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Prospects for Pluripotent Stem Cell Therapies: Into the Clinic and Back to the Bench

Laura Grabel*
Biology Department, Wesleyan University, Middletown Connecticut 06450-0170

ABSTRACT

Pluripotent stem cells, embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, both hold great promise for the understanding and treatment of disease. They can be used for drug testing, as in vitro models for human disease progression, and for transplantation therapies. Research in this area has been influenced by the ever-changing political landscape, particularly in the United States. In this review, we discuss the prospects for clinical application using pluripotent cells, focusing on an evaluation of iPS cell potential, the continuing concern of tumor formation, and a summary of in vitro differentiation protocols and animal models used. We also describe the current clinical trials underway in the United States, as well as the ups and downs of funding for ES cell work. J. Cell. Biochem. 113: 381–387, 2012.

KEY WORDS: EMBRYONIC STEM CELL; INDUCED PLURIPOTENT STEM CELL; CLINICAL TRIAL

Since the generation of the first human embryonic stem (ES) cell lines in 1998 [Thomson et al., 1998], the design and implementation of pluripotent stem cell-based therapies has been a major goal of researchers and clinicians. We have witnessed major advances, including the ability to "reprogram" adult somatic cells to return to their pluripotential roots (induced pluripotent stem (iPS) cells) [Takahashi and Yamanaka, 2006] and the first Stage I clinical trials using human ES cells [Alper, 2009; Wadman, 2011]. But this progress has been countered by a continuing threat to government funding for human ES cell research, particularly in the United States [Gottweis, 2010]. This threat is largely based on ethical concerns about the moral status of the human embryo, voiced predominantly by religious groups. These concerns have recently gained a new foothold in the form of legal challenges to federal funding for research that uses human ES cells [Levine, 2011].

In human ES cell research, ethics, and science are intimately entwined. Arguments based upon ethical considerations have clearly influenced laws and guidelines for doing human ESC work. The scientific research can also influence the ethical debate. For example, if iPS cells can truly replace ES cells for clinical application, there may be no need to use human embryos as a stem cell source for transplantation therapies [Scott et al., 2011]. This could make justifying human ES research more challenging. If the iPS cells are not the equivalent of ES cells, or are unable to provide clinical grade material, then stronger arguments can be made for continuing to support government funding of human ES cell research [Pera, 2011]. In this review, we describe the current state of progress towards human pluripotent stem cell therapies, with a focus on remaining impediments, scientific and political.

iPS CELLS: CAN THEY SOLVE THE "EMBRYO PROBLEM" AND PROVIDE GENETICALLY COMPATIBLE MATERIAL?

Until recently, the prevailing dogma in the field of developmental and stem cell biology was that progression from a pluripotent cell to a terminally differentiated somatic cell was a one way street, unless the nucleus from the somatic cell was transplanted into an oocyte [Gurdon and Melton, 2008]. The work of the Yamanaka and Thomson groups demonstrated that it is possible to reverse direction and "reprogram" a terminally differentiated cell back to the ground state of pluripotency simply by overexpressing a cohort of transcription factors: Oct4, Sox2, Klf4, and c-Myc [Takahashi and Yamanaka, 2006]; or Oct4, Sox2, Nanog, and Lin28 [Yu et al., 2007]. Since those reports, a flurry of activity has demonstrated the robustness of this phenomenon, using a variety of gene delivery systems ranging from viral vectors, to modified RNA, to small molecules [Hanna et al., 2010]. The exact molecular mechanism of reprogramming, however, remains somewhat undefined. We know that it is generally inefficient, and takes weeks, likely involving a number of stochastic events that lead to erasure of the epigenetic

*Correspondence to: Laura Grabel, Biology Department, Wesleyan University, Middletown CT 06459.
E-mail: lgrabel@wesleyan.edu
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state of the starting somatic cell [Bibikova et al., 2008; Hanna et al., 2010]. Intermediate, partially reprogrammed cells have been identified and a variety of somatic cell types have been reprogrammed, including terminally differentiated B cells [Hanna et al., 2008].

How do we know whether a given iPS cell line is pluripotent? iPS pluripotency has been tested using a variety of assays, including the ability to differentiate in vitro, the tendency to form teratomas when transplanted to ectopic sites in the mouse, and the capacity to contribute to chimera formation, including germline integration, following blastocyst injection [Jaenisch and Young, 2008]. Some iPS cell lines have even passed the most stringent test of pluripotency available, making a whole mouse using tetraploid complementation [Zhao et al., 2009]. In this assay, the cells whose developmental potential is to be tested are aggregated with blastomeres from a tetraploid embryo. The tetraploid blastomeres contribute only to extraembryonic trophectoderm, while the added pluripotent stem cells reconstitute the entire inner cell mass, and therefore the embryo.

But a number of studies have raised concerns about how closely iPS cell lines resemble ES cell lines and whether they can be safely brought into the clinic [Pera, 2011]. Genomic analyses comparing iPS and ES cell lines looking at a variety of parameters, including transcriptome and epigenome, note differences, which some interpret as significant and others as not [Pera, 2011]. iPS cell lines appear to be more subject to genomic instability than ES cells, with a higher rate of point mutation [Gore et al., 2011] and copy number variation [Hussein et al., 2011], for example. The ability to differentiate into specific lineages appears to vary more amongst iPS lines than amongst ES lines. This observation may be attributable in part to what has been termed ''epigenetic memory,'' with iPS cells derived from a particular lineage able more readily to recapitulate that lineage [Kim et al., 2010].

Additional concerns suggest iPS cells are far from clinic-ready. Many of the gene cocktails of reprogramming factors include oncogenes, and their over-expression has led to tumor formation when derivatives of these cells are subsequently transplanted into mouse hosts [Jaenisch and Young, 2008]. In addition, most protocols for generating iPS cells use viral vectors that alter the genome in a way that may affect expression of key genes, and therefore put the potential patient at risk. Attempts to minimize or eliminate genetic modification by reprogramming using small molecules or RNA [Sidhu, 2011], for example, are meeting with some success and may in the future allow generation of safer iPS cell lines.

The ability to tailor-make a patient specific iPS line could eliminate concerns about immune rejection of iPS-derived material, since the cells would be genetically identical to the prospective recipient. But recent studies, examining teratoma formation by mouse iPS cells injected into syngeneic hosts, revealed that the cells were able to elicit immune rejection, perhaps due to their genetic modification [Zhao et al., 2011]. Further investigation is required to determine the reason for the unexpected immune response. Even if patient-specific iPS cells could be generated, it is worth asking whether making a cell line for each individual to be treated would be efficacious or cost-effective, particularly given the difficulty in generating iPS lines and demonstrating they can consistently differentiate into the cell types needed for cell replacement. Others have suggested instead a human pluripotent stem cell bank approach, in which the major histocompatibiity loci would be effectively represented [Taylor et al., 2005; Yamanaka, 2010]. For now, cell replacement strategies using iPS cells await modification of protocols currently used for their generation, and therefore ES cells continue to provide a safer alternative.

iPS cells, however, are currently being successfully used to produce patient-specific models for a variety of diseases, including ALS and Angelman's syndrome [Colman and Dreesen, 2009]. Differentiation of disease-specific iPS cells can uncover the molecular mechanisms and cellular basis of the disease state and be used for drug screening. This application will likely continue to be fruitful whether or not these cells are used for cell replacement therapies.

TUMOR FORMATION IS STILL A CONCERN

Pluripotent stem cells are highly proliferative and can readily form teratocarcinomas, which contain both undifferentiated ES cells and derivatives of all three primary germ layers, when transplanted to mice. As described above, the ability to form these tumors is in fact a hallmark of pluripotency. This property presents a serious concern when thinking about using derivatives of these cells for transplantation into animals and especially human recipients [Fujikawa et al., 2005].

Ideally, prior to transplantation, the cell population should be free of undifferentiated stem cells. This can be accomplished by positive selection for the cell type to be transferred, or by negative selection against undifferentiated pluripotent stem cells, or ideally, by a combination of both [Hentze et al., 2007; Tang et al., 2011]. A number of cell sorting approaches, including fluorescence-activated cell sorting (FACS), may be used. A number of studies have demonstrated a decreased level of teratocarcinoma formation following these steps [Fukuda et al., 2006; Ko et al., 2009]. Alternatively, treatments that selectively induce apoptosis or limit growth of undifferentiated ES cells within a transplant can be used [Bieberich et al., 2004; Moretto-Zita et al., 2010], although this after-the-fact approach is less desirable.

The reality, however, is that most groups preparing ES cell-derived material for transplantation rely primarily upon efficient differentiation protocols that do not necessarily include a positive or negative selection step for eliminating ES cells. This approach may be sufficient, but it is essential to monitor the cell population carefully prior to transplant to determine if any undifferentiated ES cells remain [Germain et al., 2011]. Immunocytochemistry or flow cytometry for ES cell-specific markers is the most often used technique used to make this determination. In addition, the presence of an additional pluripotent cell population, epiblast-like cells [Cai et al., 2008], must be examined. The inner cell mass of the blastocyst stage embryo differentiates into epiblast, which will make the embryo proper, and hypoblast, which will contribute to extraembryonic yolk sacs. Epiblast cells are pluripotent and can produce derivatives of the three primary germ layers, ectoderm, mesoderm, and endoderm, but can no longer generate most extraembryonic

tissues. Epiblast cells are present in differentiating ES cell cultures and, most relevant here, can generate teratocarcinomas after transfer in vivo [Germain et al., 2011]. Therefore, efforts should be made to determine whether they are present in cell populations, and if so, these cells must be removed prior to transfer in vivo.

What is an acceptable level of pluripotent stem cell contamination in a therapeutic sample? Using animal models, the minimum number of ES cells required to promote tumor formation ranges from 2 to 100,000, depending upon the transplantation site. Fifty thousand cells were required in the mouse heart [Nussbaum et al., 2007], whereas 400 cells were sufficient for tumor formation in the cerebral cortex [Harkany et al., 2004]. As we move into the clinic, of course, extra caution must be used [Goldring et al., 2011]. The stocks of cells for transplant to human patients should be well characterized and determined to be essentially pluripotent stem cell-free. In addition, long-term animal studies must have demonstrated that animals remain tumor-free for extended periods. Even with these guidelines in mind, the fear will remain that a missed pluripotent stem cell could lead to a teratocarcinoma in a patient. This is one of many reasons the current clinical trials using human ES cell-based approaches are being watched very closely by stem cell biologists, government regulators, and prospective users.

GENERATING THE DESIRED CELL TYPES: IN VITRO DIFFERENTIATION

Many protocols for generating specific types of progenitors and differentiated cell populations from pluripotent cells have been described [Murry and Keller, 2008]. Some general themes emerge: Look to the embryo and what has been learned about the molecular and cellular cues that direct the differentiation of the desired lineage in vivo, and apply these findings to promoting production of the desired cell type in vitro. This can involve treatment with specific growth factors and signaling molecules and their antagonists, or alternatively, gain-of-function approaches directing expression of lineage-specific transcription factors. Altering cell densities, as well as cell–cell and cell–extracellular matrix interactions can be key.

Despite the observation that mouse and human ES cells have distinct growth requirements, protocols that have been established for directed differentiation of mouse cells can usually be readily adapted for human cells and vice versa. Similarly, approaches designed for the differentiation of ES cells into specific lineages can be used with iPS cells, although as noted above, iPS cell lines may vary more amongst themselves than ES cell lines in their relative ability to produce cells of a specific lineage. Beyond their clinical potential, human ES and iPS cells provide valuable material for studying human development and differentiation, given the difficulty and ethical objections connected with working directly with human embryos.

Many cell types representing all three germ layers have been efficiently generated from pluripotent stem cells. Three types of general approaches are used to promote differentiation; production of an embryoid body intermediate, monolayer adherent culture with sequential growth factor treatment, or co-culture with appropriate support cells [Cai and Grabel, 2007]. Effective derivation of desired cell types frequently requires a combination of these approaches.

The relatively recent elucidation of conditions that support production of definitive endoderm precursors has facilitated the derivation of a variety of endoderm cell types with potential clinical relevance, including liver hepatocytes and endocrine pancreatic β-cells [Murry and Keller, 2008]. Mesoderm derivatives include cardiomyocytes, as well as hematopoietic and vascular cells. Numerous approaches have been used to generate neurectoderm derivatives and a variety of neuronal subtypes, including motor neurons [Wichterle and Peljto, 2008], inhibitory GABAergic neurons [Bibel et al., 2004], and midbrain dopaminergic neurons [Lee et al., 2000], that may well prove useful for treating neurodegenerative diseases [Germain et al., 2010].

Three recent examples that exemplify the continued interest in generating cell types that may prove clinically useful are described here.

(1) *Anterior foregut*: The anterior foregut serves as a source of progenitors for a number of lineages, including thymus, thyroid, parathyroid, and respiratory tissue including lung. Previous protocols used activin treatment to induce definitive endoderm, but these cultures contain a mixture of anterior (SOX2-positive) and posterior (CDX2-positive) endoderm progenitors [Green et al., 2011]. To direct production of anterior fates, a population of ES cell-derived definitive endoderm was treated with combination of the BMP antagonist NOGGIN and the activin/nodal and TGFβ inhibitor SB-431542 [Green et al., 2011]. Interestingly, these two inhibitors induce a neurectoderm fate when added to undifferentiated ES cells, as opposed to ES-derived definitive endoderm. Withdrawal of NOGGIN and SB-431542 from ES-derived foregut progenitors and subsequent treatment with WNT3a, KGF, FGF10, BMP4, and EGF plus retinoic acid induced expression of ventral markers, including those associated with lung differentiation. Again, prior exposure first to activin and subsequently to NOGGIN and SB-431542 was essential to obtaining this lineage. Replacing retinoic acid with SHH or FGF8 leads to a parathyroid fate.

(2) *Chondrocytes*: ES cell-derived chondrocytes could be used to treat degenerative cartilage diseases such as osteoarthritis. A high-density micromass culture protocol used to produce chondrocytes from limb bud mesenchymal cells has recently been successfully adapted for use with human ES cells [Gong et al., 2011]. High density, micromass culture of ES cells alone promotes some conversion to chondrocytes, but addition of BMP2 greatly enhances the efficiency, with most of the culture expressing chondrogenic markers. Interestingly, by 14 days, cultures have not undergone hypertrophic chondrocyte maturation, suggesting they may be effective at replacing articular cartilage, which does not undergo hypertrophic conversion and is damaged in osteoarthritis.

(3) *Cerebral cortex pyramidal neurons*: Recent analysis comparing the in vitro and in vivo differentiation profile of neural progenitors generated via an initial step involving either embryoid body intermediates or co-culture on MS5 stromal cells, led to distinct outcomes [Ideguchi et al., 2011]. While

dissociation and replating of cells generated under both conditions produced roughly the equivalent levels of GABAergic and glutamatergic neurons, only the MS5 co-cultured-progenitors expressed markers of layer 5 cortical projection neurons. When transplanted to the cortex of neonatal mice, only the MS5 cells were able to project axons to subcortical brain structures, and this was a robust phenomenon. Thus, the co-culture regime can promote distinct neuronal cell fates in vitro and in vivo.

ANIMAL MODELS: REPAIRING RODENTS

Preclinical testing for pluripotent stem cell therapies must include extensive verification in animal models. Numerous animal models that reflect a variety of human disorders that could be treated using cell replacement are available using rodents, although they vary in how accurately they mimic the human condition. Depending upon the specific disease or injury, it may be desirable to deliver the cells to a single site, or alternatively, to use an approach that facilitates broader distribution. Disorders that involve a focal lesion or deficiency, such as spinal cord injury, Parkinson's disease, or type I diabetes, would likely involve localized delivery of cells via a single injection site. Disorders in which affected tissues are broadly distributed throughout the body, such as muscular dystrophies or amyotrophic lateral sclerosis, may require multiple injection sites or systemic delivery, for example, via intravenous injection. Additional measures may be needed to target cells to specific sites, for example, using chemokines known to direct the migration of the grafted cell type [Hartman et al., 2010].

Whether progenitors that retain proliferative capacity, or terminally differentiated cells unable to divide, are the best candidates for therapeutic material will also depend on the specific condition. If the graft site is an ongoing degenerative environment, as is the case for several neurodegenerative diseases and type I diabetes, for example, a population that can self renew and provide a continuous source of differentiating material over time may be desirable. Specific progenitors can respond to local environmental cues and differentiate into the desired cell type. However, a proliferating progenitor population may be able to form tumors, as has been observed with pluripotent stem cell-derived neural stem cells [Chiba et al., 2008]. Alternatively, multiple applications of a more differentiated derivative may be preferable. These concerns highlight the importance of long-term follow-up using a well-described animal model.

In a number of cases it has been convincingly demonstrated that pluripotent stem cell-derivatives can reverse the disease state as manifested in the animal model. The two clinical trials currently under way in the US using ES-derived material, described in the following section, are based upon successful demonstration of significant benefits, without concomitant disadvantages, demonstrated in rodent models. Ample evidence suggests that in rodent models of spinal cord injury, transplantation of human ES cell-derived oligodendrocytes can provide improved locomotion [Keirstead et al., 2005]. These cells populate the site of injury and promote remyelination of the lesion. In two rodent models of retinal degeneration, the RCS rat and Elov14 mouse, human ES cell-derived retinal pigment epithelial (RPE) cells were able to provide long-term rescue of visual function, replacing the degenerating retina [Lu et al., 2009]. In both models, long-term survival was not accompanied by tumor formation.

Interestingly, FDA approval for these clinical trials was granted based upon preclinical testing in rodents, without demonstrating efficacy in a large animal model, for example, primates. The ability to move directly from rodent models to human testing is advantageous for at least two reasons. First, primate testing is difficult and costly, and appropriate models have not always been developed. Second, there are many who have ethical objections to the use of primates for medical testing [Bartlett, 2011].

CLINICAL TRIALS: A BEGINNING

It is an exciting time for regenerative medicine in the United States, with the first FDA-approved clinical trials using human ES cell derivatives for cell replacement therapies currently underway [Goldring et al., 2011]. The two differentiated cell types used, oligodendrocytes and RPE cells, are well characterized, GMP grade [Alper, 2009; Wadman, 2011]. As Phase 1 trials, the primary goal is to test safety of the approach [Goldring et al., 2011], but the efficacy of the approach in treating the disease/lesion will also be determined, though on a small number of patients.

SPINAL CORD INJURY (CLINICALTRIALS.GOV IDENTIFIER NCT01217008 AND NCTO1344993)

FDA approval was initially provided to treat grade A thoracic spinal cord injuries (T3-T10) using GRNOPC1, human ES cell-derived oligodendrocytes. Two patients have been treated with a dose of 2 million cells 7–14 days post-injury. Low-dose immunosuppression was transiently administered for the first several weeks post-surgery. No significant complications, including immune rejection, have been noted thus far. Transplant regions will be monitored via MRI and motor function will be assessed. The FDA has also approved treatment of T11 injuries and a shorter waiting time between patient recruitment. Pending safety verification in treated patients, the trial will expand to include cervical region injuries and injection of increased cell numbers.

STARGARDT MACULAR DYSTROPHY AND DRY AGE-RELATED MACULAR DEGENERATION (CLINICALTRIALS.GOV IDENTIFIER NCT01345006)

Stargardt macular dystrophy is the most common form of early onset macular degeneration, and leads to decreased central vision between ages 10 and 20. Dry age-related macular degeneration is the leading cause of blindness in people over aged 60. Both conditions are associated with the death of RPE cells and photoreceptors. FDA-approved protocols for these two trials call for the injection of differentiated human ES cell-derived RPE cells (MA09-hRPE) subretinally. Twelve patients will be enrolled in each trial, with the first three receiving 50,000 cells, and the dose increased if all goes well, to 200,000 cells for the last group. One patient has been

treated in each trial at this point. In addition to monitoring for adverse effects of the surgery, the trial will also determine if there is an improvement in vision.

The preclinical and clinical trial phases of these studies has largely been funded by private corporations, with Geron supporting the spinal cord trial and Advanced Cell Technology the RPE trials. This is an interesting departure from the early development and delivery of bone marrow-based transplants, which initially was not commercialized [Rao, 2011]. Arguments have been made that a combination of government and private sector funding would optimize the development of any medical technology [Gruen and Grabel, 2006], so it will be of interest to see where support for this work comes from in the future. State initiatives are now testing the waters to determine whether they can play an active role, particularly in light of the ups and downs that characterize federal funding for human ES cell research, described below.

FEDERAL FUNDING AND LEGAL CHALLENGES

Funding for pluripotent stem cell research, perhaps more than any other area of life science investigation, has been subject to the changing political climate in the United States [Gottweis, 2010]. In 2001, shortly after the generation of the first human ES cell lines, the Bush administration issued its guidelines, which restricted federal funding to work only on those dozen or so human ES cell lines available at that point in time. This restriction was limiting from both a scientific and practical perspective. For example, the available cell lines were all generated using animal products, and this contamination could have prevented their eventual clinical use. These lines also did not reflect all histocompatibility groups, so would not satisfy the need for genetically compatible material. The guidelines also led to complicated systems of accounting and management of supplies and equipment use. A laboratory or institution that received federal dollars as well as funding to work on non-approved cell lines needed to carefully document the use of separate supplies and equipment for work done with unapproved versus approved lines.

For these reasons, the lifting by the Obama administration of the restrictions imposed by Bush was eagerly anticipated by most stem cell researchers. The changes brought by the new president, however, were not as far-reaching as many presume. While federal funding could be used to work on lines generated after 2001, pending approval of these lines by the NIH, a number of restrictions still applied. Approved lines include only those made from excess embryos donated by couples using assisted reproductive technology. Federal dollars cannot be used to work on cell lines made from an embryo generated exclusively for research purposes, for example, carrying a specific disease-causing mutation or representing a specific histocompatibility profile. Most importantly, this funding cannot be used to generate new cell lines. This is because every year since 1996, congress has approved the Dickey-Wicker Amendment, a rider added to the Department of Health and Human Services appropriations bill each year since 1996 [Gottweis, 2010]. This amendment prohibits the use of federal dollars for experimentation on human embryos. This means that funding for generating new cell lines must continue to come from other sources, including private corporations, foundations, and state stem cell initiatives.

In the summer of 2010, a court case, brought by two somatic stem cell researchers, threatened to end all federal funding for human ES cell research. While considering the case, District Court Judge Royce Lamberth issued an injunction, which temporarily stopped federal funding of human ES cell research, claiming that the research violated the Dickey-Wicker Amendment. This ruling suggested that even funding work on the cell lines produced using alternative sources encouraged the destruction of human embryos, despite the fact that 10 years of NIH-funded research indicated that it was possible to separate the generation of cell lines, from their use. The plaintiff's had standing, and could bring the case forward, because they stood to lose out on potential funding for their adult stem cell research, since the NIH would be diverting its limited resources to supporting human ES cell work. Ultimately, in the summer of 2011, Judge Lamberth decided against the plaintiffs, but other law suits are threatened and the uncertainty about funding for human ES cell research has created a concern in the community that has had a tangible impact, for example, pushing scientists to redirect their research away from this controversial area [Levine, 2011].

CONCLUSIONS

As human pluripotent stem cell work crosses the threshold and enters the clinic, it is an excellent time to take stock. The field has made significant progress in understanding the conditions that promote the growth and differentiation of these cells in vitro. Multiple rodent models have been developed and can be used to test the ability of transplanted pluripotent stem cell derivatives to incorporate into and repair diseased or injured tissue. While tremendous advances have been made in our ability to generate iPS cells from a variety of sources, their instability and genetic variability suggest that these cells are not yet clinic-ready. This means we are not yet able to abandon ES cell research, if clinical application is a goal. But the future of funding for this work in the United States is uncertain given changing political landscapes and legal challenges. The uncertainty of federal funding is particularly problematic at a point in time when, due to fiscal constraints, pharmaceutical, and biotech corporations are cutting back on research and development. Some states, including California and Connecticut, have attempted to fill the gap, but these initiatives alone cannot be expected to provide the kind of support needed for a concerted effort at the national level. A collaborative approach, including the private sector, foundations, the federal and willing state governments, could allow for a more stable source of continuous funding [Rao, 2011]. Though such an effort would be difficult to mount, it may be essential to guaranteeing that the United States remains at the leading edge of this emerging medical technology.

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