REVIEW ARTICLE

Progress and Future Challenges of Human Induced Pluripotents Stem Cell in Regenerative Medicine

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Abstract

BACKGROUND: Less than a decade ago the prospect for reprogramming the human somatic cell looked bleak at best. It seemed that the only methods at our disposal for the generation of human isogenic pluripotent cells would have to involve somatic cell nuclear transfer (SCNT). Shinya Yamanaka in August 2006 in his publication (Cell) promised to change everything by showing that it was apparently very simple to revert the phenotype of a differentiated cell to a pluripotent one by overexpressing four transcription factors in murine fibroblasts.

CONTENT: Mouse and human somatic cells can be genetically reprogrammed into induced pluripotent stem cells (iPSCs) by the expression of a defined set of factors (Oct4, Sox2, c-Myc, and Klf4, as well as Nanog and LIN28). iPSCs could be generated from mouse and human fibroblasts as well as from mouse liver, stomach, pancreatic, neural stem cells, and keratinocytes. Similarity of iPSCs and embryonic stem cells (ESCs) has been demonstrated in their morphology, global expression profiles, epigenetic status, as well as *in vitro* and *in vivo* differentiation potential for both mouse and human cells. Many techniques for human iPSCs (hiPSCs) derivation have been developed in recent years, utilizing different starting cell types, vector delivery systems, and culture conditions. A refined or perfected combination of these techniques might prove to be the key to generating clinically applicable hiPSCs.

SUMMARY: iPSCs are a revolutionary tool for generating *in vitro* models of human diseases and may help us to understand the molecular basis of epigenetic reprogramming. Progress of the last four years has been truly amazing, almost verging on science fiction, but if we can learn to produce such cells cheaply and easily, and control their differentiation, our efforts to understand and fight disease will become more accessible, controllable and tailored. Ability to safely and efficiently derive hiPSCs may be of decisive importance to the future of regenerative medicine.

KEYWORDS: iPSCs, ESC, reprogramming factor, reprogramming efficiency, somatic cell.

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Introduction

Cut off the limb of a salamander and it grows back completely. Stem-cell scientists and tissue engineers dream of unlocking the same regenerative capacity in adult differentiated mammalian cells. This dream has potentially come closer to reality with three recent reports that describe the ability of four genes to completely reprogram mouse skin cells (fibroblasts) into stem cells possessing many, if not all, characteristics of authentic embryonic stem cells (ESCs) (1-3). The ability of ESCs to integrate themselves into many different organs suggests the possibility that they may be used to repair damaged or diseased tissues (4).

Three routes have been envisioned to make patient-specific ESCs. First, akin to the process used to create Dolly the sheep (5), nuclei from adult donor cells can be transferred into egg cytoplasm (6) or ESC cytoplasm (7), yielding ESCs possessing the donor genotype. The efficiency of this approach is low, and it has not yet succeeded in humans (8). Second, fusion of adult cells with existing ESCs can reprogram the adult nuclei so that the new cell behaves as an ESC. Unfortunately, the resulting cell possesses two nuclei and thus has four copies of each chromosome instead of two (8). These approaches are ethically controversial because they require the donation of eggs or the use of human ESCs (hESCs). Third, if somatic cells from a patient can be genetically or chemically induced to return to a primordial ESC-like state, then these cells can be directly used as the source to create donor-specific ESCs. In 2006, Yamanaka and colleagues surprised the cell biology community with their finding that a core set of just four genes reprograms mouse embryonic fibroblasts (MEFs) into cells with ESC characteristics (9). Retrovirus-mediated introduction of Pou5f1 (also known as Oct4), Myc (c-Myc), Klf4 and Sox2—all genes known to be involved in maintaining the pluripotency and self-renewal of stem cells—generated "induced Pluripotent Stem Cells (iPSCs)" that acquired many ESC markers, and in transplantation experiments gave rise to cells in all three germ layers, providing evidence that they were pluripotent.

Undoubtedly, the science of iPSCs is moving forward at breakneck speed; however, with this new knowledge comes important responsibilities for the regenerative medicine community, not least the portrayal of hype and hope. Like hESCs, hiPSCs could potentially be used as therapies, disease models or in drug screening. And iPSCs have clear advantages: they can be made from adult cells, avoiding the contentious need for a human embryo, and they can be derived from people with diseases to create models or even therapies based on a person's genetic make-up. Scientists predicted that iPSCs would change the face of biology and medicine — and some would say they already have (10).

To be commercially successful, a basic discovery has to be translated into real products that are then embraced by the market. In the case of iPSCs technology, the public is already eagerly buying into the dream of future cures (11). The challenge is how to balance enthusiasm with reality for at least a decade whilst iPSCs research and translation hopefully progress through all the necessary steps in order to produce safe, effective and affordable therapies.

Hype or hope, where is iPSCs technology today? Not a single week seems to go by without reports of further breakthroughs on the IPSCs technology front. These reports are not just original papers in leading scientific journals but also articles in the regular media: web, television and press. Nor are the popular press alone in their enthusiasm; hallowed journals including Science have openly declared IPSCs as the "Breakthrough of the Year" (12).

Where is IPSCs technology today? The 'Technology Trigger' was the initial discovery in 2006 by Shinya Yamanaka in mice and then reinforced a year later when Yamanaka and James Thomson independently demonstrated the approach in man (13). Clearly the technology is not yet sliding into the Trough of Disillusionment – there are far too many positive news stories and commercial activities, for example the formation of an IPSCs-dedicated biotech company iZumi Bio (Mountain View, CA, USA) by seasoned venture capitalists (14). The technology is therefore either still on the rise (most likely) or at the peak. Indeed, there is real concern that iPSCs could fall victim to the same hype that plagued the early days of hESCs research (14,15).

Many iPSCs researchers see the field's growing pains as signs that it is reaching a state of maturity; they say that the problems are no different from those that many biomedical research fields face as they inch towards clinical application. There was this huge euphoria in the beginning, with everyone thinking iPSCs will do everything, cure all diseases, and be super-easy, but not everyone can become a stem-cell biologist overnight. It's a bit of a reality check that things are not as simple as we thought (10). "Is iPSCs technology the future of clinical medicine?"

iPSCs Controversy

iPSCS have great therapeutic potential. But genomic and epigenomic analyses of these cells generated using current technology reveal abnormalities that may affect their safe use. Several recent reports (16-20) uncover genetic and epigenetic alterations in iPSCs, stimulating debate about their future. However, will these important findings really impact what we hope to gain?

The discovery that somatic cells could be reprogrammed back to a pluripotent state (iPSCs) after the transduction of four defined transcription factors altered our initially restricted view of cellular plasticity (9). This discovery raised a number of new questions: what are the consequences, if any, of this reprogramming process? Are the genome and/or the epigenome compromised

during these cellular conversions? Are the reprogrammed cellular products functionally identical to their normal counterparts? Do iPSCs undergo additional adaptation to their culture environment? (21).

While initial reports demonstrated the overall similarities between iPSCs and their ESC counterparts (22), recent studies have revealed that intricate genomic differences exist between these pluripotent populations. Hussein *et al.* (20) studied copy number variation (CNV) across the genome during iPSCs generation, whereas Gore and colleagues (19) looked for point mutations in iPSCs using genome-wide sequencing of protein-coding regions. Lister *et al.* (18) examined DNA methylation—an epigenetic mark—across the genomes of ESCs and iPSCs at the single-base level. These studies, along with other investigations into changes in chromosome numbers (16) and CNV (17) in the two kinds of stem cell, lead to the conclusion that reprogramming and subsequent expansion of iPSCs in culture can lead to the accumulation of diverse abnormalities at the chromosomal, subchromosomal and single–base levels. Specifically, three common themes, regarding the genetic and epigenetic stability of ESCs and iPSCs, emerge.

Collectively, these findings demonstrate that both ESCs and iPSCs contain and/or acquire genetic abnormalities. The origin of these genomic alterations in iPSCs was attributed to their pre-existence in the parental somatic cells or their occurrence during reprogramming. Additionally, culture adaptations can contribute to these aberrations for both ESCs and iPSCs.

Detection of these genomic/epigenetic differences was made possible by the development of high-throughput sequencing technologies and by the generation of single-nucleotide genome-wide maps of DNA methylation. In time, these technologies will likely become even more sensitive and affordable, thus enabling additional analyses of iPSCs lines derived and maintained under a variety of conditions. Furthermore, the complete genetic and epigenetic profiles of mature cells obtained via transcription-dependent transdifferentiation, or of iPSCs generated by new methodologies, such as the miRNA-mediated reprogramming protocol described in *Cell Stem Cell* (23), have yet to be examined. Extending the analyses to at least these lengths will be required to determine whether reprogrammed cells can be derived free of, or containing minimal, genetic alterations.

Although some protocols have been presented that genetic and epigenetic aberrations can persist and/or occur during differentiation (24), a more extensive examination is warranted, since protocols will likely differ in their capacity to generate and/or maintain such variations. Importantly, in some cases, these differentiated cell types may be short-lived in vivo, limiting any possible deleterious consequences. Moreover, mutagenic events that affect pluripotent cell populations may be inconsequential to mature/differentiated cell functions.

Regardless of the future for iPSCs in the clinic, the development of iPSCs technologies has also provided methods for obtaining pluripotent cells from poorly understood or largely inaccessible disorders. Many disease-specific iPSCs have already been generated, which will not only allow us to recapitulate and study the disease phenotypes *in vitro*, but also enable screening for therapeutic candidates to minimize or prevent disease onset and/or development (21).

Just as scientists remember how our initially restricted view of plasticity was uprooted by Yamanaka's demonstration of reprogramming, we ask ourselves, should these recent findings detract from what we are aiming to gain? Let us not decide too quickly for a field that has more promise and unknowns than knowledge.

iPSCs Derivation

Pluripotency pertains to the cells of early embryos that can generate all of the tissues in the organism. ESCs are embryo-derived cell lines that retain pluripotency and represent invaluable tools for research into the mechanisms of tissue formation (24). Epigenetic reprogramming of somatic cells into ESCs has attracted much attention because of the potential for customized transplantation therapy, as cellular derivatives of reprogrammed cells will not be rejected by the donor (25,26).

Efforts to reprogram human somatic differentiated cell types to a state that resembles hESCs began with the pioneering work of Takahashi and Yamanaka (27,28). Their methods included retroviral integration of 4 vital reprogramming factors—*OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*—into adult human dermal fibroblasts. These 4 transcription factors would later become known as the "Yamanaka factors," and their roles in reprogramming are now known to be significant but not collectively necessary (29-36). Often the mission of 1 or more of these reprogramming genes was contingent on the endogenous network of the donor cell type. For example, one study found that hiPSCs derivation from keratinocytes required only 10 days, whereas neonatal skin fibroblasts required ~30 days (37). It was postulated that perhaps the keratinocytes' higher endogenous

expression levels of *c-MYC* and *KLF4* predispose them to quicker reprogramming (38). Starting cell type is thus an important consideration in the derivation process and a topic that is more thoroughly discussed elsewhere (39). Two other transcription factors, namely *NANOG* and *LIN28*, were initially shown to be able to substitute for *c-MYC* and *KLF4*, although a number of other different factor combinations have been subsequently demonstrated (1,29,36,40,41). In any event, several cocktails comprising any number of these 6 reprogramming factors, and in some cases, additional supplements such as small molecules and enzymes, have been shown to be capable of reprogramming cells to pluripotency.

A chief aim of clinical hiPSCs researchers is to achieve a high efficiency of derivation of hiPSCs, because current yields of bonafide hiPSCs can be as low as 0.001% to 0.1% of the starting cell population (42). Even in secondary reprogramming systems, using transgenic fibroblasts expressing all four transgenes simultaneously, the efficien-cy of pluripotency induction remains low, at 1% to 5% (37,43). Yamakana has proposed two mutually nonexclusive models to explain the apparent resistance to pluripotency induction, termed the "elite" and "stochastic" models (44). This proposes that only a small percentage of somatic cells, presumably resident tissue progenitor cells, are amenable to reprogramming. Evidence that hematopoietic stem cells undergo more efficient reprogramming than their differentiated progeny supported this notion (45). A stochastic model of successful reprogramming of terminally differentiated cells such as B-lymphocytes (46) and pancreatic β-islets (47) was reported, in which successive cell divisions allow rare cells to acquire the stochastic changes that are necessary for conversion to full pluripotency (48). Perhaps these seemingly contradictory hypotheses can be reconciled by a model in which adult stem/progenitor cells require fewer stochastic changes to undergo reprogramming than more differentiated cells. Further investigation of the reprogramming process using single-cell resolution imaging and other techniques is needed to undoubtedly help yield further insight into these reprogramming roadblocks.

The commodification of pluripotency by the arrival of iPSCs has not entirely diluted the value of the ESC, which is still generally held to be the ''gold standard'' by which all pluripotency should be judged, given their ability to give rise to live offspring via tetraploid complementation (in mice) and to form teratomas on transplantation into a living mouse (for hESCs). Furthermore, the processes underlying the generation of iPSCs remain relatively poorly understood, and without side-by-side study with ESCs, it is unlikely that the field will tease apart the detailed mechanisms that regulate pluripotency (49).

Companies have begun to market culture kits for hiPSCs, and given the success, irrespective of utility, of private banks for the storage of umbilical cord blood cells and stem cells from deciduous teeth, menstrual blood, peripheral blood, and bone marrow, it is almost certain that plans for a private iPSCs bank are already being laid. Indeed, when the Chinese stem cell tourism company Beike Biotechnology opened its Jiangsu Stem Cell Storage Facility in mid-2008, the company specifically cited its intent to store hiPSCs in the future.

Business issues aside, research institutions and support frameworks are already struggling to readjust to the new reality of pluripotency as a ''cheap'' and plentiful commodity, rather than a scarce and precious resource. Existing hESC cell banks, including the U.S. National Stem Cell Bank (www.nationalstemcellbank. org/) and the cell bank at the RIKEN Bio – Resource Center in Japan (http://www. brc.riken.go.jp) have already begun to bank iPSCs, and registries such as the International Stem Cell Registry at the University of Massachusetts (http:// www. umassmed.edu/iscr) and the E.U.'s hESC Registry (www. hescreg.eu) are beginning to catalog existing hiPSCs lines (49).

This trend by no means suggests that safe and effective clinical applications for these cells will soon become available, only that when the value placed on pluripotency is combined with a readily obtainable cell source and an unregulated business environment, industry is sure to follow. Given this eventuality and the significant potential risks associated with the transplantation of undifferentiated pluripotent cells, competent authorities will need to move quickly to develop quality standards and mechanisms of enforcement.

Pluripotent Reprogramming

Pluripotency and self-renewal are the hallmarks of ESCs. This state is maintained by a network of transcription factors and is influenced by specific signaling pathways (50). The transcription factors Oct4, Sox2 and Nanog are among the pluripotency-associated factors that maintain ESCs (51-55). Their targets have been mapped by chromatin immunoprecipitation (ChIP)-based technologies (56,57), revealing their extensive co-binding in both murine ESCs (mESCs) and hESCs. This has led to the proposal that these factors constitute a core transcriptional regulatory network (56).

These studies uncovered the 'Oct4-centric' and 'Myc-centric' modules. The Oct4-centric module includes Oct4, Sox2 and Nanog as well as Smad1, Stat3 and Tcf3 (58,59), which are the downstream effectors for signalling pathways controlled by Bone Morphogenic Protein (BMP), Leukaemia Inhibitory Factor (LIF) and Wnt respectively. Initially, mESCs were cultured in an ill-defined mixture of 'feeder cells' and serum, but subsequent work identified LIF and BMP4 as the key signaling factors required for the sustained proliferation and maintenance of mESCs (60-62). The LIF signalling pathway leads to phosphorylation of the transcription factor Stat3, which is required to promote self-renewal. Through the Smad signalling pathway, BMP4 seems to induce the expression of Id genes to suppress differentiation (62).

Thus, the extracellular signals communicate with the core transcriptional regulatory network, and in turn their targets. Additional pluripotency-associated factors, such as Dax1, Nac1, Zfp281, Esrrb, Nr5a2, Tcfcp2l1 and Klf4, are also linked to the Oct4-centric module (63-66). Oct4 interacts biochemically with some of these factors, including Dax1, Nac1, Tcfp2l1, Esrrb and Sox2 (67,68), which could explain their co-localization on genomic chromatin. As the depletion of Oct4 markedly reduced the co-binding of Smad1, Stat3, Dax1, Tcfcp2l1 and Esrrb (58,68), it was proposed that Oct4 acts as an anchor point for the assembly and maintenance of these multi-protein complexes on the DNA.

A second binding site module was also identified and includes c-Myc, n-Myc, E2f1, Zfx, Rex1 and Ronin (58,66,63). These factors bind to sites near the transcription start sites, and their target genes are associated with protein metabolism (66,68). In contrast to the Myc module, the Oct4 modules are found further away from the transcription start sites and have been proposed to act as enhancers. Interestingly, the Myc module has recently also been suggested to be a cancer-cell-related hub (69).

These studies additionally revealed that in mESCs, many of the key pluripotency-associated factors (Oct4, Sox2, Nanog, Esrrb, Sall4, Dax1, Klf2, Klf4, Klf5, Stat3 and Tcf3) may autoregulate their own expression (56-59,63,64,70-72). It is possible that certain transcription factors directly downregulate the transcription of their own genes to prevent over-activation of gene expression. Overexpression of pluripotency-associated transcription factors has been shown to perturb the homeostasis of mESCs; for example, overexpression of Oct4 and Sox2 triggers differentiation (51,73). Hence, the continual activation of these genes may destabilize the mESC state.

Epithelial-to-mesenchymal transition (EMT) is a developmental process important for cell fate determination. Fibroblasts, a product of EMT, can be reset into iPSCs via exogenous transcription factors but the underlying mechanism is unclear. Li *et al*. (74) showed that the generation of iPSCs from mouse fibroblasts required a mesenchymal-to-epithelial transition (MET) orchestrated by suppressing pro-EMT signals from the culture medium and activating an epithelial program inside the cells. At the transcriptional level, Sox2/Oct4 suppress the EMT mediator Snail, c-Myc downregulates TGF-beta1 and TGF-beta receptor 2, and Klf4 induces epithelial genes including E-cadherin. Blocking MET impairs the reprogramming of fibroblasts whereas preventing EMT in epithelial cells cultured with serum can produce IPSCs without Klf4 and c-Myc (74).

Temporal analysis of gene expression revealed that reprogramming is a multistep process that is characterized by initiation, maturation, and stabilization phases. Functional analysis by systematic RNAi screening further uncovered a key role for BMP signaling and the induction of MET during the initiation phase. Samavarchi *et al.* (75) showed that this is linked to BMP-dependent induction of miR-205 and the miR-200 family of microRNAs (miRNAs) that are key regulators of MET. These studies thus define a multistep mechanism that incorporates a BMP-miRNA-MET axis during somatic cell reprogramming (75).

Smith *et al.* (76) showed that Myc sustains pluripotency through repression of the primitive endoderm master regulator GATA6, while also contributing to cell cycle control by regulation of the mir-17-92 miRNA cluster. These findings demonstrate the indispensable requirement for c- or N-myc in pluripotency beyond proliferative and metabolic control (76). miRNAs add another layer of complexity to the regulation of pluripotent cells by fine-tuning gene expression (77-79).

Pluripotency-associated transcription factors Oct4, Sox2 and Nanog also regulate non-coding RNAs, including mir302 and mir290 clusters (77). These miRNAs might regulate the shortened G1 phase in mESCs through the repression of key cell-cycle regulators, such as Cdkn1a, Rbl1 and Lats2 (80,81). These miRNAs are antagonized by let-7 miRNA (82), which is negatively regulated by Lin28, a target of the core transcription factors (83). On differentiation, Lin28 is downregulated, leading to a rapid increase in mature let-7, which in turn downregulates Myc activity and suppresses the expression of downstream targets of the core transcription factors. In hESCs it has also been shown that mir302 targets LEFTY1 and LEFTY2, known modulators of the Nodal signalling pathways (84). Altogether, pluripotency factors directly activate both miRNA expression and let-7 miRNA

processing pathways to selectively maintain the gene expression program required for the ESC state.

More recently, Oct4 was shown to activate the expression of specific large intergenic non-coding RNAs (lincRNAs) of a length greater than 200 nucleotides (85). In particular, knockdown of a lincRNA, lincRNA-RoR, in hESCs led to a growth defect with elevated apoptosis. These studies reveal that the transcriptional regulatory networks are integrated with the epigenetic and non-coding RNA networks in maintaining ESCs.

Thus, the interplay between transcription factors, epigenetic modifiers, chromatin remodelers and miRNAs form the foundation of a complex regulatory network required for establishment and maintenance of the pluripotent state (86).

Barrier for iPSCs Reprogramming

The extremely low efficiency and slow kinetics of *in vitro* reprogramming suggest that further rare events are required to generate iPSCs. The nature and identity of these events, however, remain elusive. Reprogramming somatic cells to iPSCs has been accomplished by expressing pluripotency factors and oncogenes (9,24,27,31,40,87-89). But the low frequency and tendency to induce malignant transformation compromise the utility of this powerful approach. The acquisition of immortality is a crucial and rate-limiting step towards the establishment of a pluripotent state in somatic cells and underscore the similarities between induced pluripotency and tumorigenesis (90).

Normal fibroblasts, which are mature, differentiated cells, can be reprogrammed into iPSCs or tumour cells by a combination of defined factors. The transcription factors c-Myc and Klf4 promote reprogramming of fibroblasts into iPSCs in a manner that conceptually parallels their roles in transforming normal cells into tumour cells. Oct4 and Sox2, although overexpressed in cancers, are currently thought to function specifically to promote iPSCs formation. The reprogramming of fibroblasts into iPSCs is directly or indirectly limited by the p53 tumour-suppressor protein, which can be induced by $p19^{Arf}$, conversely (90-94) fibroblast in other way could be transformed into cancer cells by inducing apoptosis, or cellular senescence through its target protein, the cell-cycle inhibitor p21. Another cell-cycle inhibitor, $p16^{Ink4a}$, also promotes cellular senescence directly to limit both processes (95). The *Ink4a/Arf* locus (not shown), which encodes $p19^{Arf}$ and $p16^{Ink4a}$, is silenced during iPS reprogramming (90,92,96). The *Ink4/Arf* locus comprises the *Cdkn2a–Cdkn2b* genes encoding three potent tumour suppressors, namely $p16^{Ink4a}$, $p19^{Arf}$ and $p15^{Ink4b}$, which are basally expressed in differentiated cells and upregulated by aberrant mitogenic signals (96-98).

Genetic inhibition of the *ink4/arf* locus has a profound positive effect on the efficiency of iPSCs generation, increasing both the kinetics of reprogramming and the number of emerging iPSCs colonies. In murine cells, *Arf*, rather than *Ink4a*, is the main barrier to reprogramming by activation of p53 (encoded by Trp53) and p21 (encoded by *Cdkn1a*); whereas, in human fibroblasts, *INK4a* is more important than *ARF*. Furthermore, organismal ageing upregulates the *Ink4/Arf* locus (96,99) and, accordingly, reprogramming is less efficient in cells from old organisms, but this defect can be rescued by inhibiting the locus with miRNA. These results provide insights into new routes to more efficient reprogramming and reprogramming mechanisms while minimizing the use of oncogenes (93,94). Thus, the silencing of *Ink4/Arf* locus is rate– limiting for reprogramming, and its transient inhibition may significantly improve the generation of iPSCs (92).

The ability of stem cells to propagate indefinitely is believed to occur via the fine modulation of pathways commonly involved in cellular senescence, including the telomerase, the p53, and the mitochondrial/oxidative stress pathways. Accordingly, iPSCs exhibit alterations of p53 signaling pathways the senescence-related telomerase. However, recent data highlight that hiPSCs and hESCs, although not identical, share similar mitochondrial properties and suggest that cellular reprogramming can modulate the mitochondrial/oxidative stress pathway, thus inducing a rejuvenated state capable of escaping cellular senescence (100,101).

Similarity and Difference between iPSCs and ESCs

Identifying pluripotent cells of the highest quality is crucial to the development of therapeutic applications, so we can ensure that any transplanted cells function as well as normal cells. It's going to be important to see whether hiPSCs derived from patients have similar differences in gene expression and if they can be as good as hESCs – which continue to be the gold standard – in giving rise to the 220 functional cell types in the human body.

Although at a first glance, iPSCs and ESCs seem

to be very similar in terms of morphology, cell surface marker and gene expression levels, recent papers have demonstrated differences at the transcriptional level between the two cell types (102,103).

Several reports show that iPSCs differentiate in a manner that is very similar to ESCs in so far as their differentiated progeny seem to express similar marker genes and have similar morphology to the same cell types that differentiate from ESC (104-107). Furthermore the limited number of transplantation studies performed, suggest that iPSCs derived somatic cells may be the functional equivalents of those from ESCs (108,109).

However, a recent paper suggests that although hiPSCs use the same transcriptional network and development time course as the hESCs, their differentiation along the neural lineages is less efficient and more variable across the cell lines (110). It has been suggested (101,111) that the reduced differentiation potential may be due to low levels of transgene expression, however the recent results obtained by Hu *et al.* seem to suggest that the variable differentiation efficiency was not due to presence of transgenes with episomal transgenes established for similar results were obtained with hiPSCs (110).

Even if we can prove that there is no difference between the differentiation properties of hESCs and IPSCs we are still in the same position as we would have been if ESC were our only source of cells since we still don't have completely effective methods to direct differentiation. All of this work remains to be done whether or not we choose iPSCs (112). The overlap of expression differences decreases as more independent reprogramming experiments from different labs were compared (102). Many questions about consistent differences between hiPSCs and hESCs were proposed, then the two groups now suggest that when we are comparing many lines of hiPSCs and hESCs from different labs, we lost the consistent differences between them and find that most of them are lab specific or stochastic in nature (113,114). Chin *et al.* (102), and the latter went further to obtain additional data from new hiPSCs and compared them with a larger group of hESCs. Both groups took issue with the meta-analysis methods used in Chin *et al*. (102). Furthermore, they present data with new hiPSCs to demonstrate that reprogramming methods may affect the kinetics of this process and reconfirm that extended culturing brings hiPSCs closer to hESCs.

It is important to consider passage number, reprogramming technology, and genetic background when comparing pluripotent cells from various sources. However, it remains unclear what drives the transition of hiPSCs closer to hESCs. The data suggest that with improved technology, one can probe deeper to find expression differences between hiPSCs and hESCs not related to lab-specific differences (115).

Safety and Efficiency Issues

The development of methods to achieve efficient reprogramming of human cells while avoiding the permanent presence of reprogramming transgenes represents a critical step toward the use of iPSCs for clinical purposes, such as disease modeling or reconstituting therapies. Initial methods used to derive hiPSCs employed viral vectors, where both the vector backbone and transgenes are permanently integrated into the genome (27,40). Such vectors can produce insertional mutations that interfere with the normal function of iPSCs derivatives, and residual transgene expression can influence differentiation into specific lineages (40), or even result in tumorigenesis (1).

It has since become clear that combinations of alternative genes or chemicals can be used to substitute for some of the original four reprogramming factors, modifying the number of viral vectors required, in some cases at the expense of reprogramming efficiency (24,32,40,116). More recently, derivation of iPSCs with non-integrating vectors, plasmid transfection, or even direct protein delivery has been achieved, although with exceedingly low efficiencies that prevent reliable application for reprogramming disease-specific adult human somatic cells (65,117-120).

Regardless of the method used, somatic cells from humans appear to be more difficult to reprogram than murine cells (120). Moreover, it is becoming clear that the development of methods to achieve efficient reprogramming of cells from adult humans with disease while avoiding the permanent presence of the reprogramming transgenes, represents a critical step toward the use of this technology for clinical purposes (121-123). Importantly, such methodology should allow for the reliable and consistent reprogramming of human somatic cells, regardless of the age or disease state of the individual from whom they are derived.

Vector integration-free mouse iPSCs have been derived from liver cells with adenoviral vectors (119), and from embryonic fibroblasts with repeated plasmid transfections (118), but the low frequencies obtained make it unclear how practical these approaches will be for human cells, which generally require longer exposure to reprogramming factors (27,40).

Two alternative approaches were described to remove transgenes from mouse or hiPSCs. In one approach, Cre/LoxP recombination was used to excise integrated transgenes (120,121). This approach successfully removes transgene sequences, but leaves behind residual vector sequences, which can still create insertional mutations. A second approach used seamless excision of piggyBac transposons to produce vector and transgene-free mouse iPSCs (122). Although a promising approach, vector removal from hiPSCs produced by this method has not yet been reported, and removing multiple transposons is labor intensive.

The use of a humanized version of a single lentiviral ''stem cell cassette'' vector to accomplish efficient reprogramming of normal or diseased skin fibroblasts obtained from humans of virtually any age. Simultaneous transfer of either three or four reprogramming factors into human target cells using this single vector allows derivation of hiPSCs containing a single excisable viral integration that on removal generates hiPSCs free of integrated transgenes (124).

Before iPSCs-based therapies are applied to humans, several issues need to be addressed. One of this is related with the safety of such therapies. One concern is that since these cells are often cultured in the presence of animal products, most often mouse feeder cells and bovine serum albumin, there is a potential for incorporating animal pathogens to humans (125). Also, antigens derived from these animal products can be incorporated into the human cells and result in immune rejection of the transplant (126-128), therefore offsetting the benefits of autologous cell transplantation.

The current conditions for iPSCs derivation include animal products at different steps of the process, like derivation of the somatic cell culture, induction of pluripotency, and culture of the iPSCs. To reduce the possibility of animal-derived pathogen infection and/or immune reaction against animal antigens, hiPSCs should ideally be derived and maintained in xeno-free culture conditions (129).

Reprogramming of human fibroblasts to pluripotency can be achieved under xeno-free conditions at efficiencies similar to those obtained using animal-derived products. For this purpose, a primary culture of xeno-free human foreskin fibroblasts was established, which were used as both the source of cells for reprogramming as well as autologous feeder cells for the generation and maintenance of iPSCs. A xeno-free culture medium also been developed for hESCs/iPSCs based on a human plasma-derived serum substitute and show that it supports their long-term culture with a performance similar to those of commercially-available xeno-free hESCs media and conventional serum replacement (KO-SR)-based media (130).

There is a need to develop efficient and safe nonviral gene transfer approaches for genetic modification of adult stem cells. Unfortunately, nonviral vectors integrate very inefficiently into most primary cells and are rapidly diluted and/or degraded in a dividing stem cell population and its progeny, leading to only transient transgene expression. However, the use of nonviral gene delivery approaches in conjunction with the latest generation transposon technology may potentially overcome these limitations. Transposons derived from Sleeping Beauty (SB) are among the most promising for mammalian gene transfer (131-133). SB has been "resurrected" by molecular reconstruction from silent, ancestral transposons found in fish genomes that enabled transposition in mammalian cells (134). However, transposition of these early generation SB transposons was still relatively inefficient in most primary mammalian cells including stem cells (135-138). To overcome this limitation, novel hyperactive transposases from SB using a high-throughput, *in vitro* molecular evolution and selection paradigm (139). The particular hyperactive SB transposase SB100X, exhibited ~100-fold enhancement of transposition in human cell lines as compared with the originally resurrected SB. Most importantly, SB100X also resulted in a significant enhancement of stable gene transfer efficiencies in $CD34^+$ hematopoietic stem cells (132,139-143). Hence, this hyperactive transposon system represents an attractive nonviral gene transfer platform with broad implications for regenerative medicine, cell and gene therapy (144).

Warren *et al.* (144) describe a new methodology, using synthetic mRNA, for efficiently generating iPSCs without compromising genomic integrity. This powerful approach can also be used for directed differentiation of iPSCs, or even for trans-differentiation to generate clinically relevant differentiated cell types. The approach relies on the delivery of a cocktail of *in vitro*-generated, modified synthetic mRNA that encodes the reprogramming factors (RFs) (Klf4, c-Myc, Oct4, Sox2, and Lin28). Given that these mRNAs are translated in the cytoplasm, their transfection into human cells does not cause permanent genetic changes (144).

The new work by Morrisey and colleagues (23) shows that expression of a single primary miRNA transcript, the miR-302/367 cluster, is in itself sufficient to reprogram both mouse and human fibroblasts. The resulting iPSCs exhibit gene expression and functional properties characteristic of fully reprogrammed pluripotent cells. Approximately 10% of fibroblasts form iPSCs colonies, an improvement in efficiency of >100-fold compared

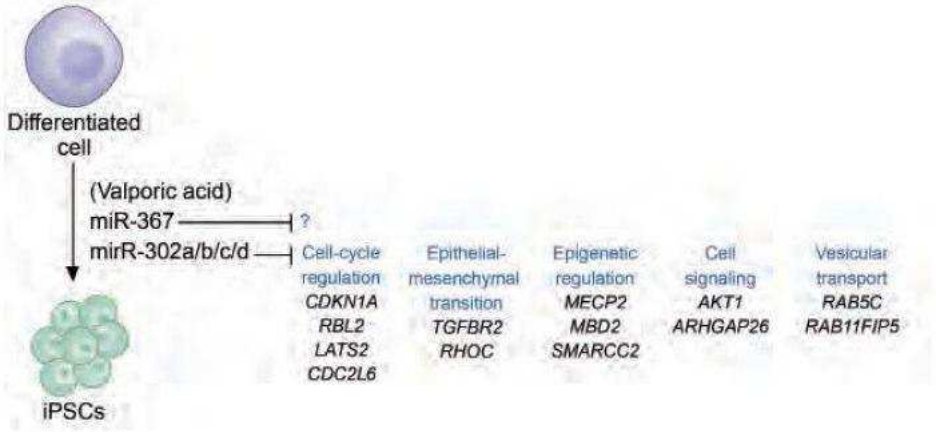

Figure 1. Reprogramming of mouse and human cells to pluripotency with miRNAs (Adapted with permission from Chang HM, *et al*, Nature Publishing Group 2011).

with Oct4, Sox2, Klf4, c-Myc (OSKM). Moreover, the appearance of iPSCs colonies and the activation of pluripotency markers occur sooner using the miR-302/367 cluster than using OSKM (23,145). miRNAs are a large family of regulatory RNAs that post-transcriptionally repress the expression of large sets of target genes and are essential for normal development and ESCs biology. ESCs express a unique set of miRNAs, which are required for rapid cell proliferation and cell-cycle progression. The majority of these miRNAs are transcribed from two genomic loci: the miR-302/367 cluster (containing five miRNAs—miR-302a/b/c/d and miR-367) and, in mice, the miR-290–295 cluster (miR-290, miR-291a, miR-291b, miR-292, miR-293, miR-294 and miR-295) or, in humans, the miR-371–373 cluster (miR-371, miR-372 and miR-373). Because ESC-specific miRNAs share a very similar 'seed' sequence, they are likely to regulate overlapping sets of target genes (146).

Blelloch and colleagues provide several lines of evidence that miRNAs promote reprogramming of human cells by targeting genes in multiple downstream pathways (Fig. 1). First, reprogramming involves a mesenchymal-to-epithelial transition, as in mouse cells. Second, inhibition of any single gene leads to a modest enhancement in reprogramming compared with the effects of the miRNA itself. Finally, reprogramming efficiency is increased by simultaneous inhibition of multiple pathways. Therefore, it is likely that miR-367 facilitates reprogramming at least in part by promoting cell proliferation (147).

Esteban *et al*. (149) report that vitamin C enhances the reprogramming efficiency of mouse and human fibroblasts transduced with three (Oct4/Klf4/Sox2) or four (Oct4/Klf4/Sox2/cMyc) factors. Vitamin C can ease cell senescence by p53 repression and may advance reprogramming by synergizing with epigenetic regulators (148,149).

Given the rapid pace of the iPSCs field, it is likely that reprogramming efficiencies will improve significantly, and that it soon will be possible to derive vector and transgene-free hiPSCs by several alternative methods. However, it will be neccesary to determine which of these methods most consistently produces iPSCs with the fewest genetic and epigenetic abnormalities, due to the impact for the application of these cells in basic research, drug development, and transplantation therapies.

Somatic Cell Sources for Generation of iPSCs

Which somatic cells are the best sources for iPSCs destined for clinical and pharmaceutical applications? In addition to fibroblasts, mouse iPSCs have been generated from bone marrow cells (9), hepatocytes and gastric epithelial cells (150), pancreatic cells (47), neural stem cells (151,152), and B lymphocytes (46). hiPSCs have been generated from skin fibroblasts, keratinocytes (88), and blood progenitor cells (153).

The first issue is to obtain somatic cells from donors simply and safely. Cells such as leukocytes meet this criterion as do epithelial cells from the oral mucosa. Generation of iPSCs from the follicle cells of a single human hair also has been reported (88). Small skin biopsy was used to obtain skin fibroblasts and keratinocytes, gastric epithelial cells by endoscopic biopsy, and BM cells and hepatocytes obtained by needle biopsy. Tissue

can also be obtained when patients undergo surgery. Other sources include cell banks such as those for cord blood; it would be extremely useful if iPSCs could be generated from cord blood cells.

The second issue is that iPSCs from different origins may have different propensities to differentiate. Certain cell types may be better for complete reprogramming with a reduced risk of teratoma formation. It may be easier to generate pancreatic-β cells and hepatocytes from iPSCs derived from somatic cells of endodermal origin such as gastric epithelial cells. Notably, iPSCs derived from mouse hepatocytes (150) or human keratinocytes (88) have fewer retroviral integration sites than do iPSCs derived from fibroblasts. These cells may be a better source for iPSCs generation; iPSCs also have been generated from mouse hepatocytes using adenoviral vectors (119).

In most cases to date, skin fibroblasts are the cell type from which patient iPSCs are generated. Acquiring a sample of this sort generally involves performing a skin biopsy, which requires patients to undergo procedures such as local anesthesia, an incision, and suturing. None of these interventions are free from potential complications, particularly risk of infection.

Another concern about using skin as a source for iPSCs line derivation is the risk that the starting cells harbor chromosomal aberrations caused by UV irradiation. After biopsy, it takes at least a month to expand fibroblasts for iPSCs induction. Undesired mutations may occur during this period. These limitations prevent many scientists from utilizing the iPSCs technology.

Three groups report the generation of hiPSCs from peripheral blood cells obtained from individuals who had received no pretreatment (153-156). Sampling of peripheral blood is one of the most commonly performed and least invasive clinical procedures. Therefore, from a scientific point of view, the achievements described in these three new papers may seem to represent a relatively small step forward. However, practically and technically speaking, their findings represent a huge and important progression in the field.

Thus, the generation of iPSCs from a small amount of peripheral blood collected from non-pretreated donors is an important step in facilitating the usage of iPSCs in the various applications described above. Instead of requiring patients to undergo an invasive skin biopsy, all we need may be a small amount (as little as 1 ml) of extra blood sample. Importantly, additional procedures are not necessary, given that blood sampling is routinely conducted on patients and also on healthy people at routine medical checkups. Furthermore, blood sampling is significantly less expensive than performing a skin biopsy. Finally, according to the methods described by the Jaenisch, Fukuda, and Daley groups, iPSCs can be induced within several days after blood sampling, thus the risk of undesired mutations can be minimized. It is reasonable to predict that the field may see a dramatic shift from using skin fibroblasts to peripheral blood as a source of iPSCs in the very near future.

Direct Reprogramming Strategy

Reprogramming of differentiated somatic cells such as fibroblasts into iPSCs using four (or even less) TFs revolutionized the understanding of cellular plasticity and accomodated a novel tool to study developmental processes and mechanisms of human disease (27). Furthermore, there are high expectations that iPSCs-derived cells might be a promising source for patient-specific cell-replacement therapies (157).

Vierbuchen and colleagues now take the concept of cell-fate reprogramming one step further and show that fully differentiated embryonic and post-natal fibroblasts can be efficiently converted into functional neurons (called iN cells) without the detour of an uncommitted pluripotent cell (158).

A former concept regarding the basic mechanisms of cell specification was that differentiated cells are bound in their cell fate in an irreversible epigenetic modifications to prevent the transcription of genes specific to other cell lineages. The development of iPSCs technology has challenged this concept by showing that in principle every cell retains the potential to dedifferentiate into a pluripotent ground state by overexpression of a few TFs (Figure 2). However, reprogramming of somatic cells into an iPSCs involves a thorough eradication of epigenetic marks, thereby allowing the cell to ''start all over again'' with an at least partially naive chromatin (3).

The findings by Vierbuchen *et al.* show that directed conversion from one differentiated cell type into another (in this case fibroblasts into neurons) with only three TFs can be achieved very quickly (within days) and efficiently, without going back to an uncommitted pluripotent cell state.

Two cardiac TFs, Gata4 and Tbx5, and a cardiac-specific subunit of BAF chromatin-remodelling complexes, Baf60c (also called Smarcd3), can direct ectopic differentiation of mouse mesoderm into beating cardiomyocytes, including the normally non-cardiogenic posterior mesoderm and the extraembryonic mesoderm of

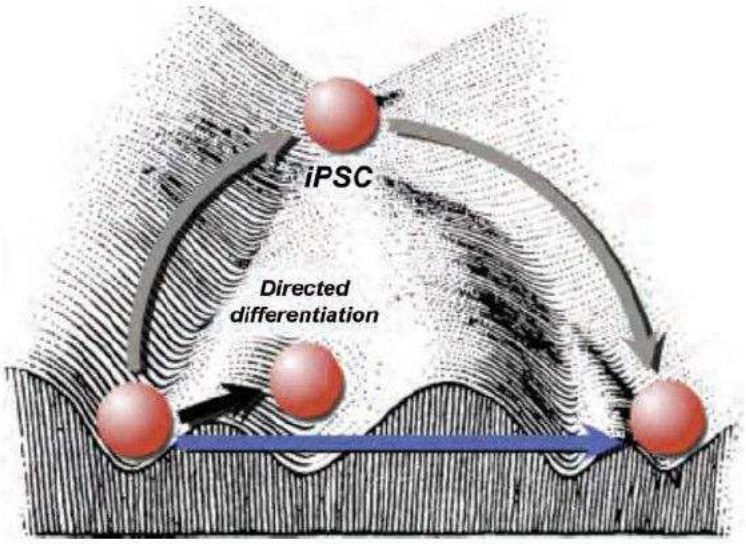

Figure 2. Directing cell fate with extopic master gene expression (Adapted with permission from Braun, SMG, *et al*, Elsevier Inc 2010).

the amnion. Gata4 with Baf60c initiated ectopic cardiac gene expression. Addition of Tbx5 allowed differentiation into contracting cardiomyocytes and repression of non-cardiac mesodermal genes. Baf60c was essential for the ectopic cardiogenic activity of Gata4 and Tbx5, partly by permitting binding of Gata4 to cardiac genes, indicating a novel instructive role for BAF complexes in tissue-specific regulation. The combined function of these factors establishes a robust mechanism for controlling cellular differentiation, and may allow reprogramming of new cardiomyocytes for regenerative purposes (160).

Efe *et al.* (161) showed that conventional reprogramming towards pluripotency through overexpression of Oct4, Sox2, Klf4 and c-Myc can be shortcut and directed towards cardiogenesis in a fast and efficient manner. With as little as 4 days of transgenic expression of these factors, MEFs can be directly reprogrammed to spontaneously contracting patches of differentiated cardiomyocytes over a period of 11-12 days. Some studies suggested that a pluripotent intermediate is not involved. This finding has wide-ranging potential implications for iPSCs-factor-based reprogramming and broadens the existing paradigm (161).

Challenges and Clinical Application for iPSCs

What is the best way of making iPSCs? First-generation iPSCs were generated by retroviral transduction (9,27). Since then, the technique has been optimized and reproduced in a number of different ways. The most important variables include choice of cell type to reprogram, choice of the cocktail of reprogramming genes, and method for gene transfer (Figure 3). Nimet Maherali and Konrad Hochedlinger recently wrote an excellent review of protocols, highlighting the details of different methodologies to make iPSCs (162).

Embryonic fibroblasts (MEFs) and tail-tip fibroblasts (TTFs) in the mouse and dermal fibroblasts in the human have been the most widely used cell types to reprogram, largely due to their availability and ease of accessibility. In addition, various other cell types have also been reprogrammed, including hepatocytes (119), stomach cells (150), B lymphocytes (46), pancreatic β cells (47), and neural stem cells (36) in the mouse; keratinocytes (88), mesenchymal cells (163), peripheral blood cells (153), and adipose stem cells (164) in the human; and melanocytes in both species (29). Variable efficiencies and kinetics of the process have been described, while the *in vitro* age of the cell type (passage number) correlates inversely with the efficiency of reprogramming (165).

Much of the focus of recent research has understandably been on the generation of clinically applicable iPSCs free of viruses and transgenic integrations. We believe that it is now critically important that iPSCs generated by distinct methods are carefully assessed for their variability, stability, and differentiation potential as well as the quality and long-term survival of differentiated cells derived from them. Ultimately, iPSCs generated by each method will need to be examined in detail at the genomic, epigenomic, and functional level in order to determine which reprogramming methods are safe for clinical cell therapy (166).

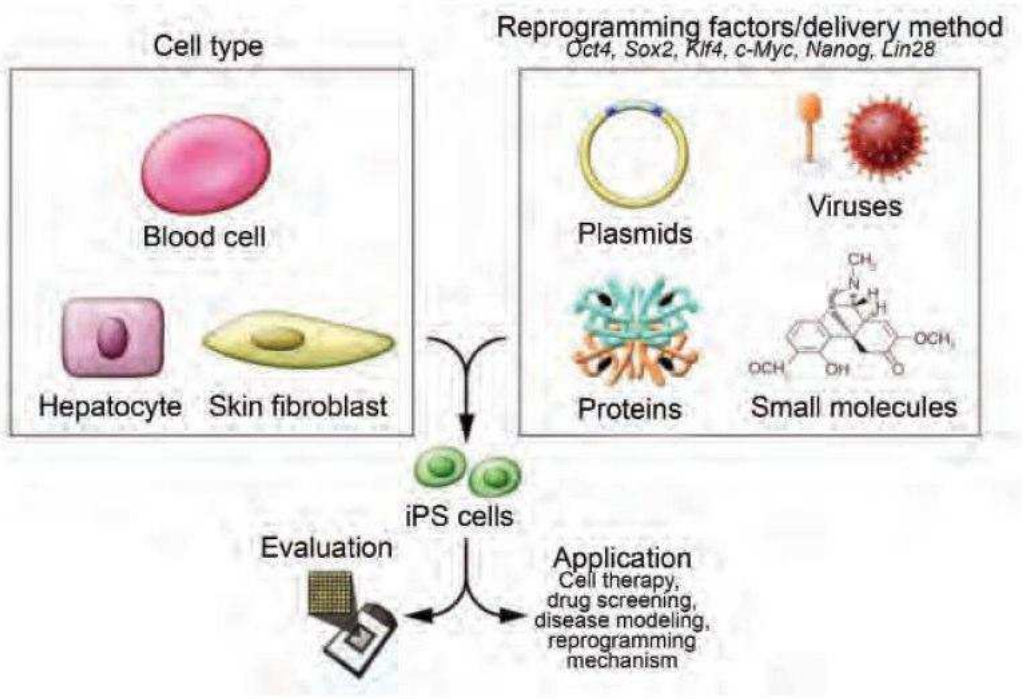

Figure 3. Generation of iPSCs(Adapted with permission from Kiskinis E, *et al*, The Journal Clinical Investigation 2010).

Although iPSCs generated using one or two factors rather than four or using recombinant proteins rather than viral expression systems may be clinically safer, it has yet to be demonstrated that this is the case. In addition, the logistical, financial, and practical aspects of each technique will need to be taken into account.

Cell replacement therapy

The convergence of stem cell research with medical application has long been a source of excitement for the scientific community and the general public alike. Pluripotent stem cells offer the hope for treatment of individuals suffering from cellular degeneration caused by either disease or injury. Various therapeutically relevant cell types have been developed successfully from ESCs *in vitro* including cardiomyocytes, motor and dopaminergic neurons, oligodendrocytes, and hematopoietic precursor cells (167). More importantly, the therapeutic potential of these ES cell–derived somatic cells has been effectively demonstrated in animal models.

The development of cell replacement therapies using ES cell–differentiated cells is, however, burdened with social and religious concerns regarding the use of human embryos, as well as issues involving immune rejection of the transplanted cells. The ability to generate PS-iPSCs by direct reprogramming of human fibroblasts overcomes these barriers and has brought the realization of personalized regenerative medicines closer (Figure 3). Patient Specific (PS) – IPSCs tailored to specific individuals should provide the opportunity for cell replacement therapy without the need for immunosuppressants, as autologous transplantation of genetically identical cells, potentially tissues and organs (164).

The rapid advancements in the field of iPSCs production during the last three years have led to the generation of clinically relevant cell lines free of genomic integration and oncogenes. The remaining challenges for clinical reprogramming are now more limited to technical issues, such as increasing the iPSCs efficiencies generation using non-integrating methods, under current good manufacturing practice (cGMP) conditions.

Disease modeling and drug discovery

Although further work needs to be done toward generating and extensively characterizing "clinical grade" hiPSCs before human cell replacement therapies can be attempted, disease modeling and drug screening are two immediate applications for reprogramming technology. The concept of utilizing hESCs and now hiPSCs to model a disease in a culture dish is based on the unique capacity of these cells to continuously self-renewing and their potential to develop to all cell types in the human body (167,168). Thus, many

could be provide limitless by pluripotent cells.

Predicting the timing and details of the first iPSCs-based therapies is difficult, as progress in the area is stochastic and the field has undergone enormous changes in just a short time. Given the rapid pace of research in this area, despite the need for a deeper understanding of hiPSCs biology, the next 5 years are likely to see real progress in drug discovery for a wide range of diseases using iPSCs platforms combined with high-throughput library screens. For cell therapies, we are optimistic that the next decade will see several hiPSCs-based therapies for debilitating and deadly diseases, such as macular degeneration and Amyotrophic lateral sclerosis (ALS), and hiPSCs-based treatment combined with gene therapy for monogenic diseases, such as sickle cell disease. It is essential that hiPSCs research does not replace hESCs research – the two cell types must be studied in parallel to provide information on the biology of both cell types. As the behavior of hiPSCs and hESCs is better understood, and their relative advantages in particular clinical scenarios quantified, it is likely that both will have futures in cell-based therapies (169).

The regulatory and ethical challenges particular to hiPSCs-based therapies were recently reviewed (170), as stem cell biologists recognize that ethical considerations did not disappear when hiPSCs came on the scene. The major ethical issues revolve around privacy, informed consent and (as with living donor solid organ donation) the proper handling of incidental, unanticipated medical information that emerges from studying hiPSCs. Furthermore, since pluripotent cells placed into cell banks can be maintained for a long time, the reach-through rights of the donor must be considered (171).

There is no question that the creation of hiPSCs is a groundbreaking, landscape-changing shake up for the field, and hiPSCs research is proceeding at breakneck speed in academics and, increasingly, in industry.

Conclusion

The reprogramming of differentiated cells to pluripotency holds great promise as a tool for studying normal development, while offering hope that patient-specific iPSCs could be used to model disease or to generate clinically useful cell types for autologous therapies aimed at repairing deficits arising from injury, illness, and aging.

The recent successes in hiPSCs derivation without viral vectors and genomic integration have brought the realization of the therapeutic potential of hiPSCs technology closer than ever. Given the scientific effort and significant achievements of the past few years, we are hopeful that hiPSCs technology will have a positive impact on therapeutic interventions.

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