Roadblocks en route to the clinical application of induced pluripotent stem cells

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Summary

Since the first studies of human embryonic stem cells (hESCs) and, more recently, human induced pluripotent stem cells (hiPSCs), the stem-cell field has been abuzz with the promise that these pluripotent populations will one day be a powerful therapeutic tool. Although it has been proposed that hiPSCs will supersede hESCs with respect to their research and/or clinical potential because of the ease of their derivation and the ability to create immunologically matched iPSCs for each individual patient, recent evidence suggests that iPSCs in fact have several underappreciated characteristics that might mean they are less suitable for clinical application. Continuing research is revealing the similarities, differences and deficiencies of various pluripotent stem-cell populations, and suggests that many years will pass before the clinical utility of hESCs and hiPSCs is realized. There are a plethora of ethical, logistical and technical roadblocks on the route to the clinical application of pluripotent stem cells, particularly of iPSCs. In this Essay, we discuss what we believe are important issues that should be considered when attempting to bring hiPSC-based technology to the clinic.

Key words: FDA, Good manufacturing practice, Induced pluripotent stem cells

Introduction

There is currently great interest in the field of stem-cell biology, as it is hoped that stem cells or their derivatives can one day be used as cell-based therapeutics for a wide range of applications. Since 1998, when James Thomson first generated human embryonic stem cells (hESCs) (Thomson et al., 1998), it was presumed that these cells would one day be applied in a variety of clinical settings. This presumption was based on the unique pluripotency of these cells, defined as their ability to generate all cell types of the human body. For these cells to be used clinically, however, it seemed to be imperative that patient-specific cell lines were developed to avoid immune rejection of transplanted cells in immunologically diverse patients. Because hESCs are derived from individual human embryos, none of the hESC lines generated would be a perfect immunological match to the many patients in need of therapy. Therefore, an intense search began to find methods for generating patient-specific stem cells. Initially, the best method seemed to be one borrowed from *Xenopus* developmental biology, known as somatic-cell nuclear transfer (SCNT) (Gurdon et al., 1975).

Four years ago, it was thought that the best method to generate patient-specific pluripotent stem cells would be SCNT (Gurdon et al., 2003; Gurdon et al., 1975). This method proved to be successful for many species, including primates (Byrne et al., 2007). However, similar success with human cells has not yet been described and, furthermore, the generation of patient-specific stem cells by SCNT requires human ova and the generation of human blastocysts, which presents logistical and ethical hurdles. In 2006, a remarkable study changed the landscape of stem-cell biology: Takahashi and Yamanaka showed that retroviral expression of four transcription factors (Oct4, Sox2, Klf4 and c-Myc) in mouse fibroblasts was sufficient to reprogram them to an apparently pluripotent state (Takahashi and Yamanaka, 2006). These transcription factors were first identified individually as factors that were important for maintaining mouse ESCs in an undifferentiated state; continuous expression of these factors was found to be sufficient to permanently induce the expression of the endogenous versions of the same genes, coincident with the onset of pluripotency. These reprogrammed cells were termed induced pluripotent stem cells (iPSCs) and seemed to have the attributes of an embryo-derived cell – namely self-renewal and pluripotency.

Just one year later, a similar strategy was shown to generate human iPSCs from fibroblasts, using two slightly different cocktails of reprogramming factors by Yamanaka and Thomson (Takahashi et al., 2007; Yu et al., 2007). Human iPSCs (hiPSCs) were shown to be highly similar to hESCs in their ability to self-renew and maintain pluripotency, as demonstrated by teratoma formation. The advent of hiPSC technology has opened up a new era in stem-cell biology, because of the perceived simplicity and minimal ethical ambiguity by which patient-specific stem cells can, in theory, be made. Because this approach does not require human egg donation or embryo destruction, it is now thought that hiPSCs are the most promising candidate for the use of pluripotent stem cells in regenerative medicine. Owing to the relative ease with which iPSCs can be derived from all types of species and the promise of this technology for clinical applications, this field has exploded, with important papers being published nearly on a daily basis.

Currently, there is great excitement about the use of hiPSCs to study human disease and tissue development in vitro (Dimos et al., 2008; Ebert et al., 2008; Park et al., 2008a). Because of the ability to generate patient-specific stem cells with relative ease from any individual, the immediate applications of hiPSCs will be in the exploration of disease etiology. For example, although many mutations that are highly correlated with human diseases have been identified [e.g. in amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease), the cell type that is primarily affected and/or the role that these mutations play in pathology are often not

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fully understood in the human context. In addition, the onset and cellular consequences of developmental disorders (such as Down syndrome, spina bifida, Rett syndrome and fragile X syndrome) can now be investigated by studying the generation of tissues in vitro with hiPSCs derived from patients with these disorders. Many reviews summarizing the potential of these types of studies have been published (Abeliovich and Doege, 2009; Chamberlain et al., 2008; Colman and Dreesen, 2009; Freund and Mummery, 2009; Koch et al., 2009; Lengerke and Daley, 2009; Nishikawa et al., 2008). Furthermore, Saha and Jaenisch recently provided a comprehensive review on the technical challenges underlying in vitro disease modeling (Saha and Jaenisch, 2009).

As hiPSCs have only recently been described, there is still much to learn about their biology. More specifically, it is still not clear whether hiPSCs have identical characteristics to hESCs. In fact, our own group and others have shown that both mouse and human iPSCs are significantly different at the transcriptional level from their embryo-derived counterparts (Chin et al., 2009; Marchetto et al., 2009). At the epigenetic level, hESCs and hiPSCs appear to be similar, although there are still significant differences between the two populations (Chin et al., 2009; Deng et al., 2009; Doi et al., 2009; Kanawaty and Henderson, 2009; Lister et al., 2009; Maherali et al., 2007; Mikkelsen et al., 2007; Wernig et al., 2007). It has been argued that mouse ESCs and iPSCs are functionally equivalent based on recent data demonstrating that some mouse iPSCs can perform tetraploid complementation to generate animals that are derived entirely from the reprogrammed cells (Boland et al., 2009; Kang et al., 2009). However, this assay obviously cannot be performed with hESCs and hiPSCs. The gold-standard assay that is available for testing the functional capacity of human pluripotent stem cells is the teratoma assay, which is not nearly as stringent as assessing the capacity of the cells to contribute to chimeras or to generate an entire animal. According to several studies that have used currently available differentiation assays, hESCs and hiPSCs have thus far been shown to be functionally equivalent. Therefore, whether the described molecular differences between hESCs and hiPSCs affect their relative functional capacity is unknown.

The safety issues surrounding iPSCs with respect to transplantation are numerous, beginning with the reprogramming method itself, which was achieved initially through retroviral integration of known proto-oncogenes (Takahashi and Yamanaka, 2006). In addition, the Yamanaka group recently showed that even the specified progeny of mouse ESCs and iPSCs have the ability to generate tumors (Miura et al., 2009). It will be essential to address these issues, as controlling both self-renewal and pluripotency will be vital in any clinical application in which these cells are used.

For iPSCs to be useful in a clinical setting, it is thought that the recipient and donor will need to be closely matched with respect to their human lymphocyte antigen (HLA) haplotype (allogenic), so that immune-mediated rejection of the transplanted cells is avoided or can be ameliorated with immunosuppressive therapy. Ideally, personalized iPSCs could be made from any person to allow the transplantation of autologous cells; however, current technologies mean that this scenario is too costly to be used in practice. Therefore, unless reprogramming methods are developed to improve the speed, efficiency and cost by which pluripotent stem cells are derived, the contents of 'hiPSC banks' will be designed to serve the needs of a diverse patient population. These banks will need to contain as many lines as possible, which have been generated using US Food and Drug Administration (FDA) good manufacturing practice (GMP) guidelines. Ideally, the contents of an hiPSC bank will be suitable for treating a diverse population of recipients while avoiding the need for long-term immunosuppressive therapy. There has been disagreement in the literature as to whether the differentiation state of a stem-cell population is related to the strength of the immune response that is induced following transplantation (Drukker et al., 2006; Magliocca et al., 2006; Swijnenburg et al., 2008), although it is generally thought that less differentiated cells induce a milder immune response. Regardless, the issue of immune-mediated rejection is an essential consideration in cell-based therapeutics. This subject has been extensively reviewed by Drukker and colleagues (Drukker, 2004; Drukker and Benvenisty, 2004; Drukker et al., 2002).

As discussed above, hiPSC technology is likely to be enormously useful for studying human disease etiology in vitro in the immediate future (Dimos et al., 2008; Ebert et al., 2008; Park et al., 2008a). If these cell lines are going to be used in a clinical setting, however, several issues must be resolved so that the cells meet the requirements of the FDA. In this Essay, we outline cell biological and logistical considerations that we believe are important for successfully and safely bringing iPSCs to the clinic. As the field learns more about the process of reprogramming and the physiology of reprogrammed cells, it is likely that additional hurdles will be discovered.

Consenting adults

To derive hESCs, it is necessary to use human embryos. There are many ethical issues surrounding the use of embryos for this purpose and, on a more practical level, there is the issue of consent. Because donors were previously given standard consent forms that did not contain specific language explaining that their embryos could be used to generate hESCs, it has been argued that many hESC lines might have been generated without appropriate consent (Cohen, 2009; Moller, 2009; Vogel and Holden, 2008). As hiPSC lines are not derived from embryos, it could be assumed that consent is not such a barrier to their clinical application. However, this will depend on the paperwork employed upon tissue donation: for example, standard consent forms given to patients that donate somatic tissue after surgery do not mention the possibility that the donated cells might be used to generate pluripotent stem cells. It could be argued that there would not be any drawbacks to using the cells of an anonymous donor to generate iPSCs. However, recent work has shown that hiPSCs can be used to generate germ-cell progenitors (Park et al., 2009) and even putative sperm (Kee et al., 2009). If this work progresses to the point that functional gametes can be generated from donated tissue, one can imagine that it would be possible to produce human embryos or even full-term human beings from excess tissue following procedures such as liposuction (Sun et al., 2009) or facelifts (Aasen et al., 2008). Therefore, although the issue of whether consent is required for the generation of both hESCs and hiPSCs is currently under close scrutiny, it seems clear that patient consent forms will need to be updated to take into account these types of applications (Aalto-Setala et al., 2009; Lo and Conklin, 2009).

Selection and collection of tissue under FDA GMP guidelines

An important starting point in reprogramming is to decide on the type of somatic cell for the process. This is an important decision when considering GMP-grade banks of cell lines and should take into account the reprogramming efficiency of a given cell type (which is linked to the proliferative rate), the relative exposure to

environmental insults that could cause DNA damage and the number of in vitro cell divisions that are required before reprogramming is achieved (to minimize the occurrence of culture-induced changes). It has been shown that a wide variety of cell types can be reprogrammed, beyond the dermal fibroblasts that are typically used (Aoi et al., 2008; Giorgetti et al., 2009; Haase et al., 2009; Hanna et al., 2008; Hanna et al., 2007; Kim et al., 2009b; Kim et al., 2008; Sun et al., 2009; Taura et al., 2009; Xu et al., 2009). As an example of the balance that must be struck when choosing a target cell, one group showed that skin keratinocytes were more efficiently reprogrammed into iPSCs than their counterparts in the dermis, the fibroblasts (Aasen et al., 2008). Therefore, it was suggested that keratinocytes would be an ideal starting material because of their easy accessibility from the donor as well as their reprogramming efficiency. However, keratinocytes are exposed to higher UV radiation and therefore have a higher mutation rate than dermal fibroblasts because of their relative location in the skin, perhaps making keratinocytes a riskier choice. More recently, it was shown that human adipocytes can be reprogrammed to a pluripotent state, providing an abundant source of starting material (Sun et al., 2009). Finally, human cord blood cells also seem to be amenable to reprogramming (Giorgetti et al., 2009; Haase et al., 2009). This source has two benefits over the others – namely, cord blood cells do not need to be cultured and cord blood banks already exist that could be tapped for the generation of iPSCs.

Selection of a starting material is also important because each type of somatic cell possesses a unique epigenetic profile that could make the cells differentially amenable to clinical application subsequent to reprogramming. Reprogramming somatic cells to a pluripotent state requires a massive rearrangement of epigenetic modifications that decorate histones and DNA and regulate chromatin compaction. The epigenetic state of the starting cell type has been hypothesized to influence both the reprogramming efficiency and the differentiation potential of the resulting iPSCs in as-yet unknown ways. For example, there is now evidence that the starting cell type can determine the tumorigenicity of iPSC derivatives in mice (Miura et al., 2009).

It will be necessary for clinical-grade iPSCs to be isolated and maintained in conditions that are acceptable under GMP guidelines, as outlined by the FDA. GMP is partially defined as the establishment of environmental conditions that are clean, secure and reproducible. In addition, to meet FDA GMP requirements, a detailed system of documentation using standard operating procedures (SOPs) is required, but these standards are not employed in most laboratories currently carrying out iPSC research. There are several characteristics that will serve as the minimum standard by which hiPSCs should be judged as suitable by the FDA. The cells must be isolated and cultured under chemically defined conditions with as few xenobiotic reagents as possible. This restriction on acceptable media components prohibits the use of standard bovine serum, which is commonly used to culture most mammalian cell lines, and requires the use of pharmaceutical-grade reagents such as trypsin and PBS. Viral vectors used in reprogramming would also have to be prepared under GMP conditions. Therefore, modifications to current protocols will be necessary and potentially difficult.

Assuming that methods can be developed to generate GMP-grade hiPSCs, there will still be an additional hurdle before their clinical application. Similar to hESCs, the growth of hiPSCs is optimal on a feeder layer with serum, which exposes them to xenobiotic factors. These methods, which were first outlined in 1998 by the Thomson group, have not dramatically changed until recently (Sidhu et al., 2008; Thomson et al., 1998; Unger et al., 2008). It has recently been shown that a new generation of pre-packaged media that claims to be chemically defined and free of xenobiotic factors can support the maintenance of hESCs and hiPSCs (Sidhu et al., 2008; Unger et al., 2008). Furthermore, two recent studies suggest that hiPSCs can be generated under feeder- and serum-free conditions (Sun et al., 2009; Totonchi et al., 2009). However, whether there are physiological differences between hiPSCs generated under standard conditions versus feeder- and serum-free conditions remains unknown. Furthermore, it should be considered that, if GMP growth conditions limit the proliferative capacity of the cells, cells with genomic abnormalities could be selected for, generating an unstable cell line.

Reprogramming without genomic disruption

The first reports of iPSCs used retroviruses to deliver reprogramming factors stably into somatic cells (Maherali et al., 2007; Meissner et al., 2007; Takahashi and Yamanaka, 2006). Owing to the potential for insertional mutagenesis and the fact that some of the reprogramming factors are known proto-oncogenes, it quickly became clear that safer strategies for gene delivery must be used if iPSCs are going to be adopted for clinical applications (Yamanaka, 2007). Many of the gene-transfer methods investigated are summarized in Table 1. Reprogramming with either transposon-based gene transfer or Cre-Lox technology, which allows integration of the reprogramming factors and their subsequent removal following reprogramming (Kaji et al., 2009; Soldner et al., 2009; Woltjen et al., 2009), has been described. Although these methods are an improvement to those that involve viral integration, it is still unclear whether removal is complete and whether the methods for removal introduce other effects into the genome. Notably, methods that avoid integration completely have recently been reported; two groups have introduced reprogramming factors with an adenovirus-based vector (Stadtfeld et al., 2008; Zhou and Freed, 2009), whereas the Yamanaka group avoided virus completely and used repeated transient transfection (Okita et al., 2008). Both of these methods were shown to successfully generate mouse iPSCs without any detectable genomic integration. The Thomson group employed a similar transient transfection approach, but with episomal vectors (Yu et al., 2009), which are known to be stable in mammalian cells for extended periods without integrating into the genome. By using nucleofection of human fibroblasts with polycistronic vectors, the Thomson group introduced reprogramming factors along with other factors that are known to promote proliferation. hiPSCs were generated without detectable integration ~30 days after nucleofection.

Another means of simplifying the reprogramming process is by minimizing the number of genes that must be delivered. This has been done either by reprogramming cells that already express one or more of the reprogramming factors (Kim et al., 2009a; Kim et al., 2008; Li et al., 2009) or by replacing some of the factors with small molecules that appear to serve as surrogates. Several groups have now shown that adding histone deactylase inhibitors or other small molecules removes the need for some of the reprogramming factors (Huangfu et al., 2008a; Huangfu et al., 2008b; Ichida et al., 2009; Lyssiotis et al., 2009; Schneider-Stock and Ocker, 2007; Shi et al., 2008). It has been speculated that these molecules act by promoting chromatin to take on more dynamic and flexible conformations, allowing the reprogramming factors greater access to their

Table 1. Methods to deliver reprogramming factors to target cells

	Benefits	Drawbacks	References
Retroviral vectors	High efficiency	Requires genomic integration; requires dividing target cells	(Maherali et al., 2007; Takahashi and Yamanaka, 2006)
Lentiviral vectors	High efficiency; target cells need not be dividing*	Requires genomic integration	(Brambrink et al., 2008; Maherali et al., 2008; Wernig et al., 2008a)
Lentiviral vectors with Cre/Lox	High efficiency; target cells need not be dividing*; can be removed post facto	Removal of integrated elements could be problematic	(Soldner et al., 2009)
Adenoviral vectors	Non-integrating	Low reprogramming efficiency	(Stadtfeld et al., 2008; Zhou and Freed, 2009)
Transposon-based methods	Can be removed post facto	Removal of integrated elements could be problematic	(Kaji et al., 2009; Woltjen et al., 2009)
Transfection	Non-integrating	Low reprogramming efficiency	(Okita et al., 2008; Yu et al., 2009)
Protein transduction	No nucleic acid	Low reprogramming efficiency	(Kim, D. et al., 2009; Zhou et al., 2009)
Small-molecule induction†	No nucleic acid	Low reprogramming efficiency	(Huangfu et al., 2008a; Huangfu et al., 2008b; Ichida et al., 2009; Lin et al., 2009; Lyssiotis et al., 2009; Shi et al., 2008)

*Recent work has shown that reprogramming efficiency is closely correlated with proliferative rate.
†Thus far, the reprogramming of human cells using only small molecules has not been demonstrated. However, small molecules have successfully been used to replace one or more factor.

transcriptional targets. Two groups have taken this finding a step further to show that adding the reprogramming factors in the form of recombinant proteins containing peptide tags that promote translocation through the plasma membrane can successfully reprogram mouse and human fibroblasts (Kim, D. et al., 2009; Zhou et al., 2009), which negates the need for gene transfer altogether. These methods are the safest for reprogramming described to date. However, it should be cautioned that, because there is still much to learn about the molecular underpinnings of the reprogramming process, even these non-integrative methods might still have as-yet unknown negative consequences.

Maintenance of genomic stability

Assuming that methods to generate FDA-acceptable hiPSC lines can be developed, the possibility that they could acquire genomic alterations that make them prone to transformation must also be considered. Decades of research based on cell culture methods have shown that many mammalian cell lines acquire genomic alterations that confer a growth advantage. For this reason, researchers in the hESC field have introduced karyotyping of cell lines at regular intervals as a standard practice. Indeed, several groups have described recurring aneuploidy genotypes that arise in hESCs, probably because they confer a growth advantage (Catalina et al., 2008; Wu et al., 2008). Notably, general karyotypic genomic abnormalities have not been pervasive as a result of reprogramming methods; almost all reports of reprogramming have shown that most cell lines generated have a normal karyotype. However, karyotyping is a rather blunt measurement of genomic abnormalities and cannot detect small amplifications, deletions, translocations or mutations. New technologies that detect genomic abnormalities at a much higher resolution are currently being developed. For example, array comparative genomic hybridization (aCGH) allows the genome of a reference sample and an experimental sample to be directly compared, and differences are highlighted on a microchip spotted with probes corresponding to the genome (Kashiwagi and Uchida, 2000); a high density of probes makes it possible to detect genomic variability with high resolution. Several groups have used aCGH to show that karyotypically normal hESC lines possess a large number of small deletions and duplications (Werbowetski-Ogilvie et al., 2009; Wu et al., 2008). Moreover, our own group recently showed that karyotypically normal hiPSC lines also possess small deletions and duplications that are detectable when they are compared to the fibroblast cell line from which they are derived (Chin et al., 2009). It is possible that hiPSC lines made with non-integrative techniques will have fewer genomic abnormalities (although this has not been formally shown), but it is doubtful that any cell line that has been cultured for a long period of time will be completely normal in this respect.

Unfortunately, the genomic abnormalities detectable by aCGH could be just the tip of the iceberg. There are presumably many more small deletions, duplications and perhaps point mutations that are undetectable using techniques such as aCGH. Perhaps the only way to completely describe the sum of genomic abnormalities in any pluripotent stem-cell line is by sequencing. With current developments in massively parallel (deep) sequencing, some technologies claim that the human genome can be sequenced within days to weeks (Wheeler et al., 2008; Ng et al., 2009; Drmanac et al., 2009). Recent studies have suggested that sequencing of hESCs and hiPSCs might become routine, and highlighted important similarities and differences between the two that would not have been appreciated with less precise methods (Lister et al., 2009). Given the current trajectory of this technology, it is not difficult to imagine that routine sequencing of complete genomes of any cell line might soon be possible in the laboratory. Although this progress would improve our ability to characterize genomic abnormalities, it will be much more challenging to understand which affect the safety of iPSCs for the purpose of clinical applications. The fact that the genome is not 'perfect' will make the task of the FDA in monitoring this aspect of iPSCs a challenge; their approach to approving the clinical use of these cells is likely to be diligent monitoring of genomic alterations combined with accurate accounting of any changes that correlate to known problems following transplantation.

Efficient directed differentiation into desired cell types

Because iPSCs have the potential to form tumors in vivo (as demonstrated by their characteristic ability to form teratomas), it is imperative that any clinical application using these cells involves transplantation of only their specified or differentiated progeny. Therefore, it is imperative that the differentiated cells used for transplantation are purified away from undifferentiated iPSCs and other cell types that have the potential to be harmful in vivo.

The advantage of pluripotent stem cells over adult stem cells is that they can generate all cell types of the body. In vitro differentiation of hiPSCs directly or through an embryoid-body intermediate has been shown to generate cells representative of all three embryonic germ layers (Lowry et al., 2008; Park et al., 2008b; Takahashi et al., 2007; Yu et al., 2007); in fact, it has been proposed that it is possible to generate any cell type of the body, although this has not been formally proven. Some cell types are easier to produce than others using existing techniques for reprogramming. Depending on the cell type and the stage of differentiation required (progenitor to fully mature), the percentage of differentiated progeny of a lineage in culture can range from 80% to less than 1%, with the remaining cells representing numerous other cell types.

Most attempts to improve the percentage of a specific cell type involve trying to replicate in vitro the differentiation conditions that are thought to exist in vivo. This can involve including different types of feeder cells and growth factors in culture. This approach has three caveats: first, it is impossible to know the precise makeup of the extracellular milieu present in vivo; second, most of what is known about in vivo microenvironments has been discovered in mice and lower organisms, and presumably is not identical in humans; and third, because hESCs and hiPSCs probably do not exactly represent any cell type found in vivo, it is not possible to predict accurately whether these cells are an appropriate starting point for generating specific cell types by replicating the in vivo conditions in which natural progenitors are found. Therefore, many differentiation protocols are based on what are presumed to be in vivo conditions and then digress into a 'kitchen sink' approach, which involves testing various factors in various combinations.

Our own experience has shown that this is a feasible approach to generate at least small numbers of differentiated cells. To generate motor neurons from hESCs and hiPSCs, we first drove the pluripotent cells towards the neural lineage (~40% efficient), and then used various growth factors and retinoic acid to replicate conditions that are thought to exist in the hindbrain and developing spinal cord (~1% efficient). After another 1 month in culture, a tiny fraction (<1%) expressed one key marker of mature motor neurons (Karumbayaram et al., 2009). Many of the current methods described to generate various cell types (including our own) require reagents or feeder cells that are not of pharmaceutical grade, so even if methods to generate large numbers of a desired cell type from hiPSCs are established, the cells would probably not be appropriate for clinical use. Therefore, modification of current protocols or entirely new protocols will be required to generate FDA-acceptable pluripotent derivatives.

Generating functional iPSC derivatives

The generation of specific cell types from hiPSCs is normally confirmed on the basis of cell morphology and the expression of markers that are expressed exclusively by a given cell type. However, it is much more challenging to determine whether a reprogrammed cell functions in the manner predicted by its phenotypic markers. This requires an appropriate assay that is quantifiable even when the cell type of interest is present as a minority in a population. In experiments in which we derived motor neurons, we used a reporter gene that expressed GFP specifically in this cell type, permitting analysis of differentiation in a mixed culture (Karumbayaram et al., 2009). The reporter was transfected early during the differentiation protocol so that, weeks later once differentiation was complete, patch clamping on only GFP-expressing cells served as a functional assay for motor neurons. However, these experiments highlighted the difficulty of generating functional cell types from iPSCs: after six weeks of differentiation, the motor neurons only fired immature evoked action potentials. Only after 8-9 weeks did full electrophysiological maturation occur in vitro. Although there are cell types that can be derived more easily and rapidly from iPSCs, it is almost certain that some cell types will prove to be even more challenging to derive. As an alternative to deriving such populations, it might be preferable to generate immature versions of the cell types of interest for transplantation and allow full differentiation to occur in the patient. However, recent work showing that iPSC-derived neural progenitor cells generated tumors following transplantation into mice suggests that a carefully considered choice based on the proliferative potential and functional capacity will be essential (Miura et al., 2009).

Many groups have now shown that hESCs and hiPSCs can be used to derive functional cell types equally well. However, studies that compare the functional capacity of the derived cells with that of their natural counterparts are rare (Marchetto et al., 2009; Song et al., 2009; Zhang et al., 2009). As a result, the function of pluripotent stem-cell-derived cells relative to naturally occurring cells in human tissues is still unclear. It is possible that pluripotent stem-cell derivatives only partially approximate the functional capacity of their natural counterparts, which might be a significant roadblock to their use in regenerative medicine. Therefore, until derivatives of hiPSCs are actually transplanted into humans, their in vivo potential will remain unknown and the field will have to rely on mouse models as a gauge of how they might function in humans.

Isolating pure populations of genomically stable hiPSC derivatives

Selective isolation of differentiated cells

Assuming specific functional cell types are successfully derived in quantities and quality that are appropriate for clinical application, it will be essential that we can isolate a given cell type from the others that are generated together with that cell type. Because of the pluripotency of hiPSCs, differentiation protocols – no matter how effective – will probably never be 100% efficient at generating one particular cell type. To eliminate both undifferentiated cells within a derived population that could potentially lead to tumors and other cell types that could have deleterious effects, purification is necessary. Most often, purification will require fluorescence-activated cell sorting (FACS) of cells that can be marked on the basis of their expression of a cell-type-specific reporter (promoter driving expression of a fluorescent protein) or by cell-surface molecules recognized by antibodies. Our own work has shown that a motor neuron reporter can be introduced transiently before FACS to enable robust purification of motor neurons (Michaela Patterson and W.E.L., unpublished observations). Notably, most work on reporters has been carried out on mouse cells, which express markers that might not be found in the same cell types in human tissue (Pankratz et al., 2007). Therefore, additional work will be necessary to identify suitable markers for purification of cells for therapy in humans. In addition, reporter labeling and antibody-based purifications are routinely performed with procedures that are not compatible with FDA guidelines. As a result, both of these methods will need to be revised to be compatible – that is, transfection reagents and antibody production will need to be standardized to comply with FDA standards, and cell-sorting procedures will need to be performed with dedicated flow cytometers that are free from

exposure to xenobiotics. The development of GMP-compatible instruments for rapid and high-volume cell purification would also be beneficial.

Determination and maintenance of genomic stability in iPSC derivatives

As discussed above for pluripotent cells, maintenance of genomic stability in the derived populations is also paramount when considering them for human transplantation. However, this has only rarely been assessed. Mutations or aneuploidy in the cells used for transplantation is just as likely as in stem-cell lines, as they are also manipulated in vitro and cultured for long periods of time. Some current methods used for the detection of genomic abnormalities could make analysis difficult for differentiated populations. Karyotyping requires the isolation of cells in metaphase, which would be rare in a population of cells that has undergone terminal differentiation. Furthermore, aCGH and sequencing approaches would require a large number of cells, and would not detect genetic changes that are not clonal in the population in a given cell line. A solution to this problem might be to perform genetic analyses at an early step during in vitro differentiation, when the hiPSC progeny are specified but not terminally differentiated; however, although this would minimize the risk that the transplanted population carried genetic abnormalities, it would not remove it entirely.

In vivo transplantation of iPSC derivatives

Perhaps the most technically challenging issue on the route towards the clinical application of hiPSCs is the procedure for the actual transplantation of the derivatives into patients. There are at least three main considerations surrounding successful transplantation: first, cell viability (survival of the transplanted cells in the host); second, the cells must functionally and physically integrate into the appropriate tissue of the host; and third, the cells must avoid detection by the immune system. The viability of transplanted cells is difficult to measure and even more difficult to regulate in vivo. In general, a substantial number of cells probably die almost immediately following transplantation because of the dramatic change in environment and inflammation associated with delivery. Some groups have shown that viability can be increased if the transplanted cells are first embedded in a physical scaffold (Bhang et al., 2007; Nakamura et al., 2005; Potter et al., 2008). These matrices – which can be made of gel (Matrigel, BD) or a more solid formulation (inorganic scaffold) – are thought to keep the transplanted cells together at the site of injection and to protect the cells from their new environment until they have the chance to adapt. Of course, if such a scaffold were used, the material would need to meet FDA requirements, which might prevent the use of one of the reagents commonly used experimentally (Matrigel, BD).

The functional integration of transplanted cells into the tissue is an issue of both geography and physiology. The cells must not only find their way to the site where they are needed in a foreign environment, but also perform their function in the context of already functioning cells of the host. For successful transplantation into the nervous system, this would require injection of neural progenitors or neurons into the brain, their migration, axon and dendrite outgrowth, and synapse formation onto the appropriate cells. Numerous groups have shown that derivatives of mouse ESCs and iPSCs can be transplanted into mouse models of tissue damage to achieve a restorative effect (Deshpande et al., 2006; Hanna et al., 2007; Nayak et al., 2006; Wernig et al., 2008b). Previous transplantation experiments using hESC-derived cells in the brains of mice have shown mixed success, whereby a portion of the cells survived and an unknown portion of the cells contributed to some degree of functional capacity (Anderson and Caldwell, 2007; Ferrari et al., 2006; Gao et al., 2006; Maciaczyk et al., 2009).

Installing a safety valve

Because of the many roadblocks discussed thus far, it would be wise to include in any iPSC-based therapy a ‘safety valve’ in the transplanted cells. Regulated activation of the safety valve could be used to eliminate or inactivate the transplanted population of cells and their progeny in the event that unwanted effects (e.g. tumour formation) occur. This safety valve could take various forms; many groups have taken advantage of so-called suicide genes that, when expressed or activated, trigger cell death. Thymidine kinase is frequently used as an inducible suicide gene; its expression is non-toxic until cells are treated with gancyclovir (Ciceri et al., 2009; Menzel et al., 2009). However, the main issue with the introduction of a suicide gene into the transplanted population is the method by which it is introduced. Genomic integration would almost certainly be required because, unlike the reprogramming factors, which get silenced as differentiation proceeds, the suicide gene must always be activatable to be effective. However, as discussed above, FDA approval would be much simpler if genomic integration were avoided. It is now possible to direct the integration of transgenes to particular loci by either homologous recombination or stable introduction to hotspots of integration (Mitsui et al., 2009; Thyagarajan et al., 2008; Zou et al., 2009; Zwaka and Thomson, 2003). These methods might be useful for directing the integration of a suicide gene to an innocuous locus in a cell population before transplantation, thereby reducing (but not eliminating) concern.

Conclusions

Although each of the steps outlined here might seem daunting, there is fantastic progress currently being made in each area. Indeed, new papers are published nearly every day that will collectively help to overcome the hurdles we have discussed here. However, there is still much work to be done. When the time comes to file an Investigational New Drug (IND) application with the FDA, the first step will be to carry out toxicology studies in animal models before carrying out safety studies in humans. At this stage, FDA regulations might permit the use of specific xenobiotic reagents (depending on the amount and their level of safety), which would make adaptation of current differentiation protocols more feasible. Even integrative methods might be permitted if the virus is prepared under GMP-grade conditions and certified by the FDA. However, as some effects of genomic instability, insertional mutagenesis or xenobiotic reagents might not arise until several years following iPSC-based treatment, it seems prudent to err on the side of caution in developing iPSC-based therapies, as such side-effects could prove worse than the condition the therapy was used to treat.

There is little doubt that each of these roadblocks will eventually be overcome to allow clinical application of hiPSCs, although the time required to achieve this remains uncertain. Taking into account the considerations outlined here will hopefully allow us to work towards the goal of generating safe iPSC lines and derivatives for clinical application.

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