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A Fresh Look at iPS Cells

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The potential of induced pluripotent stem (iPS) cells is enormous, but many obstacles remain before their medical and pharmaceutical applications can be fully realized.

In 2006, we showed that mouse embryonic and adult fibroblasts acquire properties similar to those of embryonic stem (ES) cells after retrovirally introducing genes encoding four transcription factors, namely Oct3/4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). We called these cells induced pluripotent stem (iPS) cells. The first generation iPS cells were similar to ES cells in morphology, proliferation, the expression of some ES cell marker genes, and the formation of teratomas. However, these iPS cells had a different global gene expression pattern from ES cells and failed to produce adult chimeric mice. In 2007, germline transmission was achieved with mouse iPS cells (Meissner et al., 2007; Okita et al., 2007; Wernig et al., 2007), and iPS cells were generated from human fibroblasts (Park et al., 2008b; Takahashi et al., 2007; Yu et al., 2007).

Then four groups generated iPS cells from patients with various neurodegenerative diseases—amyotrophic lateral sclerosis (ALS) (Dimos et al., 2008), spinal muscular atrophy (SMA) (Ebert et al., 2009), and Parkinson's disease (Soldner et al., 2009)—and a variety of genetic diseases with either Mendelian or complex inheritance (Park et al., 2008a). Importantly, the pathology of SMA has been recapitulated in motor neurons derived from patient-specific iPS cells (Ebert et al., 2009). In addition, iPS cells have been generated from both monkey (Liu et al., 2008) and rat (Liao et al., 2009). Here, I summarize the potential applications of iPS cell technology for drug discovery and medicine and the challenges to be surmounted to make this a reality (Figure 1). I believe that one of the most important challenges is to develop simple yet sensitive and reliable methods to evaluate the effectiveness and safety of the myriad iPS cell clones and subclones generated by many different technologies.

Long-Term Applications and Challenges

Various types of somatic cells derived from pluripotent stem cells could be used in regenerative medicine to repair tissues damaged through disease or injury. The therapeutic effects of human ES cell-derived progeny have been reported in animal models of spinal cord injury (Keirstead et al., 2005), retinal disease (Lamba et al., 2009), and Parkinson's disease (Yang et al., 2008). In January 2009, the US Food and Drug Administration (FDA) approved the first clinical trial for using human ES cells to treat patients with spinal cord injury.

The iPS cell technology potentially could overcome two important obstacles associated with human ES cells: immune rejection after transplantation and ethical concerns regarding the use of human embryos. However, the clinical application of iPS cells also faces many obstacles, some shared with ES cells and others that are unique. The first common obstacle is teratoma formation (Li et al., 2008). Even a small number of undifferentiated cells can result in the formation of teratomas (germ cell tumors comprising several cell types), so a key goal is to induce differentiation of human ES cells or iPS cells into the required cell type while leaving few undifferentiated cells behind. Should terminally differentiated cells or tissue stem/progenitor cells derived from iPS cells be used and how should they be transplanted?

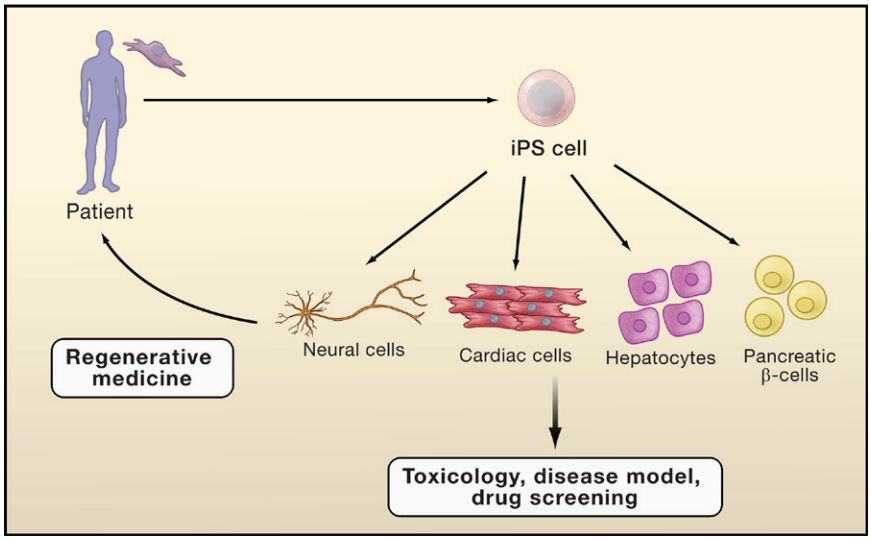

Figure 1. Applications of iPS Cell Technology
Patient-derived iPS cells can produce various somatic cells with the same genetic information as the patient. These cells could be used to construct disease models and to screen effective and safe drugs, as well as to treat patients through cell transplantation therapy. Banking of iPS cells of various HLA types would be useful for regenerative medicine.

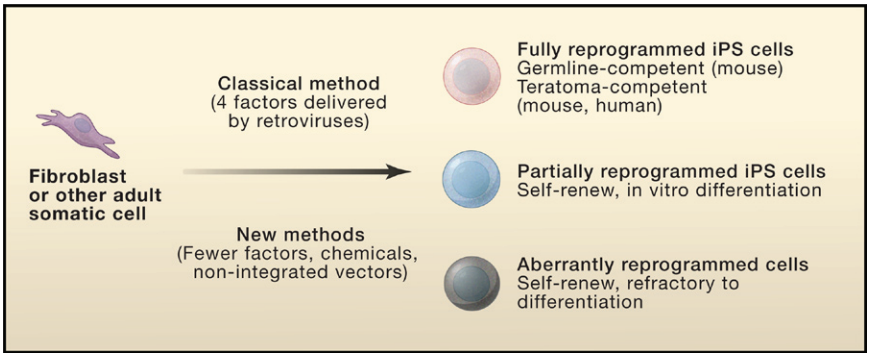

Figure 2. Old and New Ways to Generate iPS Cells
Regardless of methodology, direct reprogramming could result in fully reprogrammed iPS cells that are comparable to ES cells, partially reprogrammed iPS cells that can self-renew and differentiate into certain cell lineages, or aberrantly reprogrammed cells that self-renew but are refractory to differentiation.

There are also unique hurdles to overcome before iPS cells can be used in the clinic, primarily related to the forced reprogramming of somatic cells. We still do not know for each iPS cell clone whether nuclear reprogramming is complete (Figure 2). Aberrant reprogramming may result in an impaired ability to differentiate and may increase the risk of immature teratoma formation after directed differentiation. Notably, abnormal expression of a single gene (such as *Nat1*, *Grb2*, *Apc*, or *Nanog*) renders ES cells refractory to differentiation (Yamanaka et al., 2000). Thus, incomplete reprogramming of somatic cells to iPS cells could result in impaired differentiation of iPS cells into the required cell type.

Another key issue is the presence of transgenes in iPS cells. Most iPS cells are generated by transduction of somatic cells with retroviruses or lentiviruses carrying transgenes, which are integrated into the host cell genome. Transgenes are largely silenced in iPS cells, but the reactivation of such transgenes (especially the transgene encoding c-Myc) could lead to tumorigenesis (Okita et al., 2007). Leaky expression of these transgenes may also inhibit complete iPS cell differentiation and maturation, leading to a greater risk of immature teratoma formation.

Short-Term Applications and Challenges

A short-term goal is to use iPS cell technology for drug or toxicology screens in vitro and for creating disease models in culture (Figure 1). Liver cells (hepatocytes) generated from iPS cells derived from individuals with different cytochrome p450 enzymes would be of value for predicting the liver toxicity of new drugs. The disorder long QT syndrome (LQTS) is caused by mutations in genes involved in generating cardiac action potentials resulting in lethal arrhythmias. LQTS can also be induced by certain drugs in sensitive individuals. By generating beating cardiac myocytes from iPS cells derived from these sensitive individuals, candidate drugs could be tested in vitro.

Generating in vitro disease models using iPS cell technology will be useful not only for drug screening but also for elucidating mechanisms of disease pathogenesis. A small fraction of patients with the familial form of the neurodegenerative disease ALS carry mutations in the *SOD* gene, and transgenic mice carrying the mutant human *SOD* gene can be used to study ALS pathogenesis. Recently, Dimos et al. (2008) generated iPS cells from a patient suffering from familial ALS and derived motor neurons from the iPS cells, providing an unprecedented in vitro resource for elucidating why motor neurons die in ALS patients. Park et al. (2008a) have generated iPS cells from patients with ten different diseases including Parkinson's disease and juvenile diabetes, and Ebert et al. (2009) have done the same for SMA patients. An important challenge is how to recapitulate disease in cells derived from patient-specific iPS cells. In genetically inherited diseases with high genetic penetrance and early onset, specific pathologies may be easier to model. Indeed, motor neurons generated from iPS cells derived from an SMA patient exhibit selective deficits compared to those generated from iPS cells derived from the patient's healthy mother. But in many neurodegenerative diseases such as ALS, it takes years for symptoms to develop. We need to find ways to facilitate disease pathogenesis in patient-specific iPS cells and to mimic epigenetic changes caused by aging and the environment. Some kind of stimulation, such as oxidative stress or UV irradiation, may be required.

Another important issue is that many diseases may be cell non-autonomous, that is attributable to more than one cell type. For example, motor neurons alone derived from ALS patient-specific iPS cells may not be able to reconstitute full disease pathogenesis as they may need to interact with glial cells (Di Giorgio et al., 2007). Thus, multiple cell types may need to be generated from patient-specific iPS cells. Alternatively, motor neurons derived from ALS patient-specific iPS cells may need to be transplanted into mice to generate an effective disease model.

Predictions for the Future

The potential of iPS cell technology is enormous, but this technology is still in its infancy. To realize the full application of iPS cells, it will be essential to improve the methodologies for iPS cell generation and to precisely evaluate each clone and subclone of iPS cells for their safety and efficacy. Here, I discuss emerging technologies for direct reprogramming of somatic cells to iPS cells (Figure 2).

From 24 to Zero?

How many genes are required to make iPS cells? The first iPS cell lines were generated by cotransduction with viruses expressing 24 different factors (Takahashi and Yamanaka, 2006). Subsequent experiments narrowed the required factors down to four: Oct3/4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006), with Oct3/4 proving to be the most important. The expression of Oct3/4 is highly specific for pluripotent stem cells, whereas the other three factors are expressed in other cells (Sox2 in neural stem and progeni-

tor cells; Klf4 in skin, stomach, intestine, and skeletal muscle; c-Myc is ubiquitously expressed). In addition, Oct3/4 cannot be replaced by other Oct family members (Oct1 or Oct6) to generate iPS cells (Nakagawa et al., 2008). In contrast, Sox2 can be replaced by Sox1, Klf4 by Klf2 or Klf5, and c-Myc by N-Myc or L-Myc. Oct3/4 is absolutely required for the maintenance of ES cell pluripotency (Niwa et al., 2000). Inactivation of Sox2 leads to ES cell differentiation, but the forced expression of Oct3/4 rescues this phenotype (Masui et al., 2007). Mice lacking either Klf4 or c-Myc survive to birth, indicating that other factors compensate to maintain pluripotency. These findings argue that Sox2, Klf4, and c-Myc are not fundamentally required for iPS cell generation.

Kim et al. (2009) have generated iPS cells from adult mouse neural stem cells using Oct3/4 alone. They expressed Oct3/4 in the neural stem cells and obtained three iPS cell clones, with two clones yielding adult chimeric mice, albeit with a low contribution from iPS cells judging by the coat color. Further studies are required to determine whether iPS cells can be generated from other mouse cells and human cells using Oct3/4 alone.

Virus? Plasmid? Small Molecule?

Which reprogramming method is the most appropriate for future clinical application? Many groups have generated mouse or human iPS cells using either retroviruses or lentiviruses. Established iPS cells contain multiple viral integration sites in their genomes. During iPS cell generation, integrated proviruses are silenced and in turn the endogenous genes encoding the four factors are activated. The use of retroviruses or lentiviruses raises safety issues for iPS cells generated in this way. Viral integration often takes place within endogenous genes and can result in gene activation. When patients with X-linked severe combined immunodeficiency were treated with gene therapy using retroviruses, activation of the proto-oncogene *LMO2* resulted in leukemia (Hacein-Bey-Abina et al., 2003). However, in iPS cell clones, viral integration sites can be determined by inverse PCR enabling exclusion of clones showing dangerous retroviral integration. Each iPS cell clone may have up to 40 retroviral integration sites, but these sites could be identified efficiently by whole-genome sequencing high-throughput methods.

Another possible obstacle to using retroviruses and lentiviruses is transgene reactivation. Indeed, reactivation of *c-Myc* carried by a retrovirus resulted in tumor formation in ~50% of chimeric mice generated from iPS cells (Okita et al., 2007). Although iPS cells can be generated without *c-Myc* (Nakagawa et al., 2008; Wernig et al., 2008), reactivation of the other three reprogramming factors also may cause tumors. Furthermore, the sustained expression of transgenes might suppress the differentiation of iPS cells, resulting in a higher propensity to produce teratomas when transplanted into patients.

Two groups have shown that it may be feasible to induce iPS cells without viral integration. Stadtfeld et al. (2008b) generated iPS cells from mouse hepatocytes using adenoviruses carrying the four reprogramming factors. In an independent study, our group generated iPS cells from mouse embryonic fibroblasts using plasmids (Okita et al., 2008). We used 2A self-cleavage sequences to express Oct3/4, Sox2, and Klf4 in a single expression vector. The repeated transfection of mouse embryonic fibroblasts with this plasmid and another carrying *c-Myc* cDNA enabled generation of iPS cells. Many of these plasmid-generated iPS cells did not show integration into the host genome by either PCR or Southern blotting. More recently, iPS cells have been generated by genomic integration of the four reprogramming factors using plasmids (Kaji et al., 2009), lentiviruses (Soldner et al., 2009), or transposons (Woltjen et al., 2009), followed by transgene removal using Cre-mediated excision or re-expression of transposase. The efficiency of iPS cell generation using adenoviruses or plasmids is extremely low.

Another way to avoid viral integration is to generate iPS cells using chemicals or small molecules. Several groups have already identified chemicals that can replace one or two reprogramming factors during iPS cell generation (Huangfu et al., 2008; Shi et al., 2008). Considering the essential roles of Oct3/4, chemicals that robustly activate the endogenous *Oct3/4* gene may be able to generate iPS cells. Even if iPS cells do not exhibit transgene integration, they may have other genetic alterations, such as integration of small plasmid fragments or chemically induced mutations. It may be necessary to sequence the whole genome of iPS cell clones using the next generation sequencing technologies in order to detect these genetic alterations.

Fibroblasts? Hepatocytes? Blood Cells?

Which somatic cells are the best sources for iPS cells destined for clinical and pharmaceutical applications? In addition to fibroblasts, mouse iPS cells have been generated from bone marrow cells (Takahashi and Yamanaka, 2006), hepatocytes and gastric epithelial cells (Aoi et al., 2008), pancreatic cells (Stadtfeld et al., 2008a), neural stem cells (Kim et al., 2008; Silva et al., 2008), and B lymphocytes (Hanna et al., 2008). Human iPS cells have been generated from skin fibroblasts, keratinocytes (Aasen et al., 2008), and blood progenitor cells (Loh et al., 2009).

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 iPS cells from the follicle cells of a single human hair also has been reported (Aasen et al., 2008). Skin fibroblasts and keratinocytes can be obtained using a small skin biopsy, gastric epithelial cells by endoscopic biopsy, and bone marrow cells and hepatocytes 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 iPS cells could be generated from cord blood cells.

The second issue is that iPS cells 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 iPS cells derived from somatic cells of endodermal origin such as gastric epithelial cells. Notably, iPS cells derived from mouse hepatocytes (Aoi et al., 2008) or human keratinocytes (Aasen et al., 2008) have fewer retroviral integra-

tion sites than do iPS cells derived from fibroblasts. These cells may be a better source for iPS cell generation; iPS cells also have been generated from mouse hepatocytes using adenoviral vectors (Stadtfeld et al., 2008b).

Induced Somatic Stem/Progenitor Cells?

The ability to form teratomas is a characteristic of pluripotent stem cells, including ES cells and iPS cells. Somatic stem cells, such as hematopoietic stem cells and mesenchymal stem cells, do not form teratomas. Therefore, if we could generate somatic stem or progenitor cells directly from fibroblasts or other types of somatic cells, it might alleviate the necessity of obtaining iPS cells and hence would remove the risk of teratoma formation. Given that only a few factors are required to make iPS cells, perhaps a few transcription factors and other proteins are all that are needed to generate somatic stem or progenitor cells. Alternatively, direct transdifferentiation of one adult somatic cell into another may be the ultimate goal. Zhou et al. (2008) identified three transcription factors (Ngn3, Pdx1, and Mafa) that reprogram differentiated adult mouse pancreatic exocrine cells into cells that resemble pancreatic-β cells in morphology, size, gene expression, and insulin secretion. These new technologies may replace current technologies for generating iPS cells and ES cells for use in regenerative medicine.

Evaluation

It is extremely important that the same criteria be shared among different laboratories to evaluate technologies for iPS cell generation. The standard criterion to evaluate mouse ES cells is their ability to generate germline-competent adult mouse chimeras. Teratoma formation is considered the minimum requirement for evaluating human ES cell lines. Whether the same criteria should be used to evaluate iPS cells is controversial (Daley et al., 2009; Ellis et al., 2009). I would like to propose that two groups of technologies should be distinguished and evaluated using distinct criteria. With one group, the purpose is to recapitulate complete reprogramming of nuclear information, which is achieved by nuclear transfer or fusion with ES cells. In this case, resulting mouse stem cells should be competent for chimera formation and germline transmission. Human stem cells should form teratomas, like human ES cells. However, we note that teratoma formation does not guarantee full reprogramming as many mouse ES cell-like cell lines form teratomas but fail to produce germline chimeras. Currently, it is difficult to prove full reprogramming in human cells.

With the second group of technologies, the purpose is to produce useful stem or progenitor cells for drug discovery, toxicology, and regenerative medicine and to create disease models. In this case, the resulting cells do not have to be germline competent or even teratoma competent, as long as they can self-renew and produce useful progeny. Indeed, cells without the ability to form teratomas might be more useful and safer for regenerative medicine.

In both cases, new technologies should be evaluated by chimera formation and germline transmission (mouse) and teratoma formation (mouse and human) in addition to other standard criteria such as morphology, marker expression, gene expression, and in vitro differentiation. Scientists then have to clarify whether their new technology induces full reprogramming or partial reprogramming that yields useful stem or progenitor cells. Initial publications reporting full reprogramming should be followed up by long-term observations of chimeric mice and their progeny to evaluate the safety of the technology. For partial reprogramming, examining germline transmission and teratoma formation may not be an absolute requirement for initial publication but should be examined and reported to the community later as a sound scientific practice.

A key advantage of iPS cell technology is its simplicity: iPS cells can be generated in any laboratory using standard techniques and equipment. Each experiment generates many iPS cell clones (an advantage over many other stem cell technologies), although the best iPS cell clones need to be selected from numerous candidates. In mice, reporter systems using ES cell-specific genes, such as *Oct3/4* and *Nanog*, are useful for detecting germline-competent clones (Meissner et al., 2007; Okita et al., 2007; Wernig et al., 2007); similar reporter systems may be needed for human cells. However, we do see a substantial difference even among clones selected using reporter systems. To realize the full application of iPS cells we have to develop technologies that enable selection of the best clones.

When evaluating cells, we should realize that iPS cells are not uniform even within each clone. After retroviral integration, it takes more than 10 days before full reprogramming is achieved. A single transduced progenitor cell undergoes multiple cell divisions during this initial period, and progeny cells may be different in their reprogramming status despite the same origin. Even if only a few cells are aberrantly reprogrammed and refractory to differentiation, those cells could result in immature teratoma formation after transplantation to patients. It will be essential to develop methods to detect and remove such aberrantly reprogrammed cell populations within good clones.

Conclusions

Over the next few years, I believe we will see many advances in the realization of in vitro applications of iPS cell technology. But we cannot be too careful when it comes to applying iPS cell technology to regenerative medicine. Any iPS cells generated by any method from any cell source will have to go through vigorous examination to confirm their safety prior to clinical application. The general view is that the fewer reprogramming factors used, the safer will be the resulting iPS cells. But is it that simple? It may be difficult to achieve complete reprogramming with a smaller number of factors. Indeed, aberrant reprogramming may render iPS cells refractory to differentiation and thereby increase the risk of immature teratoma formation after directed differentiation and transplantation into patients. Even if only a small portion of cells within each iPS cell clone shows impaired differentiation, then those cells might be sufficient to produce immature teratomas. We must establish ways to precisely evaluate each iPS cell clone and to select appropriate subclones prior to clinical application.

Despite these challenges, the potential of these new pluripotent stem cells remains enormous. The biggest challenge, direct reprogramming by defined factors, has been resolved. The remaining challenges are basically technical issues, which I believe will be resolved in the near future. I sincerely hope that iPS cell technology will lead to a better understanding of nuclear reprogramming and that it will provide great benefits to many patients.

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