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Autologous blood cell therapies from pluripotent stem cells

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Summary

The discovery of human embryonic stem cells (hESCs) raised promises for a universal resource for cell based therapies in regenerative medicine. Recently, fast-paced progress has been made towards the generation of pluripotent stem cells (PSCs) amenable for clinical applications, culminating in reprogramming of adult somatic cells to autologous PSCs that can be indefinitely expanded *in vitro*. However, besides the efficient generation of bona fide, clinically safe PSCs (e.g. without the use of oncoproteins and gene transfer based on viruses inserting randomly into the genome), a major challenge in the field remains how to efficiently differentiate PSCs to specific lineages and how to select for cells that will function normally upon transplantation in adults. In this review, we analyse the *in vitro* differentiation potential of PSCs to the hematopoietic lineage discussing blood cell types that can be currently obtained, limitations in derivation of adult-type HSCs and prospects for clinical application of PSCs-derived blood cells.

Keywords

human embryonic stem cells; ESC; induced pluripotent stem cells; iPS; differentiation; hematopoietic stem cells; blood; transplantation; autologous; isogenic; erythrocytes; NK cells; neutrophils; lymphocytes

Introduction

Studies on pluripotent stem cells (PSCs) started almost three decades ago with the discovery of mouse embryonic stem cells (mESCs) by Martin¹ and Evans². mESC can differentiate into every tissue of the adult body: when reinjected in the developing embryo, mESCs chimaerize all tissues, including the germline. Over the last years, ESC technology has been extensively used for genetic manipulation of the mouse genome and creation of mutant

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mouse strains, rendering enormously valuable insights into genetic regulation of tissue function and disease pathogenesis. The discovery of human embryonic stem cells (hESCs) in 1998³ opened up exciting prospects for the use of ESC in regenerative medicine. Several organs of the adult are dependent on a stem cell pool for maintenance, and malignant or degenerative disorders affecting this cellular compartment might be treated with stem cell replacement therapies. The most prominent example is the hematopoietic system, where transplantation of hematopoietic stem cells (HSCs)⁴ is a well-established clinical tool in the treatment of malignant (e.g. leukemia and lymphoma) or genetic blood diseases (e.g. Fanconi's Anemia, immunodeficiency and hemoglobinopathy). More recently, stem cells have been identified in several other tissues of the adult (e.g. skin, gut, nervous system, lung, mammary gland). However, adult stem cells are rare, and despite attempts to drive *ex vivo* expansion, adult stem cells including HSCs remain difficult to maintain and proliferate in culture. In contrast, ESCs can be indefinitely expanded in an undifferentiated state, providing limitless amounts of cellular material. Advances in the generation of patient-specific PSCs⁵ open up prospects for autologous cellular therapies that would lack immune rejection. However, directed differentiation of PSCs into tissues of interest remains challenging. Despite intensive efforts, currently available *in vitro* differentiation protocols offer limited recapitulation of embryonic development and obtaining adult-type tissue progenitors that will function normally upon transplantation remains difficult. In this review, we discuss possible sources of histocompatible PSCs, analyse *in vitro* blood differentiation from such pluripotent cells, and discuss prospects for therapeutical applications.

Genetically customized grafts from pluripotent stem cells

Hematopoietic stem cell transplantation (HSCT) is the best established clinical cellular replacement therapy, dating back to 1957 when Thomas and colleagues first reported intravenous infusions of bone marrow in patients receiving radiation and chemotherapy⁴. In the ensuing decades transplantation of allogeneic HLA-matched bone marrow or mobilized peripheral blood CD34⁺ cells has become the standard therapy for patients suffering from a variety of malignant or genetic disorders of the hematopoietic cell compartment. However, allogeneic HSCT is accompanied by significant morbidity and mortality related to graft rejection, acute and chronic graft-versus-host-disease (GvHD), as well as infections occurring during the transition period before transplanted HSCs take over blood cell function. Autologous HSCT, in which a patient's own stem cells are harvested prior to high-dose chemotherapy, is less toxic because there is no GvHD and more rapid engraftment translates into lower rates of infectious complications. However, in patients with genetic conditions such as sickle cell anemia and thalassemia, autologous therapies necessitate correction of the genetic defect by gene therapy in the patient's HSCs, which is cumbersome due to the challenges of maintaining HSCs in culture, the intrinsic difficulties of expressing genes in HSCs, and the risk of insertional mutagenesis after gene transfer with viral vectors⁶. In contrast, generating patient's own PSCs, and using for example homologous recombination to correct genetic defects prior to differentiation into transplantable HSCs promises to overcome caveats of conventional HSCT therapies.

Classically obtained ESCs³ would face immune barriers when transplanted into (genetically non-identical) hosts. While ESCs themselves express only low levels of MHC antigens, these levels increase strongly during differentiation⁷, and grafts composed of ESC-derived progeny would provoke immune reactions and face rejection upon transplantation in genetically mismatched hosts. Thus, much effort has been invested to generate histocompatible PSCs. Early work by Briggs and Gurdon in the 1950s⁸ and 1960s^{9,10} demonstrated that replacing the nucleus of frog oocytes with nuclei from somatic cells enables development of organisms expressing the genetic information of the somatic cell donor. This principle has been successfully applied in some mammalian species where

nuclear transfer (NT)-embryos have been used to derive ESC lines. NT-ESCs are isogenic with the somatic cell donor, and thus a source of histocompatible transplant tissue. Rideout and colleagues performed a proof of principle experiment in an immunodeficiency mouse model, showing that such cells can be used for treatment of genetic disease: NT-ESCs were generated from *Rag2*^{-/-} mice; the genetic defect was corrected by homologous recombination; and the resulting ESCs differentiated *in vitro* into repopulating HSCs¹¹ capable of restoring immune function upon transplantation into *Rag2*^{-/-} mice¹².

Nuclear transfer is an elegant method for the generation of isogenic cellular products¹³, but the downside of this procedure is its very low efficiency. To our knowledge, derivation of human NT-ESCs has not yet succeeded, although there is one report on successful generation of human blastocysts via NT¹⁴ (reviewed in¹⁵). Human NT faces ethical concerns, and is further burdened by the high numbers of human oocytes that are needed as recipient cells. A more efficient method rendering histocompatible PSCs is direct oocyte activation in a process called parthenogenesis¹⁶. Parthenogenetic cells are genetically similar, but not identical to the oocyte donor. Tissue products from parthenogenetic sources may be homozygous^{17,18}, and thus susceptible to rejection through natural killer cells which recognize the missing antigens upon transplantation into the oocyte donor. However, by analysing high numbers of mouse parthenogenetic ESC lines derived in our laboratory, we found a surprisingly high degree of histocompatibility due to heterozygosity at the MHC loci, based on early recombination events during oocyte maturation¹⁶. However, histocompatible parthenogenetic ESCs would be only available for women capable of oocyte donation, and further analysis should inform whether imprinting aspects biases their differentiation into specific tissues.

During NT somatic cell nuclei are reprogrammed by the ooplasm. Significant effort has been expended to identify the mechanisms underlying these processes, in an attempt to replace the ooplasm effect by defined factors. In 2006, Takahashi and Yamanaka presented ground-breaking results from a screen on a mini-library of embryonic factors for their effect on somatic cells¹⁹: While single factors were ineffective, combinatorial retroviral transduction with four defined factors known from ESC biology (*Oct3/4*, *Sox2*, *c-Myc* and *Klf4*) was able to reprogram mouse fibroblasts to cells that resembled ESCs both functionally and molecularly¹⁹. The reprogrammed cells could be expanded as cell lines and were termed induced pluripotent stem (iPS) cells. Shortly after the initial report, three independent research groups replicated these results with human cells²⁰⁻²². During the last year and a half, a multitude of studies from numerous research groups has reported derivation of reprogrammed PSCs in mouse and human, using different somatic cells as starting populations (e.g. liver and stomach cells²³, neural stem cells^{24,25}, pancreatic beta cells²⁶, B-lymphocytes²⁷ and, most recently, human mobilized CD34+ cells from peripheral blood²⁸), transduction with different embryonic factors, treatment with histone deacetylase inhibitors²⁹ or small molecules³⁰, in an attempt to develop protocols devoid of oncoproteins and integrating viruses³¹⁻³⁴ (reviewed in¹⁵) that can be translated into clinical application.

Pluripotent stem cells have also been isolated from mouse and human embryonic gonads^{35,36} and more recently from postnatal testis³⁷⁻⁴⁰. Gonads-derived cells have been shown to form derivatives of all three germ layers and differentiate into hematopoietic cells⁴¹. To our knowledge, a careful analysis of their potential to generate distinct blood progenitors and a comparison with pluripotent stem cells from other sources is currently missing. Origin and characteristics of currently available pluripotent stem cells are summarized in Table 1.

Challenges of pluripotent stem cells *in vitro* differentiation

ESCs transplanted into immunodeficient murine recipients form teratomas, demonstrating their pluripotency. Thus, to obtain specific transplantable tissues, PSCs need to be predifferentiated *in vitro*. When removed from the specific culture conditions that sustain their self-renewal, ESCs spontaneously form cystic structures termed embryoid bodies (EBs) that contain derivatives of the three germ layers, including blood cells⁴². The presence of serum in the differentiation medium provides a mixture of growth factors that allows development of several lineages. This procedure clearly demonstrates the *in vitro* developmental potency of ESCs, but has some important disadvantages: (1) the efficiency of differentiation into specific lineages is highly variable; (2) selection for the cells of interest (e.g. by surface antigens) is required prior to transplantation; last but not least (3) the presence of bovine serum hampers clinical applications requiring protocols free of contaminating animal products.

To drive tissue formation from ESCs and to progress towards directed differentiation protocols using defined serum-free conditions, it is essential to follow a developmental biology approach (reviewed in ^{43,44}). From a developmental standpoint, ESCs are the equivalent of early epiblast cells and recapitulate *in vitro* aspects of early embryogenesis: guided by morphogens (e.g. Wnt, TGF-beta, Activin and BMP). ESCs progress through a primitive-streak like stage before forming the three germ layers, endoderm, ectoderm and mesoderm⁴⁵, which is the layer giving rise to blood. While early embryonic stages are quite faithfully mimicked⁴⁶, spontaneously differentiating EBs may not offer ideal conditions for the refined processes occurring later in development. EBs lack the anatomical structure of the embryo, and cells developing in EBs do not participate accurately in embryonic cell-to-cell interactions, and miss certain cell non-autonomous effects and exposure to physical stimuli that physiologically occur at specific stages of development (as for example the flow pressure and cellular movement occurring with onset of embryonic circulation⁴⁷). It is likely that *in vitro* differentiating ESCs are prone to generate immature, embryonic-type progenitor cells, that may not function properly upon transplantation in adults. However, these apparent inconveniences at the same time harbour the chance that – assuming specific embryonic differentiation cues are identified and provided *in vitro* – large homogenous populations of cells can be generated in this system. Therefore, *in vivo* studies of embryonic development in mice, or in developmental models such as zebrafish, *Xenopus* or chick remain a key approaches towards learning how to generate adult-type cells from ESC.

Another hurdle towards generation of ready-to-use adult-type cells from human PSC are the assays available for the evaluation of human cells. Human cells are less characterized than murine (for example with respect to surface antigens or molecular signature) and functional evaluation is confined to surrogate xenogeneic models which can introduce biases and not always faithfully reflect cell function. For example, human HSCs transplanted into mice rely on mouse cytokines and niche factors with only limited cross-reactivity to human cells⁴⁸, while human heart grafts in rodent models are exposed to atypically high heart beat frequencies. Such environmental factors bias functional evaluation of the cells and may contribute to reported differences between mouse and human cells. HSCs showing long-term engraftment were obtained from murine ESCs¹¹, and iPS cells⁴⁹ using transduction with *Hoxb4* and coculture on OP9 stromal cells, while human HSCs could not be produced following similar protocols⁵⁰.

In many transplant protocols, using iPS cells would be advantageous as the derived cells would be isogenic and would not face immune barriers in the host. The pluripotent nature of mouse iPS cells⁵¹ has been demonstrated by blastocyst chimaerisation and tetraploid complementation assays, and the proof of principle experiment that such cells can be used

for providing isogenic, genetically corrected cellular products has been performed in a sickle-cell anemia mouse model⁴⁹. Undifferentiated human iPS cells show a molecular signature highly similar to human ESC²⁰⁻²², and hESC-protocols can be used to drive their *in vitro* differentiation into derivatives of the three germ layers²². However, critical issues such as viral integration and residual levels of transgene expression⁵² may impact the differentiation potential of iPS cells. Obviously, individual reprogramming protocols can introduce distinct biases (e.g. through use of different embryonic proteins, higher transgene persistence after use of lentiviral versus retroviral vectors, off-target effects of small molecules)⁵¹. Detailed characterization of the ability of human iPS cells to form specific tissues and functional characterization of the resulting cells is still in its infancy. However, recent studies provide encouraging evidence that generation of cardiomyocytes⁵², adipocytes⁵³ and hematopoietic and endothelial cells is similar between human ESCs and human iPS^{20,21,54}. Data from our laboratory supports these observations, showing robust blood cell formation that could be further enhanced by bone morphogenetic 4 supplementation (BMP4) in differentiating human iPS cells generated by a separate protocol²² (Lengerke et al, *in press*). Since only scant data currently available on the differentiation potential of human iPS cells, in this review we will highlight results reported on differentiating hESCs.

Hematopoietic development during embryogenesis: primitive versus definitive blood

When attempting to differentiate pluripotent stem cells into adult-type blood cells, we need to assess how differentiating EBs recapitulate embryonic hematopoietic development. Hematopoietic and endothelial cells both arise from the mesodermal germ layer. Common precursors called “hemangioblasts” have been found in early stage mouse conceptuses⁵⁵ and clonally identified in *in vitro* differentiating mouse and human ESCs⁵⁶⁻⁵⁸. Moreover, vertebrate hematopoiesis occurs in two successive waves, primitive and definitive, that differ anatomically and in cell types produced. In the mouse, primitive (embryonic) blood develops transiently in the extraembryonic yolk sac, giving rise to the first blood cells consisting mainly of nucleated erythrocytes and macrophages. The definitive blood program occurs subsequently at intraembryonic sites, and lasts the life of the organism, producing hematopoietic stem cells (HSCs) capable of extensive self-renewal and multilineage differentiation into all blood lineages.^{44,59,60} Recent studies suggest that hematopoietic cells arise from hemangioblasts through a hemogenic endothelial intermediate, and show by time-lapse microscopy hemogenic endothelium from *in vitro* differentiating mouse ESCs as well as early mouse embryos^{61,62}. It is unclear at this point whether endothelial cells giving rise to primitive blood cells differ from those producing definitive blood cells.

Whether definitive HSCs arise in the yolk sac has been debated for decades. The first experiments aiming to trace the origin of blood cells were performed by Moore and Owen: chick embryo where grafted onto a donor yolk sac, and after several days of incubation, donor yolk sac derived hematopoietic cells were found in the hematopoietic organs of the recipient embryo, demonstrating the ability of yolk sac cells to participate in definitive hematopoiesis.^{63,64} However, these experiments were performed after the onset of circulation, and contamination from circulating HSCs was possible: with the onset of blood flow, cells from intraembryonic sites migrate back to the yolk sac, which then might contain definitive HSCs. Repetition of the avian grafting experiments using precirculation yolk sacs detected no contribution to host embryo hematopoiesis, arguing that indeed adult HSCs arise solely from an intraembryonic location⁶⁵⁻⁶⁷. Analyses identified intraembryonic blood sites within the chick aortic endothelium⁶⁸ and cells displaying hallmark properties of adult HSCs (long-term repopulation and multilineage differentiation upon transplantation in irradiated adult hosts) in the murine aorto-gonado-mesonephros region (AGM)^{69,70}.

However, cells from precirculation murine yolk sacs can also give rise to definitive HSCs, if they are transplanted into the supportive environment of the fetal liver of newborn mice^{71,72}.

Generating blood from PSC

Analysis of *in vitro* differentiating PSCs reveals a close resemblance to *in vivo* embryonic processes^{46,73,74}: supplementation of PSCs cultures with morphogens and inductive signals known from *in vivo* developmental models (e.g. zebrafish, frog, chick) enables directed differentiation in the absence of serum-containing medium⁴⁶ (reviewed in ⁴⁴). When directed by morphogens like bone morphogenetic protein (BMP), Wntless (Wnt) and Activin, ES cells develop into cells equivalent to the primitive streak, and give rise to cells of the three germ layers (ectoderm, endoderm and mesoderm)⁴⁵. Gene expression analysis distinguishes between the formation of anterior primitive streak-like cells, developing mostly into endoderm, and posterior primitive streak-like cells that will differentiate into mesoderm and from there through activation of patterning genes (e.g. *Cdx/Hox*), into hemangioblasts⁴⁵ and blood cells. Mouse hemangioblasts express the mesodermal marker *Brachyury* and can be identified around day 3 in differentiating EBs by expression of fetal liver kinase 1 (Flk1)⁵⁸. Human hemangioblasts⁵⁶ are phenotypically less well described, yet are found in the CD34⁺CD45⁻ cellular population⁷⁵ and express KDR (VEGF receptor 2)⁵⁶, CD31⁷⁶, and angiotensin-converting-enzyme (ACE/CD143)⁷⁷. With maturation along the blood lineage, these progenitors start expressing the hematopoietic antigen CD45⁷⁸. Emergence of CD34⁺CD45⁺ cells correlates with derivation of hematopoietic progenitor cells with colony forming unit (CFU) potential in methylcellulose assays, and sorting for CD34⁺CD45⁺ cells enriches for cells giving rise to hematopoietic colonies⁷⁶.

Two different protocols, or combinations of them, can be used for generating hematopoietic progenitors from PSCs: (1) formation of embryoid bodies (EBs) and (2) culture on supportive stromal layers⁷⁹ (e.g. OP9-stromal cells, reviewed in ⁴⁴). What type of hematopoietic progenitors are routinely generated from ESCs remains an intriguing question. Most probably, ESCs readily give rise to yolk-sac-type progenitors, generating primitive erythroid cells as well as macrophages, definitive erythroid cells, megakaryocytes and mast cell lineages, and do not routinely differentiate into specific lymphoid cells or true hematopoietic stem cells (HSC). However, if exposed to specific conditions, ESCs do form B-⁸⁰ and T-⁸¹ lymphoid cells. Thus, even though yolk-sac-type hematopoiesis is predominant, definitive hematopoietic cells may be obtained from *in vitro* differentiating ESCs.

PSCs-derived HSCs

The ultimate goal remains generating “off-the-shelf” PSC-derived human HSCs that will be capable of repopulation and durable reconstitution of the entire human hematopoietic system in adults. There are several reports on murine ESC-derived hematopoietic elements capable of longterm multilineage engraftment (reviewed in ⁴⁴, Figure 1). Burt and colleagues reported formation of c-kit⁺CD45⁺ transplantable hematopoietic progenitors, capable of longterm multilineage reconstitution of mice, following culture of mESCs in methylcellulose in the presence of serum, stem cell factor, IL-3 and IL-6⁸². Importantly, direct delivery to the bone marrow via intrafemoral instillation enabled significantly higher numbers of engrafted cells, as compared to intravenous application in the tail vein⁸². These findings are consistent with results from other studies documenting the superiority of intra-bone marrow over intravenous transplantation^{83,84}, and suggest that providing direct contact with the niche may be especially important for developmentally immature stem cells. Interestingly, applying high numbers of purified c-kit⁺CD45⁺ cells enabled engraftment even in MHC-mismatched mice, without signs of graft rejection or induction of GvHD.

Tolerance induction through high numbers of transplanted material may be another advantage offered by the PSC-system. However, the encouraging results reported in this study have not been yet independently replicated, as they also have not for the early study of Palacios and colleagues who reported back in 1995 robust multilineage repopulation following co-culture with the stromal cell line RP010⁸⁵. In this latter case, replication of results has been hampered by the fact that RP010 cells are not readily available.

Co-culture with stromal cell lines (e.g. derived from AGM or fetal liver, where HSC form and expand during their *in vivo* embryonic development) is an appealing way of promoting maturation and expansion of PSC-derived blood progenitors. A prominent example is the well-known co-culture with OP9-stromal cells, which provides a supportive microenvironment⁵⁰, generating enhanced hematopoietic activity and promoting lymphogenesis. OP9-stromal cells are derived from the calvariae of newborn *op/op* mice which lack macrophage colony stimulating-factor (M-CSF)⁸⁶. The absence of M-CSF inhibits the survival of monocyte-macrophage cells, which otherwise overwhelm other lineages. If, in addition to co-culture on OP9-stromal cell, enforced expression of the patterning genes *Cdx4/HoxB4* is performed, it is possible to generate true ESC-derived murine HSCs, capable of robust multilineage hematopoietic reconstitution in irradiated adult mice^{11,50,87}. Several groups have reported production of repopulating HSCs following genetic modification of mESC-progenitors with the homeobox gene *HoxB4* and expansion on OP9-stroma cells^{11,24,88}. Following this protocol, repopulating murine HSC have been efficiently generated also from pluripotent stem cells of different origin (e.g. parthenogenetic⁸⁹ and reprogrammed iPS cells⁴⁹, Table 1). Homeobox (*Hox*) genes are transcription factors involved in embryonic tissue patterning processes, and play important roles in developmental hematopoiesis as well as homeostasis and malignant transformation of adult blood cells (reviewed in ⁹⁰). Particularly *HoxB4* has been shown to enhance self-renewal and/or growth activity of hematopoietic progenitors and HSCs in mouse and human cells⁹¹⁻⁹³. In immature murine yolk-sac or ESC-derived hematopoietic progenitors, enforced *HoxB4* expression renders competence for longterm multi-lineage engraftment of irradiated adult hosts¹¹. However, for reasons that are not clear, lymphoid engraftment appears less robust than myeloid engraftment in these cells. Possibly, persisting *HoxB4* overexpression biases further differentiation potential of the transplanted cells away from the lymphoid lineage⁸⁸. Engraftment rates, especially also engraftment with lymphoid cells, can be enhanced by combinatorial transduction with *Cdx4*, a homeobox transcription factor of the caudal homeobox gene family. *cdx* genes have been first discovered as early patterning regulators of hematopoietic fate in zebrafish and later shown to promote hematopoiesis from murine ESC through regulation of downstream posterior *Hox* genes^{74,87,94-97}. Moreover, recent studies suggest *CDX* genes involvement in human leukemogenesis of the myeloid as well of the lymphoid lineage⁹⁸⁻¹⁰¹, reinforcing the notion that molecular pathways are shared between developmental and adult hematopoiesis (as seen also with *Hox* genes). However, this approach requires genetic modification and has not been successful with human cells: co-culture with OP9 and transduction with *HoxB4* cells promotes hematopoietic activity from human ESCs, by inhibiting apoptosis of ESCs-derived CD34⁺CD45⁺ cells; however, it does not confer stem cell function to human progenitors (see below)⁵⁰. Recently, Ledran and colleagues reported that culture of human ESC on a number of stromal cell lines and primary cells derived from the AGM and fetal liver significantly enhanced hematopoietic activity, including hematopoietic engraftment capacity into immunocompromised mice in primary and secondary transplant assays⁷⁹. However, further studies are needed to confirm these data and to improve chimaerism.

While transduction with *HoxB4* and/or *Cdx4* promotes robust engraftment, cells exhibiting the classical surface antigen phenotype of engraftable murine HSC (c-kit⁺Sca1⁺Lin⁻) have not yet been reported in this system. To our knowledge, CD41 is the earliest antigen

characterizing preformed embryonic blood progenitors, as assayed by *in vitro* colony forming assays and gene expression data^{96,102} from murine ESC and mouse embryos. In day 6 EB, hematopoietic activity is confined to cells expressing CD41⁺, which represent approximately 30% of EB-derived under differentiation in serum containing medium. Within the CD41⁺ compartment, colony forming activity is highly enriched among CD41⁺c-kit⁺ double positive cells, suggesting that c-kit marks cells with stem/progenitor capacity and may be downregulated with differentiation (CD41⁺ckit⁻ cells). Only CD41⁺(c-kit⁺) cells are able to form colonies on OP9-stroma cells, which is a necessary step in the development of transplantable cells. Ectopic expression of *Cdx4* enhances the hematopoietic activity of CD41⁺ckit⁺ cells, without conferring hematopoietic potential to CD41⁻ cells⁹⁶. The panhematopoietic antigen CD45 appears shortly after CD41 (day 6.5 to 7 in differentiating EB), presumably indicating conversion of CD41⁺ cells to more mature progenitors. However, colony forming potential declines with appearance of CD45, suggesting that further development in EB rapidly promotes terminal differentiation of the CD41⁺ progenitor cells (Lengerke C, unpublished observation). Recent comparison of the phenotype of ESC-derived repopulating HSCs with hematopoietic stem and progenitor cells derived from distinct *in vivo* developmental stages (murine yolk sac, aorta-gonad-mesonephros, placenta, fetal liver and bone marrow) suggests that ESC-derived HSCs are a developmentally immature population of cells with features of both primitive and mature HSC, defined as ckit⁺CD41⁺CD34⁻CD150⁺CD45⁺/CD48⁺)¹⁰³. Further studies are needed to characterize these cells from human ESCs.

Generating human HSC from hESC remains challenging (Table 3). As in the murine system, human ESC differentiate robustly into the hematopoietic lineage in EBs, as well as in co-culture systems with supportive stromal cells. Cells with features resembling adult-type HSC are produced: e.g. CD34⁺ expression, the ability to efflux Hoechst dye, high aldehyde dehydrogenase activity and multilineage hematopoietic colony potential in clonal assays¹⁰⁴. However, very limited repopulation ability is observed in xenogeneic transplant assays¹⁰⁵. *HoxB4* enhances hematopoietic activity of human blood progenitor cells⁹³, and OP9-stromal cell co-cultures augment survival of hESC-derived CD34⁺CD45⁺ hematopoietic precursors. However, neither *HoxB4* transduction nor OP9-stroma co-culture were able to confer HSC activity to hESC-derived cells^{50,105}. Interestingly, OP9-stroma co-culture effects the CD45⁺CD34⁺, but not the more differentiated CD45⁺CD34⁻ cellular compartment, indicating specific effects on hematopoietic progenitor cells⁵⁰. The fact that *HoxB4* does not promote HSC formation from hESC could be the result of technical issues or could reflect intrinsic differences in the biology of human and mouse ESC-derived hematopoietic cells. While differentiation in the murine system proves that derivation of HSC is possible, strategies for human ESC still need to be developed. To facilitate transition to clinical protocols, approaches involving animal products and co-culture systems should be replaced and stable transduction through inserting viral vectors avoided, replaced by strategies involving adenoviral gene transfer, protein transduction¹⁰⁶, or small molecules.

Production of specific blood lineages from PSC

While HSC remain difficult to obtain, there are several reports of successful derivation of specific lineages from ESC by supplementing cultures with cytokines or chemicals or/and using co-culture with specific stroma cell lines. Robust generation of murine erythroid cells and self-renewing immature erythroid progenitors¹⁰⁷, megakaryocytes^{108,109}, granulocytes^{110,111}, mast cells¹¹², eosinophils¹¹³, T and B lymphocytes^{81,86,114-116}, macrophages¹¹⁷, dendritic cells¹¹⁸⁻¹²⁰, NK cells¹²¹ has been reported from mESC (reviewed by Olsen and colleagues¹²²). This work in mESC has laid the foundation for interrogating detailed aspects of blood cell biology, such as the role of *GATA1* during proerythroblast maturation¹²³, and provided a basis for the development of similar protocols in hESC¹²²,

opening up prospects for use in cell replacement therapies. We will review here recent progress that has been made in blood cell production from hESC (Table 2).

Red blood cells from human PSC

Large-scale production of red blood cells from the human ESC line H1 was reported 2006 by Olivier et al. High numbers of erythrocytes (5000-fold increase in cell number) were obtained by a 14-day coculture with immortalized human fetal liver cells in the presence of serum-containing medium and subsequent expansion of CD34⁺ cells in serum-free liquid cultures supplemented with cytokines and defined factors (hydrocortisone, IL3, BMP4, Flt-3L, SCF, EPO, IGF-1, hemin)¹²⁴. However, the red blood cells obtained were mostly nucleated primitive erythroblasts, which expressed a mixture of embryonic and fetal globins but not beta-globin characteristic for adult cells¹²⁴. Two years later, the same laboratory reported that prolonging the differentiation cultures on fetal liver cells to 35 days promotes the differentiation of fetal liver-like erythroblasts which are smaller in size, express mostly fetal hemoglobin and are able to enucleate, suggesting that hESC-derived erythropoiesis mimicks human development and recapitulates accordingly the globin gene switch¹²⁵. Functional exploration of hESC-derived red blood cells showed oxygen equilibrium curves comparable to normal red blood cells and, adequate responses to pH and 2,3-phosphodiglycerate changes and suggests further *in vitro* maturation to adult-type beta-globin expressing cells^{126,127}. One study reports differentiation to functional erythrocytes under serum-free conditions, by using supplementation with cell-permeable recombinant *HoxB4* transcription factor¹²⁶. While these data provide encouraging results and a highly valuable model system to study genetic regulation of erythropoiesis, several issues need to be resolved before manufacture of red blood cells can become a clinical application¹²⁸: (1) the derivation methods need to be performed serum-free; (2) in a highly scalable fashion (up to now, the largest number reported was 2 billion red blood cells; in comparison, one 220-ml unit of packed red blood cells contains about 2 trillion cells); (3) at reasonable cost enabling industrial production; (4) with improved generation of adult-type beta hemoglobin expressing cells. Questions regarding half-life, immunogenicity, contamination with potentially tumorigenic cells need to be addressed before ESC-derived red blood cells can enter large-scale clinical applications and replace conventional red blood cell supplies.

While the issues listed above may hamper applications on a large industrial scale, there may be individual benefit for patients particularly in need of customized blood cells, for example patients requiring lifelong erythrocyte substitution therapies that over time develop alloantibodies that highly restrict the pool of matching blood cell donors. It is plausible that generating patient-specific blood cells from their own pluripotent stem cells will benefit and increase treatment possibilities in these patients.

Granulocytes and platelets from human PSCs

Granulocytes are not routinely provided by the current donation-based blood transfusion system. PSCs may represent a source for generation of large amounts of neutrophils for the treatment of patients presenting with life-threatening neutropenia. Several studies report efficient formation of myeloid cells from mouse and human ESC using combinatorial differentiation approaches in EB and coculture strategies with stromal cell lines (e.g. S17¹²⁹ and OP9^{104,78,104,105,129-132}). In a recent report, Yokoyama and colleagues provide evidence that functional neutrophils can be produced from hESC by using EB-differentiation in serum-based medium followed by coculture on OP9 cells for 7 to 14 days in medium supplemented with BMP4, SCF, Flt-3L, IL6, FP6, TPO and G-SCF. During culture on OP9, hESC-derived progenitors matured from mostly myeloblasts and promyelocytes on day 7 to mature, terminally differentiated neutrophils on day 14. The vast majority of OP9-co-

cultured progenitors expressed the hematopoietic marker CD45 at all timepoints, while expression of primitive cell markers (CD133, CD34 and CD117) was lost at later timepoints, indicating maturation to terminally differentiated neutrophils. Moreover, functional comparison of hESC- and human peripheral blood derived neutrophils showed similar phagocytosis, chemotaxis, superoxide production, bactericidal activity and oxidative burst capacity, despite observed slight differences in cellular phenotype (hESC derived neutrophils show decreased CD16 expression and aberrant CD64 and CD14 expression)¹³². Using similar approaches but different combinations of growth factors and cytokines (M-CSF, IL-3), other reports show homogeneous production of functional monocytes and macrophages from hESC, providing tools for investigating myeloid cell development and biology¹³³. Megakaryocyte formation has also been reported from human ESC^{131,134}. Using differentiation on OP9 stromal cells and supplementation with VEGF and TPO, Takayama and colleagues report generation of multipotent hematopoietic progenitors and efficient production of mature megakaryocytes expressing specific surface antigens such as CD41a, CD42a and CD42b¹³¹. Some megakaryocytes appeared to be shedding their cytoplasmic membranes, displaying demarcation membrane systems necessary for platelet formation, and platelet-like particles were detected in culture supernatants. Electron microscopy confirmed normal microtubule formation, similar to plasma-derived human platelets, although ESC-derived platelets displayed fewer granules¹³¹. Stimulation of hESC-derived platelets with thrombin and ADP induced GPIIb/IIIa activation, and filopodia formed upon adherence to fibrogen-coated dishes, indicating functionality of hESC-derived platelets. On average, after 24 days of culture, approximately 5×10^6 platelets were generated from an initial 10^5 human ESC. hESC megakaryocytes yield fewer platelets *in vitro* than their adult counterparts *in vivo*, possibly due to some microenvironment stimulus lacking in the *in vitro* differentiation system (e.g. shear flow¹³⁵). Establishing *in vitro* protocols for efficient human platelet generation from human PSC promises production of isogenic or histocompatible platelets that can potentially circumvent the need to obtain platelets through blood donation. One advantage in this system is that, as with erythrocytes, mature platelets are enucleated cells that can be irradiated, thereby eliminating safety concerns due to contamination with residual undifferentiated cells.

Lymphoid cell formation from human PSC

While erythroid and myeloid progenitor cells can be routinely generated from human PSC, there are few reports on the generation of lymphoid cells in this system. In a recent report, Woll and colleagues demonstrated generation of functional hESC-derived NK-cells capable of inducing efficient anti-tumor responses *in vivo*. hESC-derived NK-cells are uniformly CD94+CD117low/- and present higher tumor-clearing capacity than NK cells derived from umbilical cord blood¹³⁶, suggesting that they may serve as a novel cellular source for anti-tumor immunotherapy. Although NK and T cells are developmentally closely related, hESC-derived hematopoietic progenitors develop into natural killer (NK) cells^{136,137}, but typically do not form T- or B-cells¹³⁷, unless specific conditions are provided (e.g. T-cell development following injection into human thymus/fetal liver grafts in severe combined immunodeficient-humanized (SCID-hu) mice^{138,139} or coculture conditions with OP9 stromal cells expressing high Delta-1 like activity¹⁴⁰). Under such conditions, robust T-cell development was observed from hESC¹³⁸, even though in a less efficient manner than from human fetal liver, bone marrow or umbilical cord hematopoietic progenitors. Another study was able to show under OP9-coculture conditions small percentages of CD19+ cells from hESC, indicating their potential to form B-cells¹⁰⁴. Taken together, these studies corroborate the hypothesis that ESC differentiation protocols drive embryonic hematopoiesis and formation of progenitor cells resembling human yolk sac derived CD34+ cells^{47,141}, but identifying specific environmental cues will be essential to induce formation of adult-type cells.

Conclusion

ESCs and the more recently developed iPS cells hold promise for generating histocompatible or isogenic cellular transplant therapies for a variety of patients. Significant hurdles on the way to clinical application are the biased differentiation into rather immature, embryonic-like progenitor cells that may not function normally in adults. Furthermore, differentiation protocols for clinical standards need to be improved by removing serum, and other such animal products, as well as co-cultures with animal cells. However, PSCs offer an exciting new model enabling unique studies on human hematopoietic development and disease (reviewed in Lengerke C, Daley GQ. *Ann NY Acad Sci*, 2009, in press).

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Figure 1. Blood cells from pluripotent stem cells

Mouse ES cells expressing ectopic *HoxB4* differentiate on OP9 stroma into repopulating HSC (A: hematopoietic cells from day 6 embryoid body-derived cells after seven days of co-culture with OP9 stroma). Human iPS cells readily give rise to multiple blood cells including erythroid progenitors, yet have not yet been efficiently differentiated into HSC (B: BFU-E colony emerging from embryoid body-derived cells plated in methylcellulose supplemented with hematopoietic cytokines). *Abbreviations:* BFU-E (burst forming colony-erythroid); ES (embryonic stem); HSC (hematopoietic stem cells); iPS (induced pluripotent stem); OP9 (murine M-CSF^{-/-} stromal line).

Table 1
Origin, properties and hematopoietic potency of different pluripotent stem cells

Mouse and human pluripotent stem cells of different origins can differentiate into blood cells, Derivation of engraftable hematopoietic stem cells has been demonstrated for the majority of mouse pluripotent stem cells, yet remains cumbersome from human cells. *Abbreviations*: ES (embryonic stem); SCNT (somatic cell nuclear transfer); iPS (induced pluripotent stem); EG (embryonic germ); GS (germline stem).

Cell line	Origin	Chimera Formation	Hematopoietic potency	HLA-status vs recipients	Key References
Murine ES cells	Blastocyst	somatic and germline contribution	<ul style="list-style-type: none"> • Primitive and definitive hematopoiesis • Repopulating HSC (with ectopic <i>HoxB4</i>) 	Genetically identical (inbred mouse strains)	1,2,42,11
Human ES cells	Blastocyst	Not tested	<ul style="list-style-type: none"> • Primitive and definitive erythropoiesis • Multilineage hematopoietic progenitors • derivation of HSC unclear 	Genetically unique(HLA-match through banks)	3,50,56,77,78,79, 104,129
Murine parthenogenetic ES cells	Oocyte	somatic contribution	<ul style="list-style-type: none"> • Primitive and definitive hematopoiesis • Repopulating HSC (with ectopic <i>HoxB4</i>) 	Histocompatible through recombination at MHC-loci	16,89
Human parthenogenetic ES cells	Oocyte	Not tested	Not tested	Complete HLA-Matched or haploidentical	17,18
Murine SCNT ES cells	Somatic cell nucleus, oocyte	somatic and germline contribution	<ul style="list-style-type: none"> • Primitive and definitive hematopoiesis • Repopulating HSC (with ectopic <i>HoxB4</i>) 	Genetically identical	12,13
Human SCNT ES cells	Derivation has not been reported				14
Murine iPS cells	Somatic cells	somatic and germline contribution	<ul style="list-style-type: none"> • Primitive and definitive hematopoiesis • Repopulating HSC (with ectopic <i>HoxB4</i>) 	Genetically identical	19,49,51
Human iPS cells	Somatic cells	Not tested	<ul style="list-style-type: none"> • Primitive and definitive erythropoiesis • Multilineage hematopoietic progenitors 	Genetically identical	5,20,21,22,54

Cell line	Origin	Chimera Formation	Hematopoietic potency • derivation of HSC uncLEAR	HLA-status vs recipients	Key References
Murine EG cells	Embryonic gonad	somatic and germline contribution	Not tested	Genetically identical	35,41
Human EG cells	Embryonic gonad	Not tested	Not tested	Genetically non-identical	36
Murine mGS cells	Postnatal testis	Somatic contribution	Not tested	Genetically identical	37,38,39
Human mGs cells	Postnatal testis	Not tested	Not tested	Genetically identical	40

Table 2
Blood cell types generated by *in vitro* differentiation of human embryonic stem cells

Abbreviations: bFGF (basic fibroblast growth factor); BMP (bone morphogenetic protein); hESC (human embryonic stem cells); IL (interleukin); EPO (erythropoietin); Flt-3 ligand (fms tyrosine like 3 ligand); G-CSF (granulocyte colony-stimulating factor); M-CSF (macrophage colony-stimulating factor); OP9 (murine M-CSF^{-/-} stromal line); NK (natural killer); SCF (stem cell factor); TPO (thrombopoietin); VEGF (vascular endothelial growth factor).

Blood lineage	hESC lines	Differentiation protocol	Key References
HSC			
Primitive erythroid	• H1	• 14 days coculture on immortalized fetal liver cells	• hydrocortisone IL-3, BMP4, SCF, Flt-3 ligand, EPO, IGF-1, hemin 124
		• expansion in serum-free liquid cultures	
Definitive erythroid	• H1	• longer periods of culture (>35days)	• see above 125
		• recombinant iPTD-HoxB4 fusion protein	
Megakaryocytes and Platelets	• KhES-1,-2,-3 • H9, HSF6	• VEGF-induced ESC-derived sacs and coculture with OP9 or C3H10T1/2 stroma	• BMP4, VEGF, bFGF, SCF, TPO, Flt-3 ligand, EPO 131 • VEGF, TPO 134
		• coculture with OP9 stroma cells	
Neutrophils	• KhES-3	• coculture with OP9 stroma cells	• BMP4, SCF, Flt-3 ligand, IL-3, IL-6, FP6, TPO, G-CSF 132
Monocytes & Macrophages	• KCL001, KCL002, HUES-2	• EB formation, <i>no</i> cocultures	• M-CSF, IL-3 133
NK cells	• H9	• cocultures with the bone marrow stromal cell line M210-B4 ¹³⁶ or the stromal cell line SI7 ¹³⁷ followed by cocultures with the murine fetal liver line AFT024	• IL-3, IL-15, IL-7, SCF, Flt-3 ligand ¹³⁶ 136,137
		• differentiation on OP9 stroma ¹³⁸ or in EB ¹³⁹ , followed by injection into human thymic tissue implanted in immunocompromized mice	
T-lymphocytes	• H1	• coculture on Delta 1-expressing OP9 stromal cells	• BMP4, SCF, Flt-3 ligand 138,139 • SCF, IL-7, Flt-3 ligand 140
B-lymphocytes	• H1, H9	• predifferentiation in coculture with OP9, lymphomyeloid differentiation on MS-5 stroma	• SCF, Flt-3 ligand IL-3, IL-7, TPO, VEGF, BMP4 104

Table 3

Human blood cell therapies from pluripotent stem cells: Limitations and some potential solutions.

Limitations:	Possible solution
Generation of mostly primitive blood progenitors instead of definitive type-HSC	<ul style="list-style-type: none">Improving HSC generation by optimizing the <i>in vitro</i> differentiation protocols using:<ul style="list-style-type: none">morphogens⁴⁶, cytokines, small molecules andbiomechanical stimuli mimicking the <i>in vivo</i> developmental environment⁴⁷Development of non-HSC blood cell therapies:<ul style="list-style-type: none">erythroid progenitorsmegakaryocytesneutrophilsNK-cells (e.g. engineered with antitumor activity)
HSC identification	Characterization of the fetal HSC phenotype
HSC maintainance	Discovery of protocols based on cytokines & small molecules
Tumorigenicity & safety	Transplantation of well differentiated (sorted) progenitors
	Engineering of inducible suicide programs
Homing deficiencies	Transplantation directly into the bone marrow
Assessment of HSC/progenitors in xenotransplantation assays	Supplementation of non-(weakly)-cross reactive mouse cytokines with human counterparts
	Creation of a human stem cell niche by cotransplantation of human stromal cells