS cells and disease modelling

Patient-derived cell models can potentially overcome the issues with animal models and decrease the natural history of diseases such as in Alzheimer's from years to months [43]. Such patient-derived cell lines provide tremendous opportunities for explorative case-control studies and subsequent pre-clinical drug discovery [44]. Cell models of amyotrophic lateral sclerosis [45], Parkinson's disease [46], and spinal muscular atrophy have been developed and in the case of the latter, cured *in vitro*[47]. Current reports using iPSC to model genetic diseases like spinal muscular atrophy and familial dysautonomia have shown disease-specific phenotypes that may be important for modelling and drug discovery [48,49]. Importantly these cell lines represent the genomic background of each individual, a variable that would otherwise be impossible to model outside of clinical trials. Disease modelling with iPSC will be a three-part process, first the derivation of disease-specific iPSC, second differentiating these cells effectively to the cell lineage of choice like neurons, oligodendrocytes and astrocytes that hopefully express the disease phenotype and third using these derived lineage-specific cells for high-through put screening to model the disease and functional analyses if used for therapeutics down the track. None of these steps is easy and also we need to recapitulate *in vitro* key aspects of disease like neuronal degeneration in a time frame that is conducive to pathophysiological studies and, eventually, to drug screening. Transgenic animal models have taught us a great deal about disease process in some of the diseases but question remains whether therapeutically significant insights will be gained from the study of animal models. In this context, iPSC cells derived from humans with inherited neurological disorders may provide invaluable tools for elucidating the mechanisms by which the disease-causing gene products kill neurons in the human cellular context and at endogenous levels of expression. However, there are number of issues as described below that we need to address to before realising the full potential of stem cell technology.

### 5.1 iPSCs as a slippery slope for disease modelling

Critics of stem cell disease models suggest that case-control differences will merely represent the effect of the disease rather than an underlying genetic susceptibility or physiological difference. Ageing and disease effects in chronic conditions such as Alzheimer's disease are likely to be mediated via epigenetic effects on gene expression. As iPS cell technology erodes epigenetic signatures, this cell memory may be lost, both in the iPS cells themselves and their descendents, as reported recently in iPSC derived from a fragile X syndrome patient [50]. This 'ground/naïve state' appears akin to the putative inherent or embryological differences between an AD-susceptible and unaffected individuals. Alternatively one could maintain the cell memory by pursuing the culture of adult stem cells without a pluripotent stage [51,52] but here the problem would be separating disease 'cause' from 'effect'. Similarly the direct reprogramming of fibroblasts to neurons is likely to retain this 'cell memory' [53].

Derivation of iPS cells from people with diseases and their differentiation into clinically relevant cell types are only the first steps on the road to successful therapy. The identification of disease-related phenotypes in short-term *in vitro* settings might be a particular challenge, unless it turns out that the ontogenic age of the iPSC derivatives matches that of the donor rather than that of embryonic cells We could also expect that the differences in naïve iPSC from patients to manifest themselves through differentiation processes that could be traced by following a developmental paradigm. There are now the bases to recapitulate the life history of the disease through subtle perturbations with AD-related exposures, and create differentiable phenotypes *in vitro*. Of possible relevance to this issue is the observation that, thus far, disease-related cellular phenotypes have been observed in iPS models only of developmental neuropathologies: spinal muscular atrophy and familial dysautonomia [48,49]. In contrast to the models of neurodevelopmental disorders, no disease-related phenotypes have been thus far reported in iPSC from adult-onset diseases such as amyotrophic lateral sclerosis (ALS) or Parkinson's disease. Perhaps here the disease phenotype may never manifest itself under basal cell culture conditions, but it may be revealed by challenging the neural cells with stressors such as reactive nitrogen or oxygen species,

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proinflammatory factors or even toxins as explained above. Identification of these relevant stressors eliciting early neuronal phenotypes in models of adult-onset neurologic diseases will therefore be important milestones for future research.

Another important factor that may have compounding effect is the erasing of the cellular memory by the artificial reprogramming mechanisms used in creating such iPSC *in vitro*. An example of this is reported recently by Urbach *et al.*[50] that highlighted how in fragile X mutation, the fragile X mental retardation 1 (FMR1) gene is expressed in undifferentiated cells but undergoes transcriptional silencing after ESC differentiation and a significant difference exist between FX-ESCs and FX-iPSCs with regard to their expression of the FMR1 gene. The mutated FMR1 gene is expressed in FX-ESCs and transcriptionally silenced upon differentiation, whereas in FX-iPSCs the FMR1 locus remains inactive and is not reset by the reprogramming process to the transcriptionally active state. It is thus possible that other disorders related to epigenetic defects, including triplet repeat and imprinting disorders, may likewise evade the reprogramming process. However, differentiation of FX-iPSCs into neurons may facilitate the study of FMR1 in neural cells. Until a deeper understanding of the potential differences between iPSCs and hESC is delineated, the study of both iPSC from patients and human ESCs carrying the same mutation (either from preimplantation genetic diagnosis (PGD) embryos or by genetic manipulation) might, whenever possible, be the optimal approach to model human genetic disorders through cell culture. In contrast, a recent study by Tchieu and co-workers [54] indicated that female human iPSCs retain an inactive X chromosome and that has critical implications for clinical applications and disease modelling, and could be exploited for studies of X-linked diseases [50,55] and for a unique form of gene therapy for X-linked diseases.

Once disease-specific phenotypes are identified and translated into robust cell-based assays, the most consequential use of iPS cells derived from affected individuals will be the screening of candidate drugs by, for example, high-throughput platforms [56]. Such efforts at drug discovery will be greatly facilitated by the virtually unlimited supply of pluripotent cells and their derivatives. Another potential advantage in using iPS technology is that the new drugs will have already been tested on human cells, which may facilitate the identification of better therapies and accelerate their translation to clinical use.

According to Polo and co-workers [57] genetically matched iPSC retain a transient transcriptional and epigenetic memory of their cell of origin at early passage, which can substantially affect their potential to differentiate into embryoid bodies and different hematopoietic cell types. These molecular and functional differences are lost upon continuous passaging, These data also serve as a cautionary note for ongoing attempts to recapitulate disease phenotypes *in vitro* using patient-specific, early-passage iPSC lines, as the epigenetic, transcriptional and functional 'immaturity' of these cells might confound the data obtained from them. Further elucidation of the molecular indicators of fully reprogrammed iPSCs should help in the establishment of standardized iPSC lines that can be compared with confidence in basic biological and drug discovery studies.

The continuous presence of transgenes in iPSC harbours the risk of modifying the target genome and also interfereing with the subsequent differentiation process in such cells. Therefore recent protein-based hiPSC technology offers a new and potentially safe method for generating patient-specific stem cells. This system completely eliminates genome manipulation and DNA transfection, resulting in human iPSCs suitable for drug discovery, disease modelling, and future clinical translation [58,59]. Similarly other studies suggest that it may be possible to replace and/or further reduce the number of factors required for reprogramming [60-62]. To minimize/avoid chromosomal disruption, adenovirus and plasmid transfection have been successfully used to generate iPSCs in the mouse system [63,64]. Also, generation of hiPSCs by transfection with nonintegrating episomal vectors has been reported [65]. In addition, piggyback transposon [63,66] and Cre-recombinase-excisable viruses have been used to generate hiPSCs. While the transgenes can be excised by inducible gene expression once reprogramming is established [66,67], residual sequences and chromosomal disruptions may still result in harmful alterations that could pose clinical risks.

## 6. Is complete reprogramming of somatic cells essential?

Recently Vierbuchen *et al.* [53] have demonstrated that expression of only three transcription factors is required to convert mouse fibroblasts into functional neurons (iN cells) quite efficiently. Although the single factor achaete-scute complex homolog 1 (Ascl1) was sufficient to induce immature neuronal features, the additional expression of brain-2 (Brn2) and myelin transcription factor 1-like (Myt1l) generated mature iN cells with efficiencies of up to 19.5% Three-factor iN cells displayed functional neuronal properties such as the generation of trains of action potentials and synapse formation. These transcription factors were identified from a total of 19 candidates that were selected because of their specific expression in neural cell types or their roles in reprogramming to pluripotency. Future studies will have to be performed to unequivocally demonstrate that terminally differentiated cells such as mature B or T lymphocytes can be directly converted into neurons using this approach [68,69]. It will now be of great interest to decipher the molecular mechanism of this fibroblast-to-neuron conversion. Auto-regulatory feedback and feed-forward activation of downstream transcriptional regulators could then reinforce the expression of important cell-fate-determining genes and help to further stabilize the induced transcriptional program. Robust changes in transcriptional activity could also lead to genome-wide adjustments of



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repressive and active epigenetic features such as DNA methylation, histone modifications and changes of chromatin remodelling complexes that further stabilize the new transcriptional network [70,71]. It is possible that certain subpopulations of cells are 'primed' to respond to these factors, depending on their pre-existing transcriptional or epigenetic states. It has also been demonstrated that a combinatorial approach using non-genetic and epigenetic modifications of somatic cells produces intermediate pluripotent stem cells that have the capability to differentiate to neuronal populations [1,2]. The majority of iN cells described in this report are excitatory and express markers of cortical identity. A small proportion of iN cells expressed markers of GABAergic neurons, but no other neurotransmitter phenotypes were detected. The data indicates the intriguing possibility that additional combinations of neural transcription factors might also be able to generate functional neurons whose phenotypes remain to be explored. One of the next important steps will be to generate iN cells of other specific neuronal subtypes and from human cells. Future studies will be necessary to determine whether iN cells could represent an alternative method to generate patient-specific neurons.

## 7. New approaches to generate induced pluripotent stem cells: epigenetic modifications and small molecules

### 7.1 Non-integrating vectors (plasmids, adenovirus, transposons, cre–lox removal of Lentivirus)

The conventional iPSC technique that involves virus-mediated delivery of reprogramming factors, invariably leads to a permanent integration of oncogenes and potential genomic alterations. Various new methods have emerged to address the safety concerns. Such methods employed the use of plasmid transient transfection, nonintegrating adenovirus or episomal vector to generate iPSCs, albeit with low reporgamming efficiency and still with risks due to insertion of pieces of those transgenes into the target cell genome [72-75]. Similarly in order to remove the transgenes the Cre/LoxP approach is applied after complete reprogramming, but the residual loxP sites continues to pose a safety concern [76]. The same is also true for the piggyBAC transposon system that requires additional tedious efforts to complete and validate traceless removal of the transgenes in the target cells [77-78].

### 7.2 Proteins and small molecules

Recently purified recombinant reprogramming proteins fused with a poly-arginine cell-transduction domain were shown to be effective in generating iPSCs from fibroblasts. But low efficiency continued to be a limiting factor [79,80]. Attempts are being made to completely replace the reprogramming factors with a cocktail of defined small molecules that can induce or enhance reprogramming. However, long-term effects of such molecules on genetic and/or epigenetic alterations to the target cells, which can occur during the reprogramming process have not been investigated. Although we do not understand the exact mechanism of reprogramming as it entails in embryos or as in SCNT, currently studies are focussing on two main approaches: exploiting a target cell type's intrinsic properties, and treating cells with molecules that modulate reprogramming mechanisms [81,82]. Many somatic cell types (especially certain tissue-specific stem or progenitor cells) endogenously express one or more of the four reprogramming factors (e.g., cMyc, Kruppel-like factor 4 (Klf4) and sex determining region Y-box 2 (Sox2)) it was shown that such endogenous expression of the reprogramming factor(s) is sufficient to substitute its overexpression for inducing reprogramming. Also cells with an epithelial phenotype may be more favoured in reprogramming than fibroblasts, partly because iPSC reprogramming involves a mesenchymal-to-epithelial transition (MET) process. Furthermore, fetal and neonatal cells are easier to reprogram than adult cells. Two main categories of small molecules, those that are epigenetic modifiers and signaling molecules, have been identified and used in enhancing reprogramming. For example, a small-molecule inhibitor of histone methyltransferase (HMTase) G9a, BIX-01294 (BIX), was first identified to significantly enhance the reprogramming efficiency of mouse neural progenitor cells (NPCs) and fibroblasts that were transduced with only two reprogramming transcription factors (octamer-binding protein 4 (Oct4) and Klf4) [30], or functionally replace Oct4, the only gene (of the four reprogramming genes) not expressed in any somatic cells, in NPCs transduced with Klf4/Sox2/c-Myc [82]. Similarly, inhibitors of histone deacetylases (HDACs) (e.g., Trichostatin A (TSA), valproic acid (VPA), etc.), DNA methyltransfereases (DNMTs) (e.g., RG108), and a H3K4 histone demethylase (e.g., parnate) were shown to enhance reprogramming in different contexts. A combinatorial approach that incorporates epigenetic modifications and hESC extract has been shown to induce reprogramming albeit incompletely [1]. These studies highlight the important roles of dynamic epigenetic regulation involving histone and DNA modifications in reprogramming, and the utility of these small molecule modifiers. Several signaling pathways, including wingless-type MMTV integration site family member (Wnt)–β-catenin, MAPK kinase (MEK)–extracellular-signal-regulated kinase (ERK), calcium–cAMP, TGFb and Rho–Rho-kinase (Rock) pathways, have been identified to affect the reprogramming process, as demonstrated by the effects of pathway-modulating small molecules in generation of iPSCs [82-88]. For example, a specific GSK-3 inhibitor, CHIR99021, which strongly activates the β-catenin pathway, was shown to replace Sox2 and enhance reprogramming in mouse and human cells [89]. Another GSK-3 inhibitor, kenpaullone, which also inhibits cyclin-dependent kinase (CDK) and other kinases, was found to reprogram mouse embryonic fibroblasts transduced with



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Oct4/Sox2/c-Myc but in the absence of Klf4. Interestingly, neither a more specific GSK3 inhibitor (such as CHIR99021) nor a CDK inhibitor was able to replace Klf4 in the same context, indicating the involvement of other mechanisms independent of GSK-3 or CDK inhibition by kenpaullone. More recently it was shown that MEK inhibition by PD0325901 can also synergize with the TGF-β pathway inhibition in promoting generation of human iPSCs [89]. As also indicated earlier, the current reprogramming process from fibroblasts to iPSCs entails MET. The TGF-β pathway is essential for induction of epithelial-to-mesenchymal transition (EMT) and maintenance of the mesenchymal state. Conversely inhibition of TGF-β signaling can result in de-repression of epithelial fate and would benefit the reprogramming process. Consistent with this underlying notion, the demonstration that TGF-β and MAPK pathway inhibition dramatically increases reprogramming efficiency and accelerates reprogramming kinetics highlights critical roles of MET mechanisms and its players in the process.

### 7.3 Synthetic modified mRNA

Researchers at the Immune Disease Institute/Program in Cellular and Molecular Medicine at Children's Hospital Boston have reported the development of a safe and efficient technology to create human iPS cells. Perhaps even more significantly, the researchers demonstrated that their technology could also be used to efficiently steer these stem cells to form cells useful in medicine, such as blood cells, neurons and muscle cells [89].

Current reprogramming protocols for making iPS cells require viruses or DNA to reinstate the stem cell identity, which permanently alters the genome of the cells. The researchers, led by Derrick Rossi, report a novel technique that uses synthetic modified RNA to generate pluripotent stem cells without irreversibly altering the cells' genetic material. The resulting stem cells very closely recapitulate the functional and molecular properties of human embryonic stem cells, and are generated at much higher efficiencies than those produced by standard virus-based techniques. Importantly, modified RNA can also be used to direct the pluripotent stem cells into cell types that could be used clinically, the researchers show. The difficulty of differentiating iPS cells into clinically useful cell types has been a major obstacle to advancing stem-cell therapies.

Getting differentiated cells to regress, or 'reprogram' to an embryonic-stem-cell-like state requires introduction of four key proteins. These proteins are most often introduced using DNA-based viruses, an approach that carries the risk of causing mutations in the reprogrammed cells, which could trigger cancers.

To get around this problem, Rossi and colleagues thought to employ mRNA to drive expression of the reprogramming factors since mRNA does not integrate into the cellular DNA. However, they first had to overcome an obstacle: when mRNA was introduced into cells, the cells' natural defence mechanisms interpreted this as a viral infection, and responded with a potent anti-viral reaction that destroyed the RNA and killed the cells. In an attempt to reduce innate immune responses to transfected RNA, they synthesized mRNAs incorporating modified ribonucleoside bases. Complete substitution of either 5-methylcytidine (5mC) for cytidine or pseudouridine (psi) for uridine in green fluorescent protein (GFP)-encoding transcripts markedly improved viability and increased ectopic protein expression, although the most significant improvement was seen when both modifications were used together. This permitted the modified mRNA to drive protein expression effectively for days and weeks in human cells without adverse affects on the cells.

The researchers then put their method to the test, treating cells derived from human skin with a cocktail of modified mRNAs encoding the four major reprogramming proteins. With daily treatment, the cells reverted to a pluripotent state similar to human embryonic stem cells. Not only were the cells free of DNA integrations, but the reprogramming process was completed in about half the time required for standard virus-based techniques, and was up to 100 times more efficient although this technique remains very labour-intensive and not as cost-effective and thus needs further improvement.

The modified RNA technology was also effective at redirecting stem cells to form other tissue types. Currently, scientists attempt to coax iPS cells to differentiate to clinically useful cell types by changing their external environment. The new work shows, however, that the addition of a modified RNA encoding a factor important for muscle differentiation directly into the stem cells results in efficient generation of functional muscle cells. This provides a proof of concept that the RNA method could be used to generate patient-specific cells of various types for use in regenerative therapies.

### 7.4 MicroRNAs

Somatic cell reprogramming (SCR) requires global DNA demethylation to reset cell stemness, yet the mechanism underlying this epigenetic event is unclear. Current understanding is limited by co-transfection of three or four specific transcription factors, either Oct3/4–Sox2–Klf4–cMyc or Oct3/4–Sox2–Nanog–lineage 28 homolog (Lin28), to promote iPS cell formation [90-92]. Among these reprogramming factors, POU class 5 homeobox 1 (Oct3/4) alone (in concert with cells expressing Sox2, e.g., NSC, or with supplementation of small molecules drugs) is sufficient(although with low efficiency). Recent studies found that both Oct4 and Sox2 are also crucial for expressing mir-302 in human embryonic stem (hES) cells [91-93]. Mir-302 belongs to a class of small, non-coding RNAs known as microRNAs (miRNA) that function as cytoplasmic gene silencers by suppressing translation of targeted mRNA. The majority of mir-302-targeted genes are transcripts of developmental



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signals and oncogenes [94]; nevertheless, their interactions and overall functions remain unknown. The genomic sequence encoding mir-302 is located in the 4q25 locus of human chromosome 4, a conserved region frequently associated with longevity [95]. In humans, mir-302 is predominantly expressed in hES and iPS cells, but not in differentiated cells [96,97]. Loss of mir-302 has been observed prior to hES cell differentiation and proliferation during early embryonic development [96]. Analogously in mice, its homologous mir-291/294/295 family presents a similar expression profile [97,98]. Therefore, it is conceivable that embryonic-stem-cell-specific miRNAs such as mir-302 and mir-291/294/295 play a pivotal role in regulating cell stemness and pluripotency, whose functions may be applied to enhance the efficiency of SCR for iPS cell generation. Global demethylation is required for early zygote development to establish stem cell pluripotency, yet these findings reiterate this epigenetic reprogramming event in somatic cells through ectopic introduction of mir-302 function. It was reported recently [99,100] that induced mir-302 expression beyond 1.3-fold of the concentration in human embryonic stem (hES) H1 and H9 cells led to reprogramming of human hair follicle cells (hHFCs) to induced pluripotent stem (iPS) cells. This reprogramming mechanism functioned through mir-302-targeted co-suppression of four epigenetic regulators, flavin-containing amine oxidase domain-containing protein (AOF)2 (also known as lysine (K)-specific demethylase 1A (KDM1 or LSD1)), AOF1, methyl CpG binding protein (MECP)1-p66 and MECP2. Silencing AOF2 also caused DNMT1 deficiency and further enhanced global demethylation during SCR of hHFCs. Re-supplementing AOF2 in iPS cells disrupted such global demethylation and induced cell differentiation. Given that both hES and iPS cells highly express mir-302, these findings suggest a novel link between zygotic reprogramming and SCR, providing a regulatory mechanism responsible for global demethylation in both events. As the mechanism of conventional iPS cell induction methods remains largely unknown, understanding this miRNA-mediated SCR mechanism may shed light on the improvements of iPS cell generation.

## 8. Conclusion

While significant technical progress has been made in generating iPSCs from various somatic cell sources, iPSC research is still in its infancy. There remains a great deal to learn about iPSC safety, the reprogramming mechanisms and how to change the reprogramming process from its current non-specific process with poor efficiency and slow kinetics to a specific and directed process. Towards this end, a of number recent studies using small molecules, microRNAs, synthetic mRNAs and recombinant proteins are emerging as an alternative to traditional integrating system for reprogramming, which may be more acceptable for producing therapeutic cells. With current approaches using integrating system, the iPSC field will have relevance in disease modeling, and identification of new small molecules that modulate reprogramming. It is conceivable that a precise combinatorial action of signaling and epigenetic modifiers may direct a more specific and efficient reprogramming process *in vitro* or *in vivo*. In parallel, improved abilities in differentiating pluripotent stem cells as well as expanding and maintaining their lineage-specific derivatives are also keys to ultimate applications of iPSCs. The generation of disease-specific iPS cells has invigorated the prospect that disease mechanisms that underpin various human diseases, particularly the neurodegenerative disorders, could be unravelled in the Petri dish. Some recent studies have substantiated the utility of this technology in describing the initial characterization of patient-derived iPSC as a proof of concept. However, as it is becoming evident now that the cell type of origin influences the molecular and functional properties of derived iPSC. The indications that reprogramming may erase the cell memory also raises the question of whether the disease phenotype may not be correctly represented or be erased in iPSC unless coaxed by further perturbation *in vitro* culture conditions. Other associated difficulties in iPSC research, such as culture variability, selective adaptation of such cultures and the lack of robust protocols to generate homogeneous population of desired cell type, may have compounding affects in the use of these cells in disease modelling. Unless these issues are addressed properly the prospects of iPSC in disease modelling may remain a slippery slope. There is no doubt that iPSC research will continue to offer new opportunities for stem cell research and regenerative medicine for years to come.

## 9. Expert opinion

### 9.1 Generating iPSC – are new strategies essential?

- Currently the efficient way to generate iPSC is by viral transduction of somatic cells with a combination of pluripotent genes. The disadvantage is that these genes integrate into the host genome and that may cause gene mutagenesis. The iPSC produced by this method, however, are relevant in drug discovery and toxicological studies.
- For developing therapeutics with iPSC a non-integrating system of transduction is not only essential but is an absolute requirement of the therapeutic regulatory agencies worldwide.

### 9.2 Current advances in generating iPSC

- Methods are being developed for generating iPSC with minimal or no genetic modifications via excisable lentiviral and transposon vectors or through repeated application of transient plasmid, episomal and adenovirus vectors and very recently the use of small molecules, synthetic mRNA and miRNAs. The disadvantage is generally low efficiency and consistency of generating iPSC with these methods.

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- Recent developments in high-throughput assays will facilitate identification of small and synthetic molecules for reprogramming of somatic cells that may eventually eliminate the need of viral transduction.

### 9.3 Advantages of iPSC technology

- Reprogramming of somatic cells to pluripotent stage by iPSC technology takes the debate away from human embryos that are being used for a similar purpose.
- Generating patient-specific iPSC by using the prospective non-integrating system will advance the regenerative medicine field without the fear of cell/graft rejection as such cells are autologous.
- Disease-specific iPSC are paving the way for understanding the development of the disease process *in vitro* and such cells are critical in toxicological studies including drug discovery.

## Declaration of interest

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