Pluripotent Stem Cells in Research and Treatment of Hemoglobinopathies

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Pluripotent stem cells (PSCs) hold great promise for research and treatment of hemoglobinopathies. In principle, patient-specific induced pluripotent stem cells could be derived from a blood sample, genetically corrected to repair the disease-causing mutation, differentiated into hematopoietic stem cells (HSCs), and returned to the patient to provide a cure through autologous gene and cell therapy. However, there are many challenges at each step of this complex treatment paradigm. Gene repair is currently inefficient in stem cells, but use of zinc finger nucleases and transcription activator-like effector nucleases appear to be a major advance. To date, no successful protocol exists for differentiating PSCs into definitive HSCs. PSCs can be directly differentiated into primitive red blood cells, but not yet in sufficient numbers to enable treating patients, and the cost of clinical scale differentiation is prohibitively expensive with current differentiation methods and efficiencies. Here we review the progress, promise, and remaining hurdles in realizing the potential of PSCs for cell therapy.

Pluripotent stem cells (PSCs), whether embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), have the potential to form any cell type in the body. ESCs are derived from the inner cell mass of the mammalian blastocyst (Evans and Kaufman 1981; Martin 1981; Thomson et al. 1998), whereas iPSCs are somatic cells reprogrammed to a pluripotent state by exogenous expression of transcription factors responsible for conferring pluripotency on ESCs (Takahashi et al. 2007; Yu et al. 2007; Park et al. 2008b). Many diverse types of somatic cells that are readily obtained from patients—like skin fibroblasts, blood, and kerati-

nocytes—when transduced with Oct4, Sox2, Klf4, and c-Myc, will revert to a pluripotent state, albeit inefficiently (Aasen et al. 2008; Loh et al. 2009). Although the early reprogramming experiments were performed with retroviruses and lentiviruses, safer transgene-free reprogramming methods have since been developed that use synthetic mRNA or non-integrating episomes to express the reprogramming factors (Yu et al. 2009; Warren et al. 2010). In addition, although iPSCs are remarkably similar to ESCs in pluripotent function, many studies have identified subtle but potentially significant molecular differences, showing the need to continue to

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N. Arora and G.Q. Daley

study and improve reprogramming methods to achieve the closest facsimile to the naturally derived ESCs, which remain the gold standard (International Stem Cell Initiative et al. 2007; Kim et al. 2011). With regard to applications for hematologic disease, there appear to be differences in the differentiation potential of human PSC lines to form various tissues of clinical interest, particularly blood (Osafune et al. 2008; Choi et al. 2009). Lanza's group examined the blood differentiation potential of 22 human PSC lines generated at multiple institutions. The 14 human ESC lines tested showed higher hematopoietic colony forming unit (CFU) activity and a 1000-fold increase in erythroid differentiation efficiency, whereas the eight human iPSC lines displayed limited growth potential and decreased CFU activity (Feng et al. 2010).

DISEASE MODELING

PSCs are a powerful tool for modeling disease. Disease-specific human ESCs have been generated from embryos with genetic mutations causing diseases like thalassemia, Fanconi anemia, and cystic fibrosis (Pickering et al. 2005; Verlinsky et al. 2005; Mateizel et al. 2006; Ben-Yosef et al. 2008). However, affected embryos diagnosed through preimplantation testing are the prerequisite for deriving disease-relevant human ESCs, and because only a subset of predominantly Mendelian genetic disorders are subject to such testing, it is not feasible to obtain human ESCs for any specific disease of interest. On the other hand, to generate disease-specific human iPSCs, only a simple skin biopsy or blood draw from an affected patient is necessary (Park et al. 2008a; Soldner et al. 2009). Fibroblasts can be grown out of the skin biopsy or mononuclear cells collected from the peripheral blood sample and reprogrammed with the four reprogramming factors.

Disease-specific human iPSCs have already been generated for numerous diseases and are being used to model diseases in vitro. Human iPSCs generated from a patient with type 1 diabetes were differentiated into β -like cells that produced insulin and responded to glucose (Maehr et al. 2009). Parkinson's disease iPSCs were differentiated toward dopaminergic neurons, which

were functionally tested in a rat model of Parkinson's disease (Hargus et al. 2010). These studies and numerous others have shown the power of disease-specific human iPSCs in modeling diseases in vitro and represent compelling proof-of-principle for the potential of cell therapies.

HEMOGLOBINOPATHIES

Since the first demonstration of mutant hemoglobin more than a half century ago, hundreds of mutations in the α -globin and β -globin loci have been identified. Many mutations are silent or do not present a clinical phenotype, but a few are common and cause severe phenotypes. Two of the most common hemoglobinopathies caused by these mutations are sickle cell anemia (SCA) and thalassemia.

Sickle cell anemia, caused by an adenine-to-thymine point mutation in the sixth codon of the β -globin gene, changes a polar glutamic acid to a nonpolar valine (Ingram 1956, 1957). This subtle change predisposes hemoglobin to polymerization, which induces rigidity and structural deformations of red blood cells (RBCs), recognized by a characteristic sickling. Sickled RBCs cause vaso-occlusion, which leads to painful crises, organ damage, and premature death (Hofrichter et al. 1974; Crepeau et al. 1978; Dykes et al. 1979; Noguchi and Schechter 1981; Brittenham et al. 1985; Eaton and Hofrichter 1987).

Current treatments for SCA are limited. Hydroxyurea reduces the frequency of painful crises by lowering white blood cell counts, thereby reducing cellular sludging, and increasing production of γ -globin, a fetal hemoglobin, which reduces RBC sickling (Letvin et al. 1984; Platt et al. 1984). In the setting of disease complications, RBC transfusions can be used to temporarily increase the number of circulating normal RBCs, but frequent RBC transfusions result in sickle antibody sensitization and iron accumulation, which itself causes organ damage (Orlina et al. 1978; Coles et al. 1981; Davies et al. 1986; Reisner et al. 1987; Cox et al. 1988; Rosse et al. 1990; Vichinsky et al. 1990). Bone marrow transplants are a treatment option but are riskier than RBC transfusions, given the potential for graft failure and immunological complications like graft-versus-

host disease. Marrow transplant can provide a cure but remains an underutilized approach because of the considerable morbidity and mortality associated with such a heroic intervention.

Thalassemias are disorders related to imbalances in the production of the hemoglobin chains that can be caused by several different mutations. In α -thalassemia, production of α -globin chains is impaired, resulting in free β -globin polypeptides, which form an unstable hemoglobin molecule (Ingram and Stretton 1959; Ottolenghi et al. 1974; Taylor et al. 1974). In β -thalassemia, production of β -globin chains is defective, resulting in free unstable α -globin chains that are toxic to erythroid precursors (Fessas 1963; Fessas et al. 1965). As with SCA, treatment options are limited. In severe thalassemia (major), patients require regular transfusion, which comes with the risk of antibody sensitization and the complications of iron overload. Treatments like hydroxyurea, which increase fetal hemoglobin (HbF) levels, reduce the severity of β -thalassemia because they decrease the number of unpaired α -globin chains (Platt et al. 1984; Weatherall 2001; Koren et al. 2008). However, hydroxyurea is not effective in β -thalassemia patients because they need higher levels of HbF than SCA patients to ameliorate their symptoms (Alebouyeh et al. 2004; Yavarian et al. 2004). As with SCA, bone marrow transplant is a treatment option and the only chance for a cure; despite the risks of graft failure and graft-versus-host disease, it is frequently used for patients with thalassemia major.

HEMOGLOBINOPATHY DISEASE MODELS—IN VIVO AND IN VITRO

Two mouse models of β -thalassemia and one mouse model of α -thalassemia have been reported (Skow et al. 1983; Yang et al. 1995; Chang et al. 1996). These models have been used for numerous gene transfer studies to test the efficacy of lentiviruses containing various components of the human β -globin locus control region (LCR), promoter elements, and γ -globin or β -globin gene constructs (May et al. 2000, 2002; Imren et al. 2002; Nishino et al. 2006; Han et al. 2007; Lisowski and Sadelain 2007).

The studies show hemoglobin tetramers containing human β -globin at levels high enough to ameliorate the disease phenotype.

There are two humanized mouse models of SCA, the “Berkeley” mouse and the “Townes” mouse, which have proven valuable in studying disease mechanisms and testing therapeutic strategies, such as gene therapy (Pawliuk et al. 2001; Xu et al. 2011). The Berkeley mouse is a transgenic strain engineered to express normal human α -globin and γ -globin as well as β^S -globin, the sickle cell mutant (Paszty et al. 1997). The Townes mouse is a knock-in model, in which both the mouse α -globin and β -globin genes were replaced with the human genes. The β -globin locus contains $^A\gamma$ -globin and β^S -globin (Wu et al. 2006).

In 2002, the first proof-of-principle experiment for combined gene and cell therapy of blood disorders in a murine model was performed using an immunodeficient $\text{Rag2}^{-/-}$ mouse strain (Rideout et al. 2002). In this case, $\text{Rag2}^{-/-}$ ESCs were generated through somatic cell nuclear transfer, a method whereby somatic cells are reprogrammed to pluripotency via nuclear transplantation, creation of a cloned mouse embryo, and extraction of ESCs from the blastocyst stage. One allele of the Rag2 gene was repaired by homologous recombination in the ESCs, which were then differentiated toward hematopoietic precursors and transplanted back into the $\text{Rag2}^{-/-}$ mouse. Upon analysis, the engrafted mice showed mature lymphoid cells. In 2006, a similar experiment was performed with the Townes humanized SCA mouse model (Wu et al. 2006). Autologous ESCs were genetically corrected by homologous recombination, differentiated toward the hematopoietic lineage, and transplanted into the Townes mice. Analysis showed high levels of adult human HbA, reduced RBC sickling, and corrected pathology in the animals. In 2007 with the advent of iPSCs, the same experiment was performed with autologous iPSCs made from the Townes mice (Hanna et al. 2007). This was indeed an impressive demonstration of the potential for harnessing personalized stem cells for the treatment and potential cure of SCA. In a tour de force of modern cell and molecular biology, the Jaenisch

N. Arora and G.Q. Daley

group derived iPSCs from the murine model of human SCA, restored a normal allele of human β -globin through homologous recombination, then derived engraftable hematopoietic precursors through directed in vitro differentiation, and treated mice through hematopoietic transplant. Although to date the engraftment of mice with hematopoietic progenitors derived from ESCs/iPSCs remains less robust than from marrow sources, and in mice has required genetic modification of the cells, the therapeutic effect of even partial hematopoietic chimerism is quite dramatic for the hemoglobinopathies, owing to the longer half-life of the repaired cells relative to their diseased counterparts. Consequently, the peripheral blood of engrafted mice shows remarkable restoration of normal erythroid indices, compelling evidence for the potential therapeutic use of iPSCs in hemoglobinopathies.

Two groups have reported the generation of human iPSC models of SCA (Mali et al. 2008; Sebastiano et al. 2011). Zinc finger nucleases (ZFNs) have been used to correct the sickle cell mutation (Sebastiano et al. 2011). However, neither report differentiated the cells and functionally tested them to show sickling or a reduction in sickling after correction.

A handful of human PSC models for thalassemia have also been reported. Human iPSCs derived from β -thalassemia fibroblasts were used to identify genomic safe harbors for integration of gene therapy vectors (Papapetrou et al. 2010). The study reported human β -globin levels similar to those of previously reported studies that used mouse models. β -Thalassemia human ESCs have been generated through nuclear transfer using the nucleus from a fibroblast of a β -thalassemia patient, which could also be used for cell or gene therapy studies (Fan et al. 2011). Thalassemia human ESCs have also been derived from preimplantation genetic diagnosis embryos (Verlinsky et al. 2005).

CURRENT STATE OF DIRECTED DIFFERENTIATION

Before using human PSCs for cell therapy, it must first be possible to differentiate human PSCs into the desired cell type for transplanta-

tion. In the case of hemoglobinopathies, the desired cells are hematopoietic stem cells (HSCs) or mature RBCs. Second, the issue of autologous cells versus HLA matched cells from a cell bank must be addressed. If a patient's cells are to be used for autologous treatment, then gene correction becomes an extra step.

Deriving large numbers of HSCs from human PSCs is a challenge in the field. Many groups have developed directed differentiation protocols, but most reports analyze CD34⁺ cells for gene expression, presence of hematopoietic cell surface markers, and CFU potential (Kaufman 2009). High hematopoietic activity in vitro does not, however, correlate with high in vivo engraftment potential when the cells are tested in functional transplant assays (Ledran et al. 2008). One of the biggest challenges in generating HSCs from human PSCs is that HSCs are difficult to maintain in culture, making it difficult to capture, sustain, and expand to clinical scale even if a substantial number of HSCs could be generated in vitro.

Existing directed differentiation protocols use coculture with stromal cell lines or formation of embryoid bodies (EBs), disorganized tissue masses that resemble the early gastrulating mammalian embryo. In all but one report, the percent chimerism of in vitro–derived hematopoietic cells is <2%, which is significantly less than the chimerism seen from xenotransplants with cord blood cells (Wang et al. 2005; Tian et al. 2006; Ledran et al. 2008; Chicha et al. 2011). Lako's group assessed the effect of primary stromal cells and stromal cell lines from the mouse AGM and fetal liver on hematopoietic differentiation (Ledran et al. 2008). Conditioned medium or extracellular matrix from the stromal lines alone was not sufficient to induce strong hematopoietic differentiation. They reported a down-regulation of pluripotency genes followed by activation of mesodermal and hematopoietic genes. Peak hematopoietic CFU activity and accumulation of CD34⁺ cells occurred on Days 18–21 of differentiation and were enhanced by TGF- β signaling. Xenotransplants resulted in 2%–16% multilineage donor chimerism, which also engrafted secondary recipients. The highest engraftment was seen from cells cocultured with

the AGM stromal line. These data, which represent the highest standard to date, are promising yet still significantly short of what would be required before considering scale-up to a clinical stage for human transplantation.

Directly differentiating human PSCs into definitive RBCs has proven equally challenging (Chang et al. 2011). It is possible to generate large numbers of primitive immature RBCs, but generating definitive adult-like RBCs has proven elusive for the field. One group has cultured whole human PSC colonies on immortalized human fetal liver cells until CD34⁺ cells arose, then treated the CD34⁺ cells with hematopoietic differentiation cytokines and co-cultured with mouse stromal cells (Olivier et al. 2006; Qiu et al. 2008). A second group cultured whole human PSC colonies on mouse fetal liver stromal cells with hematopoietic differentiation cytokines (Ma et al. 2008). A third group differentiated human PSCs as EBs, which were then directly plated on Matrigel with hematopoietic differentiation cytokines (Chang et al. 2006). A fourth group differentiated human PSCs as EBs, which were then dissociated to single cells and plated in methylcellulose with hematopoietic differentiation cytokines (Lu et al. 2008). Although there are subtle differences among the RBCs generated under each method, they all result in similarly primitive cells. The cells accumulate hemoglobin, appear red in color, and bind oxygen. They mature appropriately as measured by cell surface markers CD71 and CD235a, but remain mostly nucleated and have a primitive hemoglobin expression pattern, expressing ϵ -globin and γ -globin with little or no β -globin. An unanswered question in the field is what mechanism will drive the switch from primitive to definitive RBCs in vitro.

GENE CORRECTION

The chief advantage of PSCs as targets for combined gene repair and cell therapy is that gene correction is performed in immortalized cells that can be characterized extensively for the fidelity and precision of gene repair and multiple other safety parameters, and the cells can be expanded from a master cell bank to clinical scale

before being differentiated into the desired transplantable cell type. A disadvantage is that gene correction is currently inefficient in human PSCs. However, recent work with ZFNs and transcription activator-like effector nucleases (TALENs) has made homologous recombination 200-fold to 1400-fold more efficient than standard transfection-based methods (Hockemeyer et al. 2009, 2011; Zou et al. 2009). As a proof-of-principle, the point mutation causing α_1 -antitrypsin deficiency has been corrected in human iPSCs with ZFNs (Yusa et al. 2011). Of the colonies that incorporated the correction vector, 54% were targeted at one allele, and 4% were targeted at both alleles. The corrected clones differentiated into normal hepatocyte-like cells that did not show the disease phenotype.

Once disease-specific human PSCs are corrected, they must be differentiated into HSCs. However, large numbers of HSCs would need to be generated and maintained long enough to transplant them into the patient, and both directed differentiation and scale-up of cell culture are current challenges for the field. Alternatively, corrected human PSCs could theoretically be differentiated into RBCs for transfusion, but this would provide only a temporary amelioration of disease, in contrast to HSC transplantation, which offers promise of a cure. For patients with antibody sensitization due to multiple prior transfusions, autologous human PSCs might provide a source of compatible RBCs. Because RBCs are enucleate and have a limited life span, such cells could be irradiated before transfusion, eliminating many of the major safety concerns associated with transplantation of stem cells that would otherwise persist in the patient. Indeed, RBC transfusions from autologous human PSCs represent one of the most appealing near-term strategies for cell-based therapies derived from autologous human iPSCs.

PROHIBITIVE COST

Another major barrier to the widespread application of human PSCs in disease treatment is the high cost of goods for derivation, maintenance, quality control, banking, expansion, and differentiation of cells, made even more cumbersome

N. Arora and G.Q. Daley

if one envisions personalized therapies. There are $\sim 2 \times 10^{12}$ RBCs per unit for transfusion, but the best of current differentiation methods generates only 10^{10} – 10^{11} RBCs per six-well plate of human PSCs, meaning that the production of one transfusable unit would require 20 to 200 plates of human PSCs (Lu et al. 2008). For RBC production on a clinical scale, $\sim 2.6 \times 10^9$ plates of human PSCs would be needed to meet the transfusion needs of only the United States.

The highly specialized maintenance and differentiation media for PSCs is prohibitively expensive, and manual passaging and cell processing are labor intensive. Moreover, for individualized therapies, the cost of the extensive quality analyses that would be required to ensure safety adds considerably to treatment costs. The advantages of immunological identity would have to outweigh the complications of using immune suppression for cells produced as an “off-the-shelf” therapy from a master cell bank, as discussed below. Because human PSCs are adherent cells, opportunities for bioreactor production of large numbers of cells for clinical application are limited.

Currently, cytokines are used to direct the differentiation of human PSCs toward hematopoietic cells, and the costs of cytokines to differentiate human PSCs to RBCs would be prohibitively expensive when considering large-scale production from a cell bank. From an individual’s autologous iPSCs, it would be less expensive because of the smaller scale, but the cost would fall on one patient, making patient-specific treatment also prohibitively expensive.

AUTOLOGOUS CELLS VERSUS CELL BANKING

For cell therapies to become financially feasible, one option is to envision mass production of cells from master cell banks that could be delivered to patients “off-the-shelf,” such that economies of scale could be realized for costs of production, maintenance, quality control, and differentiation; and costs could be distributed across a large number of patients. Such an approach would necessarily compromise the chief

advantage of personalized, individualized cell therapies—that of immunological identity—and would thus require the use of immune suppression, as currently used for organ transplantation. A major question then becomes, what number of PSCs harboring what extent of genetic diversity would be required to constitute a cell bank that might provide a suitable immunological match for most of the human population? Two groups have attempted to model the number of cell lines needed for such a cell bank, based on known immunological heterogeneity of patient populations in the United Kingdom and Japan. The approximations were made using HLA information from registries of kidney transplant recipients, assuming that HLA matching requirements and the use of immune suppression would be similar for solid organ transplants and cell transplants of human PSC derivatives. It is well known that the function and durability of renal allografts is directly proportional to the degree of immunological match, and when considering immunological identity at the human histocompatibility loci HLA-A, HLA-B, and HLA-DR, a single antigen match is considered minimally advantageous. Consequently, in the United Kingdom, a cell bank of 150 randomly chosen human PSC lines would provide a full 6/6 antigen match at HLA-A, HLA-B, and HLA-DR for <20% of the population, and 37.9% would have a beneficial match (only one HLA-A or HLA-B mismatch or better), whereas 84.9% of the population would have a minimally advantageous match at a single HLA-DR locus, or better. However, if the bank is constructed from highly selected human PSCs that harbor homozygous HLA haplotypes (the same A, B, and DR loci on both chromosomes), then only 10 cell lines would provide matches for similar percentages of the population (Taylor et al. 2005). In the Japanese population, a cell bank of 170 human PSC lines would provide a single mismatch or better for 80% of patients (Nakajima et al. 2007), whereas a cell bank of 30 highly selected homozygous human PSC lines would provide a full match for 82.2% of the Japanese population, and 50 lines would provide a full match for 73%–90.7% (Nakatsuji et al. 2008; Okita and

Yamanaka 2011). The suspected reason for the difference in beneficial matches is the low ethnic diversity in Japan compared with the United Kingdom, suggesting that the cell bank strategy would be a more reasonable option in less diverse regions and more difficult in regions with high ethnic diversity like the United States.

If a cell bank strategy were to be implemented, depending on the country or region, there would still be a significant percentage of the population for which there would be no beneficial match in the bank. Because of the considerable degree of polymorphism in the HLA loci in human populations, increasing the number of human PSC lines in the cell bank meets a diminishing return and has only a minimal effect on the percentage of the population with a beneficial match. In such cases and for severe disease, the extra effort and expense of personalized cell therapies would have to be considered.

CONCLUDING REMARKS

We have come a long way from identifying the genetic mutations that cause hemoglobinopathies, but we are still far from a cell therapy treatment or cure. Although the advent of PSCs offers an appealing theoretical platform for treating not only hemoglobinopathies but any of dozens of genetic and malignant bone marrow disorders for which current approaches of bone marrow transplant remain infeasible because of lack of suitable donors or immunological barriers, many hurdles must first be overcome. Advances are needed to improve the efficiency of transgene-free human iPSC derivation, to enhance directed differentiation toward engraftable HSCs or transfusable RBCs, and to reduce the cost of maintaining and differentiating human PSCs in order to make cell therapy treatments feasibly affordable. A hybrid effort, with the majority of the population served by off-the-shelf treatments manufactured from master cell banks, accepting the trade-offs of immune suppression, whereas others are treated with individualized strategies, may ultimately prove the only sustainable mode of delivering innovative cell therapies based on PSCs.

Pluripotent Stem Cells in Hemoglobinopathies

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N. Arora and G.Q. Daley

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N. Arora and G.Q. Daley

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