Induced pluripotent stem cells: the long-expected revolution in medical science and practice?

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Abstract

Within the matter of a few years, development of the somatic reprogramming technology to generate induced pluripotent stem (iPS) cells has contributed enormously to the stem cell research field. We learned that differentiated adult cells possess an unrestricted plasticity that allows them to be driven back to their embryonic or pluripotent state, but owing to the juvenile nature of this novel science chapter, there are many unanswered questions and dilemmas. It is indisputable, however, that iPS cells potentially could represent the jack-of-all-trades remedy in areas of medicine ranging from toxicology screening to regenerative medicine. In this review I will summarize the current strategies employed to reprogram somatic cells and the major promises and hurdles for the future of iPS cells.

Introduction

An unparalleled achievement in the study of human development and disease modeling was provided by the creation of the first human embryonic stem (hES) cell line in 1998, when Thomson and colleagues derived five hES cell lines from the inner cell mass of blastocysts cultured from donated embryos produced by *in vitro* fertilization (IVF).[1] A few years later, in August 2006, a major breakthrough in biomedical science was communicated by Takahashi and Yamanaka, describing a straightforward method for generating pluripotent stem cells from embryonic and adult mouse fibroblasts after retroviral transduction of four transcription factors (TFs); namely, Oct-4, Sox-2, Klf-4, and c-Myc (OSKM).[2] The authors of this seminal work, published in the prestigious *Cell* journal, named this novel cellular entity induced pluripotent stem (iPS) cells. One year later the same group reported the successful generation of iPS cells from human fibroblasts,[3] establishing a key landmark in the already boiling-hot area of regenerative medicine: the possibility to travel back in time in patient history and follow disease development from the pluripotent cell.

iPS cells share significant similarities to ES cells in terms of morphology, proliferation, expression of a handful of pluripotency markers, common signaling pathways maintaining the undifferentiated state, and the ability (or hazardous flaw, see below) to form teratocarcinomas *in vivo*.[4,5] However, gene expression profiles (mRNAs, microRNAs, and histone modifications) have demonstrated that iPS cells still retain a unique signature, in part owing to differential promoter binding by the reprogramming factors.[6] Nonetheless, using the OSKM factors, truly pluripotent cells have been generated and when assayed in the most stringent tetraploid complementation assay, that is, injection of foreign diploid ES or iPS cells in extra-embryonic tissue-forming tetraploid blastocysts, they produced viable, reproductively competent donor-derived progeny,[7] indicating that iPS cells can attain full pluripotency similar to that of ES cells. Stringent criteria and standards have been proposed for the generation of iPS cells to allow cross-laboratory data comparisons,[8] although some controversy exists on the feasibility of the minimal set of characterization criteria required, especially regarding the true need of *in vivo* teratoma formation assay (human) and germline competence after chimera formation (mouse) for cells that would be used for *in vitro* applications only.[9] Certainly the availability of iPS cells endowed with the ability to derive the three germ layers *in vitro* but defective of teratoma formation *in vivo* would be equally (if not superiorly) useful for tissue engineering applications.[9]

Derivation, basic biology, and efficiency of iPS cells reprogramming

The initial step in the generation of patient-specific iPS cells is to obtain individual tissue samples. iPS cells have been derived from many somatic cell types of origin, typically harvested from tissue biopsy. In addition to the standard dermal fibroblasts, iPS cells have been derived from several sources: mouse hepatocytes and gastric epithelial cells,[10] human keratinocytes contained in a single plucked hair, which also displayed a greater (>100-fold) and faster (>2-fold) reprogramming efficiency,[11] cells extracted from exfoliated deciduous teeth, stem cells from the apical papilla, and dental pulp stem cells,[12] freshly isolated human adipose stem cells in a feeder-free condition,[13] human peripheral blood $CD34^+$ cells,[14] human cord blood (CB) endothelial-derived cells[15] and $CD133^+$ CB cells, possibly more amenable to reprogramming owing to elevated endogenous expression of Klf-4 and c-Myc,[16] uniparental parthenogenetic neural stem cells,[17] and adult limbal progenitors isolated from rat limbal epithelium of the cornea.[18] All of these cell types including skin fibroblasts are potentially clinically relevant, requiring minimal invasive surgical intervention for harvesting. Undoubtedly CB-derived cells could represent one of the most attractive cell types. In particular CB cells are readily accessible and efficient to isolate, they are considered a relatively "young", immunologically immature cell type carrying minimal chromosomal abnormalities and mutations, and probably most importantly, they are bankable, offering a wide selection of HLA haplotypes for transplantation purposes.[19]

The "Yamanaka factors" $OSKM^2$ represent the prototypical factors for somatic reprogramming. Equal stoichiometric and temporal expression of the OSKM factors is critical for the successful induction of iPS cells from human fetal fibroblasts.[20] The individual role of the OSKM TFs has been investigated in the mouse by carrying out a genome-wide analysis of promoter occupancy and expression of Oct-4, Sox-2, Klf-4, and c-Myc target genes,[21] showing that fibroblast markers are repressed and early embryonic markers activated before expression of pluripotency markers. C-Myc is a major contributor to the initial reprogramming event and predominantly acts before pluripotency regulators are activated.[21] In addition to OSKM, the generation of iPS cells has been achieved by means of various combinations of TFs, arguing against the absolute requirement of some of these factors, particularly c-Myc, for iPS cell formation. In this regard, a screen for candidate reprogramming factors performed in human mesenchymal cells expressing neomycin phosphotransferase driven by the endogenous Oct-4 promoter to track reprogrammed cells successfully, uncovered that the

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combination Oct-4/Sox-2/Nanog/Lin28 is sufficient to reprogram foreskin fibroblasts.[22] Surprisingly, a factor absolutely required for the establishment of pluripotency, the homeodomain-containing protein Nanog, is not one of the canonical TFs employed to reprogram somatic cells, most likely since its window of action is restrained to the late phases of reprogramming.[23]

Another critical parameter in determining the efficiency and kinetics of reprogramming is the differentiation stage of the cell of origin.[24] Immature (stem/progenitor) cells have been shown to reprogram faster and more efficiently, possibly because their epigenetic state is more amenable to TF-induced remodeling,[24] in contrast to previous assumptions[25] reprogramming does not seem to correlate with proliferation rate.[24] This notion is supported by the recent finding that adult mouse and human fetal neural stem cells can be reprogrammed by ectopic expression of Oct-4 alone,[26,27] suggesting that the use of stem/progenitor cells, preferably obtained from CB (see above), might become the elite choice of cell source for reprogramming, since the avoidance of using multiple potential proto-oncogenic TFs would be an important advance in the overall safety of iPS cell generation (see below). A distinct view attests that reprogramming is a continuous stochastic process, where almost all the mouse donor cells eventually give rise to iPS cells on continued growth factor and TFs expression.[28] Inhibition of the p53/p21 pathway and over-expression of Lin28 increases cell division and accelerates the kinetics of iPS cell formation proportionally to the increase in cell proliferation, while Nanog accelerates reprogramming in a rate-independent cell division.[28] In fact reprogramming seems to be a stochastic and slow process, suggesting the existence of barriers limiting its efficiency. Senescence is one of these roadblocks: it has been shown that reprogramming triggers up-regulation of p53, $p16^{ink4a}$, and $p21^{cip1}$, and therefore senescence, coupled with induction of DNA damage and chromatin remodeling of the INK4a/ARF locus.[29] Ablation of different senescence effectors improved reprogramming efficiency, although from the perspective of therapeutic application this would be problematic, and transient siRNA-mediated knockdown could be at least a more feasible trial.[29] These observations have been confirmed in two separate studies demonstrating that iPS cell generation was greatly promoted either in a p53-null background or by pharmacological administration of vitamin C, further proving that the p53-p21 pathway is a critical barrier to iPS cell generation beyond its well-known function in the tumorigenic process.[30,31]

One of the major conceptual and technical challenges in somatic cell reprogramming is the efficiency of iPS cell generation. To date, the standard efficiency of reprogramming from dermal fibroblasts is ≤0.01%[3] and selecting successfully reprogrammed cells is a tedious process requiring screening of a large number of clones that need to be individually tested for multiple parameters (see above). Several different tools and improvements have been developed to refine this procedure, essentially based on exploiting three basic aspects of iPS/ES cells: epigenetic state, dependence on key signaling pathways, and exogenous TFs delivery requirement (vector design).

Initially, by screening a small collection of known compounds, Shi *et al.* found that neural progenitor cells could be reprogrammed by using Oct-4 and Klf-4 TFs in combination with the drug BIX-01294, an inhibitor of the G9a histone methyltransferase, highlighting the importance of appropriate chromatin remodeling during the reprogramming process.[32] This is also verified by the observation that DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors improve reprogramming; in particular valproic acid (VA), an HDAC inhibitor, has proven to increase efficiency by more than 100-fold using Oct-4, Sox-2, and Klf-4 TFs without the introduction of c-Myc.[33] A study employing integrative genomic analysis, including gene expression profiling, chromatin state maps, and DNA methylation analysis of mouse fibroblasts and B lymphocytes undergoing reprogramming, established a conceptual framework in addition to the above observations, showing the existence of a gradient of expression and epigenetic states from the somatic cell to the iPS cell.[34] Partially reprogrammed cells show DNA hypermethylation at pluripotency-related loci, incomplete repression of lineage-specifying TFs, and reactivation of a distinctive subset of stem-cell-related genes, while completely reprogrammed iPS cells share similar signatures with ES cells. Overall efficiency could be improved further by inhibition of DNMT1 and incompletely repressed TFs.[34]

The current effort of identifying potential signaling pathways facilitating iPS cell generation relies on the use of chemical screening platforms. A massive high-throughput small-molecule screening, aimed at identifying compounds (>500,000) that practically could replace Klf-4 in activating Nanog, detected the multikinase inhibitor kenpaullone as a functional substitute for Klf-4.[35] A similar approach, although on a much smaller scale, was used to describe an improvement of reprogramming efficiency of >200-fold by using the Alk5 inhibitor SB431542, the MEK inhibitor PDO431542, and the pro-survival agent thiazovivin in addition to the canonical OSKM factors.[36] Inhibition of TGFβ signaling appears to be a successful strategy, confirmed in two other studies: the TGFBR1 inhibitor E-616452 has been shown to replace Sox-2 in the absence of VA, whereas a second TGFBR1 inhibitor E-616451 and the Src-family kinase inhibitor EI-275 still required VA.[37] Inhibition of TGFβ signaling following administration of an Alk5 inhibitor cooperates in the reprogramming of murine fibroblasts, and bypasses the requirement for exogenous c-Myc and Sox-2.[38] Finally, an interesting report has shown recently that hypoxia (or *in situ* normoxia,[39] i.e. 5% O_2) increases the efficiency of iPS cell generation from murine embryonic fibroblasts (MEFs) and bypasses the requirement for Sox-2 and c-Myc.[40] It is conceivable that the more knowledge of normal stem cell biology is revealed, the more options will be available to find less radical and more "native" implements for somatic reprogramming.

So far, some of the most substantial progress in generating preclinical-grade iPS cells has been obtained in the development of reproducible, efficient, and integration-free methods to introduce the reprogramming factors. Several research groups around the globe are achieving outstanding progress in searching for the optimal combination and delivery of TFs. Initial attempts were made by using non-integrating adenoviruses transiently expressing the OSKM factors, however reprogramming efficiency seemed lower compared to integrating viruses;[41] analogous low efficiency was obtained in mouse by using transient transfection with two plasmids expressing Klf-4/Oct-4/Sox-2 and c-Myc, respectively.[42] An interesting attempt, although with the major caveats of very low efficiency, has been made by using a single polycistronic vector encoding Klf-4/Oct-4/Sox-2: the cistron was driven by an EF-1α promoter in a self-inactivating (SIN) lentiviral vector (LV) containing a LoxP in the 3'LTR, and between each gene was inserted a porcine teschovirus-1 2A sequence triggering ribosome skipping, allowing translation of the multiple coding sequences. Expression of Cre recombinase resulted in deletion of the LV except for a remnant 291-bp SIN LTR containing a single LoxP site.[43]

A prominent advance in the generation of transgene-free iPS cells has been reached by the use of the *piggyBack* transposon system: a moth-derived DNA transposon, highly active in mammalian cells and with a very large cargo capacity.[44] The OSKM factors were linked by sequences encoding 2A peptides in a single polycistronic unit, and the resulting transposon vector was introduced in MEFs together with *piggyBack* transposase expression plasmid to catalyze vector integration. Following generation of primary iPS, the transposon was removed on re-expression of transposase: *piggyBack* was excised without leaving genetic alterations at the excised site (footprint mutations), and transposon-free iPS cells were selected using the human herpes virus thymidine kinase (HV tk)-fialuridine (FIAU) selec-

tion system.[44] This transposon-based delivery method has been perfected further by the creation of a doxycycline-inducible *piggyBack* plasmid in which, comparably to the previous system, the individual *piggyBack* insertion can be removed from established iPS cells.[45] Presumably the instrumental handling of the *piggyBack* system marks one of the most relevant advances toward achieving clinically suitable methods of generating iPS cells in that it makes use of simple plasmid DNA preparations and delivery, has a broader spectrum of target somatic cell types, grants xeno-free production protocols, and allows accurate transgene removal.[45] An alternative ingenious method to isolate human iPS cells has been described by Hotta and colleagues using EOS LVs: they engineered a vector containing an early transposon promoter, highly transcribed in ES cells, combined with Oct-4 and Sox-2 binding motifs in a ES cell-specific core enhancer, plus puromycin resistance gene and EGFP to avoid vector silencing and mark pluripotent stem cells, respectively.[46] It is worth mentioning another non-integrating episomal expression system derived from the Epstein-Barr virus, the oriP/EBNA1 (Epstein-Barr nuclear antigen-1): this vector can replicate only once per cell cycle, and by using drug-selection can be established as a stable episome that could be removed at the end of the process.[47]

Intriguingly, iPS cells have been generated in the absence of viral vectors. In one study, the key step was fusing a poly-arginine (11R) protein transduction domain to the C terminus of the OSKM factors: these fusion proteins were expressed in *E. Coli* in inclusion bodies that were then solubilized, refolded, and purified. The resulting proteins could be administered directly to the target cells and readily entered the cell membrane after only six hours.[48] In the second study, the authors exploited the ability of the human immunodeficiency virus transactivator of transcription (HIV-TAT) to cross the cellular membrane.[49] They initially fused a short segment residing at amino-acids 48-60 of HIV-TAT, containing a high proportion of basic amino-acids (arginine or lysine) and known as CPP, to the OSKM factors. The resulting chimeric fusion protein was expressed in HEK293 and the cell extract was used to treat human newborn fibroblasts. With respect to the previous report,[48] in this case there was no need to supplement the recombinant proteins with VA for successful reprogramming, and recombinant proteins were produced in a mammalian cell line in contrast to *E. Coli*[49] The use of soluble proteins instead of viral vectors undoubtedly offers a valid option for deriving iPS cells; on the other hand, the need to repeat the delivery many times and the consequent copious costs of production could represent a serious obstacle to the feasibility of the method.

Finally, exogenous administration of ES cell-specific, cell cycle-regulating (ESCC) miRNAs miR-291-3p, miR-294, and miR-295 have been shown to increase the efficiency of reprogramming,[50] offering the attractive opportunity to enhance iPS cell generation by using small RNA-mimicking oligonucleotides.

Regenerative medicine applications: disease modeling and cell-based therapies

The remarkable yet preliminary therapeutic potential of iPS cells is evident. Albeit ES cells offer the possibility to generate disease-specific pluripotent cells although with severe limitations, at the moment iPS cells offer the prospective of unprecedented disease modeling: to recapitulate the individual patient history faithfully.[51] iPS cells could be derived from any cell type, fresh or banked, to address the pathogenesis and progression of any genetic disease from the simplest to the more complex. Furthermore, patient-specific iPS cells offer the opportunity for drug screening and discovery and could be used in cell transplantation therapies using HLA-matched or autologous cells.[52] Besides, it is superfluous to mention that use of iPS cells would avoid the ethically sensible topic of using human embryos or eggs. A further and less explored potential application of iPS cells is their use as a cancer vaccine. A recent study found that human iPS cells can trigger, even if less potently than ES cells, an immune response against a murine colon carcinoma cell line in mice probably through cross-presentation of embryonic antigens.[53] Both iPS and ES cells were able to immunize naïve mice against challenge with a lethal dose of live colon carcinoma cells and induced a tumor-specific cellular immune response with loss of $CD11b^{+}Gr\text{-}1^{+}$ myeloid-derived suppressor cells in the spleen.[53]

The record of iPS cell disease-specific models is growing at a constant rate. One of the first proof-of-principle studies for deriving and correcting a disease with autologous iPS cells has been reported for the treatment of sickle cell anemia.[54] iPS cells were derived from a humanized sickle cell anemia mouse model, the human sickle hemoglobin allele was corrected *ex vivo* by gene-specific targeting, and transplanted engineered cells partially rescued the disease phenotype.[54] A second interesting study focused on murine hemophilia, a disorder caused by mutations within the Factor VIII (FVIII) gene leading to depleted protein production and inefficient blood clotting. The authors generated iPS cells from tail-tip fibroblasts, differentiated them *in vitro* in FVIII-producing endothelial cells and progenitors, and then injected them into the liver of irradiated hemophilia-A mice, showing long-term engraftment and hemophilia phenotype correction.[55] Ebert *et al.* generated iPS cells from skin fibroblast samples collected from a child suffering from type I spinal muscular atrophy (SMA).[56] They showed that the reprogrammed cells retained the disease genotype, consisting mainly of a lack of survival motor neuron 1 (SMA1) gene expression resulting in selective degeneration of lower α-motor neurons, and could effectively differentiate in diseased motor neurons and astrocytes.[56] Disease-specific iPS cells have been derived from reprogrammed dermal fibroblasts obtained from Fanconi anemia (FA) patients.[57] In this case, restoration of the FA pathway appeared as a prerequisite for iPS cell generation, but genetically corrected cells were indistinguishable from normal iPS cells, in that they were capable of forming phenotypically normal hematopoietic cells.[57] By using an elegant doxycycline-inducible Cre-recombinase excisable LV system, fibroblasts derived from a patient with idiopathic Parkinson's disease have been reprogrammed and have been shown to differentiate into dopaminergic neurons.[58] The example offered by Ye *et al.* is particularly illustrative: they canonically reprogrammed skin fibroblasts from a patient with homozygous β_0-thalassemia into iPS cells, but most importantly they successfully reprogrammed cells from the amniotic fluid or chorionic villus normally used for prenatal diagnosis.[59] This considerable result opens the opportunity to utilize iPS cells derived after perinatal diagnosis of thalassemia, and use the disease-corrected cells as a treatment in the perinatal periods, instead of the only currently available options of pregnancy termination or logistic support of a child with a life-long illness.[59]

The feasibility of *in vitro* candidate drug screens using patient-derived iPS cells is especially visible in a study in which iPS cells were generated from fibroblasts of a familial dysautomia patient, a rare but fatal peripheral neuropathy caused by a point mutation in the I-k-B kinase complex-associated protein (IKBKAP) gene.[60] The above mutation normally results in tissue-specific exon 20 skipping and reduced IKBKAP protein levels causing reduced neuron cells motility, ultimately leading to depletion of autonomic and sensory neurons. Patient-specific iPS cells generated all three germ layers including peripheral neurons containing the tissue-specific mis-splicing of IKBKAP, demonstrating the authentic cellular origin, which recapitulated the disease pathogenesis.[60] This well-characterized iPS cell model has been used further as an assay for testing candidate drugs affecting IKBKAP levels, showing the feasibility of exploring the disease mechanism and drugs action in

patient-derived iPS cells.[60] In the future prospective of cell replacement therapy, iPS cells have been derived from type 1 diabetes patients and have been differentiated into insulin-producing cells.[61] From this study no evidence emerges on disease recapitulation, in that the derived pancreatic β-like cells apparently behaved normally, but the problem that pluripotent cells generated from the same patient perform differently surfaces, suggesting incomplete silencing or transgene reactivation and indicating the need of non-viral and more efficient TFs delivery (see below).[61] Multiple human iPS cell lines have been generated from frozen cord blood or adult $CD34^+$ cells from healthy donors or patients with myeloproliferative disorders (MPDs) with acquired JAK2-V617F somatic mutation, which can evolve to polycythemia vera.[62] Additionally, the reprogrammed cells could be redirected to hematopoietic cells while the MPDs-derived cells recapitulated the disease features *in vitro*.[62] Finally, iPS cells from a Duchenne muscular dystrophy (DMD) patient have been derived and corrected by using human artificial chromosome (HAC) carrying the dystrophin gene, providing another valuable proof-of-concept of patient-specific iPS cell's disease modeling.[63]

An additional powerful application of patient-derived iPS cells would be their *in vitro* differentiation into disease-relevant tissue-specific cells. iPS cells, similar to ES cells, can be maintained stably in an undifferentiated state *in vitro* and switched to differentiation at any moment. A copious number of well-designed protocols have been studied to induce tissue-specific differentiation of ES/iPS cells. In addition to the cell types described above,[55-57,59-62] pluripotent cells have been differentiated into midbrain dopamine and spinal motoneurons without the requirement of embryoid body formation or stromal feeder co-culture by targeting SMAD signaling.[64] Similar to the study by Ebert *et al.*,[56] neural differentiation has been obtained from iPS cells derived from a patient with amyotrophic lateral sclerosis (ALS).[65] Another two interesting studies respectively described specification of retinal cells containing functional photoreceptors[66] and hepatic endoderm-derived hepatocytes.[67]

It is tempting to speculate that iPS cell-derived differentiated cells one day could regenerate damaged tissues and organs either by implanting engineered *in vitro*-produced tissues into the patient or by directly replacing injured tissues in their native environment *in vivo*. This could even lead to whole organ replacement in the contingency of end-organ failure. Most likely the creation of transplantable tissues will require the optimization of bioactive scaffolds providing the appropriate biomechanical microenvironment to generate sufficient clinical-scale quantities of cells.[68]

Genetic modification of patient-derived iPS cells and derivatives, including disease correction or tracking, would be the ultimate goal of the theoretical disease modeling workflow. Various viral vectors have been exploited as tools to achieve exogenous expression of transgenes in iPS or ES cells, although with limited efficiency or selectivity.[69] Thankfully, advances in gene targeting of endogenous loci are rapidly developing. The host-factor independent transposon *piggyBack* has been engineered to create fully reversible genetic modifications in ES cells to generate clinical-grade ES cell derivatives.[70] This newly created *ePiggyBack* can deliver up to 18kb of genetic material, transgene expression is highly efficient and, most importantly, can be readily remobilized to a recipient plasmid by re-expression of transposase. Compared to Cre- or Flp- and Sleeping Beauty-based systems, *ePiggyBack* removal does not leave any mutations in the host genome, even though transgene integration patterns are still random.[70]

Originally developed by Naldini's group, the "genome editing" technique, based on integrase-defective LVs (IDLVs) expressing zinc-finger nucleases (ZFNs) to target specific loci in stem cells by homologous recombination (HR),[71] is evolving as a promising tool to target genes in ES and iPS cells. Well known to researchers in the field, human iPS cells grow poorly as single cells, a practice required to select rare targeted clones using plasmid-based HR. A recent work described a virus-free system to perform ZFN-mediated gene targeting at the endogenous PIG-A locus, a gene normally mutated in hematopoietic stem cells from patients with paroxysmal nocturnal hemoglobinuria (PNH).[72] By using ZFNs, HR-mediated gene targeting ensured permanent genetic alterations, increased rate of targeting efficiency, high specificity, and no karyotypic abnormalities.[72] A similar technique was perfected to create an efficient method for genetic modification using ZFN-mediated genome targeting, achieving superior site-specific integration of exogenous genes under control of either constitutively active, inducible, or tissue-specific promoters.[73]

Safety concerns

Since Takahashi and Yamanaka reported the generation of iPS cells for the first time, for many scientists somatic reprogramming was and still is surrounded by a nimbus of skepticism, for a good reason: the OSKM factors are proto-oncogenes. This is especially noticeable for c-Myc, a well-known oncogene deregulated in 70% of all cancers.[74] Several findings argue that c-Myc is not absolutely required for iPS cell generation, although the absence of the TF substantially reduces efficiency and results in longer latency of reprogramming.[75] By crossing adult chimeras, obtained from iPS cell clones derived using the OSKM factors and selected for Nanog expression, germline transmission is achievable; however, approximately 20% of the offspring developed ganglioneuroblastoma with follicular carcinomas caused by c-Myc reactivation.[76] Later, the same authors found that the use of c-Myc does not affect the teratoma-forming propensity of secondary neurospheres (SNS) derived from iPS cells, and no c-Myc or other transgene reactivation was observed in SNS or teratomas.[77] The authors reasoned that teratoma formation propensity varies significantly depending on the iPS cell's tissue of origin, showing the greater susceptibility when iPS cells are derived from adult tail-tip fibroblasts, followed by hepatocytes, MEFs, and gastric epithelial cells.[77] Unfortunately, even excluding the use of c-Myc we are far from feeling safe and sound: induced expression of Oct-4 or Klf-4 in the adult mouse epithelial compartment results in dysplasia,[78,79] while Sox-2 expression is increased in serrated polyps and mucinous colon carcinomas.[80] The sole use of integrating viral vector systems may result in insertional mutagenesis evolving in malignant transformation.[81]

The two-sided nature of the OSKM factors, stem cell and oncogenic factors, inevitably highlights the principle that pluripotency and tumorigenicity are bound together, and that iPS cell induction shares similar features to oncogenic progression.[82] Indeed, acquisition of immortality is a crucial and rate-limiting step in the establishment of the pluripotent state in somatic cells, in particular the epigenetic silencing of the INK4a/ARF locus has been shown to be a critical step for the conversion to iPS cells, and is a major contributor to the low efficiency and delayed kinetics of *in vitro* reprogramming.[83]

Improvements in the protocols for generation and characterization of iPS cells will increase safety until researchers are able to find a way to separate "stemness" from tumorigenicity. Genetically homogenous mice carrying different combinations of doxycycline-inducible reprogramming factors at minimal copy number have been generated, representing a highly valuable tool to study the reprogramming mechanism and to perform high-throughput drug screens.[84] Such transgenic systems would also cut down genetic variability caused by random integration of multiple proviral copies during iPS cell generation using integrating-vectors.[84] Xeno-free iPS cells from human dermal fibroblasts have been generated very recently, eliminating batch-to-batch variability of human serum,[85] and together with the finding that iPS cells can be produced on isogenic parental fibroblasts as

feeders,[86] is contributing to the stepwise establishment of clinical-grade cells. Using live immunofluorescence staining and flow cytometry time course analysis of fibroblasts undergoing reprogramming, Chan and colleagues identified fully reprogrammed cells over reprogramming intermediates based on TRA-1-60, DNMT38, Rex1 expression, and proviral silencing, thus establishing more rigorous standardization and characterization of iPS cells.[87]

Conclusions

At the beginning of 2009, almost simultaneously to President Obama's inaugural speech, the U.S. Food and Drug Administration (FDA) cleared the first Phase I clinical trial involving the use of hES cells to target acute spinal cord injury (www.geron.com). Shortly afterward, the Maryland biotech NeuralStem obtained the approval by the FDA for a clinical trial to test hES cells in patients with Lou Gehrig's disease (www.neuralstem.com), whereas Advanced Cell Technology will soon initiate a Phase I/II multicenter trial using hES cells to treat patients with Stargardt's Macular Dystrophy (SMD) (www.advancedcell.com). It is conceivable that in the next few years (perhaps decades) scientists will achieve conclusive progress in understanding the complexity of somatic reprogramming, so that iPS cells will substitute ES cells in most clinical settings. At present this is still considerably faroff, yet staggeringly promising.

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