

Development of Diverse Hematopoietic Cell Populations from Human Embryonic Stem Cells

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1. Introduction

Mouse and human embryonic stem cells (hereafter called mESCs and hESCs respectively) are pluripotent cells derived from the inner cell mass of preimplantation blastocyst stage embryos. mESCs and hESCs are defined by their ability to self-renew and maintain the normal karyotype after prolonged periods in culture, while retaining the capacity to generate cells of all three germ cell layers when induced or supported to differentiate.¹⁻³ While there are important differences between mESCs and hESCs in terms of morphology, growth characteristics, and culture conditions required for maintenance of the undifferentiated state,²⁻⁴ these differences will not be discussed in further detail here.

Hematopoiesis (blood cell development) has been one of the most intensively studied cell lineages using not only mESCs and hESCs but also other model systems such as the zebrafish⁵ *Xenopus*,^{6,7} developing mouse embryos,⁸⁻¹¹ mouse and human bone marrow, and human umbilical cord blood (UCB). With all these more established model systems available, there are at least three main reasons why studies of hematopoiesis from hESCs are important:

- (i) To understand the basic human developmental biology of self-renewal, differentiation, and commitment to specific lineage. hESCs

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allow easy access to a homogeneous cell population to define the earliest developmental events that occur during human embryogenesis, a stage that is difficult to obtain from primary human tissue.

- (ii) hESC-derived cells can be used to substitute and/or supplement other cell populations now typically utilized for hematopoietic stem cell transplantation (HCT) or other hematopoietic cell therapies. Much of the clinical interest in studying hESC-derived hematopoiesis comes from the fact that many malignant hematological disorders can be treated and possibly cured by HCT. Indeed, HCT is an example of a “stem cell therapy” that has been used clinically for over thirty years.^{12,13} At present there are three sources of transplantable hematopoietic stem cells (HSCs): adult bone marrow, growth-factor-mobilized peripheral blood, and UCB. Limitations to further expanding clinical use of HSCs include scarce supply, inability to expand HSCs *in vitro* without loss of stem cell function, risk of pathogen contamination, risk to donor (pain, infection, bleeding), and risk of graft-versus-host disease. These limitations could potentially be circumvented if HSCs derived from hESCs are used. Additionally, hESCs can potentially be manipulated to match or mismatch human leukocyte antigen (HLA) types for therapeutic advantage. In the situation of graft failure, patients could be given a supplemental dose of hESC-derived HSCs. Additionally, hESC-derived hematopoietic cells may be used to induce tolerance to subsequent transplantation of other hESC-derived cells and tissues (such as neurons, pancreatic islets, or cardiomyocytes) while minimizing the need for prolonged heavy immunosuppressant therapy.^{14–16} Eventually, with somatic cell nuclear transfer, we may be able to have designer isogenic cell lines for each patient. An example of this has been done to successfully create hematopoietic cells from mESCs suitable for correcting a mouse model of immunodeficiency.¹⁷
- (iii) hESC-derived cells can be used to generate other pure cell populations on a larger scale, such as red blood cells, platelets, granulocytes, or lymphocytes. These could be made potentially safer, without concern about contamination, with infectious agents such as human immunodeficiency virus (HIV), hepatitis, or prions that currently can contaminate our donor blood supply. In this manner, hESCs could also be used to potentially grow clinical grade hematopoietic cells on a large scale to create a virtually endless source of these cells for the purpose of transfusion medicine.

2. Hematopoietic Differentiation from mESCs

Studies of mESCs demonstrate that differentiation into hematopoietic cells closely mimics the ordered gene expression and development of specific cell populations that occur during normal embryonic development.^{2,18,19} During murine embryonic development, hematopoiesis develops in distinct waves with the yolk sac, giving rise to primitive hematopoietic cells followed by cells of the aorta gonads and mesonephros (AGM) region, which in turn gives rise to definitive hematopoietic cells. However, the yolk sac can potentially also generate cells of the definitive hematopoietic program, including HSCs, macrophage, definitive erythroid, and mast cells.^{8,10,11} This can become important when one is analyzing the development of HSCs and other hematopoietic cells from mESCs and hESCs, as it may not be readily apparent whether the ESC-derived cells are representative of the yolk sac (primitive hematopoiesis) or AGM (definitive hematopoiesis).

Development of specific hematopoietic lineages from mESCs has been well described. These hematopoietic cell lineages include erythrocytes,^{18,20} megakaryocytes,²¹ granulocytes,²² mast cells,^{18,23} eosinophils,²⁴ T and B lymphocytes^{25–28} macrophages, dendritic cells,^{29,30} and natural killer (NK)^{31–33} cells. Methods to generate specific lineage type cells in relatively pure populations now include addition of supplemental cytokines, chemicals (such as dexamethasone), and use of various stromal cell lines, such as S17, OP9, and MS-5. Stromal cells are thought to influence differentiation and commitment of mESCs to particular cell types by mechanisms which could include cell–cell interaction, secretion of soluble or cell-bound growth/differentiation or lineage commitment influencing factors, and/or inhibition of factors maintaining mESCs in the undifferentiated state.^{25,34–36} Embryoid body (EB) formation can also be used to induce or support differentiation of mESCs without use of stromal cell lines (37).

One example of the utility of mESCs has been to characterize the common development of the hematopoietic and vascular systems. Initially based on observation that vascular and blood islands appear in close proximity within the early developing mouse embryo, it was thought that a common progenitor cell, the “hemangioblast,” gave rise to both tissues. This hypothesis was supported by observations like common shared gene expressions [brachyury, vascular endothelial growth factor (VEGF), and Flk-1]^{38,39} in progenitors of both lineages. Subsequently, carefully timed studies of mESC-derived EBs were able to characterize the phenotype and

developmental requirements of hemangioblast cells.^{40,41} This work using mESCs was then translated into isolating a similar population of putative hemangioblasts in mouse embryos.⁴² More recently, hemangioblasts from hESCs have also been described, demonstrating the ability to extend mESC-based analyses into the human system.⁴³

3. Hematopoietic Differentiation from hESCs

An overview of hematopoietic development and analysis from hESCs is given in Fig. 1. In the initial studies of hematopoietic development from hESCs, CD34⁺ hematopoietic precursor cells expressing hematopoietic

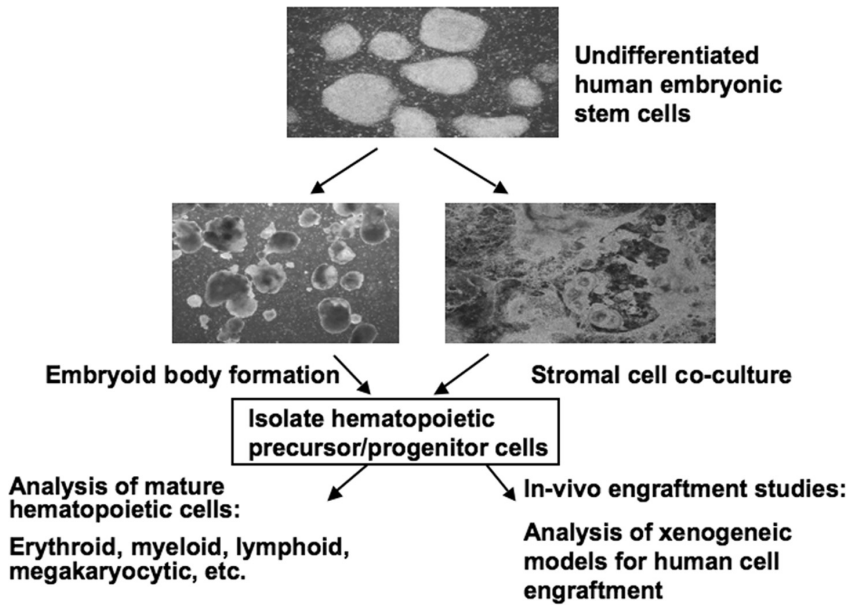


Figure 1. Overview of hematopoietic development from hESCs. Undifferentiated hESCs are typically induced to differentiate via either suspension culture or embryoid body formation, or as adherent cells by coculture on stromal cell lines. Either method of differentiation can be employed, either using FBS alone or with addition of defined cytokines and growth factors. hESCs allowed to differentiate under these conditions can be analyzed and sorted for hematopoietic cell populations based on specific phenotypes. These hESC-derived hematopoietic cell populations can be either induced to further differentiate *in vitro* into defined mature blood cell populations, or analyzed with *in vivo* models for ability to sustain long term, multilineage engraftment.

transcription factors were derived by utilizing the coculture method with the murine bone marrow cell line S17 or the yolk sac endothelial cell line C166 in the presence of fetal bovine serum (FBS), without any added cytokines.⁴⁴ Importantly, CD34⁺ selection led to enrichment of the hematopoietic progenitor cells, as confirmed by the colony-forming cell (CFC) assay giving rise to characteristic myeloid, erythroid, and megakaryocytic colonies. More mature hematopoietic cells derived from these colonies also expressed normal surface antigens corresponding to various cell types appropriately.

Subsequently, the EB method of differentiation was used for hESCs.⁴⁵ Here, treatment of hESCs during EB development with a combination of FBS and defined cytokines like stem cell factor (SCF), Flt-3 ligand, interleukin (IL)3, IL-6, granulocyte colony-stimulating factor (G-CSF), and bone morphogenetic protein 4 (BMP-4) strongly promoted differentiation into CD45⁺ hematopoietic cells. Despite the removal of cytokines at day 10, hematopoietic differentiation of hESCs continued, suggesting that the cytokines act on hematopoietic precursors as opposed to more differentiated hematopoietic cells.

Another study using EBs demonstrated that erythromyelopoiesis from hESCs in serum-free clonogenic assays parallels erythromyelopoiesis from the human embryo/yolk sac by stepwise passage through embryonic and definitive hematopoiesis.⁴⁶ Here, erythropoietin (EPO) and granulocyte-macrophage colony-stimulating factor (GM-CSF), in addition to SCF, G-CSF, IL-3, and IL-6, were used. Initially, EB-derived CD45⁺ cells differentiated into semiadherent mesodermal-hematoendothelial (MHE) colonies that formed organized, yolk sac-like structures, and subsequently led to generation of multipotent primitive hematopoietic cells, erythroblasts, and macrophages. A first wave of hematopoiesis follows MHE colony emergence, giving rise to primitive erythrocytes with brilliant red embryonic/fetal hemoglobin, expressing CD71/CD325a (glycophorin A). A second wave then gives rise to definitive-type erythroid, granulocyte, macrophage CFCs, and multilineage CFCs.

Other stromal-cell-based studies were able to obtain up to 20% of CD34⁺ cells and isolate up to 10⁷ CD34⁺ cells with more than 95% purity from a similar number of initially plated hESCs after 8–9 days of coculture with the OP9 cell line.⁴⁷ Again, as found in the previous reports,^{44,45} these hESC-derived CD34⁺ cells were expressing typical hematopoiesis-associated genes, highly enriched in CFCs, and retained clonogenic potential after *in vitro* expansion. Phenotypically these CD34⁺ hematopoietic progenitor

cells derived here were consistent with primitive hematopoietic progenitors as defined by coexpression of CD90, CD117, and CD164, along with a lack of CD38 expression. Moreover, this population contained aldehyde dehydrogenase-positive cells, as well as cells with verapamil-sensitive ability to efflux rhodamine 123.^{48–50} Additionally, when cocultured on MS-5 stromal cells with appropriate cytokines, these hESC/OP9 coculture-derived CD34⁺ cells gave rise to phenotypic lymphoid (B and NK cells) as well as myeloid (macrophages and granulocytes) lineages. However, these lymphoid cells were not tested for functional capacity, as was done in some of the lineage-specific studies described below.

Additional studies by the same group using the OP9 system demonstrated that early progenitors committed to hematopoietic development could be identified by surface expression of leukosialin (CD43).⁵¹ The appearance of CD43 was found to precede that of CD45 on all types of emerging clonogenic progenitors, and CD43 can reliably separate the hematopoietic CD34⁺ population from CD34⁺CD43⁻ endothelial and mesenchymal cells. Furthermore, these studies demonstrated that multipotent lymphohematopoietic progenitors followed precommitted erythromegakaryocytic progenitors in appearance, and their gene/transcription factor profile was representative of initial stages of definitive hematopoiesis originating from endothelium-like precursors. Once these cells acquired CD34, CD43, and CD45 expression, they were largely devoid of VE-cadherin and KDR (also termed Flk-1 or VEGF receptor-2) expression (endothelial markers) and had a distinct gene expression profile consistent with commitment to lymphohematopoietic fate.

Two studies have evaluated the effect of overexpression of the homeobox B4 gene (HOXB4) on hematopoiesis from hESCs. Several previous studies using mESCs or mouse and human HSCs have found HOXB4 to improve expansion and *in vivo* engraftment of HSCs.^{52–54} One study found that overexpression of HOXB4 considerably augmented hematopoietic development of hESCs.⁵⁵ Here, differentiation of hESCs as EBs in a serum-containing medium without the use of additional cytokines led to sequential expansion of first erythroid and then myeloid progenitor cells. However, interpretation of these results is complicated by the minimal hematopoietic development from hESC-derived control cell populations that did not overexpress HOXB4, suggesting that differentiation of these cells was not optimized. Additionally, the second study to overexpress HOXB4 in hESC-derived hematopoietic cells did not find increased hematopoietic engraftment,⁵⁶ as is the case for mESC-derived blood cells.

However, stable HOXB4 expression was not demonstrated in the transplanted cells, again complicating this analysis.

Finally, as Keller and colleagues have done for mESCs,^{40,41} they have recently reported isolation of two separate populations of cells from hESCs that meets the criteria for definition of hemangioblasts, i.e. blast colonies having both hematopoietic and vascular potential. In their experiment, 72–96 hours of BMP-4–stimulated EB differentiation gave rise to a KDR⁺ population of cells with both hematopoietic and vascular potential before appearance of the primitive erythroid cells.⁴³ Two distinct types of hemangioblasts were identified: those that give rise to primitive erythroid cells, macrophages, and endothelial cells, and those that generate only the primitive erythroid population and endothelial cells.

4. *In Vitro* Production of Specific Hematopoietic Lineages from hESCs

In addition to the studies described above that evaluated hematopoietic development from hESCs based on derivation of hematopoietic precursor and progenitor cell populations, several other studies have now characterized development of specific mature blood cell lineages from hESCs.

4.1. Erythrocytes

VEGF is well known to support hematopoietic and endothelial cell growth and development in many systems. Indeed, it was found to selectively promote erythropoietic development from hESCs.⁵⁷ Here, when hESC-derived EBs were treated with VEGF, authors found a higher frequency of cells coexpressing CD34 and KDR (hematoendothelial phenotype). These cells also expressed erythroid markers and increased expression of embryonic zeta (ζ) and epsilon (ϵ) globins, though no change in fetal/adult hemoglobin was noticed. These effects of VEGF were dependent on the presence of other hematopoietic cytokines (Flt-3 ligand, IL-3, IL-6, G-CSF, and BMP-4), and were augmented by addition of EPO. It was also noticed that the *in vitro* self-renewal potential of primitive hematopoietic cells with erythroid progenitor capacity was enhanced by the presence of VEGF.

More specific analysis of globin gene expression in erythroid cells derived from hESCs used hESCs cocultured on FH-B-hTERT (an immortalized fetal liver cell line) or S17 cells.⁵⁸ Analysis of mRNA expression

from the β -globin locus revealed that hESC-derived erythroid cells produced ϵ - and γ -globin mRNAs but no or very little amounts of β -globin expression. Over time in culture, the mean ratio of γ/ϵ increased by more than 10-fold, recapitulating the ϵ -globin to γ -globin switch but not the γ -globin to β -globin switch that occurs around birth. Subsequent studies by this group have also described an experimental protocol to produce large numbers of primitive erythroid cells from hESCs.⁵⁹ Here, a more than 5000-fold increase in primitive erythroid cells was obtained by differentiating hESC-derived CD34⁺ cells into liquid cultures. Again, these erythroid cells were morphologically and functionally similar to primitive erythroid cells present in the yolk sac of early human embryos, i.e. they did not enucleate, were fully hemoglobinized, and expressed a mixture of embryonic and fetal globins but no β -globin.

Another study also evaluated the generation of erythroid cells from hESCs, with similar results to those described above. Here, morphologically definitive erythroid cells, coexpressing high levels of embryonic (ϵ) and fetal (γ) globins at mRNA and protein levels with little or no adult globin (β), were generated from human EB-derived CD45⁺ hematopoietic development.⁶⁰ This globin expression pattern was not altered by factors like culture duration, FBS, VEGF, Flt3-Ligand, or coculture with OP-9 during erythroid differentiation. This coexpression of both embryonic and fetal globins by definitive-type erythroid cells did not faithfully recapitulate either yolk sac embryonic globins or their fetal liver counterparts. Therefore, it remains unclear if these results are spurious due to *in vitro* culture conditions, or if there is some abnormal promiscuity of globin gene expression that may be normal, but not typically observed during early human development. Regardless, this ability to achieve erythroid cells coexpressing embryonic and fetal globins generated from hESCs at high frequency can help us to understand and explore molecular mechanisms of hematopoiesis.

4.2. Megakaryocytes

The OP9 stromal cell coculture system was also used to generate megakaryocytes from hESCs.⁶¹ These platelets were functionally similar to adult human platelets with submaximal adenosine diphosphate responses augmented by epinephrine. Moreover, hESC-derived megakaryocytes undergo lamellipodium formation, actin filament assembly, and vinculin localization at focal adhesions when plated on a fibrinogen-coated

surface, characteristic of $\alpha_{\text{IIB}}\beta_3$ outside-in signaling typically seen with these cells.

Together, these studies of hESC-derived erythroid and megakaryocytic cells highlight the potential of hESCs to serve as the starting point for cells suitable for transfusion medicine therapies. While red blood cells and platelets can be readily obtained from the Red Cross and similar agencies, use of hESC-derived cells for transfusion medicine would be known to start from a homogeneous, well-characterized cell population that could be screened and guaranteed to be free of infectious blood-borne pathogens such as the HIV, hepatitis, prions, and other infectious agents. Moreover, hESC-derived cells for transfusions could be useful for patients with rare blood types or incompatibility with erythroid cell antigens.

4.3. Lymphocytes and other immune cells

Initial studies to demonstrate development of functional lymphocytes from hESCs used a two-step culture method by first sorting CD34⁺ cells derived from S17 stromal cell coculture, then using the AFT024 stromal cell line with defined cytokines known to support NK cell development.⁶² This work demonstrated that these CD56⁺CD45⁺ hESC-derived lymphocytes expressed typical markers and functioned like mature NK cells, i.e. expressed killer cell Ig-like receptors, natural cytotoxicity receptors, and CD16, and were able to lyse human tumor cells by both direct cell-mediated cytotoxicity and antibody-dependent cellular cytotoxicity.

Next, T cells were derived from hESCs by using OP9-mediated differentiation into CD34⁺ and CD133⁺ cells that were transplanted into human thymic tissues that had been engrafted into immunodeficient mice.⁶³ These hESC-derived T cells expressed surface antigens such as CD4, CD8, CD1a, and CD7 typical of T cells. Some functional capacity was demonstrated by expression of activation markers upon costimulation of T lineage cells by CD3 and CD28. Additionally, a lentiviral vector expressing enhanced green fluorescent protein (GFP) under the control of the elongation factor 1 α promoter, introduced at the hESC stage, continued to express the reporter gene at high frequency throughout thymopoiesis. These results suggest that genetically manipulated hESCs may hold promise for treatment or modeling disorders of the T cell lineage, including HIV infection.

Other cells of the immune system, such as macrophages and dendritic cells, have also been derived from hESCs. CD34⁺ cells derived by

S17-stromal cell-mediated culture of hESCs were sorted and cultured with defined cytokines [FBS, GM-CSF, and macrophage colony-stimulating factor A(M-CSF)] to derive macrophages.⁶⁴ Phenotypic and functional analyses carried out on these hESC-derived macrophages confirmed the typical phenotype of macrophages. They also displayed normal functionality by efficient phagocytosis, by up-regulation of the costimulatory molecule B7.1, and by cytokine secretion in response to lipopolysaccharide stimulation. As in the other studies described above, lentiviral transduced hESCs expressing the transgene GFP also differentiated into functionally and phenotypically normal macrophages, indicating no adverse effects due to lentivirus infection.

Dendritic cells (DCs) are another hematopoietic cell lineage that serves as principal antigen-presenting cells (APCs) in triggering and supporting immune responses. One study used EB-mediated differentiation to derive both functional DCs and macrophages. These APCs expressed high levels of HLA class II molecules and were able to stimulate mixed leukocyte reactions (MLRs) as an *in vitro* measure of immune activity.⁶⁵ A second study used the OP9 coculture system to promote differentiation of hESCs into myeloid cells.⁶⁶ First, myeloperoxidase-expressing CD4⁺CD11b⁺CD11c⁺CD16⁺CD123^{low}HLA-DR⁻ myeloid cells were generated and expanded by sequentially coculturing them first with OP9, followed by feeder free conditions and GM-CSF stimulation. When transferred to a serum-free medium with GM-CSF and IL-4, these myeloid cells led to generation of cells expressing high levels of major histocompatibility complex class I and II molecules, CD1a, CD11c, CD80, CD86, and CD40, had typical dendritic morphology, and were functional as they were capable of antigen processing and triggering T cell responses.

These studies to derive functional DCs from hESCs under appropriate conditions will facilitate future studies to better understand DC biology and potential novel DC vaccines or DC-mediated induction of immune tolerance.

4.4. Engraftment of hESC-derived hematopoietic cells

HSCs are defined by their ability to successfully support long term, multi-lineage development of all hematopoietic cell lineages *in vivo*. Since it is not feasible to test transplantation and engraftment of still poorly characterized hematopoietic cell populations such as those derived from hESCs in patients, immune-deficient mice are typically used for these analyses.

So-called SCID-repopulating cells (SRCs) are considered to be a close surrogate for HSCs, and this assay has been commonly used to test other populations of putative HSCs, such as those derived or isolated from human umbilical cord blood.

To date, three published studies have evaluated the engraftment potential of hESC-derived hematopoietic cells.^{56,67,68} All these studies were done using the H1 and H9 cell lines. Two studies utilized NOD/SCID mice, whereas one report utilized the Dorset Marino Sheep fetal sheep model (< 65 days old) for engraftment. Using an intra-bone-marrow transplantation (IBMT) technique where the hematopoietic cells differentiated cells are injected directly into the bone marrow (usually femur) of a mouse, one study was able to show successful engraftment of hESC-derived cells in 11 out of 19 mice.⁵⁶ The engrafted cells included lymphoid (CD45⁺CD19⁺), myeloid (CD45⁺CD133⁺), and erythroid (glycophorinA⁺) cells. However, the levels of human reconstitution and the frequency of detection were limited compared with UCB-derived cells. Interestingly, this same study was unable to demonstrate successful engraftment after IV (tail vein) injection of the mice with hESC-derived hematopoietic cells. Indeed, there was actually a decrease in survival of the mice after IV injection due to aggregation of the cells postinjection, resulting in pulmonary emboli. No secondary transplantation studies were done here.

The other NOD/SCID transplantation study,⁶⁸ using both IBMT and the IV injection of hESC-derived hematopoietic cells, did find successful engraftment in the mice without any decreased survival or pulmonary emboli after IV injection. This difference probably reflects variations in the cell populations derived by alternative methods (by coculture on S17 stromal cells versus by EB formation). In this study, bone marrow analyzed three or more months after IV injection showed on average 0.69% human CD45⁺ cells (compared to 2.98% of human CD45⁺ cells seen in mice injected with cells derived from UCB). In mice where IBMT was used, the level of engraftment was seen to be 1.88% in the femur directly injected with the cells and 1.79% in the contra lateral femur. Analysis of the engrafted cells found most to be CD45⁺CD33⁺ myeloid cells; however, some CD34⁺ cells were also seen, suggesting some HSC survival. Secondary transplantation studies were done to demonstrate successful long term engraftment in the secondary recipients, though at a level only detectable by very sensitive PCR analysis.

Although NOD/SCID mice are reported to have little NK cell activity, several analyses of peripheral blood mononuclear cells demonstrated

that these mice retain some NK cell activity.^{69,70} In this regard, the second study described above also evaluated mice treated with anti-asialo GM1 (ASGM1) antiserum (which depletes NK cells) the day before injection of hESC-derived cells, and subsequently every 11 days posttransplantation. This treatment led to an enhanced level of engraftment, to 1.74%. This increase in the engraftment is likely related to the fact that hESC-derived progenitors have a lower HLA class I molecule expression as compared to UCB-derived HSCs,⁶⁸ which would predispose them to NK-cell-mediated lysis.

In the fetal sheep transplantation study, hESC-derived hematopoietic cells were injected *in utero* into the peritoneal cavity of sheep at less than 65 days' gestation. Five to seventeen months after birth, approximately 0.1% human CD34⁺ or CD45⁺ cells were seen in the BM and/or peripheral blood (PB). Also, both myeloid (CD15⁺CD36⁺) and lymphoid (CD2⁺) lineage cells were identified. Overall, this level of engraftment is decreased compared to use of UCB-derived cells, where 2–3% chimerism is typical.⁷¹ However, the low level engraftment was confirmed in the hESC studies by PCR for human DNA; BM samples were chimeric in 6 out of 8 animals with 0.001% to 0.09% of cells, analyzed at 33–39 months after transplantation. Furthermore, human hematopoiesis in secondary transplanted sheep was followed for up to 22 months, proving actual engraftment rather than just transfer of the hematopoietic progenitors. Overall, successful secondary engraftment helps to confirm that the recipient animals provide an appropriate hematopoietic niche for the development of the engrafted stem cells, and that the transplanted population contains true HSCs capable of long term engraftment.

In the three published studies, one⁶⁸ used unsorted cells, though the other⁶⁷ used sorted CD34⁺lin⁻ and CD34⁺CD38⁻ cells. It is not clear whether the cells used in the other study⁵⁶ were sorted or unsorted. The hESC-derived CD34⁺ cells consist of a heterogeneous cell population that includes endothelial cells in addition to hematopoietic cells. This mixed population with fewer SRCs may account for the difficulty of demonstrating high levels of long term multilineage engraftment. Further studies are needed to directly compare engraftment and survival of mice that receive unsorted versus sorted populations of different hESC-derived blood cells in order to evaluate the strengths and weaknesses of various phenotypic populations.

The age of the recipient is likely to be an important factor for successful engraftment of hematopoietic cells. It has been shown in mouse models

that engraftment of HSCs derived from the embryonic (yolk sac) stage fails in the sublethally irradiated mice but is successful if injected into the newborn pups.⁷² It has been hypothesized that the fetal liver (which is still active in hematopoiesis in the newborn pups) may provide an age-appropriate environment for the maturation of developmentally naïve HSCs derived from the embryonic stem cells. Interestingly, these transplanted cells in the newborn pups are able to reconstitute the sublethally immune-deficient adult mice upon secondary transplantation.

As seen in the above studies, compared to UCB-derived HSCs, the levels of engraftment in using HSCs derived from hESCs are relatively poor. Studies have shown that HSCs derived from hESCs have a distinct molecular signature that more closely resembles the HSCs derived from an early developmental stage at which the yolk sac and the fetal liver are the primary site of the hematopoiesis.⁷³ It has been noted that major gene families are expressed differentially in hESCs- and UB/PB-derived HSCs.⁵⁶ They include genes associated with cell adhesion, cell migration and cell transcription, and transcriptional regulation. This differential expression of various gene families may explain unique *in vivo* properties of the hESC-derived HSCs. For example, higher expression of adhesion proteins like CKLF-1 and β_3 -integrin may reduce the ability of hESC-derived hematopoietic cells to migrate beyond the injection site and enter the circulation.

In contrast, compared to hESC-derived cells, UCB- and BM-isolated cells express higher levels of CXCR4, CD44, and I-selectin, proteins known to mediate the homing and engraftment behavior of HSCs.⁵⁶ Similarly, proteins like ADAM8⁷⁴ and ADAM17⁷⁵ that are involved in establishing HSC residence within the BM niche also have higher expression in UCB- and BM-isolated hematopoietic cells than in hESC-derived hematopoietic cells.

In addition, hESC-derived HSCs differentially express genes involved with accelerated cell cycle progression and loss of stem cell self-renewal ability, whereas somatic cells express genes required for maintenance and control of the quiescent cell cycle status, essential for the function of the transplantable HSCs.⁵⁶ As there is no single gene family which is expressed differently by hESC-derived HSCs, it is unlikely that ectopic expression of single genes like HoxB4 will make the behavior of hESC-derived hematopoietic cells similar to that of HSCs derived from somatic sources. Ectopic expression of the HoxB4 gene in mESC-derived HSCs does lead to markedly improved hematopoietic engraftment potential.¹⁶ However,

ectopic expression of the same gene was unable to improve engraftment of hESC-derived HSCs,⁵⁶ though it was difficult to determine if there was stable expression of HoxB4 in the hESC-derived blood cells. These results suggest intrinsic differences in the basic biology of mESC-derived HSCs from the hESC-derived HSCs cells. Also, the limitations in the ability of hESC-derived HSCs to activate a genetic program similar to the spectrum of genes expressed by somatic HSCs may account for their limited proliferation and migratory capacity *in vivo*.⁵⁶

To produce HSCs with better long term multilineage engraftment potential from hESCs, it is likely necessary to develop culture techniques that more closely resemble the *in vivo* microenvironment required to stimulate a genetic program needed for not only the hematopoietic specification of the hESCs, but also the transition from primitive to definitive hematopoiesis. In this regard, we need a better understanding of the pathways involved in this complex process. Several signaling pathways, like Wnt, Notch, and Hedgehog, are likely to play a prominent role in this genetic program.⁵⁶

One important but often-overlooked outcome of these three studies concerns the safety of hESC-based therapies due to the ability of the undifferentiated hESCs to form teratomas upon injection into animals. However, no teratoma formation has been seen in any of these engraftment studies done so far using hematopoietic progenitors derived from hESCs. This is despite using immune-compromised mice that were also sublethally irradiated, some of which received additional anti-NK-cell treatment. While a more rigorous study would be needed to definitively prove that transplantation of hESC-derived hematopoietic cells poses no risk of teratoma development, these studies seem to suggest the safety of this cellular therapy.

5. Hematopoiesis and Strategies to Overcome the Immune Barriers

Any future therapies derived from hESC-derived cells would typically result in the use of allogeneic cells and be subject to immune-mediated rejection. Potential means of preventing rejection of these cells have previously been outlined.^{15,16,76} These methods include the use of immune-suppressant drugs such as cyclosporin A, modification of the hESCs to make them more tolerant, and creation of “banks” of hESC lines that

would provide appropriate histocompatibility matching.¹⁶ Potentially, the most efficacious strategy would be to use somatic cell nuclear transfer (SCNT) to derive HLA-matched patient-specific hESC lines. These patient-specific hESCs could then be induced to differentiate into the lineage of choice, as needed for a particular therapy. Indeed, this type of strategy was used to demonstrate effective correction of genetic immunodeficiency in a mouse model.¹⁷

However, in addition to the unfortunate ethical/political debate and concerns, to date it has not been possible to derive hESCs via this technique, though this has been effective in other species.⁷⁷⁻⁷⁹ Many questions remain unsolved regarding the biology of the ES cells derived from SCNT. They include epigenetic influences, genomic imprinting and genomic stability of these cell lines. Furthermore, there has been an increased incidence of abnormalities in the animals derived from the technique. Therefore, it is crucial to fully understand the basic biology behind SCNT before this technique can be put to practical use.

Hematopoietic chimerism is also a very intriguing means of overcoming immune rejection of hESC-derived cells. This method is especially relevant to discussion of hESC-derived blood cells, as development of this lineage will be necessary for eventual chimerism-induced tolerance. This principle comes out of both animal experiments and human clinical experiences. Several animal models clearly demonstrate that successful immune tolerance can be achieved to the donor graft tissue by inducing mixed hematopoietic chimerism.^{80,81} This mixed chimerism results from engraftment of pluripotent HSCs, which leads to stable long term coexistence of multilineage hematopoietic cells of the donor within the host. Chimeric recipients have specific immunological tolerance to the alloantigens that are expressed by hematopoietic cells of the same donor, and do not reject a tissue or organ allograft of the donor haplotype.⁸¹ Several cases have been reported in which successful immune tolerance to solid organ transplantation was induced by prior bone marrow transplantation.^{82,83}

Similarly, it may be possible to induce mixed chimerism using hESCs differentiated into hematopoietic cells.⁷⁶ In this approach a recipient requiring a particular organ would first be transplanted with blood-group-ABO-matched (to prevent hyperacute rejection) but not necessarily completely HLA-matched HSCs to induce chimerism. After inducing stable mixed chimerism, the patient would be transplanted with a cell population or tissue (i.e. neurons, pancreatic islets, or cardiomyocytes) that the patient requires, derived from the same hESC line used to induce the

hematopoietic chimerism. One study using rat-ES-like cells suggested that such a strategy might prove more effective.⁸⁴ In this study stable chimerism was induced, and the host readily accepted a heart transplant from the same donor rat strain without any evidence of rejection.

6. Future Directions for Studies of Hematopoiesis from Human ES Cells

NOD/SCID mice have been the primary strain used to evaluate human SRCs isolated from sources such as bone marrow or UCB. However, this strain may not be optimal for engraftment of hESC-derived hematopoietic cells. NOD/SCID mice do have some innate immunity, and effectors such as mouse NK cells may recognize and kill hESC-derived blood cells.⁶⁶ Indeed, we have found that hESC-derived blood cells express lower levels of HLA class I molecules than hematopoietic cells obtained from more mature sources.⁸⁵ NK cells are especially attuned to kill cells with low class I expression. Indeed, studies of mESC-derived blood cells also have low class I expression and are better able to engraft in mice that lack NK cell activity.⁸⁶

Other strains of mice that are “more immunodeficient” have recently been utilized to demonstrate more effective development of a functional immune system when engrafted with human hematopoietic cells. These more effective mouse strains include Rag2^{-/-}/γc^{-/-} mice that have defective expression of both the recombinase-activating gene (Rag) which results in a lack of adaptive (T and B cell) immunity and the common γ chain of the IL2, IL4, IL7, and other cytokine receptors leading to a lack of NK cell activity. Also, the the NOD/SCID/γc^{-/-} mouse which combines the NOD/SCID defects with the γc deletion results in more complete immunosuppression and increased multilineage engraftment of human hematopoietic cells. Other novel mouse strains suitable for engraftment of human cells have also been described.⁸⁷ It will be of interest to now utilize these strains for studies of human-ES-cell-derived hematopoietic precursor cells. Use of neonatal mice for these transplantation studies may also be advantageous.

Additionally, nonhuman primates can be used in developing and testing several novel therapies prior to being tested in humans. Nonhuman primate ES cells differ from mESCs and resemble hESCs in several aspects, including colony morphology, growth requirements, and developmental

molecular signature.^{3,88,89} Therefore, they would serve as an excellent pre-clinical model for hESC engraftment studies. Indeed, one study has used genetically labeled (GFP+) cynomolgus ES cells and transplanted them *in utero* into the cynomolgus fetus in the abdominal cavity or into the liver.⁹⁰ While there was successful engraftment of these cells, it was surprising to also see teratoma formation in some of the animals.⁹⁰ Another group has induced mesodermal differentiation of cynomolgus embryonic stem cells and then transplanted them into the fetal sheep liver.⁹¹ Here, cynomolgus hematopoietic progenitor cells were detected in bone marrow at a level of 1–2%, with no teratoma formation.

Genetic modification of hESCs is another technology essential for better characterization of how specific genes regulate development of specific human cell lineages. While a variety of methodologies have been utilized to obtain stable transgene expression in hESCs, most of them suffer from low efficiency and gene silencing.⁹² Use of lentivirus-based vectors has allowed the most stable expression of foreign genes in hESCs, though these vectors are relatively complicated and time-consuming to produce. Recently, we have demonstrated the use of transposon-based genetic systems that allow efficient and stable transgene expression in hESCs.⁹³ We anticipate that transposons may develop into an optimal method for genetic modification of hESCs to allow future gain-and-loss-of-function analyses.

Summary

This chapter has provided a comprehensive review of studies to derive hematopoietic cells from hESCs, beginning soon after hESCs were first isolated and described in 1998. In these few years, essentially all blood cell types of the adult body and bone marrow have demonstrated feasibility in HSCs being produced from hESCs using methods that are defined to varying degrees. While it is difficult to know for sure, it is likely that more work has been published on hematopoietic development from hESCs than any other cell lineage. Indeed, as described, the strong 50-plus-year history of research on blood cell development has greatly facilitated progress in this new developmental model.

Characterization of putative HSCs capable of long term, multilineage engraftment that can be derived from hESCs remains less than satisfactory. While hematopoietic engraftment in the immunodeficient mouse and fetal sheep models shows some intriguing potential,^{56,67,68} more work

is clearly needed. The level of engraftment in these studies is reminiscent of work using mESCs, though there syngeneic mice could be utilized to reduce immune barriers. mESC-derived hematopoietic engraftment is facilitated by overexpression of genes such as HOXB4. However, similar studies using hESCs have not been as successful or clear-cut.^{55,56} This highlights the possibility that there are distinct differences in HSC development between the two models, as well as technical barriers with hESCs, that remain to be resolved.

Finally, ESC and adult stem cell research are often described as two separate and distinct systems. This is clearly not the case, since studies of isolated adult stem cells such as HSCs definitely aid in analysis of similar populations that may be derived from hESCs. Additionally, it is likely that the hESC developmental model will have positive feedback on adult stem cell models. Future progress in both the basic science and clinical applications of hematopoietic cell development will require optimal use of all available resources.

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