WHITE PAPER: TRANSLATION ISSUES FOR hESC-DERIVED THERAPIES

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This document focuses on the pre-clinical studies that should reasonably be expected of sponsors/investigators before initiation of Phase I studies applying hESC-derived cells, and acknowledges areas for which pre-clinical studies, no matter how extensive, cannot completely resolve safety issues before translation. After each section, opportunities for research are highlighted, to direct attention to gaps in knowledge that should be targeted for funding.

Introduction.

Virtually all position papers dealing with human embryonic stem cells start with a sentence like this one: "Human embryonic stem cell (hESC)-derived cell therapies hold enormous promise for the relief of pain and suffering associated with a wide range of pathologic processes." 'Promise' is generally considered to be far off. Recent progress in the handling of hESCs in vitro, expansion of hESC biologists facilitated by iPS technology, and importantly, the publication of impressive therapeutic findings in animal models all suggest that the promise of hESC translation may not be that far away. The FDA hearing held in April 2008 also highlighted the progress of three commercial groups in bringing hESC-derived therapies closer to clinical realization and of course, the 2009 approval of Geron's clinical trial to study safety of hESC-derived preoligodendrocytes in patients with acute thoracic spinal cord injury is a major landmark in the field. For these reasons it is critical to continuously assess the translational needs and unknowns surrounding hESC-derived therapies, especially as the FDA regulatory framework for facilitating safe translation is an important priority in advancing the therapies. California Institute of Regenerative Medicine (CIRM) is committed to working with all appropriate regulatory agencies to safely facilitate clinical application of hESC-derived therapies. Safety concerns are *the* top priority in anticipation of novel hESC-derived cell therapies, but safety considerations must be thought out within the framework of risk/benefit assessments of novel hESC-derived cell therapies.

Some safety concerns raised about hESC-derived cell therapies are common to all cell-based therapies (sterility in the manufacturing process, transmission of human or non-human infectious diseases, appropriate genetic tests) and current regulations and practices in place for other cell-based therapies can be applied to hESC-derived therapies, with little modification of current regulatory guidelines (documented in multiple CFR reports). Other safety concerns are unique to cells derived from hESC because of the distinctive biologic properties of these cells and inexperience with these cells in the clinic. The unique safety concerns connected with cells derived from hESC are the potential for teratoma formation, for malignant transformation, unknowns regarding their immunogenicity, and the inability to completely predict and directly control and monitor the fate of injected cells. Our position is that the safety issues of concern for hESC-derived therapies are not 'solved' but that a complete 'solution' to all these issues (an absolute guarantee that there will be no adverse events) before FDA allows Phase I trials is not in the best interest of progress in patient care. For example, a full understanding of hESC self-renewal also implies a complete understanding of tumor cell self-renewal, in other words, a complete solution to very long-standing refractory biologic issues; expecting such an advance before allowing regulated translation of hESC-derived therapies would unnecessarily stall advances in treatment of many diseases. On the other hand, demonstration that potentially teratoma-forming cell populations

have been functionally removed from the hESC-derived transplanted cells and a long-term follow up in an appropriate animal model is a reasonable expectation during pre-clinical studies.

The purpose of this commentary is not to offer precise details of the necessary safety studies that should precede Phase I trials of hESC-derived therapies, but to reaffirm the general recommendations made by regulatory experts (Halme and Kessler, 2006; Fink http://www.fda.gov/Cber/genetherapy/stemcell012907df.htm) in public commentary. The details of end-points for safety studies (what studies are performed and how long animals are followed) and therapeutic end-points studied in animal models are ideally planned by investigators working together with FDA-CBER, and will be different for each cell therapy application. Phase I clinical studies should then only follow pre-clinical studies in which biological and statistically significant benefit is demonstrated from the hESC-derived cell therapy.

Preclinical safety and efficacy studies must be performed with acknowledgement that animal models of disease processes cannot give full information about safety of hESC-derived therapies in humans: Animal models insufficiently reflect the heterogeneity of the human disease microenvironment, and often do not capture the entire spectrum of disease signs and symptoms. Nonetheless, animal models should be capitalized on, and used to their full extent as necessary, in an effort to minimize unanticipated adverse events from transplantation of cells derived from hESC.

Furthermore, because of the unknowns and calculated risks in translation of hESC-derived therapies, the patient populations studied should be those with disease or injury that has high mortality or morbidity and for whom adequate therapies do not currently exist. [These populations may be especially vulnerable, that is, inherently coercible, because of their disease state. For this reason again, special attention to staged informed consent is warranted, analogous to the practice in living donor organ donation (Colardyn, 2003)]. This consideration of the appropriate populations for initial Phase I testing also implies that testing of hESC-derived cell therapies in normal human volunteers before Phase I tests in diseased subjects is not justifiable since real benefits of therapy cannot be weighed against risks in normal subjects.

The discussion that follows is focused only on hESC derived from the inner cell mass of blastocysts, and not on parthenogenetically-derived hESC, or those derived from pre-blastocyst stages of development, or cells that have been obtained by somatic cell nuclear transfer, or hESC lines that are induced pluripotent lines derived after retroviral-mediated gene transfer in adult cells. The vast majority of relevant pre-clinical studies in the literature have been conducted with primary hESC lines derived from blastocyst-stage embryos, and therefore a large body of scientific literature appropriate to pre-clinical testing is only available from primary hESC lines derived from blastocysts.

Specific safety issues.

In order to obtain a biologics license for any stem cell-based product, the following inter-related characteristics of the product must be demonstrated: Sterility, purity, potency, identity, stability, safety, efficacy (Fink http://www.fda.gov/Cber/genetherapy/stemcell012907df.htm)

Fortunately, many regulations established by CBER for tissue and cell therapies, and to blood banking, are applicable to hES cell-derived cell therapies, and can be readily adopted to assure safety of hES cell-derived transplanted cells.

In the derivation, expansion, and differentiation of any hESC-derived cell therapy, all components used in the cell product and their handling must be documented and standardized procedures for the production and characterization of the hESC-derived cell therapy are required. Exquisite documentation is a major feature of GMP standard operating procedures. Regulations around blood and organ donor handling have evolved because the safety and efficacy of these products is dependent on stereotypic, controlled handling protocols, just as hESC-derived cell handling will significantly impact safety and efficacy of the transplanted cells. The FDA has issued many applicable rules to assure sterility and safety of cells for transplantation by addressing the donor information necessary to assure safety (as covered in CFR Title 21 640.3.)

Donor-derived infections. The medical history and blood testing for blood- or cell-borne infectious disease can be patterned on that used by blood banks for blood donors (Mushawar, 2007) and for islets and solid organs derived from brain dead donors. Fortunately, for development of hESC-derived therapies, even more rigor than used for solid organ transplantation can and should be applied regarding the safety of the donor source(s), since more time is available for testing than in solid organ donor allocations. For all infectious disease testing, FDA-licensed kits and protocols should be followed for infection documentation. Evidence of active infection with the following precludes use of the cells in clinical applications: HIV-1, HIV-2, HTLV-1, HTLV-2, hepatitis B, hepatitis C, cytomegalovirus, Ebstein Barr Virus, West Nile virus (by nucleic acid testing), syphilis, or a history of Creutzfeld-Jacob disease or variant Creutzfeld Jacob disease (Alter, 2007). For infectious diseases for which standardized testing is not available, blood banks have worked out extensive surrogates to help eliminate risky donors (travel to certain areas, etc.), again readily applicable to analysis of donor sources of hESC-derived cell products. Testing standards are not static, and communication with FDA is important for maintaining currency.

Donor sources with a history of inherited diseases (or family history of inherited disease) should not be used unless the particular disease is ruled out in the donor cells by standardized genetic tests. Considerable controversy surrounds the extent of genetic testing that should be done on hESC, but safety concerns mandate as extensive a characterization as possible within the law.

Sterility in handling and cultivation of cells before transplantation. HESC-derived cell therapies are likely to require prolonged expansion of undifferentiated cells, derived intermediate proliferative populations, and then further prolonged expansion and maintenance in culture of cells used for implantation, transplantation, or infusion. The prolonged expansion in the undifferentiated state is a major theoretical advantage of hESC over adult stem cells, since properly maintained undifferentiated hESC do not undergo proliferative senescence (Zeng and Rao, 2007) and since proliferation of adult stem cells in culture (especially hematopoietic stem cells) is limiting. Even if proliferation of adult stem cells in culture could be enhanced, primary adult stem cells will ultimately be limited as a source of cells for master cell banks because of proliferative senescence.

FDA has developed extensive regulation for collection and handling methods to ensure sterility and safety of the blood supply, and these regulations are applicable to collection and handling of hESC-derived cells. Similarly, pancreatic islets are cultured in some centers before they are infused, and the FDA regulations used to guide the islet culture facilities are applicable to hESC-derived therapies. All facilities involved in processing, storage, or distribution of the hESC-derived should be guided by the extant FDA rules for blood banking and islet transplantation (21 C.F.R. 601.12 and 18, 2006). These regulations cover personnel, equipment, supplies and reagents, and record-keeping. Maintenance of sterility during long, complex hESC differentiation protocols will be challenging compared to the shorter-term cultures used currently in clinical settings.

Contamination with xenogeneic products. A unique concern surrounding hESC-derived therapies is the potential for xenogeneic product contamination, since some protocols for hESC handling have included trophic mouse fibroblast feeder layers, bovine serum, and/or Matrigel, enzymes derived from non-human sources, and other medium additives derived from non-human sources. Transfer of non-human material with a cell transplant product could lead to severe immune reactions or to infection with non-human viruses or prions. Since undifferentiated hESC can be expanded without bovine serum or mouse feeder layers, and virtually all additives can be replaced with human products, these particular issues should not present a safety problem in the long-term, provided documentation of the provenance and complete handling history of the cells (Xu et al, 2001; Amit et al, 2004; Ludwig et al, 2004). For example, irradiated human fibroblasts, if feeder layers are necessary for the expansion/differentiation protocol, can provide trophic support equivalent to murine embryonic fibroblasts (Amit et al, 2003; Hovatta et al, 2003). In the future, then, use of materials in cell handling that can *possibly* contain infectious agents (such as collagenase from bovine sources) should be avoided, in favor of GMP-grade materials (CFR 7 (8):1581-1619). Matrigel, a murine tumor product, is used in many expansion protocols, and therefore can contaminate cell preparations with murine xenoproteins. Furthermore, the protein composition of Matrigel is not defined with poorly characterized lot-to-lot variation. Recently, newly-derived hESC lines were isolated and grown without Matrigel by substituting human laminin as the matrix (Fletcher et al, 2006). (Geron's master bank was set up with Matrigel in the protocol, but was FDA approved.) If human serum is used in expansion of human embryonic stem cells, the serum should be clinical grade, subject to the same testing as clinically available blood products. However, human serum can be avoided in many protocols, replaced with defined factors in the medium (Skottman et al, 2006).

Replacement of all medium and culture components with human products or chemically defined products that carry zero infectious risk is currently prohibitively expensive. Furthermore, the commercial sponsors nearing ability to enter clinical trials all used NIH registry lines exposed to non-human species reagents in manufacturing their cell products. For these cell products, extensive testing for all known murine/bovine retroviruses should be sufficient to ensure safety of the products in terms of transfer of xenoviruses. Similarly, the immune xenoreaction to contaminant non-human proteins can be partially anticipated with individualized mixed lymphocyte reactions, using recipient patient lymphocytes and serum exposed to the final cultured cell product. The reactions are not standardized, and would have to be performed for each recipient separately. Nonetheless, properly designed and controlled in vitro reactions with

lymphocyte activation, proliferation, and secretion end-points should be able to anticipate adverse immunologic reactions to non-human proteins (Ubiali et al, 2007), and so, the use of non-human cells or proteins in the expansion of the transplanted cell product should not be an absolute contraindication to use of these cells clinically.

RESEARCH OPPORTUNITIES:

1. Efficient expansion/differentiation methods for hESC without non-human products
2. GMP-compatible scale-up procedures for growth and differentiation of hESC products
3. In vitro assays to detect immune responses to non-human protein contaminants

Non-living adjuvants to cell therapy. In order to contain cells at a particular site in the body, the stability of the cellular implant may require engineered scaffolds, including matrices or hydrogels (Hannouche et al, 2007; Potter et al, 2008). These agents must undergo the same sterility and safety testing as the cell products (and are addressed in CFR regulations). Pre-clinical and animal studies are necessary to determine the patterns of proliferation, cell death, and differentiation of hESC-derived cells in the presence of these scaffolds, and the effect of the scaffolds on the host immune system, and the functional lifespan and biodegradation patterns of the scaffold.

Purity/identity. The identity of each hESC-derived cell type and the representation of this cell type in the transplanted cell population (purity) must be established for normalization across clinical trials and clinical applications. The presence of undifferentiated hESC in the final transplanted cell product is a particular concern of regulators, because the undifferentiated cells can generate teratomas after transplantation (see below). The absence of undifferentiated stem cells in the transplanted cell product must be documented and the sensitivity of the assay for detecting undifferentiated cells validated. Heterogeneity in gene expression is expected even in 'characterized' cell populations. Furthermore, effective therapies can emerge from cells that are not 100% pure. (For example, hESC-derived cardiomyocytes where only 70% of the differentiated cells were cardiomyocytes, are effective in treating experimental rodent heart disease.) There may also be a clinical benefit to treating well-defined ratios of mixed cell types (for example immature neurons mixed with immature oligodendrocytes), but the numbers and proportions of these cells in the transplant must be characterized completely.

Therefore, it is critical that investigators work with FDA regulators to develop a rigorous consensus for purity and identity of cells differentiated from hESCs, and as new (better) markers emerge as consensus cell identity markers, they should be incorporated into the quality control protocols for manufacture. Identity is critical in order to quantify dose-response relationships. The identification of cells for transplant will include selected positive markers (markers associated with a specific stage of differentiation and specific differentiated function of the cells) and negative markers to ensure absence of unwanted cells (undifferentiated hESC or other undifferentiated, proliferating populations). Negative markers should also guarantee absence of immunologically competent cells in the mixture, to prevent graft vs. host disease. Product is characterized and released based on acceptance criteria that should include purity and impurity

profiles, product integrity/viability under different conditions, identity by markers and functional assays.

Identity of the final product also implies that the cells are responsive to drugs that will be used either to treat the disease processes of the patients or as adjuvants for the cell therapy, including immunosuppressive drugs. So functional identity may involve assays such as electrophysiological assessment, secretion profiles in response to a drug, or contractile assays in response to a drug.

RESEARCH OPPORTUNITIES:
1. Non-toxic, efficient methods for sorting undifferentiated cells (GMP compatible)
2. Differentiation methods that guarantee loss of undifferentiated cells (GMP compatible)
3. HESC toxicity studies of necessary drug therapies in clinically relevant doses.

Potency. Both in vitro (efficiency of generating a particular cell type under defined culture conditions) and animal studies should be used to estimate quantifiable end-points of therapy attributable to hESC-derived cell transplants. It is important that clinical domain experts in particular disease processes are involved in evaluation of the pre-clinical animal studies, including expert subspecialty pathological evaluation. In making these considerations, we acknowledge that many animal models inadequately reflect human disease processes. (For example, pharmacologically-induced Parkinson's disease does not capture the entire spectrum of human Parkinson's disease, and macular degeneration is studied in animals that lack maculae. Certainly the psychologic ramifications of disease are not captured adequately in rodent models.) Whenever possible, pain behavior should be quantitatively analyzed in animal models after transplantation, even in disease processes for which pain is not a major reason for transplantation, because pain behaviors may be an indication of mass effects or inflammatory reactions. Animal models will have to be used extensively in preclinical hESC studies, for documentation of changes in the pathologic disease markers, functional outcome, and overall well-being (growth, weight, appetite, behavior, motility) of animals and the stability of these changes over months. Dose-response curves using varying numbers of cells as the 'dose' are not going to be possible or practical in many human clinical trials using hESC-derived therapies. Here animal models will be particularly useful in helping to define optimal doses for clinical trials.

In addition to serial pathologic examination afforded by animal models, some animal models can yield important information about transplanted cell fate – information that simply will not be accessible in human subjects. For example, imaging modalities are able to penetrate the short distance between the surface of the body and the heart to follow survival of marked cells transplanted into the myocardium (Swijnenburg et al, 2007). Similar resolution for human studies of cells transplanted into myocardium is currently not technically feasible. However, the use of imaging, if available, in animal models represents another use of animals that should be exhausted as part of both potency and safety evaluation of hESC-derived cell therapies. In particular, viability of cells transplanted into animals in parts of the body that are feasible to image over time is important pre-clinical information, and animal studies are critical to linking cell viability with specific outcome parameters.

Potency assays will also be dependent on site of injection. Animal models will be useful for determining the optimal delivery sites. For example, for stem cells targeted to skeletal muscle, intraarterial vs. intramuscular delivery of the same number of cells may have different quantitative effects on muscle regeneration.

Stability. Enzymatic treatment of cells for passaging results in some cell loss. Many clinical investigators note dramatic loss of cells after transplantation in animals, likely because cells survive only after finding appropriate niches and matrices, and attachment sites. Massive cell death at the time of implantation can cause localized acidosis and electrolyte disturbances; animal models should be used to develop methods that minimize acute loss of cells after transplantation (Robey et al, 2008). Long-term viability of cells is critical to mechanism in many applications, and long-term viability after transplantation can only be assessed in animal models. For example, the oligodendrocyte precursor cells developed by Geron for anticipated clinical application mediate their therapeutic effect by myelinating damaged neurons after spinal cord injury (Keirstead et al, 2005) and by release of neuroprotective factors. Loss of the transplanted cells over time in vivo may or may not result in loss of therapeutic benefit, depending on the major mechanism by which they exert their therapeutic effect. For these reasons, long-term stability of cells after transplantation is a critical pre-clinical end-point of analysis.

Scale up. Given the technical difficulties in safe expansion of hESC in culture, the ability to generate sufficient banks of cells for clinical use is a technical challenge. GMP methods of large scale fermentation applications were developed for suspension cultures, and hESC are generally maintained and differentiated in monolayers. Recent technologic advances using murine cells suggest that undifferentiated ES cells can be grown in suspension (Andang et al, 2008), that the size of EB can be engineered in culture (Carpenedo et al, 2007), and that the size of EBs is a critical factor in their differentiation pattern (Bauwens et al, 2008). For clinical application of hESC, such new approaches to growing hESC will have to be developed and standardized, and these kinds of tools will require collaborative efforts of biologists and engineers. The inability to sort undifferentiated cells from mixed populations without significant loss of cells also creates challenges for scale-up and purity. HESC are difficult to get into single cell suspension, and do not tolerate single-cell manipulation well. Cell sorting also presents special challenges for maintaining sterility of cells, but the precedents for cell handling developed for bone marrow transplantation can be helpful in designing clinical sorting protocols. Furthermore, identification of antibodies that are toxic to undifferentiated but not differentiated ES cells suggests that active exclusion of undifferentiated cells, rather than just reliance on differentiation protocols, may be technically feasible (Choo et al, 2008).

Scale-up of large numbers of defined differentiated cells is a recognized hurdle for translation of hESC-derived therapies (Li et al, 2008).

RESEARCH OPPORTUNITIES

1. Expansion/differentiation methods that result in improved viability/efficiency than current methods
2. Methods to increase survival of cells after transplantation (including identification of factors that can increase survival at the time of transplantation)

3.Optimized large-scale suspension cultures of undifferentiated and differentiating hESC
4.Optimized cryopreservation procedures, including those that allow for direct injection after thawing
5.Development of hESC-specific GMP protocols and hardware

Chromosomal instability of hESC in culture. Chromosomal instability (Buzzard et al, 2004; Draper et al, 2004) in long-term cultured hES cells is a concern that has not been addressed by FDA guidelines to date, because fetal or adult cell therapies are generally not extensively manipulated in vitro, while hESC are prone to chromosomal instability during prolonged culture. Culture conditions may contribute to karyotypic instability, though investigators disagree strongly about the frequency and causes of karyotypic instability in cultured hESC. For example, some investigators believe that manual (microdissection) passaging of hES cells is essential to maintenance of normal karyotypes (Mitalipova et al, 2005). Some investigators have pointed to particular medium formulations as causes of karyotypic instability (Denning et al, 2006), and others have noted that lowered oxygen levels in culture are associated with less chromosomal instability (Forsyth et al, 2006). Undifferentiated hESC are dependent on adherence to a matrix to maintain their integrity, though the optimal matrix has not yet been identified. Enrichment of hES cells with ability to grow directly on plastic (without matrix coatings) selects for cells with abnormal karyotypes (Imreh et al, 2006).

Collectively, these studies suggest that the details of expansion protocols used to grow undifferentiated hESC can affect the stability of their genome, and that the precise details to optimize stability have yet to be defined. The studies also suggest that karyotypic stability is achievable with current methodologies, at least using certain cell lines and in the hands of experienced investigators. However, simple karyotyping will not pick up potentially important (tumor-mediating) mutations that emerge in long-term culture. Similarly soft agar assays are gross assessments of transformation, and inadequate. Importantly, careful mutation analysis of hESC and validation of the mutation-associated tumor risk after transplantation has not been done. Neither has a comparison of the incidence of karyotypic instability (or other mutations) in expansion of adult stem cells vs. that in adult stem cells derived from human embryonic stem cells been performed. Collectively, animal studies in which cells derived from hESC were transplanted have not uncovered an obvious propensity for tumor formation (other than teratomas), but in consent processes the risk of tumor formation must be communicated as an unknown. Safety studies should be conducted with the latest passage hESCs intended for production of the hESC-derived cell product for clinical trials.

RESEARCH OPPORTUNITIES:
1.Optimal methods to maintain chromosomal integrity of hESC during expansion
2. Identification of common mutations acquired in hESC culture and their consequence
3. Novel methods to identify pathologic growth of transplanted cells in vivo.
4.Development of suicide genes appropriate for clinical transplantation

Teratoma formation. Teratoma formation from transplanted undifferentiated hESC into immunocompromised mice is a standard assay of pluripotency of the cells. Recipients of hESC-derived therapies will likely require immunosuppression, increasing the risk for teratoma (and

other tumor) formation. Teratomas, though 'benign' can cause harmful mass effects in closed compartments like the CNS, and CNS sites of transplantation will not be accessible for protocol biopsies like those done in solid organ transplantation. Furthermore, CNS is considered extremely permissive for teratoma formation (Lebkowski J, FDA meeting) and any teratoma formation in CNS as a consequence of hESC-derived cell transplants would be disastrous for individual patients (since the tumors will not be surgically resectable) and for the field of stem cell transplantation generally. For this reason, exclusion of undifferentiated stem cell populations using sorting is an important research goal for lowering tumor formation after transplantation (Shibata et al, 2006; Fukada et al, 2006), though technically not feasible at this time.

The ongoing proliferative potential of hESC-derived transplanted cells (exclusive of undifferentiated hESC in the population) also raises concern that intermediate progenitor cells may also be sources of inappropriate proliferation, even when the cells have not undergone transformation (Kim et al, 2002). Ability to predict (and control) proliferation after transplantation, independent of transformation, is particularly critical for some applications where mass effects alone are detrimental. Again, the methods used in culture may impact later tumor-forming potential of the cells after transplantation. For example, murine ESCs differentiated as substrate adherent neural aggregates did not generate tumors over a 4-month follow-up following transplantation into rodent brain, a significant improvement over previous differentiation protocols (Dihne et al, 2006). Data from this study suggest that the improved safety was due to a purer (or more differentiated) population of neurons generated using the adherent aggregate culturing method, without complete elimination of the nestin-positive progenitors.

The issues of teratoma formation dominated discussion of safety of HESC-derived therapies at the April 2008 FDA meeting. The practical question of how long animals should be monitored after hESC-derived cell transplants was addressed but not answered. On the one hand, some consultants felt that years and years of animal follow-up were appropriate and must be done to allay concerns about teratoma formation in patients. On the other hand, animal models (including non-human primates) may not accurately predict tumor formation in patients. Further, the pressing need for therapies of some diseases combined with reasonable efforts to exclude undifferentiated stem cells from the final cell product leads some consultants to suggest a shorter, defined time for monitoring animals for teratoma formation is appropriate. Further complicating this discussion, the anatomic location of transplanted cells and the disease target are also important factors in this consideration. For example, if cells are transplanted in a site where (in theory) they can be retrieved if a complication develops, then following animals for many years as a preclinical prerequisite seems unnecessary. In terms of disease target, patients with ALS (and regulatory agencies) may be willing to accept a higher risk of teratoma formation and only months-long follow-up of animals, because life-span is so shortened by ALS, whereas stable spinal cord injured patients with long life expectancy may reasonably expect longer follow-up of animals to feel comfortable about the risk of CNS teratomas caused by a cell therapy.

What is reasonable for CIRM to recommend as far as the thorny question of length of time animals should be followed for teratoma formation after transplantation of hESC-derived cells (that may contain a few undifferentiated cells)? For all anticipated clinical applications, it is reasonable to expect that the latest practical methods to exclude undifferentiated hESC from the final cell

product are adapted in the differentiation procedures. Months of follow-up of animals transplanted exactly as patients would be transplanted (same site, same technique, same immunosuppression) is also reasonable, six months of tumor-free graft survival, for example. Such a recommendation recognizes that the near future is not likely to yield the critical information necessary to make a scientifically-grounded decision; no matter how long animals are free of teratomas after transplantation, some teratomas may form in patients. For this reason, further research on the kinetics of teratoma formation and methods to prevent and treat the tumors should be priorities for research. Furthermore, clinical assays should be developed to detect early tumor formation (Lawrenz et al, 2004) analogous to the AFP assays used to follow patients with end-stage liver disease for development of hepatocellular carcinomas.

RESEARCH OPPORTUNITIES

1. Better estimates of numbers of cells that can lead to teratoma formation over time, kinetics of tumor formation
2. Immunologically humanized animal models to study teratoma formation
3. Identification of the factors that are permissive for tumor formation in particular environments
4. Development of clinical assays to detect early teratomas

The fate of infused stem cells in humans cannot be followed directly in patients. This gap in ability to directly monitor cell transplants is shared by all stem cell populations in clinical applications, especially if the cells are delivered into the circulation. For solid organ transplants, biopsies are a gold standard for diagnosing rejection, not an option for most stem cell applications. Clinically, hematopoietic stem cells delivered intravenously (bone marrow transplants) eventually establish a niche in bone marrow, but monitoring of this process is indirect, relying on recovery of peripheral blood counts. An enormous literature also shows that marrow-derived stem cells can fuse with a variety of cell types including peripheral nerve and hepatocytes, and the stimuli for fusion are not known. Stem cells generally are migratory which further complicates tracking after infusion or transplantation. Similarly, differentiation patterns (and transdifferentiation)--the phenotype of transplanted cells--and survival of cells cannot be followed <u>directly</u> in most anticipated clinical applications, but must be inferred from clinical examination and laboratory studies.

Migration of stem cells is not well-understood, and current technologies do not afford control over migration of cells after transplantation. For some applications, migration is undesirable (stereotactic transplantation into a particular brain site) and for some applications migration is highly desirable (migration of muscle stem cells to muscle throughout the body after injection into the circulation). The knowledge gap surrounding migration of stem cells is also not likely to be solved before clinical application of hESC-derived therapies, but also deserves considerably more research.

RESEARCH OPPORTUNITIES

1. Development of labeling techniques to follow stem cells after infusion/transplantation in animal models (and clinically)
2. Migration assays, molecular mechanisms that control migration of stem cells

Immunogenicity. Currently commercial entities are testing adult mesenchymal stem cells that the sponsors have been able to demonstrate are immune privileged in allogeneic transplantation, and may be immunosuppressive in the special clinical setting of graft vs. host disease (Osiris). Undifferentiated human embryonic stem cells express low levels of major histocompatibility (MHC) class I proteins and undetectable levels of MHC class II proteins (Drukker et al, 2002), and MHC complex is the major player in solid organ transplant rejection. However, cytokines can elicit upregulation of MHC class I in hESC (Drukker et al, 2002), and cytokines may be present in the disease microenvironment. More importantly, differentiated cells derived from hESC would be expected to express the MHC class I and II molecules usually expressed in the differentiated lineage. Not surprisingly, MHC expression increases with differentiation of hESC (Drukker et al, 2002). The role of minor histocompatibility antigens (only recently being studied systematically in solid organ transplantation) in cell transplant immunogenicity is largely unstudied, but recent work suggests that minor histocompatibility antigens in ES cells can elicit a vigorous immune response (Robertson). A recent small study of islet transplant recipients suggests that autoreactive T cells may also be a significant mediator of cell graft rejection (Huurman et al, 2008) independent of alloreactivity. Recent studies also suggest that cells transplanted in the relatively immune privileged site (brain) are more likely to function well long-term if they are immunologically identical to the recipient (Tabar et al, 2008), pointing to a relative but not perfect immune privelege of the CNS. The complexity and unpredictability of the alloimmune response mean that human subjects should be treated conservatively, i.e. protected pharmacologically from cytotoxic immune responses to implanted hESC-derived cells, acutely and over the life of the cellular transplant, unless compelling evidence of tolerance can be demonstrated. Not all investigators agree on this point, and the Geron protocol, for example, incorporates withdrawal of immune suppression within a year of transplantation.

So, despite evidence for relative ease of inducing tolerance to ES-derived cells in mice (Robertson et al, 2007), extensive HLA testing is appropriate for both donor and recipient of hESC-derived cells. HLA typing should be performed as for solid organ transplantation (by immunology labs experienced in typing) for HLA-A, HLA-B, HLA-C, HLA-DRB, and HLA-DQB loci. Though this tissue typing may not alter the immune suppression regimen of the recipient (as is the case in liver transplantation), the typing will facilitate understanding of the immune response to hESC-derived cell therapies over time. Minor histocompatibility antigens should also be measured and studied in the first donors and recipients of hESC-derived therapies.

Based on the current state of knowledge, the safety of hESC-derived cell therapies across allogeneic barriers for many applications will require immune suppression, and transplant immunologists should be involved in developing the optimal drug regimen for transplanted human subjects. Methods to detect rejection without biopsy are needed to help guide and optimize immunosuppression regimens. Immune suppression is associated with significant side-effects including infection and malignancy, and so, informed consent for hESC-derived cell therapies must include informed consent for these potent drugs. In the long-term, the derivation and banking of an immunologically diverse collection of hESC lines could lead to wider use of hESC-derived cell therapies without the downside of pharmacologic immunosuppression.

Ideally non-pharmacologic strategies to induce tolerance to transplanted cells will be identified and translated. These include T cell depletion (Robertson et al, 2007), or use of hES-derived dendritic cells that are capable of inducing immunological tolerance when administered in advance of cell transplants (O'Neill, 2006) the use of hESC-derived hematopoietic cells with other hESC-derived cells for transplantation could also be used to develop therapies based on immune tolerance through mixed chimerism, an area of renewed interest in solid organ transplantation (Starzl, 2008). These and other tolerogenic strategies (cell surface modifications) should be a high priority area for research that will benefit solid organ as well as cell transplantation. Based on the current knowledge and practice of immunosuppression for solid organ and bone marrow transplantation, and the wide range of drugs available for immunosuppression, rejection of hESC-derived cell grafts is a manageable problem, but mandates involvement of transplant immunology specialists in the study teams. Side-effects of life-long immunosuppression must be weighed against the potential benefits of cell transplantation. Withdrawal of immunosuppression after patients have stabilized should also be done cautiously, and only if a clear end-point of rejection can be measured. At first, the assays for rejection will have to be adopted from the solid organ transplant literature, and such assays are currently not validated (Stordeur, 2007).

Experience with solid organ transplantation suggests that the design of Phase I studies for cell-based therapies should incorporate distinct plans for acute rejection and for chronic rejection.

RESEARCH OPPORTUNITIES

1. Identification of optimal immunosuppression pharmacologic regimens for particular differentiated cell types.
2. Tolerogenic strategies for replacing pharmacologic immunosuppression.
3. Development of non-biopsy based assays for cell transplant rejection.

The disease environment. Animal models of disease are generally more homogeneous than the clinical counterpart diseases. Quite commonly, animals used in preclinical studies are young, and many patients requiring cell therapies may be older, subjecting cells to an oxidized environment (Jones et al, 2002). Some disease targets also cause significant oxidant stress systemically, notably diabetes (Goh and Cooper, 2008), and in the diseased tissue, such as muscular dystrophy and Parkinson's disease (Tidball and Wehling-Henricks, 2008; Olanow, 2007). Ideally pre-clinical studies will subject cells to an environment that mimics the disease environment into which they will be transplanted. Each disease-induced environment must be considered carefully with the help of domain experts. For example, the inflammatory nature of end-stage liver disease promotes carcinogenesis, and hepatocytes transplanted into that environment would be subject to the pro-carcinogenic signals, whereas end-stage renal disease is not associated with massively increased renal tumor risk. Autoimmune processes that cause disease may recur in transplanted cells. Some disease environments may be so toxic for transplanted cells that adjuvant therapies to down-regulate pathologic signals may be needed, and clinical research to further define the pathologic signature at various stages of disease should be considered as part of 'disease team' strategies.

RESEARCH OPPORTUNITIES

1. Development of better animal models of disease
2. Identification of key pathologic signals in specific disease microenvironments

Clinical considerations in the design of Phase I trials.

Efficacy end-points. Because of the novelty and risks inherent in hESC-derived cell therapies, ideal design of a Phase I trial will go beyond safety issues, and include some end-point(s) of therapy. A Phase I/II design has the potential to speed translation. The end-points must be quantitative and objective, as stem cell therapy is associated with a significant placebo effect (Roncalli et al, 2008). The precise characteristics of the patient population chosen for these studies are critical. For example, at the FDA hearings, concerns were raised that patients with a complete transection of the spinal cord (ASIA A lesions) were less likely to have a positive therapeutic response to cell therapy than patients with incomplete spinal cord lesions. (Complete lesions are less likely to respond to any other interventions when compared to incomplete lesions.) Nonetheless, finding a homogeneous population of diseased subjects, for which any therapeutic improvement can be rigorously attributed to cell therapy and not to natural variation in the disease process, is a challenge for the first Phase I/II studies. In some cases, a well-characterized database may be used to judge therapeutic response of an individual transplanted patient against historical standards. (For example, the ALS research community has a large and useful database). Professional psychiatric evaluation of patients may be extremely useful in determining which patients are best able to participate in detailed examinations during clinical trials.

Animal studies that lead to clinical trials should show both statistically significant and biologically significant differences in an objective end-point of disease, since subtle changes in disease progression are not enough to warrant risks of hESC-derived therapies. For example, a large number of patients have received mesenchymal stem cells for experimental treatment of myocardial infarction. Collectively the studies seem to indicate a short-term but small improvement in left ventricular ejection fraction. The small change in ejection fraction is statistically significant, but may not be biologically significant, and would not justify hESC-derived (more risky) therapy. A single animal study also is generally insufficient justification for proceeding to hESC-derived therapies, and ideally, several studies are performed in different labs in pre-clinical work.

Thus the choice of clinical end-points of therapy must be quantitative, designed by clinical experts to facilitate population analysis of therapy, and importantly, pre-clinical animal studies whenever possible should be analyzed using the same clinically-relevant quantitative end-points. The animal model (stage of disease) should mirror as closely as possible the patient population that will receive the cell therapies.

Ongoing treatment for the targeted disease. For most applications of hESC-derived therapies, the medical regimen of the patients will be continued in parallel with the cell therapy. For this reason, sponsors are obligated to determine that the common drug therapies used for a particular disease process are not toxic to the cell product. If anesthetics are needed for surgical placement of cells, the anesthetics should be standardized across patients, and also tested on the cells in preclinical studies. In addition, the design of immunosuppression regimens must take into account the pharmacologic interactions between the immunosuppressants and the other drugs the patients will receive. Ideally, drug regimens are standardized across the patients in Phase I trials.

Choosing the first patients for hESC-derived therapies. The translation of hESC to the clinic will certainly be driven by the sponsor who develops a suitable cell product first. But all of stem cell biology will be affected by the outcome of the first studies, and adverse events in the first Phase I trials could hamper the field for a long time. For this reason, the ideal first applications of hESC into clinical therapies are those in which the cells do not have to integrate into tissue in order to perform their therapeutic function (for example, encapsulated cells performing a hormonal function for diabetes, or a synthetic function for hemophilia), and are retrievable. Cells which can be retrieved if they do not function properly will be considered to have a safer risk profile than cells which have to integrate into tissue to mediate their therapeutic response.

Other specific considerations for Phase I trial design. It is hard to generalize about trial design since the trials will be disease-specific and each disease presents unique considerations for trial design. However, some general considerations for pre-clinical studies are likely to be uniformly required before regulatory bodies will agree that safety concerns have been adequately addressed:

1. Surgical procedures-The precise equipment and procedures to be used in patients must be tested in animal models that are about the same size as the human recipients. All details for surgery (training of surgeons on specialized equipment, bore of needles, flow and injection rates, optimal delivery site including stereotax axes, need for perioperative antibiotics and analgesics) should be worked out in detail in appropriate animal models.
2. Quantitative pain testing-For any CNS application, and for patients at risk for peripheral neuropathies as part of their underlying disease (diabetes, MS), experts in quantitative pain measures should evaluate patients before cell therapy and periodically afterward. (Unfortunately in the U.S., these specialists are not easy to find.)
3. Long-term follow up of patients should be part of the consent process for any hESC-derived cell therapies

Conclusions. CIRM is an advocate for the translation of hESC-derived cell therapies, but not at the expense of patient safety. We believe the current foundations of pre-clinical and basic stem cell science combined with decades of international experience with solid organ transplantation provide a data set sufficient for establishing consensus goals defining the necessary preclinical studies and clinical protocols to guide safe Phase I analysis of hESC-derived cell therapies. Rigorous, quantifiable safety and therapeutic end-points are essential, especially because the mechanism of action underlying hESC-derived cell therapies for many applications is not completely understood. In addition to evaluating the effectiveness of hESC-derived therapies in replacing a missing physiologic function lost with a disease process, well-designed Phase I trials will critically analyze patients for systemic complications of both the cell transplants and the adjuvant therapies.

Finally, the **basic ethical principles** underpinning research using human subjects must guide the planning of Phase I trials for hESC-derived cell therapies: respect for human dignity, human rights, justice, autonomy, equity, and beneficence. Consent processes should acknowledge gaps in knowledge that could lead to adverse events, and follow privacy and confidentiality standards for information exchange, as outlined in HIPAA legislation. Every reasonable effort to minimize risk

to human subjects is expected. Since the disease processes to which hESC-derived therapies will first be applied are disease with significant mortality/morbidity for which few therapeutic options exist, time is available for a staged consent process, allowing patients and their families time to adequately absorb the possible risks vs. benefits in proceeding with cell transplant therapy.

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