Developing safe therapies from human pluripotent stem cells

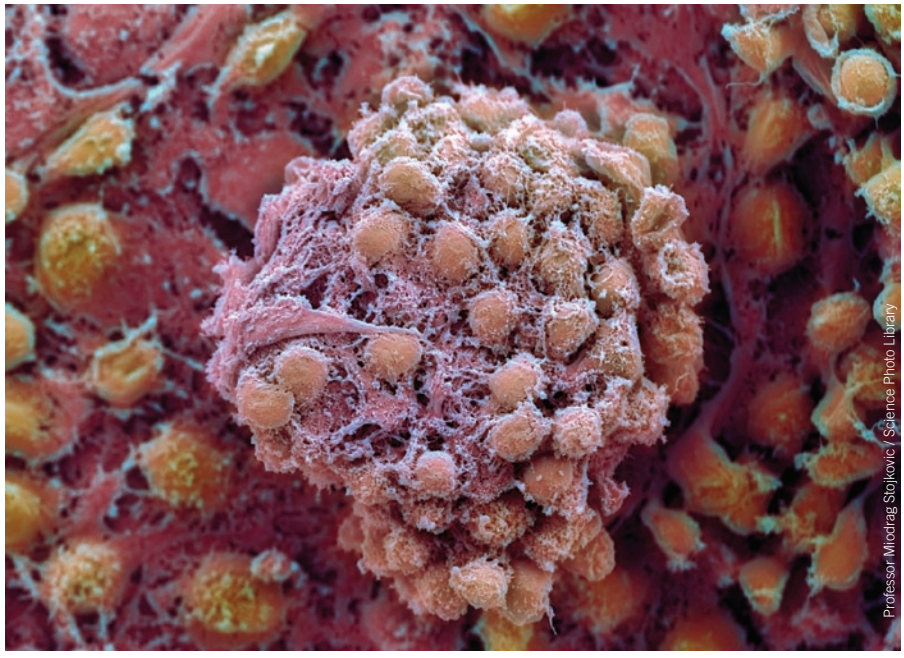
Melissa K Carpenter, Joyce Frey-Vasconcells & Mahendra S Rao

Translation of human pluripotent stem cells into cell therapies will require the development of standardized tests for product consistency, stability, tumorigenicity, toxicity and immunogenicity.

Stem cell therapies are one of the most promising areas of medicine, and many such therapies are now in development by industrial and academic groups. Therapies based on adult stem cells have been in use for several decades, beginning in 1968 with the first successful bone marrow transplant[1,2]. As the technologies for cell therapies have advanced, so have the regulatory systems to oversee them. Currently, cell therapies are regulated under the US Food and Drug Administration's (FDA's) Good Tissue Practices Final Rule, which uses a tiered system based on the level of risk associated with the cell product (**Table 1**). A few cell products, such as Carticel and skin replacement therapies, have received FDA approval (**Table 2**). Although there are no approved stem cell products, several are in late-stage clinical trials (**Table 2**).

The general strategy for many adult stem cell therapies and all pluripotent stem cell therapies is the scale-up of undifferentiated stem cells followed by differentiation to a specific cell type and delivery to the patient, where the cells may reside indefinitely. Some of the regulatory issues surrounding this strategy arise from the fundamental characteristics of living cells. Cells change over time both *in vitro* and *in vivo*, generally exist in a heterogeneous environment, integrate and migrate after transplantation and interact with host systems, such as the local milieu and the immune system. But cell products derived from pluripotent stem cells present additional issues that result from the two defining features of these cells: extensive replicative capacity and pluripotency. The ability of the cells to proliferate apparently indefinitely allows production of the very large quantities of cells needed for commercial cell products but also imposes a requirement to assess the stability of the cells over time *in vitro*. The pluripotency of the cells allows generation of a wide array of differentiated cell products but also entails the possibility of teratoma formation and the presence of unwanted cell types. This commentary addresses the regulatory issues specific to therapies with cells derived from pluripotent stem cells[3,4]. As the first clinical trials will use human embryonic stem cells (hESCs), we focus on this class of therapies; a penultimate section discusses several unique issues relevant to induced pluripotent stem cells (iPSCs).

Demonstration of the safety of hESC-derived therapies will require evaluation of the starting material, demonstration of reproducible differentiation and cell processing, assessment of the identity of the final cell product and characterization of *in vivo* properties, such as biodistribution, tumorigenicity, toxicity and immunogenicity. Some of these



A cell product derived from hESCs (pictured) was approved for clinical testing in the US earlier this year.

Melissa K. Carpenter is at the Carpenter Group, San Diego, California, USA; Joyce Frey-Vasconcells is at Pharmanet Consulting, Princeton, New Jersey, USA; and Mahendra S. Rao is at Invitrogen Corp., Frederick, Maryland, USA.
e-mail: m_carpenter@mac.com

issues have been discussed previously in the context of cell therapies[5,6]. Here we highlight those issues that pose particular challenges, including derivation of hESCs, product consistency, cell stability, tumorigenicity, toxicity and immunogenicity.

Derivation of hESCs

Most of the hESC lines generated to date were destined for research and were derived using mouse feeder layers as a substrate. Currently, there are no FDA guidelines specific to the generation of hESCs for clinical use. Researchers must therefore work within the existing guidelines for somatic cell therapies. Under these guidelines, hESCs destined for clinical applications would be derived under Good Tissue Practice (GTP) and Good Manufacturing Practice (GMP) requirements. This includes meeting donor eligibility rules and ensuring GMP compliance in the recovery, screening, testing, processing, storing, labeling, packaging and distribution of hESCs, as described in 21CFR 210 & 211 (see **Table 3** for links to FDA guidances and reference material). At present, however, derivation of hESCs according to GTP/GMP requirements is impractical. Embryo creation in *in vitro* fertilization clinics, although suitable for enabling successful pregnancies, is not GTP/GMP compliant. Moreover, GTP donor eligibility rules include donor screening in the form of acquiring family history and blood samples from each donor. This is not often done at *in vitro* fertilization clinics and cannot usually be accomplished subsequently, when frozen embryos are donated for hESC derivation.

Considering the logistical challenges of generating GTP/GMP-compliant hESC lines, we would emphasize that the spirit of these regulations is to ensure that cell products are not contaminated with adventitious agents. It should therefore be sufficient to test the starting cellular material and the final cell product for adventitious agents, in accordance with FDA's Points to Consider in the Characterization of Cell Lines guidelines (**Table 3**). Testing hESCs directly mitigates any lack of information from donor screening. Given the extensive proliferative capacity of hESCs, it is possible to generate large master and working cell banks containing sufficient numbers of cells for testing of adventitious agents, even though exact compliance with GTP guidelines has not been accomplished. Indeed, prospective generation of a GTP-compliant cell line does not appear to be required for clinical entry: in January, the FDA approved an investigational new drug application from Geron (Menlo Park, CA, USA) for the use of oligodendrocyte progenitor cells derived from one of the first hESC lines generated in an academic laboratory on mouse feeders[7]. Nevertheless, as the field moves forward, it is likely that new cell lines that are more consistent with GTP/GMP guidelines will be generated for clinical applications and product development. Toward this end, several groups have worked to generate 'clinical-grade' hESC lines (that is, lines that have been adequately tested and produced under GMP conditions), and one group has reported the generation of six such lines[8].

Table 1 Hierarchical regulatory approach

Category	Regulations	Qualifications	Examples
Category 1	Not regulated through FDA 21 CFR 1271	Cell-based products not considered human cell/tissue-based products No or minimal manipulation Homologous use Not combined with drug or device	Organ transplants Whole blood Blood-derived products Bone marrow for transplant after high-dose chemotherapy and/or total body irradiation
Category 2 '361'	Regulated under section 361 of the Public Health and Safety Act	Human cell/tissue-based products that are minimally manipulated Homologous use Not combined with drug or device Autologous use or first- or second-degree blood relative	Surgical replacement of left knee cartilage with right knee cartilage Specific cell purification of $CD34^+$ hematopoietic cells from peripheral blood for hematologic malignancies Cryopreserved cornea for transplantation
Category 3 '351'	Regulated under section 351 of the Public Health and Safety Act	Human cell/tissue products that are cultured or manipulated Not intended for homologous use Combined with drug or device Allogeneic use	Use of expanded human neural stem cells to deliver lysosomal enzymes Use of cardiomyocytes derived from human embryonic stem cells in myocardial infarction

Product consistency

The goal in manufacturing an hESC-derived product is the consistent generation of sufficient cell numbers in an aseptic environment with adequate safeguards, sterility and traceability. This will require developing a process that allows adequate propagation to obtain sufficient numbers of undifferentiated cells, a defined method of differentiation and validated testing methods to ensure that the product is consistent from lot to lot. Such consistency is generally approached by manufacturing in a GMP environment using

Table 2 Examples of cell therapies on the market or in late stage of development

Product	Description	Indication	Current Status
Carticel	Autologous chondrocytes	Cartilage replacement	Approved
Epicel	Autologous fibroblasts	Skin replacement	Approved
Dermagraft	Allogeneic fibroblasts	Skin replacement	Approved
Transcyte	Allogeneic fibroblasts	Skin replacement	Approved
Apligraf	Allogeneic fibroblasts	Skin replacement	Approved
Prochymal	Human adult mesenchymal stem cells	Graft versus host disease	Phase 3
MyoCell	Autologous expanded skeletal myoblasts	Congestive heart failure	Phase 3
ALD-101	Subpopulation of umbilical cord blood cells	Inherited metabolic diseases, lysosomal storage disorders, peroxisomal storage disease, hematopoietic stem cell transplantation	Phase 3

Table 3 Reference material for developing stem cell therapies

Document	Link
Guidance for Reviewers Instructions and Template for Chemistry, Manufacturing, and Control (CMC) Reviewers of Human Somatic Cell Therapy Investigational New Drug Applications (INDs)	http://www.fda.gov/OHRMS/DOCKETS/98fr/03d0349gdl.pdf
Guidance for FDA Reviewers and Sponsors Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)	http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Xenotransplantation/ucm092705.pdf
Guidance for Industry: Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans	http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Xenotransplantation/ucm092707.pdf
Guidance for Industry Regulation of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps) Small Entity Compliance Guide	http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/ucm062592.pdf
Guidance for Industry Current Good Tissue Practice (CGTP) and Additional Requirements for Manufacturers of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)	http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/ucm091408.pdf
Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (1993)	http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/OtherRecommendationsforManufacturers/UCM062745.pdf
Guidance for Industry: Guidance for Human Somatic Cell Therapy and Gene Therapy	http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm072987.htm
Guidance for Industry: Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products	http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/ucm073964.htm

standardized protocols and by developing validated tests to monitor consistency over the entire production cycle. Defining the consistency of the product is particularly challenging because, unlike small molecules, cells are living entities and change over time. Ensuring consistency will require particular care to minimize source variability, to regulate the differentiation and manufacturing process and to rigorously test the function and composition of the final product. **Figure 1** shows many of the steps involved in manufacturing a stem cell product.

Ensuring product consistency in light of source variability. Current models of hESC-based therapies envisage initiation of the cell population from a single culture followed by considerable propagation *in vitro* to produce a validated hESC master cell bank. If a cell product is generated from a single cell line, as will occur initially, variability may exist between vials of hESCs in the master cell bank. Thorough testing of cell composition in these banks should address this issue. However, consistency concerns will increase as multiple cell banks are created from multiple donors to allow for human leukocyte antigen (HLA) matching. Hundreds of different hESC lines have been isolated, and although there are few detailed comparative studies[9], considerable differences in differentiation capacity have already been observed[10,11]. This is not surprising given that other stem cell populations harvested at the same stage from different individuals can behave differently (unpublished data). Differences between hESC lines may arise from differences in isolation procedures, isolation stage (e.g., morula, epiblast) or individual allelic differences, which result in differences in growth rates, differentiation capacity, karyotypic stability or the ability to integrate and function *in vivo*.

Similar variability on a more limited scale has been seen with sourcing biologics, such as erythropoietin, and these problems have been resolved by limiting production to a single source or developing strong assessment criteria that provide predictive power. Applying the same strategies with hESCs is somewhat more challenging. Although progress has been made in defining the characteristics of pluripotent stem cells, the relationship between these characteristics and safety remains unclear. Characterization of hESCs has been performed with standard methods such as detection of pluripotency markers by immunocytochemistry and PCR, evaluation of karyotype and differentiation to embryoid bodies and teratomas. In addition, comparative analysis of different hESC lines has been carried out using microarrays, serial analysis of gene expression (SAGE) analysis and transcriptional profiling[9,12–15]. One of us (M.R. and colleagues[12]) proposed a set of pluripotency genes based on array analysis. Most recently, an unbiased set of genes to assess hESC lines was generated from a meta-analysis of multiple cell populations[16]. These tests, while intriguing, are neither universally accepted nor suggested to have the requisite precision or validation for clinical use. Given the potential for variability between hESC lines, we suggest that testing of master cell banks should include phenotypic, genotypic and functional assessment. Possible characteristics to be tested are shown in **Table 4**.

Ensuring lot-to-lot consistency during the differentiation and manufacturing process. Although progress is being made in developing efficient differentiation protocols[17,18] or purification[19] and selection[20] processes, the final cell population is unlikely to be absolutely homogeneous. Therefore, reproducible differentiation protocols that provide cell populations with similar cellular composition will be required. Some of the characteristics that should be measured at critical control points during differentiation and manufacturing include cell phenotype, doubling time, viability, activity, heterogeneity of the cell population and safety tests such as sterility, endotoxin level and mycoplasma contamination.

Ensuring consistent function by defining product composition. In general, cell products

can be thought of as a heterogeneous population of cells comprising the active ingredient (the active cell type(s)) and contaminants. The contaminants may be supporting or accessory cells, undifferentiated cells or other cells unrelated to the therapeutic activity. The manufacturing process should produce lots containing consistent amounts of the active ingredient and of the contaminants.

The presence of unwanted cell populations and the importance of defining acceptable levels of these cells is discussed in detail below. Functional evaluation of the cell product involves developing surrogate measures of efficacy and potency that allow prediction of activity *in vivo*. This type of assay can be complex because the active cell type or its mechanism of action may not be known, or there may be multiple mechanisms (as in spinal cord injury, in which therapeutic cells can remyelinate or provide growth factors or both). The development of predictive *in vitro* assays will substantially facilitate the development of manufacturing processes by reducing the cost and time involved in testing various product formulations or optimizing manufacturing conditions.

The relevant considerations for characterizing the final product will depend on the cell population and its mechanism of action. For example, a population of dopaminergic neurons for cell replacement in Parkinson's disease should express appropriate markers, such as neurofilament and tyrosine hydroxylase, while not expressing inappropriate markers of undifferentiated cells, endoderm or mesoderm. Functional tests could include the capacity to release dopamine after depolarization and maintenance of a nonmitotic phenotype after implantation. In another example, if the cell therapy were designed to deliver factors that protect endogenous cells or replace a missing endogenous factor, characterization would focus on assessment of factor secretion.

Epigenetic profiles may also prove useful in evaluating cell products. To date, FDA has not required expression profiles of key genes or epigenome testing for cellular products. Even so, once this sort of evaluation has been validated, it may be helpful in characterizing cell populations. As a first step, epigenetic stability could be assessed by evaluation of histone modification and DNA methylation[21,22].

Defining a consistent product is inherently challenging because of the very characteristics that make pluripotent stem cells attractive as a source of therapeutic cells. Pluripotent stem cells are dynamic, respond rapidly to the environment and can grow indefinitely in culture, making it difficult to manufacture a consistent product whose cellular composition

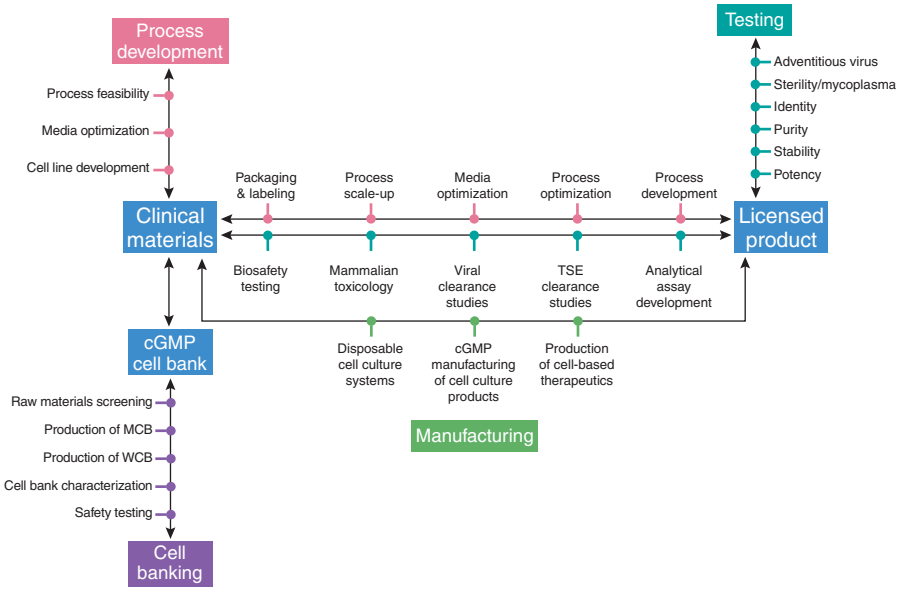


Figure 1 Schematic showing processing and manufacturing steps involved in developing a stem cell product. cGMP, current Good Manufacturing Practice; MCB, master cell bank; WCB, working cell bank; TSE, transmissible spongiform encephalopathy.

remains the same from lot to lot and whose *in vivo* activities can be predicted from *in vitro* assays. For the individualized therapies envisaged from iPSCs, each lot will be specific to one patient, and the starting material will change with each lot, complicating evaluation of product consistency. Nevertheless, strategies are being developed to define and monitor the critical variables.

Cell stability

The issue of cell stability applies to cell therapies generated from dividing cell populations. The specific concern is whether cells become 'unstable' or transformed over many population doublings. Indeed, a rare complication of hematopoietic cell transplantation (the only stem cell therapy in use on a large scale) is donor-cell leukemia[23]. As cells are maintained for long periods in culture, they may begin to 'drift' or acquire genetic and epigenetic changes.

Prolonged propagation of hESCs in culture may select for cells harboring genetic changes that enhance self-renewal. In some cases, hESCs apparently maintain a stable phenotype, karyotype and imprinting status over long-term culture[24–28], whereas in other cases, they acquire abnormal karyotypes similar to those of human embryonal carcinoma cells, such as trisomy 12 and 17 (ref. 26), or other mutations[29]. Two groups have recently identified recurrent chromosomal abnormalities in hESCs maintained over long-term culture, including amplification at 20q11.21, which is associated with oncogenic transformation[30,31]. In general, such abnormal cultures retain pluripotency and expression of standard hESC markers[25,26,32]. Although it is unclear whether the presence of rare karyotypically abnormal cells in hESC cultures will alter bulk characteristics, such as growth rate, cell cycle regulation or differentiation capacity, the identification of these cells highlights the need for careful monitoring. The development of predictive tests to identify aneuploid cells would be of great assistance in safety testing.

Concerns about the epigenetic stability of hESCs arose initially in the context of their source material, human embryos. Culture of human embryos for assisted reproduction has been associated with epigenetic disorders[33,34]. Several groups have begun evaluating the epigenetic profile of hESCs by analyzing the status of X-inactivation, imprinting and methylation. X-inactivation status appears to vary in different hESC lines[35,36]. Studies of imprinting show a substantial degree of genomic stability in culture[27,28,33]. New tools are being developed to assess the methylation signatures of pluripotent cells[37, 38]; the next step will be to determine whether these signatures are stable over long-term culture.

These concerns about genetic and epigenetic drift in cultured hESCs have several implications for safety testing. Most importantly, evaluation of hESCs and of the differentiated cell product must be carried out over sufficiently long periods of time to reach robust conclusions about stability. Thus, the basic assays described above for determining product consistency (phenotype, karyotype, and genetic and epigenetic

profiles) should be applied longitudinally. A stability profile covering a discrete number of passages or population doublings could include expression of key genes (e.g., telomerase, cell cycle, key hESC markers), genomic stability and epigenetic stability (methylation, miRNA, histone acetylation and X-chromosome inactivation). Coupling such assays with *in vivo* testing of the stability of the therapeutic product may eventually result in the use of genetic and epigenetic profiles as surrogate markers for *in vivo* stability.

Although we believe that the optimal hESC populations for cell therapies will be euploid and stable, it is worth noting that FDA-approved clinical trials have been carried out using the human embryonal carcinoma cell line NT2 (refs. 39,40). The neurons delivered in these trials carried out by Layton Bioscience (Sunnyvale, CA, USA) were prepared from the NT2/D1 cell line. NT2/D1 cells were exposed to retinoic acid for 6 weeks followed by exposure to an antimitotic cocktail for 6 days, and the resulting neurons were cryopreserved until the time of transplant. At transplantation, cells were thawed, processed and injected into stroke patients. The patients received 2–10 million cells and were evaluated for 52 weeks after transplantation. NT2 cells were reported to have 56–61 chromosomes with a multitude of abnormalities[41]. However, separate rodent studies showed that NT2N cells (cells prepared in essentially the same manner as the cells for the clinical trials) implanted into the central nervous system of nude mice were not tumorigenic or mitotically active for up to 14 months after transplantation[42]. Furthermore, tumorigenesis has not been a reported outcome in these trials. These data indicate that an abnormal karyotype or gross aneuploidy may not be predictive of tumorigenesis.

Tumorigenicity

One of the most important issues in the development of safe therapies from pluripotent stem cells is ensuring that the cell product does not form tumors after implantation. There are two primary concerns. First, the cell product might contain contaminating undifferentiated cells that would eventually proliferate and form a teratoma. Second, the cell product may not be stable and may 'de-differentiate' or transform to produce a benign or malignant tumor. We suggest that concerns around tumorigenicity be addressed in the context of the risk/benefit ratio for the individual. If the patient has a fatal, devastating disease, such as amyotrophic lateral sclerosis or Huntington's disease, the risk tolerance may be considerably higher than for someone who will live many years with a reasonably good quality of life, such as an individual with diabetes.

Table 4 Characteristics of hESCs[a]

Features	Criteria
Population doubling time	~36 h
FGF dependence	Yes
Ability to differentiate	Yes
Stable karyotype	Yes
SSEA-1	Absent
SSEA-3	Present
SSEA-4	Present
TRA-1-60	Present
TRA-1-81	Present
Oct4	Present
Nanog	Present
Sox2	Present
E-cadherin	Present
Brachyury	Absent
Pax6	Absent
APF	Absent

[a]Modified from ref. 34.

Issues to be considered when evaluating tumorigenicity. Pluripotency of hESCs is evaluated by the ability of the cells to generate teratomas in immunocompromised mice. These teratomas are generally understood to be benign tumors that contain derivatives of all three germ layers. Although hESCs are derived from the inner cell mass of the embryo, the teratomas they form are remarkably similar in phenotype to spontaneously occurring germ cell teratomas. It is possible that we can use the extensive literature on germ cell tumors and their clinical outcomes to better understand and predict the behavior of teratomas derived from pluripotent stem cells.

Spontaneously arising teratomas are a germ cell tumor defined as a benign tumor containing cells from one or more germ layers[43]. The behavior of these tumors is different depending on the site of origin and whether the patient is prepubertal or postpubertal. Most ovarian teratomas are benign with a diploid 46, XX karyotype. These tumors are generally cystic and comprise differentiated cells in organized structures. Mature testicular tumors often are cytogenetically abnormal solid tumors with a complex profile and are classified as teratocarcinomas[44]. Testicular teratocarcinomas often contain complex cytogenetic abnormalities including a 12p amplification in the form of an isochromosome [i(12p)]. Immaturity of a teratoma usually manifests as the presence of immature neuroepithelium. Those patients with less neuroepthelium in their teratomas or teratocarcinomas show a higher survival rate[43].

These characteristics of germ cell tumors suggest that sex, karyotypic stability and the type of teratoma formed in immunocompromised mice will inform us about the degree of risk and the level of testing required. In particular, the type of tumor formed by a pluripotent population will be an important part of the cell therapy's safety profile. An hESC line that is stably euploid and generates a mature, fully differentiated teratoma is superior to an aneuploid hESC line that generates an immature teratoma containing neuroepithelium.

Testing the starting material. After determining that an hESC line is competent to form a teratoma, the next questions to be answered are: how many hESCs are required to make a teratoma, and is teratoma formation affected by environment?

Evaluation of the cell dose required for teratoma formation will help to establish safety parameters, such as the number of hESCs that the cell product can contain without risk of teratoma formation. Teratoma generation usually requires injection of 1–10 million hESCs. However, the efficiency of teratoma formation likely depends upon the site of transplant and the strain of the animal. In addition, considerable cell death, probably at the time of injection, substantially reduces the 'effective dose' of cells. Finally, hESCs are more stable when cultured as colonies or clumps of cells[45], and dissociation to single cells can result in cell death or aneuploidy. This is supported by recent work showing that survival after dissociation to single cells is improved by a ROCK inhibitor that affects cell-cell interactions[46–48]. Thus, testing the absolute cell number for teratoma generation requires understanding the configuration and environment of the cells.

The importance of implant site is evident from a study in which injection of one line, NCL1, subcutaneously or into the liver, resulted in teratomas with different growth and differentiation characteristics[49]. Liver teratomas were cystic, contained a mixture of immature and differentiated cells, appeared much more quickly and grew to a larger size, whereas subcutaneous teratomas grew more slowly, were smaller and contained mostly differentiated cells. It is unclear whether these findings were due to differences in cell survival at the time of injection, proliferation rates after injection, environmental influences or a combination of these.

The influence of microenvironment is further emphasized by the findings of Shih *et al.*[50], who transplanted hESCs together with human fetal thymus, pancreas and lung tissues. Transplanting hESCs with fetal thymus led to teratomas containing primitive and undifferentiated cells rather than differentiated cellular structure typical of a mature teratoma.

In contrast, injection of the same cell line into the hind limb of mice produced differentiated teratomas. These authors also examined the number of cells required to generate a teratoma in the context of transplantation with fetal thymus or lung. Injection of 5,000 cells consistently produced teratomas, and none of the animals injected with 50 cells presented with teratomas. Of course, delivery of hESCs and fetal tissues has not been envisaged as a therapeutic approach. Perhaps a more relevant test would involve transplantation of hESC-derived cells and mature human tissues. Regardless, these data indicate that cell products derived from hESCs must be tested in the relevant environment. In a clinical setting, the microenvironment may often be a disease background and perhaps immunosuppression. We suggest that the cell dose required for teratoma formation for each hESC line should be assessed in multiple sites, including the site of delivery of the intended product.

Testing the cell product. Thus far, methods for *in vitro* differentiation of hESCs are not 100% efficient. Therefore, the differentiated cell populations used for therapies will likely be heterogeneous and may contain contaminating undifferentiated or partially differentiated[51] hESCs that proliferate inappropriately. It will be essential to rigorously test the cell product to ensure that it is not tumorigenic. The evaluation required will be dependent upon the cell product, cell dosage, implant site, clinical indication and the availability of animal models.

Several studies have shown that hESC-derived cell populations can be delivered into animal models without the appearance of teratomas. This appears to be correlated with the purity of the cells, the site of implantation and the maturity of the cells. If hESCs are not fully differentiated, teratoma formation becomes more likely[52]. No teratoma formation was observed after implantation of various hESC-derived populations into the heart[19,53,54], the spinal cord[17,55] and the brain[56]. However, Kroon *et al.*[10] reported teratomas in 15% of animals implanted with a pancreatic endoderm population. The authors note that this population was not enriched and likely included mesodermal and ectodermal cells at the time of transplantation. Therefore, it is difficult to determine whether the teratomas identified were a result of contaminating hESCs in the population or of implanting derivatives of three germ layers. In another study, implantation of hESC-derived dopaminergic neurons into Parkinsonian rats resulted in grafts with expanding cores of undifferentiated mitotic neuroepithelium that may be tumorigenic[51]. In contrast, Ravindran *et al.*[56] reported that implanted hESC-derived neural progenitor cells did not form teratomas, even 1 year after implantation. These two groups used different hESC lines, maintained in different culture conditions, differentiated with different protocols and implanted into different brain regions.

These early studies indicate that the tumorigenicity of an hESC-derived product will be influenced by the choice of hESC line, the purity of the cell population, the maturity of the cell population, the number of cells implanted and the site of implantation. Safety testing should therefore assess all of these factors. In addition, it should take into account the intended clinical situation. For instance, some therapies may require transient or long-term immunosuppression, which may alter the survival of the implanted cells or the host response to the implanted cells.

The goal of these tests is to determine whether the cell product will ultimately form a tumor. Therefore, these will be long-term studies (>1 year), with the length depending upon the patient population to be treated and the risk/benefit ratio. The cell number tested will depend on the expected human doses. It is generally anticipated that the human dose should be tested tenfold. Therefore, if the patient dose is 10 million cells, then these experiments would test 100 million cells. It is straightforward to deliver this quantity of cells to a small animal. If the cell dose is envisioned to be a billion or more, however, the number of cells required may not be compatible with small-animal models. In this case, surrogate assays may be needed.

Determining the appropriate animal models for such studies can be challenging. The advantage of using mice and rats is that immunocompromised strains are available; the disadvantage is that these animals have a fairly short lifespan (12–18 months) and begin to form spontaneous tumors as they age. Large, immunocompetent animals require immunosuppression, which is generally incomplete and not suitable for long-term studies. In such studies, lack of tumor formation may result from immune rejection or toxicity of the immunosuppressive agents rather than from the characteristics of the cell product. The choice of animal model must ultimately be determined by the cell population and the clinical target.

Finally, biodistribution studies should be conducted to determine whether the hESC-derived cells have migrated to other locations in the body. If the cells have migrated, it will be necessary to determine whether the new environment affects their differentiation and stability. Laflamme *et al.*[19] performed this type of analysis after implanting hESC-derived cardiomyocytes into rat hearts. Four weeks after implant, PCR analysis of human ALU sequences showed no human cells in other organs, indicating that there was little or no migration and survival of the cardiomyocytes in organs other than the heart. The assessment of cellular migration will be important for each cell product.

Toxicity

Although tumorigenicity is the main safety concern for hESC-derived products, toxicity and stability must also be evaluated. Toxicology studies should test for acute and chronic toxicity to major organs and for changes in blood chemistry and blood counts after cells are delivered to the intended site. These studies will likely include surrounding tissues and organs. For instance, if the cell population secretes growth factors or neurotransmitters, this may affect tissues surrounding the site of implantation. Although such studies are generally conducted in normal animals, in some cases, toxicities might be seen preferentially in the disease state, and testing in disease models should be considered. In addition, adjunct therapies such as immunosuppression may be a component of this evaluation.

Functional stability of the differentiated cells is also essential. If the therapeutic effect results from secretion of factors in a regulated manner, this regulation must be shown to be stable over time. For instance, if a cell product for diabetes secretes insulin in response to glucose, it is critical that this regulation be stable over the lifetime of the cells as the dysregulation of insulin secretion would be harmful to patients.

Immunogenicity

Although early reports indicated that hESCs and their derivatives may be 'immune-privileged'[57,58], because they do not express HLA class II molecules and show limited expression of HLA class I molecules, recent findings show that hESCs can induce immune rejection in immunocompetent rodents[59]. It is clear that the immune status of each hESC-derived cell product will need to be carefully assessed and that, in most cases, some level of immune suppression will be required. This has raised several issues. One question is whether an effort should be made to match major HLA phenotypes (as with organ transplants) in orderto use less-aggressive immunosuppression. This strategy would require having many hESC lines available, with estimates

ranging from less than a hundred to several thousand[60,61]. Even if it were feasible to generate such large numbers of lines, the regulatory issues would be daunting, as each line would likely be considered a separate product that must be qualified separately. Another proposal is to engineer transplanted cells to delete their immunogenicity; although intriguing, this strategy has not yet been demonstrated.

The need for immunosuppression will be influenced by the implant site. Implantation into an 'immune-privileged' site, such as the central nervous system, may require only a short course of immunosuppression, whereas implantation into a peripheral site will likely require a full immunosuppressive regimen. The immune status of each cell product must be tested in the appropriate site. Using iPSCs in a patient-specific paradigm is expected to solve issues of immunogenicity. In this case, iPSCs would be generated from the patient's somatic cells, expanded, differentiated and delivered back to the individual. This very promising research is still in its early stages and will face other regulatory concerns.

iPSC-derived cell products

The discovery that human somatic cells can be reprogrammed into pluripotent cells has had a great impact on how we view the future of stem cell therapies. The ability to readily generate patient-specific stem cell populations presents many opportunities and challenges. Similar to hESCs, iPSCs have extensive proliferative capacity and are pluripotent. The development of safe therapeutics from iPSCs will entail consideration of all the issues discussed above for hESCs, except immunogenicity, as well as several additional issues related to the reprogramming technologies used, the extent of reprogramming required, the tissue source, lot-to-lot variability and donor-to-donor variability.

The technology for generating iPSCs is still in its early stages, although it is moving remarkably quickly. Initially, iPSCs were generated by the introduction of retroviruses or lentiviruses carrying the transgene combinations *Oct4*, *Sox2*, *Klf4* and *c-Myc*[62] or *OCT4, SOX2, NANOG* and *LIN28* (ref. 63). Safety concerns arose from the use of oncogenes and the potential for insertional mutagenesis by integrating viruses. In addition, although transgenes are largely silenced in iPSCs, reactivation of *c-Myc* and tumorigenesis have been detected after germline transmission[64]. To minimize genomic integration, subsequent studies demonstrated the generation of iPSCs by adenovirus[65], plasmid transfection[66], episomal vector transfection[67], the *piggyBac* transposon[68] and Cre-recombinase-excisable virus[69]. Most recently, iPSCs have been generated by directly introducing the four reprogramming proteins Oct4, Sox2, Klf4 and c-Myc by engineering proteins capable of penetrating the cell membrane[70,71]. Despite considerable improvements in the technologies for iPSC generation, the yields and consistency remain quite low. It is likely that the 'dose' of individual reprogramming factors and the ratio between them are important. Several groups have reported incomplete reprogramming, which may result from inadequate levels of the reprogramming factors or exposure times. Incomplete reprogramming in the nuclear cloning field results in death or serious abnormalities of cloned animals[3]. It is possible that partially reprogrammed iPSCs may be unstable and more likely to result in immature teratomas. Therefore, evaluation of the completeness of reprogramming will be needed when assessing the safety of iPSC-derived cell therapies. Going forward, the generation of iPSCs for clinical applications will require consistent generation of iPSCs, which will likely be dependent on the consistent 'dosing' of the reprogramming proteins in a manner that achieves complete reprogramming.

Although iPSCs are similar to hESCs, propagation of these cells for many years has not been reported yet. It will be important to determine whether iPSCs are stable over long-term culture. Determination of phenotypic and karyotypic stability will be critical in determining the suitability of these cells for therapeutic applications. In addition, given the issues related to partial reprogramming discussed above, it will be necessary to measure the epigenetic stability of these cells, as described above in the section 'Cell stability'.

Another unresolved issue is the ideal tissue source for iPSCs. To date, human iPSCs have been generated from fibroblasts[63,72], keratinocytes[73] and blood progenitor cells[74]. The choice of tissue source will be influenced by the ability to safely and reproducibly acquire tissue biopsies and the ability of cells from different origins to be efficiently reprogrammed. It has already been demonstrated that mouse hepatocytes[75] and human keratinocytes[73] required fewer retroviral integration sites or a lower 'dose' of the reprogramming factors. Although it is too early to determine which tissue source will be best for reprogramming, these data indicate that there are differences between tissue sources.

In addition to differences between cell sources, there will likely be differences between donors. Patient-specific therapies envision harvesting starting material from patients of different ages and states of health. These fundamental differences may affect the success or yields of reprogramming and the ability of the cells to differentiate to the envisioned cell product.

Although iPSCs offer exciting opportunities for stem cell therapies, many questions must be addressed before these technologies will be suitable for clinical applications. The knowledge base that is currently being generated with hESCs will undoubtedly accelerate the development of iPSC-derived therapies.

Conclusions

As we survey the pluripotent stem cell field, we see rapid progress and some important regulatory challenges as FDA and other authorities attempt to build appropriate regulatory pathways that take into account the unique issues related to hESCs and iPSCs. Given the current technological constraints, we recommend that careful attention be given to ensuring consistency of the cell product. This consistency can be achieved only by ensuring that variability is minimized at all steps of the manufacturing process, which depends on consistent testing throughout the process. Testing the end product alone is not sufficient; the quality and stability of the hESC master cell bank must also be assured. Rather than adding an unnecessary burden or delaying therapy, these experiments will enable cell lines to be evaluated before allocating substantial resources to establishing and validating tests and animal studies. The qualified master cell bank could then be used (along with additional data) as starting material for cell products. Our emphasis on product consistency and standardization of tests was dictated by the realization that the tumorigenic potential of hESC-derived products may vary dramatically depending on the cellular composition of the cell product and the host environment and that, as a result, testing for tumorigenicity in animal models should be performed after establishing defined processing protocols and a defined cell product. If feasible, implantation of the cell product should be performed in the context of the appropriate disease and immunosuppressive therapy. Although our discussion has focused on one class of pluripotent cell—the hESCs derived from the inner cell mass—many of our arguments are equally applicable to other pluripotent cell populations, such as iPSCs[76,77].

The determination of the safety of any pluripotent stem cell product is inseparable from an assessment of the risk/benefit ratio in each clinical indication. As with any other therapy, it is wise to acknowledge that it is generally not possible to eliminate all risk. Our goal is to ensure that the regulatory authorities, patients and clinicians have access to the relevant risk/benefit ratios to make appropriate decisions in the context of available treatments and technologies.

1. Bach, F.H., Albertini, R.J., Joo, P., Anderson, J.L. & Bortin, M.M. *Lancet* **2**, 1364–1366 (1968).
2. Gatti, R.A., Meuwissen, H.J., Allen, H.D., Hong, R. & Good, R.A. *Lancet* **2**, 1366–1369 (1968).
3. Jaenisch, R. & Young, R. *Cell* **132**, 567–582 (2008).
4. Yu, J. & Thomson, J.A. *Genes Dev.* **22**, 1987–1997 (2008).
5. Hentze, H., Graichen, R. & Colman, A. *Trends Biotechnol.* **25**, 24–32 (2007).
6. Preti, R.A. *Nat. Biotechnol.* **23**, 801–804 (2005).
7. Thomson, J.A. *et al. Science* **282**, 1145–1147 (1998).
8. Crook, J. *et al. Cell Stem Cell* **1**, 490–494 (2007).
9. Adewumi, O. *et al. Nat. Biotechnol.* **25**, 803–816 (2007).
10. Kroon, E. *et al. Nat. Biotechnol.* **26**, 443–452 (2008).
11. Osafune, K. *et al. Nat. Biotechnol.* **26**, 313–315 (2008).
12. Bhattacharya, B. *et al. Blood* **103**, 2956–2964 (2004).
13. Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R.C. & Melton, D.A. *Science* **298**, 597–600 (2002).
14. Richards, M., Tan, S.P., Tan, J.H., Chan, W.K. & Bongso, A. *Stem Cells* **22**, 51–64 (2004).
15. Sato, N. *et al. Dev. Biol.* **260**, 404–413 (2003).
16. Muller, F.J. *et al. Nature* **455**, 401–405 (2008).
17. Nistor, G.I., Totoiu, M.O., Haque, N., Carpenter, M.K. & Keirstead, H.S. *Glia* **49**, 385–396 (2005).
18. Chambers, S.M. *et al. Nat. Biotechnol.* **27**, 275–280 (2009).
19. Laflamme, M.A. *et al. Nat. Biotechnol.* **25**, 1015–1024 (2007).
20. Yang, L. *et al. Nature* **7194**, 524–528 (2008).
21. Spivakov, M. & Fisher, A.G. *Nat. Rev. Genet.* **8**, 263–271 (2007).
22. Wu, H. & Sun, Y.E. *Pediatr. Res.* **59**, 21R–25R (2006).
23. Flynn, C.M. & Kaufman, D.S. *Blood* **109**, 2688–2692 (2007).
24. Rosler, E.S. *et al. Dev. Dyn.* **229**, 259–274 (2004).
25. Brimble, S.N. *et al. Stem Cells Dev.* **13**, 585–597 (2004).
26. Draper, J.S. *et al. Nat. Biotechnol.* **22**, 53–54 (2004).
27. Rugg-Gunn, P.J., Ferguson-Smith, A.C. & Pedersen, R.A. *Nat. Genet.* **37**, 585–587 (2005).
28. Sun, B.W. *et al. Hum. Mol. Genet.* **15**, 65–75 (2006).
29. Maitra, A. *et al. Nat. Genet.* **37**, 1099–1103 (2005).
30. Lefort, N. *et al. Nat. Biotechnol.* **26**, 1364–1366 (2008).
31. Spits, C. *et al. Nat. Biotechnol.* **26**, 1361–1363 (2008).
32. Sun, X. *et al. Human Reproduction* **23**, 2185–2193 (2008).
33. Rugg-Gunn, P.J., Ferguson-Smith, A.C. & Pedersen, R.A. *Hum. Mol. Genet.* **16**, R243–251 (2007).
34. Pannetier, M. & Feil, R. *Trends Biotechnol.* **25**, 556–562 (2007).
35. Hoffman, L.M. *et al. Stem Cells* **23**, 1468–1478 (2005).
36. Dhara, S.K. & Benvenisty, N. *Nucleic Acids Res.* **32**, 3995–4002 (2004).
37. Ball, M.P. *et al. Nat. Biotechnol.* **27**, 361–368 (2009).
38. Deng, J. *et al. Nat. Biotechnol.* **27**, 353–360 (2009).
39. Kondziolka, D. *et al. Neurology* **55**, 565–569 (2000).
40. Kondziolka, D. *et al. J. Neurosurg.* **103**, 38–45 (2005).
41. Mostert, M.M. *et al. Cancer Genet. Cytogenet.* **87**, 95–102 (1996).
42. Kleppner, S.R., Robinson, K.A., Trojanowski, J.Q. & Lee, V.M. *J. Comp. Neurol.* **357**, 618–632 (1995).
43. Ulbright, T.M. *Mod. Pathol.* **18** Suppl 2, S61–S79 (2005).
44. Andrews, P.W. *Biochim. Biophys. Acta* **948**, 17–36 (1988).
45. Ginis, I. *et al. Dev. Biol.* **269**, 360–380 (2004).
46. Watanabe, K. *et al. Nat. Biotechnol.* **25**, 681–686 (2007).
47. Harb, N., Archer, T.K. & Sato, N. *PLoS One* **3**, e3001 (2008).
48. Martin-Ibanez, R. *et al. Hum. Reprod.* **23**, 2744–2754 (2008).
49. Cooke, M.J., Stojkovic, M. & Przyborski, S.A. *Stem Cells Dev.* **15**, 254–259 (2006).
50. Shih, C.C., Forman, S.J., Chu, P. & Slovak, M. *Stem Cells Dev.* **16**, 893–902 (2007).
51. Roy, N.S. *et al. Nat. Med.* **12**, 1259–1268 (2006).
52. Brederlau, A. *et al. Stem Cells* **24**, 1433–1440 (2006).
53. Leor, J. *et al. Heart* **93**, 1278–1284 (2007).
54. Caspi, O. *et al. J. Am. Coll. Cardiol.* **50**, 1884–1893 (2007).
55. Cloutier, F., Siegenthaler, M.M., Nistor, G. & Keirstead, H.S. *Regen. Med.* **1**, 469–479 (2006).
56. Ravindran, G., Ramnath, R.L., Rao, H.S. & Viswanathan, C. *Biochem. Biophys. Res. Commun.* **373**, 258–264 (2008).
57. Drukker M. *et al. Proc. Natl. Acad. Sci. USA* **99**, 9864–9869 (2002).
58. Li, L. *et al. Stem Cells* **22**, 448–456 (2004).
59. Swijnenburg, R.J. *et al. Proc. Natl. Acad. Sci. USA* **105**, 12991–12996 (2008).
60. Taylor, C.J. *et al. Lancet* **366**, 2019–2025 (2005).
61. Rao, M.S. & Auerbach, J.M. *Lancet* **367**, 650 (2006).
62. Takahashi, K. & Yamanaka, S. *Cell* **126**, 663–676 (2006).
63. Yu, J. *et al. Science* **318**, 1917–1920 (2007).
64. Okita, K., Ichisaka, T. & Yamanaka, S. *Nature* **448**, 313–317 (2007).
65. Statfeld, M. *et al. Science* **322**, 945–949 (2008).
66. Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T. & Yamanaka, S. *Science* **322**, 949–953 (2008).
67. Yu, J. *et al. Science* **324**, 797–801 (2009).
68. Woltjen, K. *et al. Nature* **458**, 766–770 (2009).
69. Soldner, F. *et al. Cell* **136**, 964–977 (2009).
70. Zhou, H. *et al. Cell Stem Cell* **4**, 381–384 (2009).
71. Kim, D. *et al. Cell Stem Cell* **4**, 472–476 (2009).
72. Takahashi, K. *et al. Cell* **131**, 861–872 (2007).
73. Aasen, T. *et al. Nat. Biotechnol.* **26**, 1276–1284 (2008).
74. Loh, Y.H. *et al. Blood* **113**, 5476–5479 (2009).
75. Aoi, T. *et al. Science* **321**, 699–702 (2008).
76. Rao, M. & Condic, M.L. *Stem Cells Dev.* **17**, 1–10 (2008).
77. Condic, M.L. & Rao, M. *Stem Cells* **26**, 2753–2758 (2008).