Minireview

Induced human pluripotent stem cells: promises and open questions

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Abstract

Adult cells have been reprogrammed into induced pluripotent stem (iPS) cells by introducing pluripotency-associated transcription factors. Here, we discuss recent advances and challenges of in vitro reprogramming and future prospects of iPS cells for their use in diagnosis and cell therapy. The generation of patient-specific iPS cells for clinical application requires alternative strategies, because genome-integrating viral vectors may cause insertional mutagenesis. Moreover, when suitable iPS cell lines will be available, efficient and selective differentiation protocols are needed to generate transplantable grafts. Finally, we point to the requirement of a regulatory framework necessary for the commercial use of iPS cells.

Keywords: cell therapy; differentiation; induced pluripotency; in vitro reprogramming; tumour formation.

Introduction

Since 1989 hundreds of pluripotent human embryonic stem (ES) cell lines have been established from embryos after in vitro fertilisation (IVF). However, the use of human IVF-embryos to generate ES cell lines is controversially discussed and regarded as ethically problematic. Therefore, both the scientific community as well as the public were looking for alternative strategies for the generation of pluripotent cells without using human oocytes or embryos.

A breakthrough came in 2006, when Shinya Yamanaka and his co-worker demonstrated for the first time in vitro reprogramming of murine fibroblasts into induced pluripotent stem (iPS) cells by retroviral integration of pluripotency-associated genes (Takahashi and Yamanaka, 2006). In the meantime, scientists worldwide confirmed the induction of pluripotency in somatic mouse and human cells by retroviral transfer of specific transcription factors. These iPS cells were proven to be functionally and molecularly quite similar to ES cells (for review, see Amabile and Meissner, 2009; Hagedoorn and Plath, 2009).

It is generally accepted that in vitro reprogramming of somatic cells into induced pluripotent stem cells offers new applications in basic research, diagnosis and cell therapy. The most attractive application would be the production of patient-specific donor cells for cell replacement and/or tissue substitution. For example, skin fibroblast-derived iPS cells showed the potential to differentiate into islet-like clusters and to release insulin in response to glucose thus potentially providing a strategy for the treatment of diabetes (Tateishi et al., 2008). Another exciting strategy may be the combination of iPS cell generation combined with somatic gene therapy similarly as shown for mouse cells by Jaenisch and co-workers (Hanna et al., 2007).

However, other applications of iPS cells are also feasible, such as the use of iPS cells to study pathomechanisms of diseases, including Parkinson’s disease and Huntington’s disease, juvenile-onset type 1 diabetes mellitus or amyotrophic lateral sclerosis (Dimos et al., 2008; Park et al., 2008). Recently, skin fibroblasts from a patient with spinal muscular atrophy were reprogrammed into proliferative iPS cells that maintained the disease genotype and were able to differentiate into motor neurons. The iPS cells showed selective deficits typical for the disease as well as drug responsiveness (Ebert et al., 2009). This example demonstrates that iPS cells may be a promising tool to study the mechanisms of diseases and the effects of drugs thus enabling the development of new therapies.

Moreover, similarly to human ES cells, iPS cells could be applied in toxicology and pharmacology. For instance, iPS cells differentiating in vitro may be used as an alternative system for screening of embryotoxic and/or teratogenic substances (Caspi et al., 2008).

Generally, the fast-growing field of in vitro reprogramming enhances the hope of cures for injured people as well as the commercial interest of companies. However, before iPS cells can be used in diagnostic and therapeutic applications, several criteria have to be fulfilled.

Requirement #1: removal of genome-integrating viruses

One of the most critical steps in generating iPS cells is the application of viral vectors for reprogramming differentiated cells into pluripotent cells. The first human iPS cell lines were generated by transduction of adult fibroblasts with retroviral vectors expressing the reprogramming transcription factors Oct4, Klf4, Sox2, c-Myc (the
‘four pluripotency factors’; Takahashi et al., 2007) and Oct4, Sox2, Nanog and Lin28 (Yu et al., 2007), respectively. Although adult cells have been reprogrammed by non-integrating adenoviral vectors expressing the ‘four factors’ (Stadhelfeld et al., 2008), the genetic transfer of viral vehicles into somatic cells is critical. Specifically, because of uncontrolled insertions into the genome, lentiviral and retroviral vectors may potentially activate endogeneous oncogenes when iPS cells generated by such methods would be transplanted (Nienhuis et al., 2006).

Which techniques can be regarded as alternatives? At first, the use of non-viral vectors may be an alternative. Repeated transfections of expression plasmids containing the cDNA of the four pluripotency-associated genes into mouse embryonic fibroblasts resulted in virus vector-free iPS cells without evidence of plasmid integration (Okita et al., 2008). An optimal solution would be to induce reprogramming directly by chemical factors (‘small molecules’), which specifically modulate the epigenetic status of the cells. For example, a specific inhibitor of histone methyltransferases (BIX 01294) in conjunction with the transduction of only two transcription factors (Oct4 and Klf4) enabled mouse foetal neural progenitor reprogramming into iPS cells (Shi et al., 2008). This demonstrates that there are specific cell types which may be more easily reprogrammed into iPS cells and would allow reprogramming by using only one or two transcription factors. Recent data showed that adult mouse neural stem cells (which endogenously express Sox2) could be reprogrammed by viral transfer of only one transcription factor, Oct-4 (Kim et al., 2009). Another molecule, the histone deacetylase inhibitor valproic acid, enabled pluripotency reprogramming of human fibroblasts with two pluripotency-associated transcription factors (Huangfu et al., 2008).

A novel approach was recently reported to reduce the number of viruses by delivering reprogramming factors in a single virus using ‘self-cleaving’ peptides, which facilitated efficient polycistronic expression from a single promoter (Carey et al., 2009). In this case, the four reprogramming factors, Oct4, Sox2, Klf4 and c-Myc, could be expressed from a single virus to generate iPS cells. The study showed that insertion of the transduced pluripotency genes is not required for in vitro reprogramming. In addition, a virus-free approach was applied with the direct delivery of transcription factor proteins to somatic cells by the use of protein transduction domains or immunoliposomes (Heng and Richards, 2008).

Moreover, genetic strategies that allow removal of the viral vectors after successful transfer of pluripotency-associated genes from the iPS cells were reported. Two groups demonstrated successful reprogramming of mouse fibroblasts by using doxycycline-inducible transcription factors delivered by ‘piggyBac’ transposition (Woltjen et al., 2009) and showed that the individual vector insertions needed for introducing the reprogramming factors could be removed from the iPS cells (Kaji et al., 2009).

A further step forward to successful reprogramming of adult human cells was the derivation of reprogramming ‘factor-free’ iPS cells by the use of Cre-recombinase excisable viruses (Soldner et al., 2009) and the use of episomal vectors (Yu et al., 2009). The data presented evidence that iPS cells after removal of transducing viral vectors or episomes were free of transgene sequences. Although these elegant techniques avoid the permanent integration of viral vectors, cells may still be genetically or epigenetically altered by the previous vector integration.

Therefore, the recent report on protein-induced reprogramming of mouse embryonic fibroblasts without genetic modification indicated a further breakthrough (Zhou et al., 2009). The authors demonstrated the generation of protein-induced pluripotent stem (piPS) cells by repeated transductions of four recombinant cell-penetrating reprogramming proteins (Oct4-11R, Sox2-11R, Klf4-11R and c-Myc-11R) in combination with valproic acid treatment (Figure 1). However, further methodical improvements will be necessary to increase the reprogramming efficiency, a requirement for routine application.

Moreover, another intrinsic problem of the reprogramming technology has to be solved: detailed molecular analysis of data showed that quite often reprogrammed cells keep the genetic/epigenetic ‘memory’ of adult cells even after reprogramming into the pluripotent state.

In summary, by applying protein-induced reprogramming methods to human cells, requirement #1 could be fulfilled in the near future. But many open questions related to regulatory mechanisms of human cell reprogramming, standardisation and quality control of iPS cells have to be solved before therapeutic applications will be feasible.

**Requirement #2: eliminating the risks of tumour formation**

Not only integrating viruses are critical with regard to the potential induction of tumours by reprogrammed iPS-derived cells in therapeutic applications. Undifferentiated iPS-derived cells themselves would be tumorigenic in vivo (as comparable to ES cells), when donor cell grafts would be contaminated by pluripotent undifferentiated cells.

Importantly, in both human ES and iPS cells, the genetic and epigenetic stability during expansion and long-term cultivation is highly significant for further developments and future application. It will be necessary to screen iPS-derivatives during in vitro cultivation not only for potential major chromosomal aberrations (karyotype analysis) but also for non-visible minor genetic or epigenetic modifications that could be a risk for tumour formation.

Moreover, several positive and negative selection techniques have been proposed to eliminate from the graft any contamination by undifferentiated cells. As suggested for ES cell derivatives, iPS-derived cells processed for transplantation should be screened for any undifferentiated cells that remained in the graft. To eliminate undifferentiated iPS cells, FACS depletion of contaminating Oct4-positive cells (see Cantz et al., 2008) or the use of cytotoxic antibodies that identify and destroy undifferentiated cells expressing specific embryonic cell surface antigens may be applied (for review, see Hentze et al., 2007). In principle, it is believed that these problems may
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After isolation of human donor material (e.g., dermal fibroblasts), cells have to be expanded in vitro. The introduction of reprogramming factors by transfection of the cells with various combinations of pluripotency-associated genes is achieved by viral vectors. Selection and propagation of reprogrammed cells are necessary to generate induced pluripotent stem (iPS) cells. Successful reprogramming of adult human cells was first published 2007 by Takahashi et al. and Yu et al. by using different combinations of pluripotency-associated genes. Dashed lines (-----) indicate methods that have been established for generating mouse and only partially for human iPS cells. These methods include strategies to reduce the number of reprogramming viral vectors and/or to enhance the reprogramming efficiency by small molecules (i.e., valproic acid, BIX 01294 or BayK8644), and in addition principal techniques to remove viral vectors after successful reprogramming. Dotted lines (.....) outline the potential strategy for the generation of protein-induced pluripotent stem (piPS) cells, shown at present for mouse cells by Zhou et al. (2009). Both, iPS and piPS cell clones have to be carefully characterised before any application in diagnosis or therapy would be feasible. An overview of donor cell types used for reprogramming and the combination of reprogramming factors and reprogramming efficiencies is given by Amabile and Meissner (2009), and chemicals used for epigenetic modification are listed in the paper by Feng et al. (2009).

Requirement #3: establishment of efficient differentiation protocols

Considering therapeutic applications of human iPS-derived cells, the graft has to fulfil disease-specific requirements, including correct cell integration, migration and survival within the surrounding tissue of the recipient. Therefore, one of the most important questions at present is whether human iPS cells will generate functional cells of the desired phenotypes at sufficient amounts. Methods have to be developed or adapted from existing human ES cell protocols to evaluate the differentiation potential of reprogrammed cells and to scale up the amount of iPS-derived donor cells. Such methods established for human ES-derived cells are lineage-specific directed differentiation and selection techniques to generate pure populations of specialised cells. For example, human ES cells have been used to generate functional neurons essential to cure senso-motoric deficits in an animal model of Parkinson’s disease (Cho et al., 2008) or to develop insulin-producing cells that reverted glycaemia in diabetic animals (Kroon et al., 2008). At first, these techniques have to be adapted to reprogrammed iPS cells. However, sometimes experimental data showed reduced efficiency of iPS cell differentiation, which may be due to yet unknown mechanisms related to the genetic/epigenetic ‘memory’ of reprogrammed cells (see above).

Another essential problem that has to be considered is the problem of cell ageing in somatic tissues of adults. Tissue cells with increasing age are known to enrich recessive mutations due to mutagenic effects of stress factors and reactive oxygen species. Such mutations would be present also in reprogrammed iPS cells. Therefore, it has to be clarified whether it will be possible to generate high quality patient-specific iPS cells also from old-age donors (therefore, reprogramming of individual frozen cord blood-derived cells could be an alternative to overcome this problem).

In summary, to utilise the full therapeutic benefit of iPS cell technology, it will be necessary to develop standar-
disd differentiation and selection methods and to establish improved quality standards which are also required to determine the exact cellular state of iPS cells (Daley et al., 2009).

Outlook and future developments

Here, we have addressed some critical aspects concerning the generation and potential use of human iPS cells for future diagnosis and cell therapy. Based on these issues, it is evident that our knowledge of the processes and mechanisms of reprogramming to pluripotency is still insufficient and far-off from understanding.

However, with regard to the generation of cells for therapies, we should not only consider reprogramming to pluripotency but also lineage reprogramming or reprogramming by de- or transdifferentiation (Gurdon and Melton, 2008). In the latter cases, one would avoid complete reprogramming to a status of pluripotent (potentially tumourigenic!) cells, but reprogram defined adult cells into another cell type of related somatic lineages.

Although, some years ago, the concept of phenotypic ‘transdifferentiation’ of adult cells had to be revised (see Wagers and Weissman, 2004), transcription factor-induced ‘re-differentiation’ or ‘transdifferentiation’ mechanisms have currently been described. For instance, the enforced expression of C/EBPα and β in differentiated B cells led to their rapid and efficient reprogramming into macrophages, i.e., the switch from one lineage to another (Xie et al., 2004). Transdifferentiation was demonstrated by adenovirus transfection of transcription factors involved in pancreatic differentiation (Pdx1, Ngn3 and MafA) resulting in the direct conversion of exocrine cells of the pancreas into endocrine β cells (Zhou et al., 2008).

Transdifferentiation of murine mesoderm into beating cardiomyocytes was also achieved by transfection of two cardiac transcription factors, Gata4 and Tbx5, and a cardiac-specific subunit of BAF chromatin-remodelling complexes, Baf60c (Takeuchi and Bruneau, 2009). These cardiac-specific subunit of BAF chromatin-remodelling cardiac transcription factors, Gata4 and Tbx5, and a cardiomyocytes was also achieved by transfection of two cardiac transcription factors, Gata4 and Tbx5, and a cardiac-specific subunit of BAF chromatin-remodelling complexes, Baf60c (Takeuchi and Bruneau, 2009). These data thus supported earlier findings of MyoD-induced conversion of fibroblasts into muscle cells as a consequence of MyoD autoactivation (Davis et al., 1987). It is suggested that such transcription factor-induced transdifferentiation processes may function only between cells of related lineages, such as mesenchymal cells and muscle cells representing cells of the mesenchymal/mesodermal lineage, or between pancreatic cells of the exocrine and endocrine compartment. However, this direct lineage switching by transcription factor-induced transdifferentiation provides another strategy of changing cell fates and might eventually avoid the critical pluripotent (and tumourigenic) state for generating cells for therapy.

Another aspect of the future diagnostic and therapeutic use of iPS cell technology is related to the existing regulatory framework. In the European Union, the Directive 2004/23/EC of the European Parliament and of The Council (called ‘Tissue and Cells Directive’, from March 31, 2004) and Commissions Directives (i.e., 2006/17/EC and 2006/86/EC) regulate standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. The International Society for Stem Cell Research (ISSCR) provides a survey on regulations regarding the therapeutic use of stem cells worldwide (www.ISSCR.org) and recently approved Guidelines for the Clinical Translation of Stem Cells (December 3, 2008). In most countries, the derivation and use of human ES cells is controlled by specific guidelines or by law. For instance, in Germany, the embryo protection act (ESchG, 2000) and the stem cell act (StZG, 2002 and 2008) regulate the import and use of pluripotent human ES cells in basic research.

With regard to their generation, pluripotent iPS cells do not require specific legal consideration. However, in the future there may be implications connected with the use of iPS-derived cells that would require regulatory framework (Condic and Rao, 2008). This may be the case for the commercial use of patient-specific iPS cells in drug and toxicity testing, or the allogeneic use of iPS-derived cells as a ‘cell therapy product’. Moreover, specific regulations would be necessary with regard to the potential application of personalised iPS-derived germ cells (see Park et al., 2009) – although not yet available – in infertility treatment (see Consensus Statement of the Hinxton group: Consensus Statement, 2008).

In conclusion, the strategies of reprogramming adult tissue-specific cells into pluripotent stem cells or the direct transcription factor-based transdifferentiation of somatic cells offer exciting opportunities for basic research and medical application. However, before any of these strategies may become reality in the clinic, many open questions remain to be solved.

References


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