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REVIEW

Human ES cells – haematopoiesis and transplantation strategies*

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Abstract

Human embryonic stem (ES) cells provide a novel opportunity to study early developmental events in a human system. We have used human ES cell lines, including clonally derived lines, to evaluate haematopoiesis. Co-culture of the human ES cells with irradiated bone marrow stromal cell lines in the presence of fetal bovine serum (FBS), but without other exogenous cytokines, leads to differentiation of the human ES cells within a matter of days. A portion of these differentiated cells express CD34, the best-defined marker for early haematopoietic cells. Haematopoietic colony-forming cells (CFCs) are demonstrated by methylcellulose assay. Myeloid, erythroid, megakaryocyte and multipotential CFCs can all be derived under these conditions. Enrichment of $CD34^+$ cells derived from the human ES cells markedly increases the yield of CFCs, as would be expected for cells derived from adult bone marrow or umbilical cord blood. Transcription factors are also expressed in a manner consistent with haematopoietic differentiation. This system now presents the potential to evaluate specific conditions needed to induce or support events in early human blood development. Human ES cells are also a novel source of cells for transplantation therapies. The immunogenicity of ES cell-derived cells is unknown. The unique properties of ES cells afford the opportunity to explore novel mechanisms to prevent immune-mediated rejection. Potential strategies to overcome rejection will be presented, including creation of haematopoietic chimerism as a means to successfully transplant cells and tissues derived from human ES cells.

Key words embryonic stem cells; haematopoiesis.

Introduction

Twenty years of studies using mouse embryonic stem (ES) cells, and preceding work on mouse and human embryonic carcinoma (EC) cells have been instrumental to elucidating basic aspects of mammalian developmental biology (Andrews et al. 2001; Smith, 2001). The ability to delete specific genes in mouse ES cells permits well-defined analysis of the role of specific proteins on the developmental fate of an organism. The isolation of ES cells from blastocytsts of non-human primates, and more recently from human pre-implantation blastocysts, sets the stage now to define the nature of the earliest stages of human development (Thomson et al. 1995, 1998). Because mouse ES cells have been so extraordinarily useful, the promise and the hope of human ES cells is great. These cells will not only permit studies of basic human developmental biology, but they also have the potential to revolutionize medical therapies.

The study of blood development, haematopoiesis, serves as another model of achievement with stem cells. Research beginning in the 1940s and 1950s established bone marrow as the source of transplantable haematopoietic cells. This research eventually led to the establishment of the clinical field of bone marrow transplantation (BMT), now better referred to as

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haematopoietic cell transplantation (HCT) (Thomas & Blume, 1999). Studies by Till, McCullough and colleagues first demonstrated the clonal nature of haematopoietic development by showing that single bone marrow-derived cells could give rise to colonies of differentiated blood cells (Till & McCullough, 1961; Becker et al. 1963). Thousands of patients a year now receive haematopoietic cell transplants as the most effective means to treat and cure certain haematological malignancies such as laeukemia, lymphoma and myeloma, as well as some non-malignant conditions such as aplastic anaemia and genetic disorders.

A central question in the study of haematopoiesis is how the transplantable HSCs develop from earlier precursor cells. To date, most research on haematopoiesis focuses on one of two main experimental models. A large number of studies use mouse ES cells, or other embryonic mouse tissue derived from a particular day of fetal development (Keller, 1995; Palis & Yoder, 2001; Yoder, 2001). Other groups analyse human (or mouse) post-natal haematopoietic precursor cells typically isolated from bone marrow or umbilical cord blood (Morrison et al. 1995; Akashi et al. 2000). These experimental models have produced much insight into the genes and proteins involved in haematopoietic development (Weissman, 2000; Orkin, 2001). These models have also been successful at characterizing haematopoietic stem cells (HSCs) and other haematopoietic progenitors based on cell surface antigen expression and gene expression (Morrison et al. 1995; Phillips et al. 2000; Orkin, 2001). However, while sites of haematopoietic development such as yolk sac, fetal liver and bone marrow are clearly defined, other basic issues such as the relationship between primitive and definitive haematopoiesis remain less well understood.

Mouse ES cells have served as an excellent *in vitro* model of haematopoietic development. Work by Keller and other groups demonstrates that the appearance of blood cells derived from mouse ES cells follows the same time course as prenatal blood development *in vivo* (Keller, 1995). The ability to modify these ES cells genetically permits intricate understanding of the mechanisms of early haematopoiesis. For example, deletion of the gene *SCL/tal-1* prevents haematopoietic differentiation of mouse ES cells *in vitro* (Robertson et al. 2000). Moreover, the deletion of individual genes such as *flk-1* leads to death at approximately day 9 of mouse embryogenesis due to lack of haematopoietic development (Shalaby et al. 1995).

The direct application of *in vitro* studies with mouse ES cells to development of transplantable mouse ES cell-derived HSCs has been problematic. While HSCs can clearly be derived from ES cells, this has been best demonstrated by evaluating HSCs within chimeric animals derived from ES cells and not directly from ES cells maintained in culture (Forrester et al. 1991; Nagy et al. 1993). Why mouse ES cell-derived blood cells are refractory to long-term engraftment remains unclear. However, as described in more detail below, some recent studies suggest there are ways to get around these barriers by either direct injection of the cells into the neonatal liver, or by genetic modification of the ES cells to derive transplantable cells. These techniques may overcome problems due to improper homing or other deficiencies of mouse ES cell-derived HSCs.

The derivation of ES cells from rhesus monkeys and humans opens new methods to understand haematopoietic development. Interestingly, the first characterization of these ES cells described fundamental differences between human (and rhesus) ES cells when compared to mouse ES cells. The human ES cells grow as flatter colonies, divide at a slower rate and express different cell surface antigens (Thomson et al. 1998; Andrews et al. 2001). Perhaps most importantly, whereas mouse ES cells require laeukemia inhibitory factor (LIF) or other agonists of the gp130 signalling pathway to prevent differentiation of the cells, the human and rhesus ES cell do not require LIF to be maintained as undifferentiated cells (Thomson et al. 1995, 1998). Therefore, while mouse ES cells can be induced to differentiate into blood (and other lineages) simply by removal of LIF from the culture media, other strategies are needed to promote differentiation of the human ES cells.

Haematopoiesis from rhesus and human ES cells

A recent study by Li reported production of haematopoietic cells from rhesus monkey ES cells (Li et al. 2001); and our group has described haematopoietic cells derived from human ES cells (Kaufman et al. 2001). The similarities and differences between these two studies make comparison quite instructive. Both groups coaxed haematopoietic development by co-culture of the ES cells on bone marrow-derived stromal cells. These stromal cells are known to support growth of human haematopoietic precursor cells obtained

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from bone marrow or umbilical cord blood. The human ES cells were cultured with these stromal cells in media containing fetal bovine serum (FBS) but no other exogenous cytokines. This led to the development of $CD34^+$ cells, presumed haematopoietic precursor cells. When placed in a secondary culture system in a semisolid methylcellulose-based medium containing haematopoietic growth factors, these $CD34^+$ cells produced colonies of mature haematopoietic cells. These colonies of erythroid (red blood) cells, myeloid (white blood) cells and megakaryocyte (platelet precursor) cells appear identical to colonies of blood cells derived from marrow or cord blood. In contrast, haematopoietic differentiation of the rhesus ES cells was done by culture of the cells on bone marrow stromal cells in media containing FBS and a cocktail of cytokines and growth factors. While a variety of individual, terminally differentiated blood cells were obtained (red blood cells, white blood cells and megakaryocytes), no haematopoietic colony-forming cells (CFCs) could be demonstrated. This suggests that the conditions used in the rhesus system (FBS plus added growth factors) pushed the differentiation pathway past the early precursor stage of development. Therefore, only with less mitotic and developmental stimulation of the human ES cells (FBS alone) could the CFC progenitor cells be isolated. Since the eventual goal is to develop HSCs with long-term engraftment potential, developmental and growth stimuli may be best kept to the minimum that will support early stages of differentiation down a particular developmental pathway without excessive induction of more terminally differentiated cells. This balance between self-renewal, growth and differentiation may be difficult to maintain.

It now becomes important to demonstrate the *in vivo* potential of these human ES cell-derived haematopoietic cells. Haematopoietic stem cells (HSCs) are best defined by their ability to sustain long-term engraftment when transplanted into a syngeneic, congenic or immunodeficient host (all these experimental models are designed to prevent immune-rejection of the transplanted cells). NOD/SCID (or related) mice now serve as the best recipients for xenogenic human HSC transplants (Lapidot et al. 1997; Dao & Nolta, 1999). Multiple studies demonstrate that HSCs derived from human bone marrow or cord blood can be transplanted into these immunodeficient mice. After a short time, multiple types of differentiated human blood cells (erythrocytes, myeloid cells and lymphoid cells) can all be isolated from the blood or marrow; and these human blood cells are sustained for the life of the mouse. Bone marrow containing human HSCs from the engrafted NOD/SCID mice can then be transplanted into secondary and tertiary hosts as the most compelling evidence of an original HSCs population with long-term self-renewal and repopulating potential. This type of study will be required to prove the development of HSCs from human ES cells. As described above, to date work on haematopoiesis from mouse ES cells has not convincingly derived HSCs with this long-term engraftment potential. Clearly, mouse ES cells have this potential since chimeric mice derived from mouse ES cells have normal haematopoietic cells (Nagy et al. 1993).

While all the details of attempts to derive HSCs from mouse ES cells remain murky (negative data are not typically published), multiple theories exist as to why this *in vivo* engraftment is so difficult. One barrier may be that *in vitro*-derived blood cells do not 'home' or 'traffic' to the proper haematopoietic environment when injected intravenously into adult mice. In an attempt to overcome this problem, a recent study suggests that injection of the mouse ES cell-derived HSCs into the livers of neonatal mice does lead to engraftment (Schroeder et al. 2001). Also, production of blood cells from mouse ES cells that express the oncogenic BCR/ABL protein can sustain long-term engraftment, although the transplanted cells are leukemogenic (Perlingeiro et al. 2001). Clearly, the potential to produce HSCs from human ES cells should exist. However, the best strategy to derive and identify these cells remains to be determined.

Strategies to avoid immune-mediated rejection of ES cell-derived cells

Once research groups have identified methods to derive cells and tissues of choice from human ES cells, the next hurdle to overcome will be to prevent immune-mediated rejection when these cells are transplanted into hosts that are unlikely to be histocompatible with the ES cell-derived cells. While the transplant recipient could be given immunosuppressive drugs such as cyclosporin or tacrolimus, there are considerable side-effects from these medications. Fortunately, the novel properties of ES cells will allow exploration of strategies to prevent rejection without requiring pharmaceutical immunosuppressants. Many of these potential methods have been described in recent reviews and are summarized in Table 1.

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Table 1 Strategies for preventing immune-mediated rejection of human ES cell-derived cells and tissues

Strategy	Potential benefits	Potential problems
Immunosuppressive medications	Currently available Efficacy known	Requires lifelong use Risk of infection Risk of lymphoproliferative disorders Risk of organ toxicites
HLA-defined ES cell 'banks'	Readily available cell lines Model after cord blood banks	May require thousands of stored ES cell lines Requirement for HLA matching unknown
Deletion/insertion of HLA genes	Tissue would express no HLA antigens, or 'Self' HLA molecules Should prevent rejection by T cells	May be subject to rejection by NK cells May not be suitable for autoimmune diseases Large regions of DNA may need to be altered
Insertion of genes for immune-modifying proteins	Single gene may be effective May only require limited number of ES cell lines	Currently difficult to transfect human ES cells stably May require multiple gene replacements
Deletion of gene(s) for co-stimulatory immune response proteins	Single gene may be effective May only require limited number of ES cell lines	Currently no effective homologous recombination in human ES cells May require deletion of multiple genes
Nuclear reprogramming to produce HLA-matched ES cells	Tissue would express 'self' HLA molecules	Would require ES cell lines specific for each individual Currently no reliable method for nuclear transfer for humans May not be suitable for autoimmune diseases
Haematopoietic chimerism	May require only limited number of ES cell lines Suitable model with current transplantation methods	Requires derivation of multiple cell types Requirement for HLA matching unknown

Haematopoietic chimerism is one method to prevent rejection of ES cell-derived cells and tissues without reliance on immunosuppressive drugs (or minimal or short-term use of the medications). Studies of HCT recipients show that engraftment of HSCs can lead to tolerance of a second tissue (typically a kidney graft) obtained from the same donor as the haematopoietic cells. In these cases, a patient with leukaemia or other haematological malignancy receives a bone marrow transplant from an HLA-matched sibling as a means to cure the underlying cancer. In some cases the therapy to undergo a successful HCT has significant nephrotoxicity leading to renal failure. Therefore, a kidney graft from the same sibling will treat the renal disease and prevent the need for dialysis therapy. Since the patient's blood cells, including lymphocytes that mediate transplant rejection, are now derived from the same source (sibling) as the transplanted kidney, the renal graft is not seen as foreign tissue and no additional immunosuppressant medications are needed. A recent review of the literature describes 28 patients who have received both an HCT and solid organ transplant (Dey et al. 1998) and many other unreported cases likely exist. The clinical success of these dual transplants has led to recent studies that prospectively transplant blood cells and a kidney from the same donor as a means to induce tolerance without long-term immunosuppression. Initial reports of these studies have been encouraging (Spitzer et al. 1999; J. A. Shizuru, personal communication).

This concept of haematopoietic chimerism has special relevance to use of ES cells as the starting point for cell replacement therapies. Since multiple cell types can be derived from the same starting ES cell population, it will be possible to derive HLA-matched blood cells and a second tissue such as pancreatic islet cells or cardiomyocyte. Using the concept described above, engraftment of the ES cell-derived blood cells should permit transplantation of the second cell type without the need for additional immunosuppressive therapies, since both cell populations will be HLA-matched (from the same starting ES cell population). This dual transplant may be particularly important when treating autoimmune diseases such as type 1 diabetes mellitus by transplantation of pancreatic islets. If these islets (or insulin-secreting beta cells) are derived from ES cells and made to be HLA matched (for example by nuclear reprogramming, or genetic engineering of HLA genes), the host may still reject these cells by the same autoimmune process that led to destruction of the original beta cells. However, if the host receives a population of HSCs derived from the same ES cell line as the insulin-secreting cells, then tolerance should

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develop and prevent rejection of the beta cells and cure of the underlying diabetes.

Conclusion and future directions

Multiple hurdles will have to be overcome before ES cell-based therapies become a clinical reality. The first step is to demonstrate that specific lineages of cells can be derived from ES cells in an efficient manner. As described at the conference on Embryonic Stem Cells – Prospects for Human Health (University of Sheffield, UK, 10 September 2001) and recent publications (Kaufman et al. 2001; Odorico et al. 2001; Zhang et al. 2001), significant progress toward this goal is being rapidly obtained. Haematopoietic, neural, cardiomyocyte and other lineages have all be described from cultures of ES cells. While these results are encouraging, the next step to demonstrate *in vivo* function of these ES cell-derived cells will likely be even more challenging. The path to clinical trials of ES cell-based therapies will not be linear, and many twists and turns will present themselves as research on these cells progresses. The best means to ensure future success is to use human ES cells to learn about the earliest stages of human developmental biology – how do human ES cells differentiate into HSCs? Better understanding of these basic principles, what genes and proteins are essential for this developmental progression, is crucial. With this knowledge, new therapeutic strategies may be pursued that do not specifically involve transplantation of ES cell-derived cells. Perhaps novel proteins that improve expansion of umbilical cord blood cells can be derived based on the basic studies of ES cell differentiation. Other indirect applications may also be applicable to a variety of medical fields. With multiple groups now working on various aspects of human ES cell studies, this field should continue to produce exciting advances for years to come.

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