Stem Cells International

Ex Vivo Generated Red Cells as Transfusion Products

Guest Editors: Anna Rita Migliaccio, Giuliano Grazzini, Michel Sadelain, and Christopher D. Hillyer

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Editorial Board

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Editorial

Ex Vivo Generated Red Cells as Transfusion Products

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This issue of the Stem Cell International journal contains papers from many of the leading scientists in the emerging field: ex vivo expansion of hematopoietic progenitor cells into erythrocytes for transfusion.

Blood transfusion, the first form of successful cell therapy and, at least to some, "transplantation", was inspired by the discovery of the circulation by Richard Harvey in the 1600s [1] and begun in earnest later in that century. The development of this clinical practice into the safe and routine therapy we all know today has been both an exciting scientific adventure and the foundation for a number of other scientific disciplines. More specifically, the *immunology* of transfusion and transplantation began with the discovery of the heterogeneity of human blood group antigens by Dr. Karl Landsteiner in 1901 (recognized with a Nobel Prize in 1930). The discovery of clinically relevant infectious diseases transmitted by transfusion played an important role in the development and advancement of *virology*. The inheritance of certain form of anemias was discovered during blood transfusion practice and led to development of the *genetics* of human red cell disorders. In the 1940–1950s, the establishment of blood banks followed by the development of rigorous donation criteria and standardization of blood manufacturing processes has made transfusion safe and widely available and has provided a paradigm for the development of emerging therapies using ex vivo expansion and differentiation of many cell types. An example of one such therapy is represented by the tumor immunotherapy described by Lapteva and Vera.

The blood supply of industrialized countries is adequate overall. Nearly one hundred million donations are made every year worldwide (http://www.who.int/mediacentre/factsheets/fs279/en/index.html). The availability of blood and blood products in these nations has permitted the development and implementation of numerous life-saving surgical procedures (open heart surgery, organ transplantation, damage control resuscitation for trauma, and others) and cancer treatments which were not even imaginable without assurance that blood for transfusion would be readily available and safe. However, blood is not an unlimited resource and its potential need as the world rapidly develops requires a significant increase in blood donation. By some estimates (CDH), given the world's population and given the per capita transfusion of Canada as a utilization benchmark, nearly 250 million whole blood donations would be needed. Furthermore, and despite its high level of safety, human donated, unit-by-unit-derived blood donation/transfusion (i.e., without batched blood manufacturing into an aliquoted and homogenized pharmaceutical product), still leads to morbidity and mortality of its own accord and has significant variation from product to product based on the nature of the collection, manufacturing and storage processes, and the antigenic variation of any given donor, amongst others. Finally, it is not known what effect the aging of the world's population will have both on per capita utilization and on the ability of the smaller, younger populations to donate [2]. These issues, and the nearly 20-year-old search for alternative products to meet the transfusion need are discussed in the paper by Whitsett et al.

Scientific research is inspired by the prospect of a clinical goal. In recent years, a revolution in stem cell biology has occurred that has far reaching implications, specifically,

the discovery that it is possible to generate a potentially unlimited supply of stem cells by epigenetic/genetic treatments of somatic cells (T cells, fibroblasts, others) from any individual (see Pourcher et al., Hyroyama et al., and Chang et al.). In addition, techniques have been discovered to reprogram any cell into another cell type avoiding the induction of pluripotency. These techniques are fascinating though there are numerous scientific, safety, and scaling-up issues to be resolved before cells which have been genetically "altered" in the laboratory may be considered ready for widespread clinical use. As red blood cells do not have a nucleus, it is possible that they will be accepted as genetically safe. Indeed, it is this notion that supports that red blood cells from Hematopoietic stem/progenitor cell expansion or redifferentiation may represent the first therapeutic product to be generated by genomic reprogramming technology.

Reprogramming technology is still under development. Therefore, red blood cells expanded *ex vivo* from primary stem cell sources currently discarded (buffy coats produced during the blood manufacturing processes and low-volume umbilical cord blood) are being considered for first-in-man studies. Tirelli et al. identify the cell populations present in adult blood which are responsible for massive production of red blood cells *ex vivo*. The first-in-man proof-of-principle study for the use of *in vitro* expanded red blood cells for transfusion was reported on September 1st 2011, by Luc Douay and colleagues [3], who have also coauthored Pourcher et al. This paper reported that red blood cells generated *in vitro* from mobilized $CD34^{pos}$ cells collected by apheresis have normal survival (determined by ^{51}Cr labeling) when transfused into an autologous recipient [3]. This first-in-man autologous transfusion described also what would be the most likely safety data necessary for a larger clinical study with such products [*in vitro* characterization (blood group antigen expression profiling, deformability, hemoglobin content and O_2 dissociation curves) and *in vivo* functional studies in animal models (survival and morphology); http://www.clinicaltrials.gov/ct2/show/NCT00929266]. *In vivo* functional studies of human red blood cells in animal models will likely allow more complete characterization in many ways [4]. Ghinassi et al. describe an improved animal model which allows *in vivo* imaging and cell fate determination of human erythroid cells by labeling the cells before transfusion with a fluorescent reporter gene by retroviral technology.

Although red blood cells do not have nuclei, their immediate precursors the erythroblasts do. The terminal maturation of erythroblasts into functional red cells requires a complex remodeling process which ends with extrusion of the nucleus and the formation of an enucleated red blood cell [5]. These late stages of maturation are intrinsically controlled by epigenetic/genetic expression programs of the erythroblast itself. Cell reprogramming methodologies may (and at present appear to) disrupt these programs, leading to inefficient enucleation. Keerthivasan et al. discuss novel insights into the critical mechanisms of terminal maturation of a red blood cell and strategies to improve the efficiency of these processes.

As represented by all the information, data, and in fact vision contained in this issue, we are clearly at the beginning of a rapidly expanding field. The papers herein provide a broad and comprehensive overview of the most relevant areas of research which have been pursued and are needed to advance the field. Still, as state of the art as this issue is presently, the field is moving so rapidly that one may predict that new knowledge will rapidly follow.

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Review Article

Alternative Blood Products and Clinical Needs in Transfusion Medicine

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The primary focus of national blood programs is the provision of a safe and adequate blood supply. This goal is dependent on regular voluntary donations and a regulatory infrastructure that establishes and enforces standards for blood safety. Progress in ex vivo expansion of blood cells from cell sources including peripheral blood, cord blood, induced pluripotent stem cells, and human embryonic stem cell lines will likely make alternative transfusion products available for clinical use in the near future. Initially, alloimmunized patients and individuals with rare blood types are most likely to benefit from alternative products. However, in developed nations voluntary blood donations are projected to be inadequate in the future as blood usage by individuals 60 years and older increases. In developing nations economic and political challenges may impede progress in attaining self-sufficiency. Under these circumstances, ex vivo generated red cells may be needed to supplement the general blood supply.

1. Introduction

In the last 30 years, transfusion medicine has evolved from a field focused on blood component therapy and red blood cell serology to include advanced cellular therapies produced by ex vivo expansion [1, 2]. The broadest applications of cellular therapies to date have been in the area of regenerative medicine and have involved ex vivo cultured fibroblasts, keratinocytes, and chondrocytes (reviewed in [3]), but substantial progress has also occurred with cellular therapies involving lymphoid and hematopoietic cells. Infusions of donor lymphoid cells to enhance engraftment following allogeneic stem cell transplantation is a common practice [4], and progress has occurred in the development of adoptive T-cell therapies and cellular vaccines [5, 6]. Sipuleucel-T, an autologous cancer vaccine prepared from peripheral blood mononuclear cells (PBMCs) that have been activated and expanded ex vivo with a recombinant fusion protein consisting of prostatic acid phosphatase fused to granulo-cyte-macrophage colony-stimulating factor (GM-CSF), has been licensed for treatment of castration-resistant prostate cancer [6, 7]. Experimental therapies using ex vivo expanded cells are also being evaluated to improve the results of umbilical cord blood transplantation in adults [8, 9]. Infusion of notch-mediated ex vivo expanded cord blood progenitors shortens the time to neutrophil recovery [8] and infusion of ex vivo expanded T regulatory cells appeared to decrease the severity of acute graft versus host disease [9]. Substantial progress has also occurred in developing culture systems for ex vivo generation of red blood cells [10–17]. The cell sources under evaluation include peripheral blood mononuclear cells (PBMCs), CD 34^{pos} mobilized cells, cord blood (CB), induced pluripotent stem cells (iPSCs) and human embryonic stem cell (hESC) lines [10–16] (Table 1).

The United States Department of Defense is supporting research focused on ex vivo generation of red blood cells from cord blood on an industrial scale. Arteriocyte, a biotechnology company in Cleveland, Ohio, with a coalition of academic and industrial scientists supported in part by research funding from the United States Department of Defense recently provided red cells generated from cord blood to the United States Food and Drug Administration

Table 1: Cell sources for ex vivo generation of red blood cells*.

Cell type	Source	Special consent required	Advantages	Disadvantages
Peripheral blood mononuclear cells (PBMCs)	By product of leukoreduction	No	Donor with known phenotype	Limited expansion capacity
	Apheresis collection	Yes	≥3 products from single collection	Donors may become ineligible
	Mobilizing agent before apheresis	Yes (for mobilization and apheresis)	Expansion similar to cord blood with humanized media	Quality control requirement greater than blood
Cord blood	Low volume units unsuitable for transplantation	Yes	Hematopoietic progenitor cell number > PBMC 3–50 products phenotype must be determined	Multiple donations not possible Quality control moderately complex
iPSC	Fibroblast cultures from autologous or allogenic donors of known phenotype	Yes (for skin biopsy, fibroblast culture, and induction of pluripotency)	Unlimited expansion capacity	Quality control highly complex
hESC	Human embryos ≃1200 cell lines available	Yes	Unlimited expansion capacity	Ethical limitations on development and use Quality control highly complex

* Bacterial, mycoplasma, fungal, or viral contamination possible.

(US FDA) for evaluation [ABC newsletter July 23, 2010]. On March 8, 2011, Cellectis, a French biotechnology company, announced StemRed a joint venture with Etablissement Francais du Sang that will develop red cells ex vivo from induced pluripotent stem (iPS) cells [http://www.dailymarkets.com/stock/2011/03/08/cellectis-and-the-etablissement-francais-du-sang-launch-stemred-a-program-to-produce-red-blood-cells-from-stem-cells/%5d.]. These efforts to produce red blood cells in vitro using existing biotechnologies recently led to the first-in-man injection of ex vivo generated red cells in an autologous situation [17].

Scientists studying ex vivo production of platelets have refocused on animal models to clarify the optimal cell product for transfusion, that is, megakaryocytes or mature platelets [18] while efforts continue to improve ex vivo platelet production from umbilical cord blood hematopoietic progenitors using immobilized glycosaminoglycans to enhance megakaryocyte progenitor expansion and platelet release from $CD41a^{pos}$ megakaryocyte progenitors in vitro by inhibiting megakaryocyte apoptosis [19].

Efforts to generate neutrophils are also continuing, perhaps encouraged in part by clinical trials to determine if larger doses of granulocytes collected by apheresis from donors stimulated with dexamethasone and granulocyte colony stimulating factor (G-CSF) would improve clinical outcomes in neutropenic patients with sepsis [20–22]. In this regard it is important to note that a Phase I/II trial sponsored by Cellerant Therapeutics and the Department of Health and Human Services is evaluating the safety, tolerability, and activity of ex vivo expanded human myeloid progenitor cells (CLT-008) administered after up to 5 days of "standard of care" cytarabine-based consolidation chemotherapy in acute lymphoblastic leukemia, acute myeloid leukemia, and high-risk myelodysplasia [http://clinicaltrials.gov/ct2/show/NCT01297543]. Given the enormous effort currently focused on ex vivo expansion of blood cells in general and the progress in ex vivo expansion of red blood cells in particular, the potential clinical benefits to be derived from such alternative products should be considered.

2. Materials and Methods

The authors conducted searches on PubMed and Google using the following search terms: adverse reactions, allergic transfusion reactions, alloimmunization, blood group antigens, blood safety, blood supply, blood collection, blood donors, blood transfusion, developing countries, developed countries, emerging diseases, HLA antigens, hemovigilance, history of blood transfusion, neutrophil-specific antigens, platelet-specific antigens, transfusion medicine, transfusion medicine research, clinical trials, transfusion related acute lung injury, and transfusion-transmitted infections. In addition the following websites were consulted: European Medicines Association (EMEA), World Health Organization, United States Department of Health and Human Services Committee on Blood Safety and Availability, Centers for Blood Evaluation and Research, US FDA, Serious Hazards of Transfusion (SHOT). Consensus on needs in transfusion medicine was developed by a process that involved expert opinion as presented in the scientific literature, position

papers and statements by governmental and non-governmental organizations involved in areas of blood safety and availability, and discussions among the authors.

3. Results

3.1. Perspective on Needs in Transfusion Medicine. Since the beginning of modern transfusion therapy extraordinary efforts have been required to maintain a safe and adequate blood supply [23–25]. Governments have acknowledged the importance of these efforts to the overall public health by supporting the development of national blood programs and creating administrative and regulatory structures to assure the health of blood donors and the safety of blood products [26–28]. The goal for nations has been to attain national self-sufficiency, a target that may be unobtainable for some nations with the current economic, political, and social challenges. For more than a decade efforts to develop national blood programs were supported by the World Health Organization (WHO) Global Collaboration on Blood Safety, a voluntary partnership of internationally recognized organizations with expertise in various aspects of transfusion medicine [29]. WHO emphasized five important strategies considered critical to the development of effective national blood programs. These were recently reiterated in a forum addressing the role of regulatory agencies [27]: (1) "establishment of well-organized, nationally coordinated blood transfusion services with legislation and effective mechanisms for oversight, to ensure the timely availability of safe blood and blood products for all patients requiring transfusion," (2) "collection of blood from voluntary non-remunerated blood donors from low risk populations," (3) "performing testing for (a) transfusion-transmissible diseases [including HIV, hepatitis B, hepatitis C, and syphilis] (b) major blood groups and (c) blood compatibility," (4) "safe and appropriate use of blood and a reduction in unnecessary transfusions," and (5) "quality systems covering the entire transfusion process, from donor recruitment to the follow-up of the recipients of transfusion." Alignment of a safe and adequate blood supply with achievement of specific WHO Millennium Development Goals (MDGs) assisted developing nations in establishing priorities [30, 31]. The blood supply impacts MDG 4 reduction of the underfive child mortality rate by two-thirds, MDG5 reduction in the maternal mortality rate by three-quarters, MDG 6A halting and reversing the spread of HIV by assuring that younger individuals have comprehensive correct information about HIV and HIV prevention obtained at school and MDG6C decreasing death rates associated with malaria, often caused by severe anemia. The global economic crisis has required that WHO reassess priorities, and The International Society for Blood Transfusion (ISBT) has established the ISBT Working Party on Global Blood Safety to continue the work begun by the WHO [32].

Adverse reactions following blood transfusion that could not have been predicted based on current scientific knowledge have been a concern throughout the history of transfusion medicine [23–25]. Prevention of transfusion-transmitted infections [33–38] and characterization of human blood groups to assure transfusion of compatible blood products [39–43] have always been priorities, but adverse reactions following transfusion continue to occur despite scientific and technical advances and regulatory harmonization [44–50]. Risks posed by new and emerging infections increase [51–53], and evolving scientific knowledge about the immunologic and physiologic consequences of transfusion [54–59] make the descriptors safe and adequate moving targets.

3.2. The Blood Supply. In developed nations the blood supply is adequate overall [60–62], but extreme weather conditions and natural disasters such as the recent earthquake and tsunami in Japan and flooding in the United States disrupt routine blood collection activities leading to temporary shortages. The WHO 2008 Blood Safety Survey, which included data from 164 nations representing 92% of the global population, reported on 91.8 million donations. Forty-eight percent of donations were collected in high-income countries representing only 15% of the world population [63]. The accepted standard for evaluating blood availability is blood donations per 1000-population. In this report the donation rate for high-income countries was on average 36.4 (range 13.3–64.6) donations/1000 population, but the rate was lower in middle income and low income nations: 11.6 (range 1.65–36.2)/1000 population and 2.8 (range 0.40–8.2)/1000 population on average, respectively. While there have been substantial improvements in blood safety as developing countries have implemented the WHO recommended testing for syphilis, hepatitis B, hepatitis C, and human immune deficiency virus (HIV), some countries continue to rely on family/replacement and paid donors (36% low income and 27% middle income, resp.) whereas high-income countries reported only 0.3% of such donors.

The 2009 National Blood Collection and Utilization Survey Report provided information on blood collection and utilization in the United States for 2008 [61]. The whole blood/red cell collection rate per thousand population was 85.8, and 14,855,000 units of red cells were transfused for a transfusion rate of 48.8 units/1000 population. Benchmarking data presented at a 2009 meeting of the Alliance of Blood Operators indicated that for 2007/2008 the transfusion rate was on average lower in the European Union (40/1000 on average, with rates in Germany and Denmark closer to United States) and in Canada (32/1000) compared to the United States (48-49/1000 population) [62]. The average transfused dose of platelets was 5.8/1000 for the US, 4.3 for the National Health Service Blood and Transplant, and $\leqq$4/1000 for France. Plasma supplies also appear to be adequate although shortages of AB plasma and plasma from IgA-deficient donors are occasionally reported to be in short supply. In 2006, 4,010,000 units of fresh frozen and apheresis plasma were transfused in the United States with the median volume of plasma for a single episode being 300 mL. Plasma is also collected for fractionation. There is growing concern among patient advocacy groups that the amount of plasma available for fractionation to produce coagulation factor concentrate, intravenous immune globulin, and other plasma products for rare diseases will be inadequate to meet

clinical needs. Most nations permit only non-remunerated blood and plasma collections whereas others (including the United States) permit remunerated as well as non-remunerated plasma donations. Patient advocacy groups are concerned that efforts to collect needed plasma from paid donors may compromise donor standards and disrupt routine blood collections. The issue of paid plasma donation is reviewed elsewhere [64]. The Dublin Consensus Statement summarizes these concerns [65]. Recombinant products have been developed to replace some but not all therapeutic plasma proteins. Movement of source plasma and recovered plasma across borders is quite common and perhaps serves as an example of how blood reserves could be shared across borders with standardized regulatory and manufacturing standards. While these data indicate that the blood supply is adequate in developed countries at the present time, there is growing concern that in the future the blood supply may become inadequate [66–74].

Several factors are responsible for these concerns. Firstly, the number of conditions for which donors are temporarily or permanently deferred is increasing [48, 51–53, 64, 66]. Globalization and emerging infections are implicated in many of these deferrals [33]. A study that estimated donor eligibility in the US population using 31 exclusionary factors corresponding to AABB standards concluded that conventional methods overestimate eligible donor prevalence by approximately 59% [67]. In addition the aging of the population is expected to impact blood availability by increasing blood usage [68–73]. The world population, currently estimated at 6.8 billion, is projected to reach 7 billion in 2012 and surpass 9 billion by 2050 [74]. Most of the population increase (2.3 billion) will occur in developing countries where individuals 15–59 years old will account for 1.2 billion and individuals 60 years and over will account for 1.1 billion of the expected increase. The population in developed countries is expected to increase from 1.23 billion to 1.28 billion. However, the worldwide population of individuals of 65 years and over is projected by the US Census bureau to increase from 7.7% (2010) to 16.5% in 2050. Blood donations will also be affected by aging of the population [68–80].

The American Red Cross reported that donations by repeat donors 50 years or older increased from 22.1% of total donations in 1996 to 34.5% of total donations in 2005 [80]. During the same period a decrease in the numbers of male and female donors between 20 and 49 years of age was observed. Data from Germany reflects similar donation patterns in that repeat donors are more likely to belong to older age groups (35–44 years and 45–54 years) [70, 71]. The issue of an aging donor base has been resolved in part by countries such as the United States, the United Kingdom, and Australia by eliminating the upper age limit of 70 and allowing younger donors (16 years) to donate with parental permission [75–79]. The Club 25 concept originally developed in Zimbabwe [79] is being adapted for use in developed countries. Despite these efforts, increased blood usage by older populations remains an issue.

Most transfusions in older individuals are related to diagnoses of cardiovascular disease, cancer, and the need for orthopedic surgery [68, 69]. Investigators from the Finnish Red Cross observed that many developed nations had similar age-standardized mortality rates for cancer, cardiovascular disease, injuries, and noncommunicable disease, and using these data simulated red cell usage per 1000 population between 2010 and 2050 for several developed countries based on age-distributed variation in blood usage in Finland between 2002 and 2006 [72]. The simulation predicted substantial increases in blood usage in developed countries associated with a decrease in the size of the population eligible to donate. Earlier studies had predicted a shortfall in blood collections in the United States [68]. Independent assessments using alternative data sources confirm the projected increase in demand for blood caused by increases in the population of individuals over 60 years of age [71, 73].

3.3. Prevention of Transfusion-Transmitted Infections: Global Perspective. Progress in preventing transfusion-transmitted infections was recently reviewed [35] Implementation of restrictive donor eligibility criteria and use of volunteer non-remunerated donors, along with specific testing for syphilis and hepatitis B, hepatitis C, HIV1/2, and HTLV1/2, are critical steps in assuring blood safety. In developing countries recruitment of volunteer non-remunerated donors is impacted by cultural definitions of community [81–83] instead of geographic proximity. As a result, efforts to recruit volunteer donors in some nations have been ineffective. Research conducted on the impact on blood safety of converting family/replacement donors to allogeneic donors in South Africa has demonstrated that when such donors are repeat donors, the blood provided is safer than first-time volunteer donors [84–86]. These observations are consistent with previous studies in developed countries indicating a lower risk of infection for repeat donors and should encourage more innovative approaches for donor recruitment in Sub-Saharan Africa.

The most recent published data from the American Red Cross (using NAT testing) indicate that the residual risk per donated unit is 1 : 1,149,000 for HCV, 1 : 357,000 to 1 : 280,000 for HBV, and 1 : 1,467,000 for HIV [36]. By contrast, the estimated residual risk of transfusion-transmitted HIV in a multinational collaborative study involving five nations in Sub-Saharan Africa which used either an antibody assay or combined p 24antigen/antibody assays reported a residual risk for HIV of 34.1 per 1 million donations which represents 1 in 29,000 donations [86]. In addition to these traditional transfusion transmissible diseases for which all donor blood should be tested, donors are also screened for other diseases based on the geographic location, such as for malaria (either by history or using antibody tests [87, 88]) and for local viral and parasitic diseases such as Chagas' disease [89, 90], dengue [91, 92], West Nile virus, [93–95] and chikungunya [96]. In addition, epidemic viral diseases such as H1N1 and yellow fever must also be considered.

Asymptomatic healthy blood donors may also transmit common viral infections which do not cause harm to the average transfusion recipient but which may represent significant risks in selected patient populations. For example, latent cytomegalovirus infection (CMV) is common in

normal donors. Depending on the geographic location and socioeconomic status of donors, 70–100% of donors may be seropositive. Pregnant women, neonates, and other immuno-compromised CMV-negative patients may develop serious infectious complications due to transfusion-transmitted CMV. Currently, leukoreduced blood products and products from CMV seronegative donors are used for populations vulnerable to CMV [97–101]. A meta-analysis comparing leukoreduction and antibody screening as CMV prevention strategies reported that both techniques were effective in reducing transmission but indicated that antibody screening may be more effective [98]. Studies of the natural course of CMV infection in blood donors indicate that CMV DNA may persist up to 269 days following infection [97, 99] and suggest that blood donors who have recently seroconverted be deferred for a year.

Parvovirus B19, an erythrotropic virus that uses the P blood group antigen to infect erythroid progenitors, may cause severe anemia, red cell aplasia, and congenital anomalies when transmitted by transfusion [102–107]. Neonates, pregnant women, and patients with congenital and acquired forms of hemolytic anemia are most vulnerable to this infection, but routine screening of blood donors for parvovirus B19 is not performed. However, plasma pools for fractionation are screened for parvovirus B19 because current fractionation/inactivation techniques do not completely inactivate the virus and transmission of parvovirus by coagulation factor concentrate is well documented. Although the risk of transmission of parvovirus B19 appears low in transfusion recipients because of preexisting protective antibody from past natural infections in many transfusion recipients and blood donors, transmission of parvovirus B19 by transfusion has been recently documented [103–105]. Data from the National Heart Lung and Blood Institute Retrovirus Epidemiology Donor Study-II which tested pre- and posttransfusion samples found no transmission to 24 susceptible recipients from transfusion of components with parvovirus B19 DNA containing less than 10^6 IU/mL [102]. Data from the Japanese Red Cross hemovigilance system provide an alternative perspective which suggests that specially screened products may be needed for selected populations [104]. Between 1999 and 2008, when parvovirus B19 donor screening was conducted using a cutoff similar to that of the REDS study, eight patients with transfusion transmitted parvovirus B19 DNA were identified and sequence identity between patient and the linked donor was confirmed in 5 cases. Red cell aplasia developed in 3 of the 5 confirmed cases. This report suggests that alternative blood products with lower risk of parvovirus B19 infection should be provided for at-risk patient populations.

3.4. Alloimmunization. As the scientific basis for producing better and safer products for transfusion through donor screening and product testing has made transfusion safer, alloimmunization resulting from transfusion has emerged as a major issue in clinical care. The magnitude of this problem led to a recent NHLBI sponsored conference to identify areas in which additional research was needed to better understand factors contributing to alloimmunization as well as research on prevention and management of alloimmunization [108]. Alloimmunization in females may result from exposure to paternal antigens during pregnancy. In the general population alloimmunization occurs following transfusion, solid organ or tissue transplantation, or following hematopoietic stem cell transplantation with partially matched donors.

Blood contains many cells and proteins expressing polymorphic antigen systems that may lead to alloimmunization. Erythrocytes alone may express antigens from 30 blood group systems for which over 300 different antigens have been identified [40–43, 109–112]. Small numbers of residual red cells and leukocytes may contaminate plasma and platelet products causing alloimmunization (anti-D developing following transfusion of Rh positive plasma to Rh negative individuals or anti-HLA following platelet transfusion). Typically blood products are matched for ABO group and Rh (D) only unless patients are alloimmunized. Once alloimmunization occurs subsequent transfusions must be matched unless the antibody is considered clinically insignificant [112]. It is estimated that 1–3% of the general population is immunized to blood group antigens.

Alloimmunization is more common in chronically transfused patients such as patients with hemoglobinopathies or transfusion-dependent myelodysplasia [113–116]. Alloimmunization also occurs more often in communities where blood donors and blood recipients are from different ethnic groups [112]. Investigators observed that the rate of alloimmunization in sickle cell disease (SCD) patients in Jamaica where more blood donors were of African ancestry was lower than the alloimmunization rate of SCD patients in the UK where most blood donors were caucasian. Historically, twenty- to thirty-five percent of chronically transfused patients with sickle cell disease or thalassemia are immunized to blood group antigens [113–116]. When these patients experience a hemolytic transfusion reaction, autoantibodies as well as alloantibodies to transfused cells may be produced, causing a syndrome referred to as hyperhemolysis [117–121]. Limited matching strategies such as matching for Kell, Rh (Cc, D, E, e), Fy, and Jk have reduced overall alloimmunization rates in most chronically transfused patient populations [113, 114] but have led to alloimmunization to variant blood group antigen differences which testing with serological reagents does not identify [122–125]. For example, in the Blue Tag program at Children's Hospital in Philadelphia, established to identify African-American donors antigen-matched for African American patients with SCD, alloimmunization to other more subtle antigen differences such as D-deletion variants developed requiring more precise matching with DNA-based technology [122]. Similar observations have been made in other sickle cell patient populations [123]. Patients with myelodysplasia and myeloproliferative neoplasms may also require chronic transfusion support and become alloimmunized. DNA-based methods are now being used to screen large numbers of blood donors to identify compatible blood for alloimmunized patients [126–137]. In the United States, these methods are not FDA approved and phenotypes are confirmed with serological reagents. While most of this work is occurring at blood centers, a recent study suggests that transfusion service laboratories

may be able to identify some units in their existing inventory [138]. Identifying matched blood for patients alloimmunized to multiple blood group antigens and patients with rare blood types (variably defined as 1/1000 or 1/10,000 or fewer compatible donors) is a challenge. Although reference laboratories around the world cooperate in this effort [132], blood for such patients is often in short supply. Blood centers are now using information about rare blood types and the populations most likely to contain compatible donors to develop strategies to recruit such donors. For example, Life Share Blood Centers (Louisiana, USA) has published on its website statistics about the ethnicity of donors testing negative for the blood group antigens S, s, and U (a high-incidence antigen), a phenotype more common in African-Americans than Caucasians. Publication of this data has increased minority participation in blood donation. New York Blood Center has a similar program to recruit donors called Precise-Match, tailored for outreach to the various ethnic groups in New York City.

Platelets express A and B blood group antigens, class I HLA-A and HLA-B locus antigens (HLA-C locus antigens are not expressed well on platelets), and platelet-specific antigens 27 of which are well characterized [139–141]. Antibodies to HLA-A and HLA-B locus antigens are most often implicated in platelet refractoriness, but antibodies to platelet-specific antigens are also implicated [142, 143]. Neonatal thrombocytopenia usually involves platelet-specific antigens (PLA 1–17), but several cases implicating antibodies to HLA antigens have been described. Class I and or class II HLA antigens are also expressed on various lymphocyte and monocyte populations, and granulocytes express unique polymorphic antigens [144, 145]. Plasma proteins also express polymorphisms, but serious transfusion reactions have been documented most often in IgA-deficient and haptoglobin-deficient individuals who develop class-specific antibodies to IgA and haptoglobin, respectively [146, 147]. Immunization to HLA antigens affects not only survival of transfused platelets but also prevents engraftment of hematopoietic stem cells and may lead to allograft rejection of solid organs as well as hematopoietic stem cells. Until recently only alloimmunization of transfusion recipients was thought to be a problem, but recently alloantibodies to HLA and granulocyte antigens in donor plasma (and apheresis platelets) have been documented to be the cause of transfusion-related acute lung injury (TRALI) [140, 148]. Because women are more likely to be alloimmunized, TRALI mitigation strategies include use of male donors, identification of never pregnant female donors, and identification of female donors testing negative for anti-HLA antibodies. Implementation of this strategy has substantially reduced morbidity and mortality from TRALI in developed countries but has once again reduced the numbers of individuals available to donate certain blood components.

Platelet transfusions have steadily increased over the past eight years in developed countries. Transfusion of ABO identical platelet products is preferred, but such products are not always available. Platelet recovery in ABO-incompatible transfusions may be slightly lower, but platelet survival is normal. However, transfusion of ABO-nonidentical platelets has caused serious hemolytic transfusion reactions in some patients because of high-titer anti-A and anti-B in donor plasma [149–153]. While hospitals have policies to manage such transfusions, blood centers have not routinely performed anti-A and anti-B titers to identify high-risk group O donors. In Europe, blood centers are testing products and restricting use to group O recipients (reviewed in [154]). The use of platelet additive solutions on a large scale could also lower the risk of hemolysis because such products contain less plasma. A recent publication reported that de novo HLA allosensitization in patients on ventricular assist devices is lower when patients receive leukoreduced ABO identical products, suggesting that even small amounts of hemolysis might facilitate alloimmunization to HLA [151]. Thus matching both for ABO and HLA may be important in providing transfusion support for highly alloimmunized patients [155].

Refractoriness to platelet transfusion is usually caused by alloimmunization to HLA antigens [142, 143, 155, 156]. The TRAP study, a large randomized clinical trial designed to compare leukoreduction and UVB irradiation in preventing refractoriness to platelet transfusions related to alloimmunization to HLA antigens, reported that 45% of AML patients receiving control platelets but only 18% of patients receiving leukoreduced and 21% of patients receiving UVB-treated platelets were alloimmunized [156]. Following this study, leukoreduced platelet products became the standard of care even though UVB irradiation was also effective. Followup of patients who became alloimmunized to HLA revealed that 56% of patients subsequently became antibody negative [157]. Since UVB irradiation and riboflavin are incorporated in current pathogen inactivation methods for platelets, licensed in Europe but not yet in the US, there will likely be renewed interest in this method to prevent alloimmunization [158]. However, a meta-analysis of randomized controlled trials of pathogen-reduced platelets indicates that posttransfusion-corrected count increments were lower, an observation that will affect dosing strategies [159].

Granulocytes play a crucial role in protecting individuals from a number of pathogens. In the early 1970s, the first studies suggesting that granulocyte transfusions might decrease mortality associated with bacterial infections were published [160]. However, collection of adequate numbers of granulocytes and provision of these products to patients in a timely way is a logistical challenge. The introduction of many new and more effective antibiotics and antifungal agents as well as the routine administration of G-CSF and GM-CSF has substantially reduced mortality from infections. However, considerable mortality and morbidity still occur in neutropenic patients following hematopoietic stem cell transplantation. Clinical trials using granulocyte transfusions have provided mixed results [161]. Currently granulocytes are collected by apheresis from prescreened donors (often regular plateletpheresis donors) using dexamethasone or a combination of dexamethasone and G-CSF and transfused as soon as possible but never more than 24 hours after collection. The logistics of collection, variable yields, and limited information on the best conditions for storage have contributed to the variable outcomes in clinical studies

published to date. Alloimmunization to granulocyte-specific antigens appears to inhibit the function of transfused granulocytes [160]. Three clinical centers have recently published retrospective analyses of their experience with granulocyte transfusions using optimal mobilization techniques and reported improved survival [154, 162, 163]. An NHLBI-sponsored phase III clinical trial examining the effectiveness of granulocyte transfusions in individuals with neutropenia and infection following dose-intensive chemotherapy or hematopoietic stem cell transplantation is underway [164]. Should this trial provide support for the effectiveness of granulocyte transfusions, a less labor-intense and more cost-efficient method to produce granulocytes would be desirable.

3.5. The Red Cell Storage Lesion. Since the publication in 2008 by Koch et al. [55] of a retrospective study reporting increased risk of postoperative complications and reduced short-term and long-term survival in cardiac surgery patients receiving blood stored for more than two weeks, there has been considerable debate about the benefits of transfusing fresh versus older units of blood [57, 59, 165]. Biochemical changes during red cell storage in currently licensed additive solutions are well documented. However, the primary criteria for licensure are hemolysis (below 0.8% in EU and 1% in the US) and red cell survival not less than 75% at the end of storage [165, 166]. In an editorial discussing the potential clinical relevance of this low level of hemolysis on transfusion recipients, potential differences in the effects of free hemoglobin compared to hemoglobin in microvesicles are discussed referencing studies of thrombin generation by red cell supernatant [166]. It is not currently known if clinical outcomes are affected by the age of blood transfused. However, a retrospective cohort study of individuals in Sweden and Denmark transfused between 1995 and 2002 as recorded in the Scandinavian Donations and Transfusions (SCANDAT) Database which analysed 404,959 transfusion episodes revealed a small (5%) excess mortality for recipients of blood stored for 30–42 days and mixed-age compared to recipients of blood stored for 10–19 days [167]. The authors noted no dose response pattern or effect of leukoreduction and concluded that the differences observed were "compatible with a higher baseline risk among recipients of the very oldest units than with an actual deleterious effect of the oldest units." However, should either clinical or basic research studies provide conclusive evidence that inferior outcomes are associated with transfusion of blood stored for longer periods, the shift toward usage of fresher blood would reduce blood inventories and create significant blood shortages.

The editorial by Simone A. Glynn, MD, MPH from the Division of Blood Diseases and Resources at the National Heart Lung and Blood Institute summarizes the situation well by stating that, despite the numerous publications, there is "genuine uncertainty as to whether transfusing fresher blood is more, less, or equally beneficial as transfusion of older blood" [59]. The editorial identifies four large randomized clinical trials related to the age of transfused blood that are underway in North America. The Canadian Institutes of Health Research is funding two trials, one in intensive care patients randomized to receive either less than 8-day or standard issue red blood cells (2–42 days) in The Age of Blood Evaluation (ABLE) Study which has as the primary outcome 90-day all-cause mortality and a second study in which premature infants ($\leq$1250 g) will be randomized to receive either less than 8-day or 2–42-day aliquots with a primary endpoint being a 90-day composite measure of all cause mortality and organ dysfunction. The Cleveland Clinic is conducting the Red Cell Storage and Outcomes in Cardiac Surgery Trial (NCT00458783) in which individuals 18 yrs and older undergoing cardiopulmonary bypass for primary and reoperative coronary artery bypass grafting, coronary artery bypass grafting with a valve procedure, and isolated valve procedures are randomized to receive blood transfusion with storage duration less than 14 days or greater than 20 days. NHLBI is also sponsoring through the Transfusion Medicine Clinical Trials Group the Red Cell Storage and Duration Study (RECESS)—NCT00991341 in which individuals 12 years or older undergoing complex cardiac surgery and likely to need transfusion are randomized to receive blood $\leq$10 days storage duration or $\geq$21 days storage duration. The primary outcome is the change in the composite multiple organ dysfunction score (MODS). Dr. Glynn also identified research projects funded under the NHLBI program "Immunomodulatory, Inflammatory, and Vasoregulatory Properties of Transfused Red Blood Cell Units as a Function of Preparation and Storage" designed to provide information on mechanisms via which transfusion of older blood might lead to adverse physiological outcomes and to design interventions to eliminate them. Current data are inadequate to determine if transfusion of older units of blood is associated with either short- or long-term adverse outcomes. Basic research in animal models and prospective randomized clinical trials are needed in this area.

3.6. Impact of Ex Vivo Generated Blood Cells on Needs in Transfusion Medicine. Red blood cells have been successfully generated ex vivo from peripheral blood mononuclear cells, cord blood, and human-induced pluripotent stem cell (hiPSC) and human embryonic stem cell (hESC) lines. The expansion potential of cord blood and peripheral blood is limited, but that of hiPSC and hESC is infinite. Multiple products from a single collection (blood or skin fibroblasts) would be generated from well-characterized donors under GMP conditions. Irrespective of the cell source, the donor will be selected to be negative for known transmissible diseases and will represent either a rare phenotype (1/1000 or $\leq$1/10,000) or a phenotype much in demand (O Rh negative or O Rh positive). The primary product will be almost devoid of other contaminating blood cells and suspended in defined media designed to support viability and cell function but lacking immunoglobulins and other plasma components which precipitate TRALI and allergic transfusion reactions (IgA, haptoglobin). Theoretically, the equivalent of 10–50 therapeutic units of blood (2×10^{12} cells) can be generated from a single cord blood unit. The development of cord blood banks for hematopoietic stem cell transplantation has created an infrastructure that facilitates collection of cord blood for other uses (i.e., endothelial cells for regenerative

medicine) [168]. Cord blood can also be cryopreserved for up to 23 years with recovery of adequate numbers of hematopoietic progenitors and the ability to generate iPS [169]. Although the expansion potential of peripheral blood is considered to be lower than that of cord blood, at present peripheral blood is more readily obtained because it is a byproduct of leukoreduction.

The first in-man transfusion is likely to be from one of these two cell sources with studies of red cells generated from hESC or hiPSC coming later [170–172]. Currently 1200 hESC lines have been established worldwide, and 375 of these are deposited in two international registries [172]. Investigators at the forefront of ex vivo expansion studies expect that GMP compliant facilities to produce red cells ex vivo will be available within the next 2-3 years. Location of manufacturing facilities will be critical given the complexity of the manufacturing process and the need for reliable transport. The potential impact of such products on blood manufacturing operations is substantial.

The first clinical use of ex vivo generated red cells is likely to be for highly alloimmunized patients and for patients with rare blood types [170, 171]. Theoretical calculations to determine how many iPSC lines would be needed to support alloimmunized patients in France have already been performed [171]. Although a large number of embryonic stem cell lines have been developed, the expression of blood group antigens by erythrocytes from these cell lines is not known [172]. Forty percent of rare units in France were provided to patients with sickle cell anemia [171]. Data from WHO suggests that worldwide patients with hemoglobinopathies are represented among patients requiring chronic transfusion. Hemoglobinopathies occur in over 332,000 conceptions or births annually and account for 3.4% of under-5 mortality [173, 174]. Worldwide an estimated 275,000 have a sickle cell disorder and 56,000 a major thalassaemia, approximately 30,000 of which will require chronic transfusion to survive. In the WHO America region, 52.4% of transfusion-dependent patients receive transfusions, but in the Eastern Mediterranean region and South-East Asian region, which have a higher number of transfusion-dependent patients, 17.8% and 9.6%, respectively, of transfusion-dependent patients received transfusions [174]. These data suggest that, as healthcare improves in developing nations, the number of patients with hemoglobinopathies requiring transfusions will increase and these individuals will likely need special antigen-matched products.

Once manufacturing facilities are established for ex vivo generation of red cells, the general inventory of blood in both developing and developed countries could be increased by ex vivo generation of red cells. Theoretically, if peripheral blood mononucleated cells (PBMCs) from each whole blood donation were to be expanded ex vivo, each donation would produce not only one but possibly 3–10 or more units of blood. This would allow blood donors to donate less often, decrease concerns about iron deficiency, and reduce costs for donor recruitment. PBMC generated by leukoreduction during the manufacturing process for red cells are the easiest product to obtain. The least expensive way to prepare leukoreduced products is the buffy coat method, but recovery of PBMC from in-line or sterile docked filters is also possible. In 2006, prestorage leukoreduction was performed on approximately 11.3 million units of red blood cells in the United States. Currently, cord blood units are being collected as a source of hematopoietic progenitor cells for transplantation, but cord blood contains other cell populations that have value in regenerative medicine such as naïve immune cells, mesenchymal cells, and endothelial progenitor cells. Accurate figures for donations to public banks are available. Donation rates to public banks vary from 0.1% of births to 0.34% of births [168]. Some families choose to bank cord blood for autologous/family use, and these units are not available for ex vivo generation of blood cells. Overall, these data indicate that a potentially vast but untapped resource is available for ex vivo generation of blood cells. At present there is no data to suggest that ex vivo products are needed in developed nations for the general inventory but should projections about increased blood usage in individuals age 60 years and older be correct such products may be needed in the future.

The supply of safe blood in developing countries has improved in recent years but is still inadequate. Anemia related to trauma, pregnancy, or malaria is the most common indication for transfusion, and currently blood is not always available when needed. It is unrealistic to propose that a country that cannot effectively run a national blood program would have the resources to expand blood cells ex vivo. However, a manufactured blood product produced elsewhere could be imported. Manufactured products are more uniform because methods and product specifications can be standardized with quality monitoring implemented to assure that such standards are met. Under these circumstances, cellular products might move freely across national borders as is the case with some pharmaceuticals. Alternatively, one might maintain the current not-for-profit business model for whole blood collection and model ex vivo expansion on a for-profit model where economies of scale might work to reduce overall cost.

Progress in ex vivo generation of megakaryocytes and platelets is not as advanced as development of red cells. The value of platelet transfusions in preventing hemorrhage in thrombocytopenic patients and patients with thrombocythemia is not disputed although the level at which platelet transfusions should be given prophylactically to thrombocytopenic patients to prevent hemorrhage was the subject of recent clinical trials. Platelets may be manufactured from whole blood by centrifugation to produce platelet concentrate or prepared by apheresis. An adult dose of platelets would require pooling of 4–6 units of platelets depending on local practice or a single apheresis product. Both products are usually leukoreduced. There is ongoing concern about the adequacy of platelet inventories for two reasons, the shelf life is short (maximum of 5 days) and platelet products stored at room temperature are more likely to be contaminated with bacteria [37, 38]. Refractoriness to platelet transfusions is usually caused by anti-HLA antibodies. Antibodies to platelet-specific antigens may also be implicated. In neonatal thrombocytopenic purpura, the offending antibody is typically directed at

platelet-specific antigens. Refractoriness to platelet transfusions is a significant problem for multitransfused patients with hematologic malignancies receiving chemotherapy or undergoing hematopoietic stem cell transplantation. Identification of suitable products for alloimmunized patients involves HLA typing of the patient (typically performed with DNA-based techniques), antibody screening for anti-HLA class I and anti-platelet-specific antibodies (solid phase or flow cytometry) as well as platelet crossmatching (solid phase or flow cytometric techniques). Complex computer algorithms for selecting HLA compatible donors based on epitope sharing have been developed to identify compatible products [142, 143]. Thus the ability to generate platelet products ex vivo and platelet products lacking HLA antigens in serum free media would have great clinical value. Methods have been developed to generate platelets ex vivo from cord blood CD34 positive cells and from embryonic stem cells [175]. Ex vivo generation of platelet products deficient in HLA class I antigen expression could have an enormous impact on the provision of platelet products to refractory patients. Using an RNA-interference-based mechanism (RNAi) in which a lentiviral vector was used to express short-hairpin RNA targeting β2-microglobulin transcripts in CD34 positive cells, Figueiredo et al. generated platelets demonstrating an 85% reduction in class I HLA antigens compared with platelets generated in CD34 positive cells transduced with a lentiviral vector containing a nonsense shRNA [175]. These platelets appeared to have normal function in vitro. Alternatively, hESC or hiPSC could be used to generate products for highly immunized patients.

Granulocyte transfusions have not consistently demonstrated improved clinical outcomes in infected neutropenic patients. However, these inconsistent results may reflect heterogeneity in patient populations, failure to transfuse an adequate number of granulocytes, limited information on optimal storage conditions for granulocyte products, and difficulty in identifying a rapid reliable assay for determining granulocyte compatibility (other than ABO). Studies of ex vivo generated neutrophils may provide valuable information on optimal storage conditions for neutrophils and may ultimately produce a product with a longer shelf-life.

Theoretically, ex vivo generated red blood cells will be produced on demand on a predictable schedule, will provide cellular products of more uniform composition with limited contamination by other cell types, will be associated with lower rates of transmission of infectious diseases, cause fewer allergic reactions, produce a lower rate of alloimmunization because extended matching will be possible, and will have longer in vivo survival while producing less iron overload than currently available products. It is not possible to predict when these products will be approved for clinical use. Many complex issues related to scale-up production and the potential immunogenicity (neoantigen formation) of products produced in vitro remain to be resolved. However, the development of these products will inform and transform quality control and manufacturing processes for traditional blood products.

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Review Article

Generation and Characterization of Erythroid Cells from Human Embryonic Stem Cells and Induced Pluripotent Stem Cells: An Overview

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Because of the imbalance in the supply and demand of red blood cells (RBCs), especially for alloimmunized patients or patients with rare blood phenotypes, extensive research has been done to generate therapeutic quantities of mature RBCs from hematopoietic stem cells of various sources, such as bone marrow, peripheral blood, and cord blood. Since human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) can be maintained indefinitely in vitro, they represent potentially inexhaustible sources of donor-free RBCs. In contrast to other ex vivo stem-cell-derived cellular therapeutics, tumorigenesis is not a concern, as RBCs can be irradiated without marked adverse effects on in vivo function. Here, we provide a comprehensive review of the recent publications relevant to the generation and characterization of hESC- and iPSC-derived erythroid cells and discuss challenges to be met before the eventual realization of clinical usage of these cells.

1. Introduction

Medical progress, specifically in the fields of hematology/oncology and transplantation surgery, as well as an overall aging population, has led to an ever-increasing demand for erythrocytes for transfusion to currently approximately fifty thousand RBC concentrates per million population per year in countries with a high standard of health care. Currently, the exclusive source for these is volunteer donors, who obviously are subject to the same societal changes as the recipients, that is, they are aging also. Recruitment of new donors from the shrinking pool of eligible individuals is challenging and additionally hampered by ever-increasing restrictions, predominantly recipient-directed exclusion criteria for donors. Perceived lack of safety of blood products also is a highly sensitive issue in the population, particularly since the emergence of HIV in the eighties, as a consequence of which a whole generation of hemophilia patients was infected. The desire to counter these challenges has led to the extensive effort in the generation of red blood cells (RBCs) in vitro from a variety of sources, such as bone marrow, peripheral blood, and cord blood. More recently, utilizing embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) to generate universal donor RBCs has been envisioned [1, 2].

hESCs are pluripotent stem cells derived from the inner cell mass of the blastocyst [3], and iPSCs are ESC-like cells generated by reprogramming somatic cells, most often via forced expression of a combination of transcription factors, such as Oct3/4, Nanog, KLF4, c-Myc, LIN28, and SOX2 [4, 5]. Both hESCs and hiPSCs can be maintained indefinitely in culture and can be induced to undergo differentiation to give rise to any cell types of all three germ layers. These characteristics made them not only a valuable tool for the study of developmental biology, but also a potential source for providing unlimited numbers of cells for cell replacement

therapies. While our laboratory has previously shown that the then existing hESC lines are not conducive to culturing universal donor RBCs [6], many additional hESC lines have been generated ever since. Furthermore, the recent advances in hiPSCs have partially obviated this obstacle, as exemplified by the generation of hiPSCs from a Bombay individual whose RBCs lack ABH antigens expression due to the absence of the H gene (FUT1-) and Secretor gene (FUT2-) encoded α 1,2 fucosyltransferase activities [7]. It has been proposed by French researchers that based on their database, as few as 15 hiPSC clones would cover 100% of the needs of all Caucasian patients with rare blood phenotypes/genotypes in France [2]. In addition, they suggested that one single hiPSC clone would meet 73% of the needs in alloimmunized patients with sickle cell disease for whom rare cryopreserved RBC units are required [2]. In this review, we summarize the recent development in the in vitro generation and characterization of erythroid cells from either hESCs or iPSCs and point out areas of further investigation needed before their use for clinical purposes.

2. Methods for the Generation of Erythroid Cells from hESCs and iPSCs

hESCs and hiPSCs represent renewable, potentially unlimited cell sources, in contrast to the hematopoietic stem cells originating from bone marrow, cord blood, or peripheral blood, that require donors to be constantly secured, for the ex vivo generation of RBCs. The potential charm of autologous hiPSCs in the context of erythrocyte generation is the identical genetic identity between the cells and the recipient for all blood group antigens, since the prevalent clinical problem is the polysensitization against foreign erythrocyte antigens, and these patients are at risk for acquisition of additional antibodies. Several laboratories have established protocols to derive erythroid cells from the hESCs and hiPSCs. While each protocol varies in technical details, they can generally be divided into two main categories: those that coculture stem cells with stromal layers to induce hematopoietic differentiation, and those that culture stem cells in suspension to form embryoid bodies (EBs) (Table 1). One exception is a recent report by Salvagiotto and colleagues who circumvent EBs and stromal coculture by utilizing a matrix protein to generate a 2-dimentioinal culture system to support the generation of hematopoietic cells from hESCs and hiPSCs [25].

2.1. Stromal Coculture. Several types of stromal layers have been reported in the coculture system, including murine bone marrow (BM) cell line S17 [9, 26] and OP9 [8, 10, 27], the yolk sac endothelial cell line C166 [9], murine fetal liver-derived stromal line (mFLSC) [12, 13], murine aorta-gonad-mesonephros (AGM) stromal lines [12], primary murine stromal cells from AGM and FL [12], immortalized human fetal liver hepatocyte line FH-B-hTERT [1, 14, 15], and primary human stromal cells derived from aorta- AGM, FL, and fetal BM (FBM) [11]. A side-by-side comparison finds that FH-B-hTERT cells are more effective than S17 cells in stimulating $CD34^+$ cells generation from hESCs [14]. The direct contact with stromal layers increases hematopoietic differentiation efficiency [11, 12, 26], although it is not an absolute requirement [11, 26]. In general, when culture media are supplemented with fetal bovine serum (FBS), no additional growth factors are required during coculture [1, 9, 12, 14, 15, 26]. To generate large quantities of relatively pure erythroid cells, often a further purification of $CD34^+$ cells from the coculture population is performed prior to seeding them in media supplemented with growth factors for erythroid expansion [1, 8], or the mixture of coculture cells is placed in methylcellulose cultures for hematopoietic colony assays, and then individual erythroid colonies are extracted from semisolid culture for further analysis [13]. While these studies have provided important insights into the erythroid development from hESCs or hiPSCs and have the potential for large-scale production [1, 8, 13], the utilization of murine cells in coculture by some protocols may make them unsuitable for clinical applications in the future.

2.2. EB Formation. Alternatively, successful hematopoietic induction can be achieved by EB formation, usually by placing clumps of hESCs or hiPSCs in nontissue culture-treated plates, or ultralow attachment plates. It has been reported in mouse EBs that cells undergo self-organization to establish anteroposterior polarity and form a primitive streak-like region via the Wnt signaling pathway [28]. For robust hematopoietic differentiation, good quality EB formation is essential. While Lu et al. observe that it is essential to use high-quality hESCs with minimal signs of differentiation and uniform stem cell marker expression for EBs formation and for high-efficiency erythroid generation [22], Ungrin and colleagues find that aggregates formed from populations of cells with the highest levels of Oct4 actually exhibit the least stability in terms of EB formation [29]. We find that hESCs or hiPSCs cultured under murine embryonic fibroblast (MEF) feeder-free conditions on matrigel-coated plates with MEF-conditioned media or a chemically defined media, such as mTeSR (StemCell Technologies), can also be used for EB formation, although they tend to be more temperamental with less consistent EB formation for unknown reasons (unpublished data). EB formation can be further controlled by plating a defined number of cells in untreated U bottom or V bottom, 96- or 386-well plates, or AggreWells and forcing the formation of aggregates by centrifugation [30, 31]. Because the forced aggregation of a defined number of hESCs or hiPSCs requires the generation of single cell suspensions prior to aggregation, we find that the addition of p160-Rho-associated coiled coil kinase (ROCK) inhibitor Y-27632 before and during the first 24 hours of EB formation is essential for the survival of these stem cells, consistent with previous reports [29, 32]. However, while forced aggregation leads to uniformly sized EBs, it has been shown that in fact, the traditional suspension culture of placing clumps of hESCs directly into low-attachment plates generates EBs with higher levels of hematopoietic differentiation than forced aggregation or hanging drop culture does [33]. Regardless of the EB formation method employed, it is important

Table 1: Summary of generation and characterization of erythroid cells from human embryonic stem cells and induced pluripotent stem cells.

Author[a]	Stem cell lines	Method	Stromal cells[b]	Major globin chains	Hb	Enu (%)	Yield[c]	Culture length (days)[d]	Note	Ref.
Dias J.	H1, hiPSCs	coculture	OP9 → MS5	ε, γ	N/M	2–10	200,000	75		[8]
Kaufman DS.	H1	coculture	S17, C166, MEF	α, β, δ	N/M	N/M	N/M	42		[9]
Klimchenko O.	H1, H9	coculture	OP9 → MS5	ζ, ε	N/M	no	N/M	38		[10]
Lee KY.	H1, H9, H14	coculture	AGM, FL, FBM	α, γ, δ	N/M	no	N/M	32		[11]
Ledran MH.	H1, H9, hES-NCL1	coculture	AGM, FL	$\alpha, \varepsilon, \gamma$	N/M	N/M	N/M	39		[12]
Ma F.	H1	Coculture	mFLSC	$\varepsilon, \gamma, \beta$	N/M	6.2	1,200	34	Time-dependent β globin increase at clonal level. Oxygen dissociation curve and G6PD activities are more similar to CB than to adult PB	[13]
Qiu C., Olivier EN.	H1	Coculture	FH-B-hTERT, S17	$\zeta, \alpha, \varepsilon, \gamma$	N/M[e]	1.5–16	80	59	Maturation switch and lineage switch noted. Minor increase in β globin expression	[1, 14, 15]
Cerdan C.	H1, H9	EB	N/A	ε, β	HbA > HbF	N/M	N/M	29		[16]
Chang KH.	H1, BG02, hiPSCs	EB	N/A	$\alpha, \varepsilon, \gamma$	N/M	N/M	N/M	21		[17–19]
Lapillonne H.	H1, hiPSCs	EB	N/A	α, γ	HbF	4–66	200–3,500	46	CO rebinding kinetics similar to CB	[20]
Zambidis ET.	H1	EB	N/A	$\zeta, \alpha, \varepsilon, \gamma$[f]	HbF > HbA	N/M	N/M	36	Time-dependent increase in β globin expression observed. Primitive to definitive switch noted	[21]

Table 1: Continued.

Author[a]	Stem cell lines	Method	Stromal cells[b]	Major globin chains	Hb	Enu (%)	Yield[c]	Culture length (days)[d]	Note	Ref.
Lu SJ.	H1, MA01, MA99, HuES-3	EB → BL	MSC, OP9[g]	$\zeta, \alpha, \varepsilon, \gamma$	N/M	10–30; 30–65[h]	800–8,000	25	Oxygen dissociation curve slightly displaced to left of normal RBCs. Decreased response to 2,3-DPG depletion as compared to adult RBCs. Comparable Bohr effect at physiologic pH	[22]
Honig GR.	H1, H7, MA01	EB → BL	N/A		Hb Gower I > Hb Barts	N/M	N/M	25	Low $(\alpha+\zeta)/(\gamma+\varepsilon)$ synthesis ratio	[23]
Liu YX.	H1	EB → coculture	hFLSC	$\zeta, \alpha, \varepsilon, \gamma$[f]	N/M	N/M	N/M	29	β globin expression increases with culture time. Oxygen dissociation curve slightly displaced to left of normal RBCs	[24]

[a] Publications by the same laboratory are grouped together. [b] Arrow indicates sequential exposure to different stromal cells. [c] Number of erythroid cells generated per hESCs or hiPSCs. [d] Culture time is approximate as it is often variable even within one particular study. Culture length is for generating hemoglobinized erythroblasts and may not include time required for enucleation. [e] The authors speculated the hemoglobin tetramer species based on globin chain results. [f] The major globin chains expressed cannot be determined based on the method employed. [g] For enucleation purpose only. [h] Higher enucleation efficiency is associated with stromal coculture. Hb: hemoglobin tetramer; N/M: not mentioned; Enu: enucleation; N/A: not applicable; CB: cord blood; PB: peripheral blood; BL: hemangioblasts; MSC: mesenchymal stem cells.

to note that care should be taken to avoid fusion among individual EBs as active migration of cells and the expression of transcription factor Cd×2, known for its role in affecting cell fate decisions [34], have been observed in a subset of cells at the junction of fusing EBs [29]. While housing individual EBs in separate wells can prevent the aggregation, it is not recommended, as the paracrine effect enhances hematopoietic differentiation of EBs [33]. Encapsulating them in size-specified agarose capsules [35] may provide an alternative, although the hematopoietic differentiation of encapsulated EBs has not been fully examined.

An array of media has been used for the generation of EBs. Our laboratory has routinely used a medium essentially composed of Iscove's Modified Dulbecco's Media, fetal bovine serum (FBS), and protein-free hybridoma medium. Originally, we included basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in the medium [17] and later found them to be unnecessary in FBS-supplemented media, although VEGF-165 has been reported to augment erythropoiesis during EB formation [16]. Lot specific effects of FBS on hematopoietic differentiation during EB formation have been noted and thus require prescreening of FBS for the EB assay (unpublished observations). Several laboratories have established serum-free media for EB formation, in which growth factor supplementation is required [26]. Bone morphogenetic protein 4 (BMP4), VEGF, granulocyte colony-stimulating factor (G-CSF), interleukin-3 (IL-3), stem cell factor (SCF), Flt3 ligand, IL-6, insulin-like growth factor-2 (IGF-II), thrombopoietin (TPO), and bFGF have been used in an assortment of combinations to achieve induction of hematopoietic differentiation of EBs [22, 26, 30, 33, 36, 37]. A recent publication utilizing human plasma finds the addition of BMP4 and VEGF to be indispensable, consistent with the finding by Tian and colleagues [26], and the inclusion of SCF, TPO, Flt3 ligand, erythropoietin (EPO), IL-3, and IL-6 in the EB medium gives the optimal erythropoietic stimulation [20].

2.3. From EBs to Erythroid Cells. Different laboratories harvest EBs at different time points for further erythroid differentiation. Our protocol originally included an adherent culture step for erythroid induction, but was later streamlined to seeding dissociated day-7 EBs directly into a serum-free erythroid induction medium in which a large number of relatively pure erythroid cells (>90%) can be obtained with 21 days of total culture time [17–19]. We find that EPO and SCF are absolutely required for the survival and differentiation of erythroid progenitors in EBs; IL-3 and IL-6 augment the expansion of erythroid cells, but G-CSF and GM-CSF are dispensable (unpublished data), similar to the conclusion of others [10]. If added, FBS has significant inhibitory effect on the growth and expansion of these erythroid progenitors from EBs, as previously noted by Zambidis et al. [21]. Furthermore, while dissociated day-7 EBs give rise to almost exclusively erythroid cells (>90%) in the serum-free erythroid induction medium, day-14 EBs generate a mixture of erythroid and myeloid cells [17]. Interestingly, while ∼5–10% cells of hESC line H1-derived EBs express glycophorin-A between 7 and 14 days in our FBS-based system [17, 19], very little glycophorin-A expression is detected in the H1-derived EBs up to 20 days in the human plasma-based cultures in spite of the hematopoietic cytokines supplementation in the medium [20]. The differences in culture time and cytokine supplements notwithstanding, Lapillonne et al. also find the unpurified, dissociated EBs from their human plasma-based cultures generating exclusively erythroid cells when placed in an erythroid expansion medium that also contains human plasma. Lu and colleagues take a different approach by placing dissociated day-3.5 EBs in blast-colony growth media with the addition of bFGF and recombinant tPTD-HoxB4 fusion protein to expand hemangioblasts prior to transfer into an erythroid differentiation and expansion culture [22]. A mixture of erythroid and nonerythroid cells is apparently obtained, which requires an overnight adhesion culture to remove nonerythroid cells.

2.4. From Erythroblasts to RBCs. Extended ex vivo propagation of cells is associated with acquisition of genetic alterations, including transformation. Safety concerns about in vivo use of such cells therefore have been raised, specifically in the autologous setting. As terminally differentiated (and physiologically enucleated) cells, in this respect, erythrocytes possess considerable advantages over transplants of immature cells or cells with proliferative capacity in spite of their maturity, such as T cells. Several studies show that hESC- and hiPSC-derived erythroblasts are capable of enucleation and become fully mature RBCs. A prolonged culture appears to be an important factor with or without the involvement of stromal cells. Qui and colleagues find enucleation can be obtained by sequential exposure of $CD34^+$ cells sorted from FH-B-hTERT/H1 coculture to two sets of cytokines, followed by a 2-step 10-day coculture with MS-5 stromal cells [15]. The enucleation efficiency varies from 1.5% to 16% for the day-35 H1/FH-B-hTERT coculture, and 0% for the day-14 H1/FH-B-hTERT coculture. A 3-step culture that involves a 15-day coculture of H1/mFLSC coculture, followed by a 14-day erythroid burst colony formation, and then a 7-day liquid culture expansion also produces clusters of enucleated RBCs with enucleation efficiency ranging from 1.3% to 11.4% [13]. A higher degree of enucleation is achieved by Lu et al. with their hemangioblasts protocol composed of EB formation, hemangioblast expansion, sequential cytokine exposure of the blast cells, and then coculture with human mesenchymal stem cells (MSCs), with OP9 mouse stromal cells, or without stromal cells [22]. The enucleation efficiencies are 10%–30% without stromal cells, ∼30% on MSC stromal cells, and 30%–65% on OP9 cells, suggesting that the presence of stromal cells significantly facilitates the enucleation process. On the other hand, a recent finding by Lapillonne and colleagues shows that by supplementing culture media with human plasma, a significant degree of enucleation is attained without stromal cells in H1 hESCs-derived erythroid cells (52%–66%) [20]. However, much lower degrees of enucleation (4%–10%) are obtained with erythroid cells derived from 2 separate hiPSC lines using the same protocol, suggesting that the reprogramming process or epigenetic memories retained, may impact the

ability of hiPSC-derived erythroid cells to enucleate. Given the currently still incomplete efficiency of enucleation of erythroblasts and incomplete lineage determination in vitro and if these ex vivo generated erythroid cells were to be used in transfusion, it might be reasonable to filter them through conventional leukocyte filters, followed by irradiation with 3,000 cGy, as erythrocytes tolerate irradiation without critical loss of function.

2.5. Large-Scale Production of Erythroblasts or RBCs from hESCs or hiPSCs. Several publications have claimed that their respective protocol results in large-scale generations of erythroid cells from hESCs or hiPSCs, which is a prerequisite of any potential clinical usage. With the H1/FH-B-hTERT coculture system, Qiu et al. obtain approximately 80 erythroid cells per hESC although it is estimated that a 50×10^3-fold expansion is achieved by a single $CD34^+$ cells from the coculture [15]. Using H1/mFLSC coculture, Ma and colleagues generate large erythroid bursts consisting of $\sim 0.2 \times 10^6$ erythroid cells per burst [13]. Taking into consideration the colony forming unit frequencies and further expansion potentials in liquid culture, roughly 1,200 erythroid cells are generated per hESC. A slightly higher production efficiency is achieved by using EB formation and human plasma supplementation that shows 1,500–3,500 erythroid cells are produced per hESC, but only 200–400 erythroid output per hiPSC [20]. Using recombinant tPTD-HoxB4 fusion protein and hemangioblast approach, Lu and colleagues reported the generation of up to 8,000 erythroid cells per hESC [22]. The highest production efficiency is reported by Dias and colleagues employing the OP9 coculture system, followed by dissociation and reaggregation on low attachment plates with the addition of cytokines, and then coculture on MS-5 feeders [8]. Up to 200,000 erythroid cells are reported to be generated per hESC, and a similar level of expansion is also obtained with hiPSCs, although the requirement of murine cells for coculture throughout the differentiation procedure, in addition to the significantly longer culture time (70–120 days), remains a setback for potential clinical applications. Together, these data show that without significant progress in generating erythrocytes from hESCs or hiPSCs more cost effectively, the magnitude and cost of replacing donor-derived erythrocytes with these in vitro generated RBCs will be prohibitive, given that one RBC concentrate contains approximately 2×10^{12} erythrocytes, that is, in a country like Germany, 8×10^{18} (10^{11} per head of the population) red blood cells are transfused in any given year.

3. Characterization of hESC- and hiPSC-Derived Erythroid Cells

3.1. Globin Chain Expression. Amongst all erythroid-associated characteristics, the globin expression phenotype has been studied most extensively in the hESC- and hiPSC-derived erythroid cells. Our laboratory finds that the α globin, rather than embryonic ζ globin, is the major α-locus globin expressed, although expression of ζ globin can be detected in all erythroid cells from H1 hESC at the protein level, suggesting a low, but pancellular expression of embryonic ζ globin [17], differing from the finding of Cerdan et al. that shows ζ globin is not expressed by hESC-derived erythroblasts [16]. The expression of ζ globin is also confirmed by studies in erythroid cells generated from other hESC lines as well as H1 line [15, 21, 22, 24], and hiPSCs originated from either fetal or adult tissues [20]. We find the α/ζ globin ratio does not change whether the erythroid cells are derived from day-7 or day-14 EBs [17], although Qiu and colleagues find that by extending the coculture time between hESCs and immortalized human fetal liver hepatocyte line FH-B-hTERT from 14 days to 35 days, a lineage switch occurs and these hESCs-derived erythroid cells express α locus globins with an increased α/ζ globin ratio [15]. Furthermore, individual clonal erythroid expansion and maturation apparently is accompanied by a maturation switch characterized by an increasing α/ζ globin ratio over time as the hESC-derived erythroid cells proceed from the stage of proerythroblasts to the stage of orthochromatic erythroblasts [15].

With the exception of the first two studies examining the hematopoietic/erythroid differentiation of hESC that claim the adult β globin to be the major globin expressed by hESC-derived erythroid cells [9, 16], all other studies followed find embryonic ε and fetal γ globins to be the predominant β-locus globin expressed by these cells, at least initially, regardless of the differentiation methods employed or the ontogenic origin of the hiPSCs [1, 8, 13, 15, 17–22, 24]. While some studies find little changes in ε/γ ratio in mature erythroid cells derived from different time points of culture [8, 17, 24], Qiu et al. reported that similar to their finding in the changes in α/ζ globin ratio, increasing coculture time also leads to the generation of erythroid cells expressing mainly fetal γ globin [15]. An increase in the adult β globin expression with prolonged coculture time is also observed albeit its expression level is low ($\sim$2% of all β-locus globins), consistent with the findings of others [8, 22, 24]. Interestingly, Ma et al. show a more drastic increase in β globin expression with their erythroid cells derived from H1/mFLSC cells coculture. The globin expression in the erythroid cells of individual clones changes in a time-dependent manner: embryonic ε-globin-expressing erythroid cells from individual clones decrease from $\sim$100% to $\sim$50%, whereas adult-type β-globin-expressing cells increase to $\sim$100% in all clones examined [13]. It is not known, however, whether the adult β globin actually is the major β-locus globin expressed, given that 100% of the cells are also expressing γ globin, and $\sim$50% of the cells are expressing ε globin. Nevertheless, these studies provide supporting evidence that with prolonged coculture, and/or prolonged erythroid expansion, the erythroid cells adapt a more developmentally advanced globin expression pattern.

The transcriptional controls of the globin expression have not yet been studied in these cells, but a limited number of studies have been done to analyze the epigenetic landscape of β globin locus of hESC-derived erythroid cells. It is found that domains of DNA hypomethylation spanning thousands of base pairs within domains of acetylated histones are

established around the most highly expressed genes during each developmental stage when comparing hESC-derived erythroblasts to uncultured FL and bone marrow cells [38, 39]. In addition, our laboratory also finds that throughout development, human primary erythroblasts employ similar histone codes including enrichment of H3K4me3 and selective depletion of H3K27me1 to ensure proper temporal expression of specific globin genes (unpublished data). Furthermore, a looping mechanism is also at play to bring the hypersensitive sites of the LCR into physical contact with the ε globin promoter, presumably to initiate transcription via the formation of an active chromatin hub, in the ε globin producing hESC-derived erythroid cells.

3.2. Hemoglobin Analyses and Functionality. As compared to the globin chains, few studies have been conducted to study the hemoglobin tetramers in the hESC- or hiPSC-derived erythroid cells. Using CE-HPLC, it is found that the mature erythroid cells, derived from both hESCs and iPSCs based on the EB/human plasma culture system, express mostly HbF ($\alpha 2\gamma 2$) with little Hb Gower I ($\zeta 2\varepsilon 2$) or Gower II ($\alpha 2\varepsilon 2$) [20]. Furthermore, the CO-rebinding kinetics, which probe the allosteric equilibrium of R and T states, of hemoglobin from hESC- and hiPSC-derived erythroid cells, are almost identical to those of cord blood, with or without potent allosteric effector inositol hexaphosphate, suggesting that the HbF in these erythroid cells is functional [20]. In addition, the oxygen dissociation curve of hESC-derived erythroid cells is displaced to the left, similar to that of the human cord blood, as compared to that of adult RBCs, consistent with the higher affinity of HbF to oxygen than HbA [13, 22, 24]. The decreased response to 2,3-diphosphoglycerate (2,3-DPG) depletion as compared to adult RBCs is consistent with the lack of interaction between HbF and 2,3-DPG [22]. In addition, hESC-derived erythroid cells also have high glucose-6-phosphate dehydrogenase activity, confirming that they are protected from oxidative damage [13]. Together, these data indicate that hESC- and hiPSC-derived erythroid cells will function as oxygen carriers like cord blood or fetal blood. In contrast, a recent study using the hemangioblast approach shows that hESC-derived erythroid cells produce mainly Hb Gower1 ($\zeta 2\varepsilon 2$) with low levels of HbF, Hb Portland ($\zeta 2\gamma 2$), and Hb Gower-2 ($\alpha 2\varepsilon 2$), each represents less than 5% of the total hemoglobin [23]. Moreover, these hemangioblast-derived erythroid cells also have an elevated amount of Hb Barts ($^{G}\gamma 4$) that accounts for 12%–18% of the total hemoglobin. As Hb Barts has extremely high affinity to oxygen, this finding appears to contradict the previous report by the same group that hESC-derived erythroid cells have an oxygen dissociation curve only slightly displaced to the left of the adult RBCs, and that they show a comparable Bohr effect at physiologic pH [22]. In addition to not releasing oxygen into the tissue due to extremely high oxygen affinity, Hb Barts is moderately insoluble, and therefore accumulates in the red blood cells. Hence, this study would argue that hESC-derived erythroid cells, despite the large-scale production using hemangioblast protocol [22], can not be used for transfusion. The authors do offer that, however, with further maturation that leads to developmentally more advanced erythroblasts or enucleated RBCs, the level of Hb Barts may decrease in these hESC-derived erythroid cells.

4. Future Challenges

The magnitude and cost of replacing donor-derived erythrocytes with in vitro generated RBCs is prohibitive and requires significant progress in the expansion, maturation, and terminal differentiation/enucleation of erythrocytes to make the transfusion products cost effective. A recently published method of culturing hESCs and hiPSCs in suspension may aid in the scaling up production of hESCs and hiPSCs [40]. In addition, the proposed utilization of synthetic 3D structures mimicking bone marrow structure [41] and the manipulation of transcriptional environment, such as ectopic expression of engineered Nup98-HoxA10 fusion protein [42] and downregulation of microRNAs-126/125* [43], may improve the erythroid production efficiency.

However, even if a cost-effective method of generating erythrocytes from hESCs or hiPSCs were achieved, significant challenges remain to be met before the therapeutic use of these cells. While not expressing HLA, mature RBCs express many surface antigens against which regular (present in all individuals not expressing the antigen) or irregular (acquired after contact with the antigen) antibodies can be directed. Thirty blood group systems, or antigen families, with almost 400 antigens have been documented, and antibodies against all of these antigens have been described. If ignored, such antibodies cause acute, potentially deadly, or delayed hemolytic transfusion reactions. Therefore, the presence of such antibodies necessitates selection of antigen-negative RBCs. Since antigens are expressed from genes located throughout the genome, any combination of blood group antigens can occur, thus in theory, an almost infinite number of hESC or iPSC lines would be required to generate compatible erythrocytes for all individuals. Generation of null variants for blood group antigens via genetic manipulation is not conducive as null variants have been described for many blood group antigens, but all of which are associated with structural defects and/or with the generation of alternative antigens, with the exception of Rh dd (Rh-negative, the null variant which represents the counter antigen to Rh D) and O (absence of both A and B transferase). It may be conceptually reasonable to generate a single hiPSC line from an O Rh negative ccddee K negative donor heterozygous for as many antigens as possible (to decrease foreign antigen dose and hence, antigenicity) and propagate it in huge biogenerators as a source for nonimmunized patients. For allo-(poly)sensitized or at risk, chronically transfusion-dependent patients, specifically those of rare blood types, autologous iPSC lines could be established, from which individualized erythrocytes could be generated and stored. It must be stressed, however, that the membrane protein and glycosylation profile of RBCs produced in vitro, even from autologous iPSC lines, may be altered compared to that of the in vivo produced RBCs, due to the lack of complex environment found in vivo, or an imbalanced transcription factor expression [44]. The important finding that iPSCs and their progeny are rejected in the autologous setting [45]

appropriately brings to the forefront the issue of antigenicity of ex vivo generated cells due to expression of neoantigens. It may be plausible to enzymatically remove surface antigens. However, such approach has not been achieved except for A and B substances [46].

Furthermore, rigorous studies must be conducted to fully characterize these in vitro generated RBCs including their membrane surface potential, pliability, and half-life in vivo, in addition to profiling their hemoglobin packing, gas exchange properties, and immunogenicities. The sensitivity of the system is well illustrated by the recent evidence that has come forth about effects of ageing of erythrocytes on their therapeutic benefit—free hemoglobin, increased membrane rigidity, membrane damage, and altered O_2 affinity of stored red cells have all been implicated in adverse outcomes observed with older erythrocytes [47]. Thus, even after all the challenges of devising suitable in vitro systems for expansion, maturation and terminal differentiation/enucleation of erythrocytes from primitive cell sources have been mastered, all of these biophysical parameters will still need to be carefully compared between in vitro and in vivo generated erythrocytes before they can be used clinically.

5. Conclusions

Significant progress has been made in generating erythroid cells from hESCs and hiPSCs. The production efficiency of up to 200,000 cells per hESC or iPSC has been achieved [8]. Unfortunately, the methodology that generates the highest number of erythroid cells per hESC is the least defined and relies heavily on the presence of murine stromal cells: first OP9, then MS-5. The hemoglobin expression pattern and enucleation efficiency are also less clear with erythroid cells derived from this particular method, than from the other mass production protocols [13, 20, 22, 23]. Further investigation of the hemoglobin tetramer composition is critical, as it is shown that erythroid cells generated from the protocol with the second-highest production efficiency (8,000 erythroid cells per hESC) have mostly Hb Gower I, followed by an elevated level of Hb Barts, which would not be suitable for transfusion [23]. The method proposed by Lapillonne and colleagues, without using animal products or stromal cell lines, generates moderate number of erythroid cells from hESCs (1,500–3,500 erythroid cells per hESC) that enucleate efficiently (~60%) with functional HbF tetramers as the major hemoglobin (~93%). Unfortunately, this method appears to work less efficiently with hiPSC, which is important since one of the major goals of generating RBCs is for the transfusion needs of patients with rare blood phenotypes or those that have been alloimmunized. The decreased efficiency of erythroid generation may be offset by prescreening for hiPSC lines with increased erythroid differentiation propensity, as line-to-line variation has been documented [18, 19], or by simply increasing the input number of hiPSCs. Alternatively, generating immortal erythroid progenitor lines capable of terminal differentiation and enucleation, which have been shown to be successful in murine ESC-derived cells [48], may be a viable option. Finally, little is known about these hESC- and hiPSC-derived erythroid cells other than their hemoglobin composition, gas exchange properties, and their growth and expansion pattern in response to various methods. Membrane compositions, surface antigen expression, immunogenicity, deformability, and half-life in vivo all require further and vigorous investigations before the transition from bench to bedside can occur.

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Review Article

Erythroblast Enucleation

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Even though the production of orthochromatic erythroblasts can be scaled up to fulfill clinical requirements, enucleation remains one of the critical rate-limiting steps in the production of transfusable red blood cells. Mammalian erythrocytes extrude their nucleus prior to entering circulation, likely to impart flexibility and improve the ability to traverse through capillaries that are half the size of erythrocytes. Recently, there have been many advances in our understanding of the mechanisms underlying mammalian erythrocyte enucleation. This review summarizes these advances, discusses the possible future directions in the field, and evaluates the prospects for improved ex vivo production of red blood cells.

1. Introduction

Blood transfusions are a common practice to treat severe anemia and shock. Even though developed countries by and large have sufficient supplies of transfusable blood, an increase in the incidence of complications from allogenic immune reactions provides impetus to search for alternatives that are less immunogenic [1–3]. Moreover, developing and third world countries battle a shortage of blood units for transfusions [4]. One alternative under investigation is the ex vivo derivation of red blood cells from autologous hematopoietic stem/progenitor cells. In addition to enhancing the supply of transfusable blood, this approach may decrease the incidence of allogenic immune reactions in chronic transfusion-dependent patients. Human embryonic stem cells, CD34+ cells from umbilical cord blood, adult hematopoietic stem/progenitor cells, peripheral blood CD34+ cells, or human-induced pluripotent stem cells (iPS cells) can all be used as source for synthesizing RBCs [5]. In all these systems, however, the efficiency of enucleation is low. Enucleation is an important criterion because nucleated erythroblasts are not as efficient in oxygen transport and because they are likely to undergo hemolysis as they traverse through narrow capillaries. Enucleated cells also offer the benefit of lacking DNA and the ability to divide, obviating the risk of introducing a malignancy into the recipients [6, 7].

Enucleation remains one of the critical rate-limiting steps of in vitro RBC synthesis. Lu et al. achieved 60% enucleation of erythroblasts derived from human ES cells (hES cells). The RBCs produced expressed mainly fetal and embryonic hemoglobin and were comparable to normal RBCs in terms of oxygen delivery [8]. Recently, Lapillonne et al. have reported the production of red blood cells from human iPS cells. Although the majority of the culture consisted of orthochromatic erythroblasts, enucleated RBCs accounted for only between 4 and 10% of the culture [9]. Thus, there is a long way to go in terms of achieving 100% enucleation in vitro. This review will summarize the current mechanistic understanding of enucleation, propose a new model, and discuss future research directions.

2. Defining Erythroblast Enucleation

Mammals have evolved to enucleate their erythroblasts while other animals maintain circulation of RBCs with condensed pyknotic nuclei. Of note, however, the circulation of early mammalian embryos includes nucleated primitive erythroid cells. These cells mature in the bloodstream between embryonic days (E) 14.5 and E16.5 of mouse gestation and eventually enucleate likely within the fetal liver [10]. Definitive erythropoiesis, which leads to exclusive

production of enucleated reticulocytes, begins in mid gestation. RBCs derived from definitive erythropoiesis originate in the fetal liver or bone marrow depending upon the age of the fetus [11].

In all cases, erythroblasts are derived from hematopoietic stem cells (HSCs). The first cell committed towards the erythroid lineage is the burst forming unit-erythroid (BFU-E), which further proliferates and matures to the colony-forming unit E (CFU-E). The BFU-E stage is the most proliferative segment of the differentiation program followed by the CFU-E stage. Acquisition of EPO receptors occur in the mid-to-late stage of BFU-E, and by the time these cells reach CFU-E stage maximum numbers of EPO receptors are present on their surface [12]. During this time period, cells are completely dependent on erythropoietin (EPO) for their survival [13]. The CFU-E then undergoes a series of maturational steps named proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, and eventually orthochromatic erythroblasts, at which point they exit the cell cycle. By the time, the cells reach late polychromatic erythroblast stage the cells are independent of EPO for their survival [14]. Other cytokines and humeral factors that may be required to promote differentiation beyond the polychromatic stage has not been identified although autocrine production of several cytokines have been observed [15, 16]. Whether these cytokines provide the necessary signals for terminal differentiation including enucleation is unclear at this point. Throughout the differentiation program, erythroid progenitors and erythroblasts undergo numerous morphological changes. These include (1) a decrease in cell size, (2) nuclear condensation, and (3) an increase in the cytoplasmic nuclear ratio.

As red cells mature, chromatin becomes condensed, and transcription in general is suppressed. A network of chromatin factors and histone modifying proteins likely contribute to this process. For example, the nonhistone nuclear protein mature erythrocyte nuclear termination stage specific protein (MENT) has been shown to promote chromatin condensation and nuclear collapse at the terminal stage of maturation of chicken erythrocytes [17]. Similarly, the condensin II subunit mCAP-G2 represses transcription by recruiting class I histone deacetylases (HDACs) and promotes terminal differentiation of mammalian cells [18]. During maturation of murine erythroblasts, histone H3(K9) dimethylation was found to increase while histone H4(K12) acetylation was dramatically decreased [19]. Consistent with a requirement for decreased acetylation, treatment of murine erythroblasts with the HDAC inhibitors trichostatin A or valproic acid blocked chromatin condensation and enucleation [19, 20]. Moreover, knockdown of HDAC2 led to a prominent block in condensation and nuclear extrusion. Recent studies have further revealed that downregulation of miR-191, which occurs during normal differentiation, contributes to chromatin condensation by allowing the upregulation of Riok3, an atypical protein kinase, and Mxi1, a c-myc antagonist [21]. Downregulation of myc appears to be required not for cell cycle arrest, but rather to allow for nuclear condensation and histone deacetylation [22]. Together these studies show that chromatin condensation is an integral part of erythroblast maturation.

After exiting the final cell cycle, the nuclei of orthochromatic erythroblasts are polarized to one side of the cell. Eventually these cells enucleate to form reticulocyte and a "pyrenocyte," the extruded nucleus with a thin rim of cytoplasm surrounded by a plasma membrane [10, 23]. Pyrenocytes are engulfed by macrophages in erythroblastic islands within the fetal liver and bone marrow. During terminal differentiation, cells undergo multiple cellular processes including protein sorting, autophagy, membrane maturation, vesicle trafficking, and cytoskeletal remodeling. Important questions to the field include the following (1) To what extent do these processes contribute to enucleation? (2) Which of these steps are required for enucleation? (3) At which point in the differentiation continuum does enucleation start? (4) Can enucleation occur in a cell that is not completely matured, such as a polychromatic erythroblast? (5) Can enucleation proceed before nuclear condensation is completed? At this point, it has not been demonstrated whether enucleation can occur in an immature erythroblast. Based on the model that chromatin condensation is a prerequisite for enucleation [19, 20, 24], however, one would assume that this is unlikely. Examining the nuclei engulfed by macrophages within genetically modified mice that have a block in maturation of late erythroblasts may help answer whether enucleation can take place in erythroblasts other than orthochromatic erythroblasts. Since nuclear condensation and other morphological changes are progressive processes that proceed from proerythroblasts through orthochromatic erythroblasts, and since primitive RBCs and nucleated peripheral blood RBCs of lower animals have condensed nuclei, for the purposes of this review, we will consider enucleation to be a process that begins with polarization of nucleus in an orthochromatic erythroblast.

3. Mechanism of Enucleation

3.1. Apoptosis, Asymmetric Cytokinesis, or Other?. Historically there have been two prominent models of enucleation: apoptosis and asymmetric cytokinesis. The presence of partial karyolysis and leakage of nuclear material into the cytoplasm visualized by electron microscopy favors the apoptosis model [25]. Additional evidence includes the prevailing model that lens epithelial cells and keratinocytes undergo a mechanism similar to programmed cell death to eliminate their nuclei [26, 27]. To directly test this whether apoptosis is required for enucleation, Carlile et al. studied the effect of siRNA-mediated caspase knockdown on enucleation and found that there was a 50% decrease in enucleated cells in the knockdown condition as compared to control. However, the authors noted that maturation was blocked at a stage between proerythroblasts and basophilic erythroblasts, suggesting a role for caspases at an earlier stage of erythroblast development [28]. Furthermore, Krauss et al. observed that critical nuclear structures such as the nuclear matrix protein NuMA (nuclear mitotic apparatus) and the splicing factors Sm and SC35, as well as the

interaction between lamin B with the nuclear envelope and DNA persisted during late erythroblast development prior to enucleation, consistent with an absence of caspase activity [29]. In addition, treatment of enucleating erythroblasts with pan-caspase inhibitors did not block enucleation [23]. Together, these findings strongly suggest that apoptosis is not involved in enucleation per se.

The cytokinetic model posits that nuclear extrusion is a form of cell division, in which the nucleus is separated from the cytoplasm by an active process that involves cytokinetic machinery. By this definition, an enucleating cell should have a well-defined cleavage furrow, a contractile actomyosin ring, and a stage of completion with abscission [30]. Indeed, many studies including ultra-structural observations from the 1960s lend support to this model. For example, Skutelsky and Danon and others noticed that the pyrenocytes had a thin rim of cytoplasm surrounded by an intact plasma membrane [23, 25, 31–34], a finding that confirms that the nucleus is not extruded out or exocytosed, but rather separated in a well-orchestrated process. Moreover, one can see a constricted surface resembling a cleavage furrow on enucleating erythroblasts [32, 33, 35, 36]. Further, there are numerous studies showing that cytochalasin D, an actin-depolymerizing agent which blocks filamentous actin formation, inhibits enucleation of late erythroblasts in-vitro [23, 33, 35, 37]. It has also been shown by immunofluorescence that actin accumulates in the region between the nucleus and the cytoplasm close to an anatomic constriction zone [33, 38, 39]. Finally, elegant studies with murine primary erythroblasts have demonstrated that Rac1 and Rac2 function through mDia2 to contribute to actin accumulation in the constriction zone [36]. While these findings provide strong evidence to support a role for the actin cytoskeleton in erythroblast maturation, it is unclear regarding the specific role actin is playing in enucleation, including whether it interacts with nonmuscle myosin II to form a contractile actin ring similar to cytokinesis (Figure 1).

In addition to the actin cytoskeleton, microtubules play an important role in cell division including cleavage furrow formation [30]. Studies by Koury et al. showed that the inhibition of microtubules with various toxins such as colchicine, vinblastine and taxol did not affect enucleation [33]. Ji et al. showed that enucleation does not depend on RhoA activity, using dominant negative mutants of RhoA and C3 exoenzyme, a specific inhibitor of RhoA [36]. It is well known that RhoA is involved in the formation and ingression of cleavage furrow. RhoA when activated accumulates in the cleavage furrow and further activates downstream effectors including Rho kinase (ROCK), citron kinase, LIM kinase, and formins [40–43]. Hence a lack of role for RhoA and microtubule in enucleation suggests that the anatomical constriction zone that is visible on enucleating cell may not be actually a cleavage furrow.

What about intermediate filaments? Using murine splenic erythroblasts infected with the anemia-inducing strain of Friend virus (FVA), Koury et al. found that erythroblasts approaching enucleation downregulate expression of vimentin [33]. Further, Xue et al., using immunofluorescence techniques, observed that vimentin anchored the nuclear lamina to the center of the cell as well as to the plasma membrane periphery and was expressed during the periods of 12, 24, and 36 hours of FVA in vitro culture but lost by 36–48 hours [39]. This loss of vimentin could release the nucleus and enable actin to push it towards one end of the cell close to plasma membrane. Of note, circulating avian erythrocytes continue to have intact vimentin, which might be one of the reasons why they resist enucleation [44].

To test whether actin functions as contractile actomyosin ring in nuclear extrusion, Keerthivasan et al. treated primary murine and human erythroblasts with blebbistatin at different time points and assayed the effect on cell division and enucleation [34]. Blebbistatin is a specific nonmuscle myosin II ATPase inhibitor that blocks the contractility of the actomyosin ring in cytokinetic cells and results in polyploidy of dividing cells without affecting the formation of actin cytoskeleton [45]. Keerthivasan et al. showed that blebbistatin potently inhibited enucleation when added to cultures at 24 hours, when the majority of cells were undergoing cell division. In this case, blebbistatin resulted in cell cycle arrest and the accumulation of polyploid cells. In contrast, blebbistatin had little effect on enucleation when it was added to cells at 38 hours, a time when the majority of cells are postmitotic [34]. These findings suggest that although actin accumulates in the region between the nucleus and cytoplasm during late stage erythroblasts, its role as a contractile actomyosin ring similar to cytokinesis in enucleation is questionable.

Abscission is the final stage of cytokinesis and involves trafficking of vesicles to the midbody region and fusion of these vesicles to lead to separation of the daughter cells [46, 47]. Throughout cytokinesis, membranes are supplied to the progressing tip of the cleavage furrow and the abscission site through golgi and recycling endosome-derived vesicles [48, 49]. Several lines of evidence support the model that vesicle trafficking directly contributes to enucleation, in large part by providing membranes to facilitate the separation of the pyrenocyte from the reticulocyte. First, electron microscopy has revealed the presence of vesicles and U-shaped tubes in the region between the nucleus and incipient reticulocyte [25, 34, 50]. Second, there is an accumulation of transferrin laden vesicles/vacuoles in the region between the nucleus and the cytoplasm [34, 50]. Third, disrupting vesicle trafficking by a battery of chemical inhibitors inhibited accumulation of those vesicles and blocked erythroblast enucleation [34]. Finally, siRNA-mediated knockdown of clathrin inhibited enucleation of human primary erythroblasts [34]. These findings strongly suggest that vesicle trafficking is a key component of erythroblast enucleation and that at least a part of nuclear extrusion process is similar to abscission (Figure 1).

3.2. Protein Sorting and Enucleation. An important event during enucleation is the differential sorting of proteins to the pyrenocyte and the reticulocyte. Geiduschek and Singer studied this phenomenon by an immunofluorescence technique and followed the distribution of lectin receptors and spectrin through erythroid differentiation. They found that spectrin completely sorts to the incipient reticulocyte

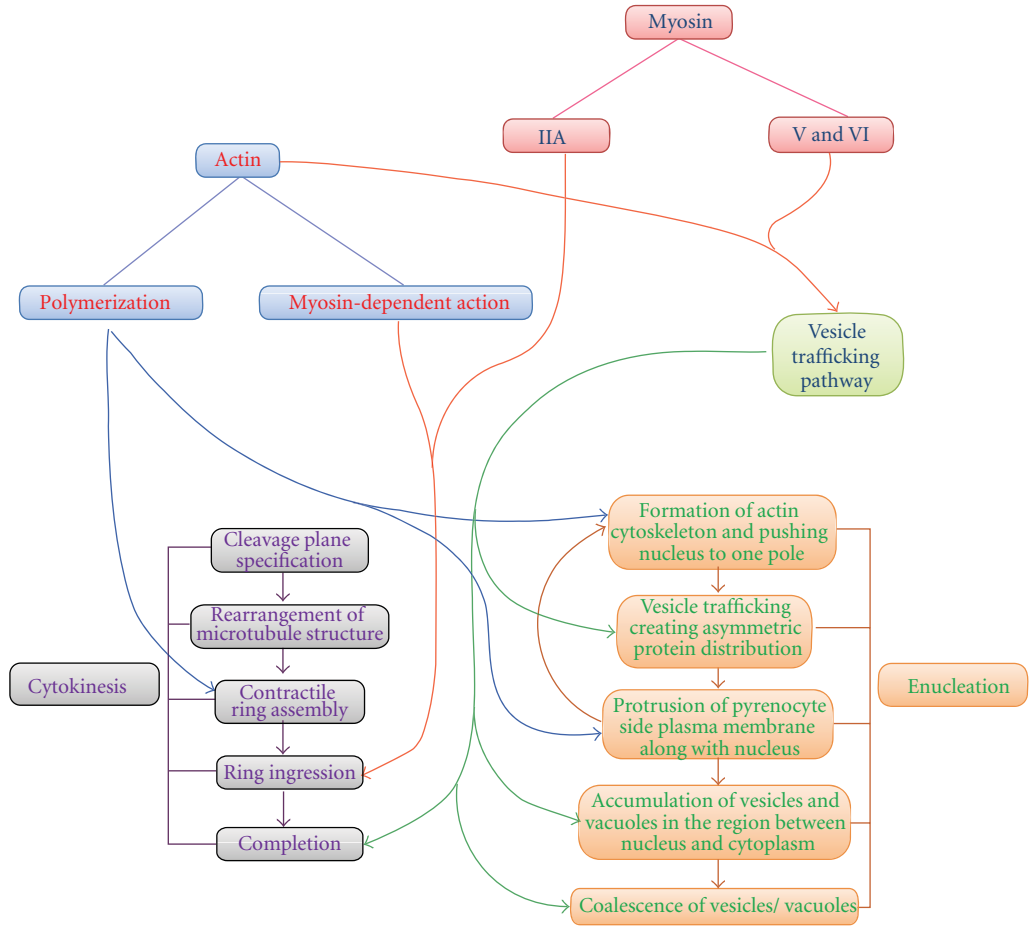

Figure 1: Key events in cytokinesis and enucleation. Even though the final stages of cytokinesis and enucleation are both driven by vesicle trafficking, the preceding events are substantially different between the two processes.

while receptors for concanavalin A are restricted to the plasma membrane surrounding the extruding nucleus [51]. Using murine erythroleukemia (MEL) cells, Patel and Lodish reported that enucleated cells detach from a fibronectin matrix due to the loss of the fibronectin receptor while both Band 3 and ankyrin are enriched in the reticulocyte [52]. Transferrin has been found to differentially sort to the pyrenocyte membrane while glycophorin A/TER119 segregates to the reticulocyte membrane [53, 54]. Inhibition of vesicle trafficking by MiTMAB (dynamin inhibitor) prevented sorting of the transferrin receptor (CD71) towards pyrenocyte side of membrane of enucleating erythroblasts [34]. Thus, sorting of proteins and enucleation appear to be coupled in that both require vesicle trafficking. Primitive erythroblasts also differentially sort proteins, such as TER119 (reticulocyte) and α4-integrin (pyrenocyte) in a manner similar to definitive erythroblasts [55]. This difference in composition of the membranes of pyrenocyte versus reticulocyte likely assists pyrenocytes to attach to macrophages while allowing reticulocytes to move into the circulation.

Further, nuclear positioning, an essential component of enucleation (Figure 1), can be speculated to depend on protein sorting and vesicle trafficking. Interestingly, the observations by Skutelsky and Danon [31] and Ji et al. [56] about the nuclear positioning supports this notion. The former group, using fixed sections, noticed that some erythroblasts were having protrusion of the plasma membrane along with a portion of nuclei. They saw a variety of sizes of this protrusion, including in some cells the nucleus completely occupied inside the cavity. Using these pieces of fixed section observations, they constructed a model in which the nucleus at first occupies an eccentric position adjacent to the cell membrane and a cytoplasmic protrusion ensues taking along the nuclei until the protrusion completely holds the nuclei. Ji et al. observed a similar process in murine fetal liver erythroblasts using live cell imaging. These findings suggest that the visible constriction zone on the surface of enucleating erythroblast is indeed a junction region in the plasma membrane that separates pyrenocyte and reticulocyte membrane. The membrane that is destined to enclose pyrenocyte that is in close proximity to nucleus lacks actin cytoskeleton, spectrin, and other critical proteins and as a result can be visualized to balloon out without resisting the pressure exerted by the cytoskeletal activity (Figure 2).

Figure 2: Model of enucleation. A schematic representation of the enucleation process in mammals is shown. Yellow arrows denote the direction of force applied over the nucleus by actin cytoskeleton. Blue arrows and vesicles denote the protein trafficking, which directs the proteins that are destined to reach the pyrenocyte. The blue membrane adjacent to the nucleus is the part of the pyrenocyte membrane lacking spectrin, glycophorin A, actin cytoskeleton, and other reticulocyte-specific proteins that are differentially sorted. The green arrow represents the direction of the force exerted on the pyrenocyte by a bound macrophage while the red arrow indicates the movement of the reticulocyte away from the center by lamellipodia and filopodia.

3.3. Macrophages and Enucleation. Pyrenocytes gradually start expressing phosphatidyl serine on their surface, providing an "eat me" signal for macrophages, which engulf them [23]. The engulfed nucleus is then digested in lysosome where DNase II digests the DNA within the engulfed nuclei [57]. The importance of this pathway for continued erythropoiesis is highlighted by the phenotype of DNase II knockout mice, which die in utero due to embryonic lethal anemia. When DNase II null fetal liver progenitors are transplanted into lethally irradiated recipients, the progenitors give rise to normal erythrocytes, showing that the defect is noncell autonomous, attributable to macrophages. The important role of macrophages in enucleation is also supported by the phenotype of retinoblastoma (Rb) tumor suppressor gene knockout mice. Mutant embryos exhibited a defect in enucleation, which was attributed to a lack of suppression of Id1 (a helix-loop-helix protein) by Rb in macrophages [58]. In vivo, definitive erythropoiesis takes place within erythroblastic islands. Macrophages reside at the center of island, with erythroblasts at various differentiation stages layered around the outside [59–61]. Hanspal and Hanspal found that the interaction between erythroblasts and macrophages is needed for normal proliferation of erythroblasts as well as for enucleation [62]. This interaction is mediated by EMP (erythroblast macrophage protein), which functions to prevent apoptosis of developing erythroblasts [63]. While these studies point to an essential role for macrophages during erythropoiesis, many groups have shown that erythroblasts cultured in vitro in the absence of macrophages undergo complete differentiation including nuclear extrusion [16, 64]. Hence we can conclude that enucleation formally can occur without macrophages.

However, in vivo macrophages appear to play important functions in enucleation and erythroid homeostasis.

3.4. Autophagy and Enucleation. Another important phenomenon that takes place during enucleation is autophagy, a process by which cellular components such as organelles and protein aggregates are catabolized [65–67]. Autophagy proceeds in multiple steps. First, a double membrane develops around the cytoplasmic cargo to be degraded by autophagy. This double membrane can be derived from either the endoplasmic reticulum [68, 69] or the plasma membrane [70, 71]. Fusion of these membranes to one other sequesters the cargo to form an autophagosome, which in turn fuses with multivesicular body/late endosome/lysosome, leading to degradation of the cargo and the inner bilayer of the double-membrane. This vacuole that contains digested cytosolic contents is called autophagolysosome [72–74].

In erythroblasts, mitochondrial clearance has been shown to be accomplished through autophagy [75]. Multiple studies have shown that Nix (Bnip3L), a Bcl-2 family member, is required for mitochondrial clearance in reticulocytes and that loss of Nix leads to anemia [76, 77]. The lack of clearance has been proposed to be the result of defective entry of mitochondria into autophagosomes [77–79]. Although loss of Nix did not affect enucleation [77, 80], it is interesting to consider the dependence of autophagy on vesicle trafficking. The inhibition of vesicle trafficking blocked formation of autophagolysosomes and resulted in enucleation defects (G.K, A.W, and JDC, unpublished data). Further studies on the relationship between autophagy, vesicle trafficking, and enucleation may shed additional light on erythrocyte maturation.

3.5. Possible Roles of Actin in Enucleation. Actin has multiple roles in a cell, including cell division, migration, junction formation, chromatin remodeling, transcriptional regulation, vesicle trafficking, and cell shape regulation [81]. In an enucleating cell, actin could be involved in maintaining the shape of the cell, and/or in maintenance of polarity of the nucleus. Actin may also assist in the formation and movement of endocytic vesicles [82, 83]. Indeed, actin has been proposed to mediate the short-range movement of vesicles and may work together with myosins V and VI and members of the kinesin family [84], to direct vesicles during enucleation. Actin is regulated by Rac proteins that play a role in the formation of lamellipodia, filopodia, membrane ruffles, and cell movement [85]. Taken together, actin likely participates in erythroid maturation by polarization of nucleus, by promoting the accumulation and coalescence of vesicles/vacuoles, and by inducing migration of the reticulocyte away from the pyrenocyte.

4. Model of Enucleation

Erythroblast enucleation is a unique process that incorporates multiple aspects of cytokinesis and vesicle trafficking (Figure 2). First, one or more cellular signals initiate the process of enucleation. At least one study suggested that p38 mitogen-activated protein (MAP) kinase (MAPK) signaling is involved in late erythroid differentiation and enucleation [86]. However, if extracellular factors either secreted by the bone marrow stroma or by erythroblasts themselves play a role in engaging yet unidentified receptors to initiate an intracellular signaling cascade/s leading to the activation of signaling molecules such as the p38MAP kinase and/or the Rac-1 GTPase that may begin the enucleation process is yet to be determined. Once the process is initiated, the actin cytoskeleton polarizes the condensed nucleus, free from intermediate filament attachments, to one side of the cell. Note that this polarization need not be random, and there may well exist novel factors that determine the polarity. At this time, the region of plasma membrane in close proximity to the nucleus yields to form a small extrusion that includes a portion of the nucleus. We speculate that vesicle trafficking and other protein-sorting pathways provide additional membrane to the pyrenocyte region, allowing expansion and further extrusion of the nucleus. The subsequent formation and coalescence of U-shaped channels and vesicles that have accumulated in the region between the nucleus and incipient reticulocyte allows for separation of the reticulocyte from the pyrenocyte. In vivo, this process is likely complemented by attachment of the pyrenocyte to a nearby macrophage coupled with actin-mediated movement of the reticulocyte away from the pyrenocyte. Thus, multiple pathways, including chromatin condensation, actomyosin motors, and vesicle trafficking work in concert to ensure orchestrated terminal maturation of red cells.

5. Future Directions

Although there have been many advances in the past decade, several aspects of enucleation remain unclear. First, what are the signaling pathways that trigger enucleation in vivo and in vitro? Second, what, if any, factors determine polarity in enucleating cells? Third, what are the contributions of macrophages and how can these cells be harnessed to improve ex vivo enucleation? Finally, which motor proteins are responsible for coordinating the movement of the nucleus and cytoplasmic vesicles? It is likely that these questions will be answered in the next decade of research.

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Research Article

Phenotypic Definition of the Progenitor Cells with Erythroid Differentiation Potential Present in Human Adult Blood

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In Human Erythroid Massive Amplification (HEMA) cultures, AB mononuclear cells (MNC) generate 1-log more erythroid cells (EBs) than the corresponding $CD34^{pos}$ cells, suggesting that MNC may also contain $CD34^{neg}$ HPC. To clarify the phenotype of AB HPC which generate EBs in these cultures, flow cytometric profiling for CD34/CD36 expression, followed by isolation and functional characterization (colony-forming-ability in semisolid-media and fold-increase in HEMA) were performed. Four populations with erythroid differentiation potential were identified: $CD34^{pos}CD36^{neg}$ (0.1%); $CD34^{pos}CD36^{pos}$ (barely detectable-0.1%); $CD34^{neg}CD36^{low}$ (2%) and $CD34^{neg}CD36^{neg}$ (75%). In semisolid-media, $CD34^{pos}CD36^{neg}$ cells generated BFU-E and CFU-GM (in a 1 : 1 ratio), $CD34^{neg}CD36^{neg}$ cells mostly BFU-E (87%) and $CD34^{pos}CD36^{pos}$ and $CD34^{neg}CD36^{low}$ cells were not tested due to low numbers. Under HEMA conditions, $CD34^{pos}CD36^{neg}$, $CD34^{pos}CD36^{pos}$, $CD34^{neg}CD36^{low}$ and $CD34^{neg}CD36^{neg}$ cells generated EBs with fold-increases of ≈9,000, 100, 60 and 1, respectively, and maturation times (day with >10% $CD36^{high}CD235a^{high}$ cells) of 10–7 days. Pyrenocytes were generated only by $CD34^{neg}/CD36^{neg}$ cells by day 15. These results confirm that the majority of HPC in AB express CD34 but identify additional $CD34^{neg}$ populations with erythroid differentiation potential which, based on differences in fold-increase and maturation times, may represent a hierarchy of HPC present in AB.

1. Introduction

Hematopoiesis is defined as the orderly sequence of events that replenishes the cellular elements of the blood on a daily basis [1]. Under steady-state conditions, the bone marrow provides the microenvironmental cues that allow hematopoietic stem cells to generate a hierarchy of cells (the hematopoietic progenitor cells, HPCs) progressively more restricted in their proliferation and lineage maturation potential [2]. In addition, bone marrow contains very rare precursor cells with the potential to generate hematopoietic stem cells [3]. Human stem cell precursors and stem cells are functionally defined by surrogate assays in animal models [4], while HPCs with different proliferation/maturation potential are defined by semisolid cultures that model the hematopoietic process in vitro [5]. These functional in vitro assays provided the basis for the identification and prospective isolation of a hierarchy of different hematogenic populations present in bone marrow [6]. Based on number and lineage of the cells generated and of the time required for their generation, semisolid assays identify a series of HPCs: HPCs able to generate large colonies (>30,000 cells) comprising cells of multiple lineages (the colony-forming unit, granulocytic-erythroid-megakaryocitic-monocytic, CFU-GEMM) by day 15–18, those which generate erythroid bursts (approximately 5,000 cells, burst-forming unit erythroid BFU-E) and granulomonocytic colonies (colony forming unit, granulomonocytic, CFU-GM) by day 12–15, and finally those which generate clusters (50–200 cells) composed only by erythroid (colony-forming unit, erythroid, CFU-E), granulocytic (CFU-G) or monocytic (CFU-M) cells by day 8 [5].

CD34 is an antigen expressed by HPCs of all types whose expression is lost at the CFU-E level [5, 6]. CFU-GEMM express also CD38 but do not express the α subunit of

the interleukin-3 (IL-3) receptor, which is acquired during the transition of these cells to BFU-E, CFU-GM, and CD45RA [7, 8], which is specifically expressed by BFU-E [5, 6]. CD36 is an antibody that recognizes thrombospondin, the receptor for the malarial parasite whose expression is activated within a few hours of exposure to erythropoietin (EPO) [9]. Although it is conceivable that CD36 is expressed by erythroid cells of all types, how its expression is modulated during the transition from CFU-GEMM to CFU-E is not known. HPCs may egress from the bone marrow into the circulation [2]. However, since maturation alters the adhesion receptor profile of the cells and their affinity for the marrow niches, HPCs are released from the marrow with different efficiencies and their frequency in blood may not correspond to that of the marrow [10]. The majority of erythroid HPCs in the marrow are CFU-E, but the majority (>90%) of those in blood are BFU-E [11].

The HPCs present in adult peripheral blood (AB) are discarded during the leukoreduction process used to prepare red blood cells for transfusion. Discarded AB HPCs are used in several liquid culture systems to generate great numbers of lineage-restricted precursors to study hematopoiesis [12, 13]. More recently, it has been realized that AB HPCs discarded in the buffy coat from a single donation cultured in the presence of dexamethasone (DXM) and estradiol (ES), and in addition to stem cell factor (SCF), IL-3 and EPO (human erythroid massive amplification, HEMA, culture) [14] may generate erythroblasts (EBs) in numbers sufficient for 3–50 transfusions [15], paving the way for an important area of translational medicine: production of alternative transfusion products ex vivo. Although both AB mononuclear (MNC) and $CD34^{pos}$ cells generate great numbers of EBs in HEMA culture, the total number of erythroid cells generated by $CD34^{pos}$ cells is on average 1-log lower than that generated by MNC [13]. This observation has been ascribed to loss of HPCs with erythroid differentiation potential (erythroid precursor cells, EPC) during the CD34 selection procedure and/or to the existence of circulating $CD34^{neg}$ EPC. The second hypothesis is supported by a recent report indicating that AB $CD34^{neg}$ cells may differentiate into EBs under HEMA conditions generating more EBs than the corresponding $CD34^{pos}$ cells [16]. The phenotype of the $CD34^{neg}$ cells with erythroid potential present in AB buffy coats is not known.

The aim of our study was to further clarify the phenotype of the HPCs/EPC present in AB MNC and to evaluate their contribution to the generation of EBs under HEMA conditions. Flow cytometric profiling for CD34 and CD36 expression of AB MNC followed by functional characterization (colony-forming ability in semisolid media and fold increase in HEMA) of the prospectively isolated populations was perfomed. The results presented indicate that CD34/CD36 profiling identifies a hierarchy of EPC in AB.

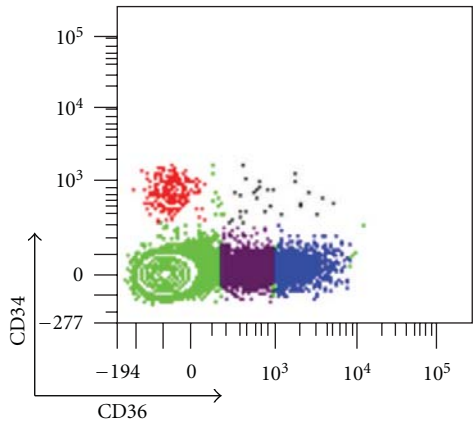
2. Materials and Methods

2.1. Human Subjects. Peripheral blood was collected from 10 normal adult donors at the transfusion center of "La Sapienza" University (Rome, Italy) according to guidelines established by institutional ethical committees.

2.2. Cell Separation. Mononuclear cells (MNCs) were separated by centrifugation over Ficoll-Hypaque (Amersham Pharmacia Biotec, Uppsala, Sweden). MNC were first antigenically profiled for CD34/CD36 expression by standard flow cytometric techniques and MNC populations with different CD34/CD36 profiles subsequently separated by a combination of magnetic bead separation and sorting as described in Figure 1. For flow cytometrical profiling, MNC were suspended in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline, supplemented with 1% BSA, 2 mmol/L ethylenediamine tetraacetate (EDTA), and 0.01% NaN3, stained with either allophycocyanin- (APC-) conjugated CD36, phycoerythrin- (PE-) conjugated CD14 (monocyte differentiation antigen 14 antibody), or fluorescein isothiocyanate- (FITC-) conjugated CD42a (which recognize GPIb) [17], or appropriate isotype controls (all from Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) and analyzed with the FACS Aria (Becton Dickinson Biosciences) equipped with three air-cooled and solid-state lasers (488-nm, 633-nm, and 407-nm). Dead cells were excluded by SYTOX Blue (0.002 mM, Molecular Probes, Carlsband, Calif, USA) staining. MNC were then divided into $CD34^{pos}$ and $CD34^{neg}$ populations using Magnetic MultiSort Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The $CD34^{pos}$ fraction was further divided into $CD36^{neg}$ and $CD36^{pos}$ by sorting with the FACS Aria. The $CD34^{neg}$ fraction was enriched for $CD36^{pos}$ and $CD36^{neg}$ cells with Magnetic MultiSort Microbeads coated with CD36. All the bead-based cell enrichments were performed as described by the manufacturer. $CD36^{pos}$ cells were further divided into $CD36^{low}$ and $CD36^{high}$ by sorting. Whenever the cell number allowed, the purified populations were reanalyzed for purity and found >90% pure. Results were analyzed by BD FACSDiva Software version 5.0.3.

2.3. Colony-Forming Assay. The colony forming ability of unfractionated and sorted cells was evaluated in standard semisolid methylcellulose cultures (40%, Fluka Biochemika) stimulated with human SCF (10 ng/mL), IL-3 (10 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF, 10 ng/mL), granulocyte colony-stimulating factor (G-CSF, 100 ng/mL) and EPO (5 U/mL) [18]. The cultures were incubated at 37°C in a fully humidified 5% pCO_2 atmosphere and scored after 14 days for the growth of hematopoietic colonies. CFU-GEMM-, BFU-E-, and CFU-GM-derived colonies were recognized according to standard morphological criteria [18, 19].

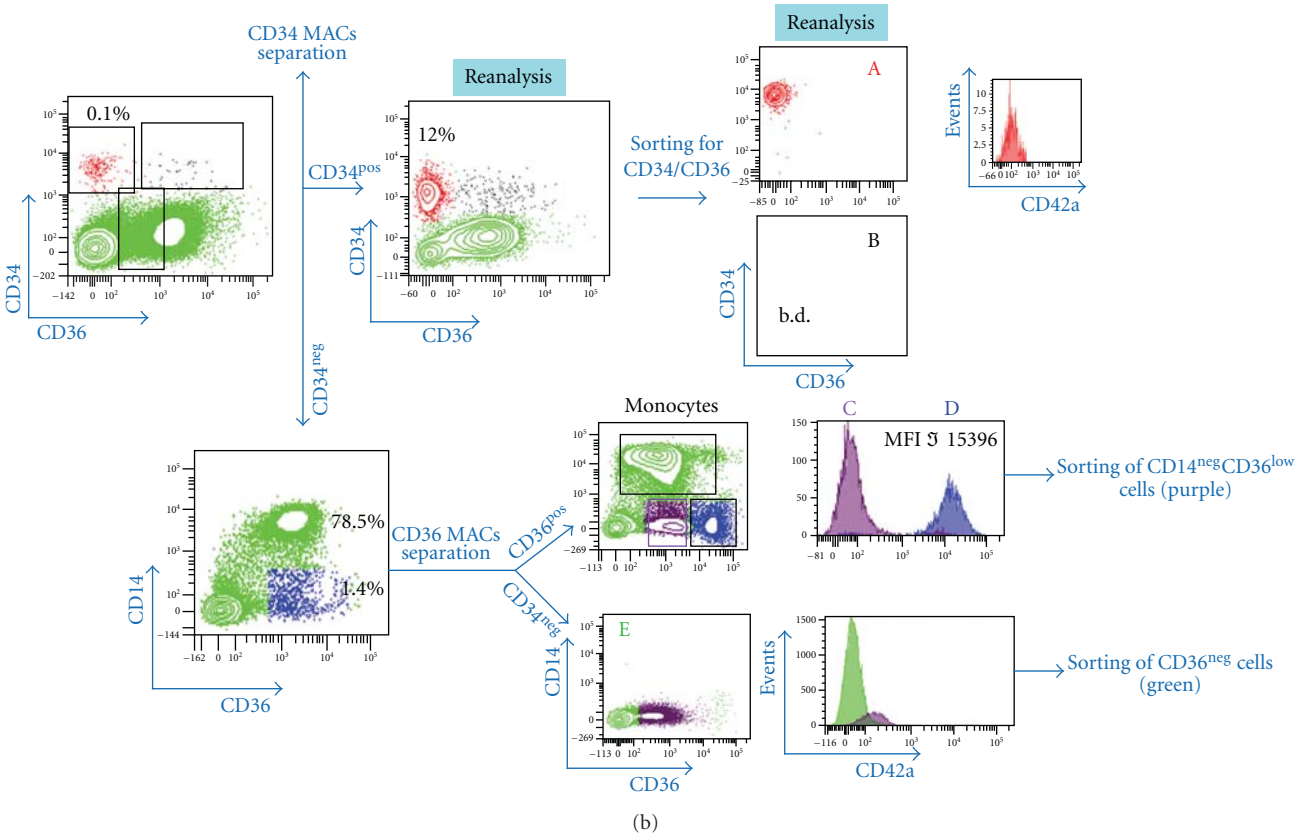
2.4. Ex Vivo Expansion of Human EBs under HEMA Conditions. MNC (10^6 cells/mL) and prospectively isolated cells (5×10^4 cells/mL) were cultured under HEMA conditions, as described [14]. Briefly, the cultures contained Iscove's modified Dulbecco's medium (IMDM, Lonza Group Ltd, Basel, Switzerland) supplemented with fetal bovine serum (FBS, Sigma-Aldrich) (20% v/v), detoxified human serum albumin (HSA) (25%, Baxter International Inc, Deerfield,



Summary of CD34/CD36 profiling of AB MNC

	Phenotype	Frequency
A	$CD34^{pos}CD36^{neg}$	0.1 ± 0.05%
B	$CD34^{pos}CD36^{pos}$	Barely detectable-0.1%
C	$CD34^{neg}CD36^{low}$	2 ± 0.8%
D	$CD34^{neg}CD36^{high}$	1 ± 0.6%
E	$CD34^{neg}CD36^{neg}$	74.3 ± 21.8%

(a)



(b)

Figure 1: CD36/CD34 expression profiling of AB MNC. (a) Representative coulter plot analyses for CD36/CD34 expression of MNC from a representative AB and summary of the frequency of the different populations identified by this analyses. CD36/CD34 profiling identified five populations: $CD34^{pos}CD36^{neg}$ cells (population A, red), $CD34^{pos}CD36^{pos}$ cells (population B, black), and $CD34^{neg}CD36^{neg}$ cells (population E, green). A fourth $CD34^{neg}CD36^{pos}$ population contained numerous $CD14^{pos}$ cells which are represented by monocytes (see Figure 1(b)). Exclusion of these $CD14^{pos}$ cells from the analyses revealed two $CD34^{neg}/CD36^{pos}$ populations which express CD36 al low ($CD34^{neg}CD36^{pos}$, population C, purple) and high ($CD34^{neg}CD36^{high}$ cells, population D, blue) levels, respectively. The table on the right summarizes the mean frequency (±SD) of each population among MNC obtained from 3 different donors. All the results presented in this figure and in Figure 2(a) are presented with the same color code. (b) Prospective isolation of AB MNC on the basis of CD34/CD36 expression. MNC were first divided in two populations enriched or deprived of $CD34^{pos}$ cells by CD34-coated magnetic bead adsorption. The $CD34^{pos}$ population was further purified and divided into $CD36^{neg}$ and $CD36^{pos}$ cells by sorting. The CD34 beads flow-through fraction (enriched for $CD34^{neg}$ cells) was further divided into $CD36^{pos}$ and $CD36^{neg}$ cells by magnetic bead isolation. The cells eluted from the beads were purified by sorting on the basis of lack of expression of CD14 and low level of CD36 expression (population C, purple). The $CD14^{neg}CD36^{high}$ cells (population D, blue) were not isolated because expressed high levels of the megakaryocytic marker CD42a. Finally, the CD36 beads flow-through fraction was enriched for $CD36^{neg}$ cells by sorting. These $CD36^{neg}$ cells were also $CD34^{neg}$ upon reanalyses (not shown). Whenever feasible, the prospectively isolated cells were reanalyzed for purity. Results are representative of those obtained in 3 independent purifications.

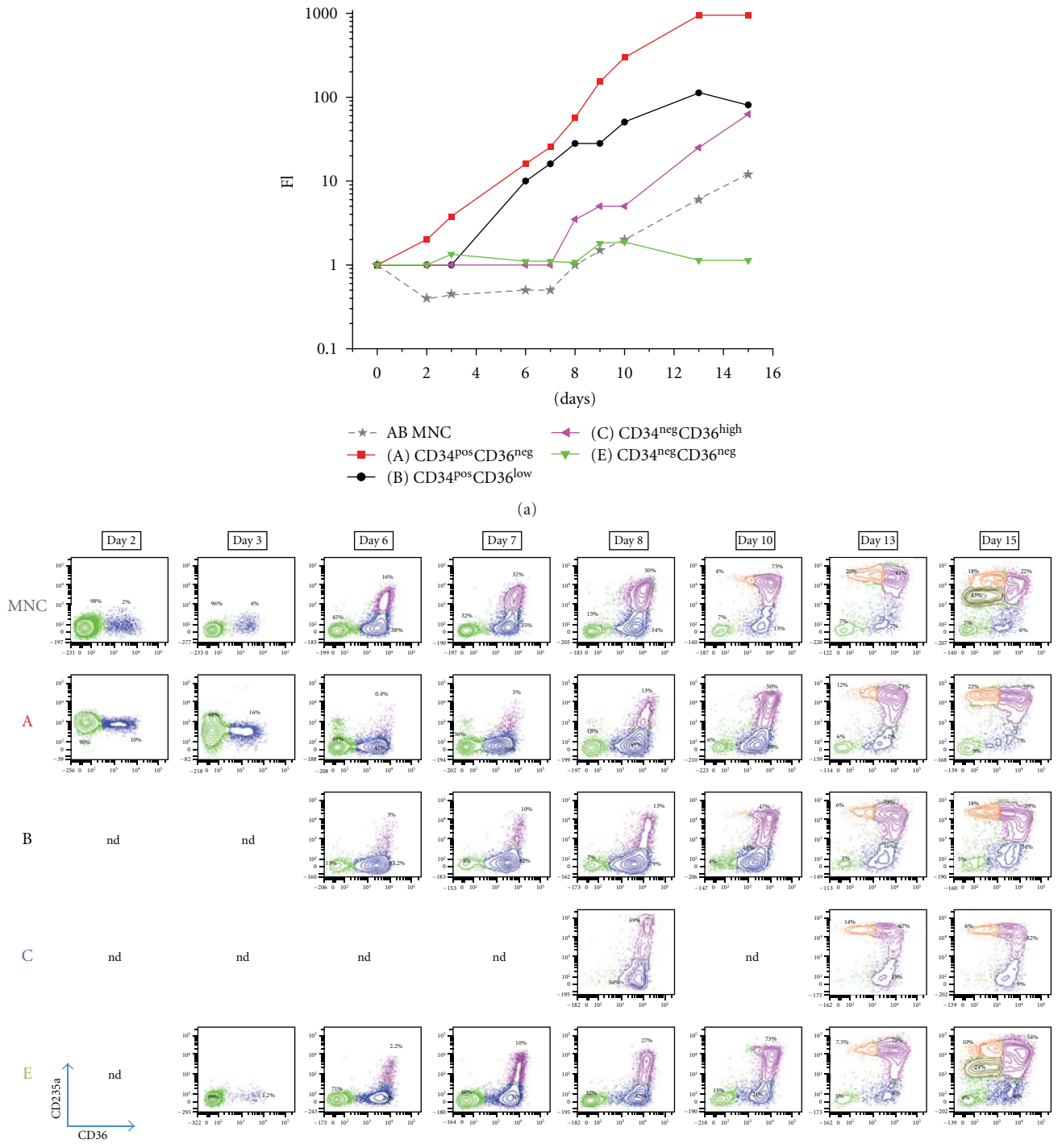


Figure 2: Growth and erythroid maturation of MNC prospectively isolated on the basis of CD34/CD36 profiling under HEMA conditions. (a) Growth curve of cells prospectively isolated from AB MNC under HEMA conditions (the same color code as in Figure 1). MNC (grey dotted line) were cultured in parallel as control. The number of cells present in the cultures is expressed as fold increase (FI). Results from a representative experiment are shown. Similar results were observed in 2 additional experiments (see also Table 1). (b) Time course of the maturation of EBs in HEMA cultures seeded with either AB MNC or with populations A, B, C, or E, as indicated. EBs maturation was defined on the basis of CD36/CD235a profiling which divides EBs into three populations: $CD36^{pos}CD235a^{neg/low}$ (proerythroblasts, blue); $CD36^{pos}/CD235a^{med/high}$ (basophilic erythroblasts, purple), and $CD36^{low}CD235a^{high}$ (orthochromatic erythroblasts, red). Forward and side scatter analyzes identified a fourth population of small $CD36^{low}CD235a^{low}$ cells, probably represented by pyrenocytes (yellow). Cells which do not express EB markers are indicated in green. The numbers within each quadrant indicate the frequency of the different subpopulations. Results are representative of those obtained in three independent experiments. nd = not done, due to low cell numbers.

Table 1: Summary of the number of cells recovered in each fraction after CD36/CD34-based purification and of their growth in HEMA culture. Results with MNC and population A are presented as mean (±SD) of those obtained in three separate experiments. Results with population E are representative of two independent experiments, while a complete data set for populations B and C is available only from one experiment.

	Cell population	Cells obtained from1 buffy coat	Recovery (%)*	FI in HEMA (Day 14)	Total theoretical number of EBs generated in HEMAat day 14**
	MNC	$272\ (\pm 86) \times 10^6$	100%	10.1 ± 1.4	2.7×10^9
A	$CD34^{pos}CD36^{neg}$	$75\ (\pm 53.5) \times 10^3$	27.5%	$9,347 \pm 950$	7.0×10^8
B	$CD34^{pos}CD36^{pos}$	9,500	3.5%	113	1.1×10^6
C	$CD34^{neg}CD36^{low}$	7,500	1.4×10^{-3}%	62.5	5.0×10^5
E	$CD34^{neg}CD36^{neg}$	$2–10 \times 10^6$	1.0–4.9%	1.14–3.5	$0.2–3.5 \times 10^7$

* Recovery was calculated by dividing the total number of cells obtained after the purification per the theoretical number of cells present in MNC. The theoretical cell number of each fraction was calculated by multiplying the frequency of the population, presented in Figure 1(a), per the average number of MNC obtained from an AB buffy coat (272×10^6).

** The theoretical total number of EBs obtainable at day 14 from each fraction was calculated by multiplying the total cell number of the fraction per the corresponding FI.

iLL, USA) [15], human SCF (50 ng/mL, Sigma-Aldrich), EPO (3 U/mL, Neorecormon, Auckland, New Zealand) and IL-3 (10 ng/mL, Biosource, San Jose, Calif, USA), DXM (10^{-6} M) and ES (10^{-6} M) (both from Sigma-Aldrich), L-glutamine (200 mM, Euroclone SpA, Siziano, Italy), antibiotics [penicillin (10,000 units/mL), streptomycin sulfate (10,000 μg/mL), fungizone (25 μg/mL), Lonza Group Ltd], and β-mercaptoethanol (10^{-6} M). The cultures were kept for up to 10–15 days at 37°C and 5% pCO_2 in a fully humidified incubator.

2.5. Cell Viability, Phenotypic Analysis and Sorting. Cell numbers and viability were assessed by microscopic evaluation after trypan blue (Boston Bioproducts, Ashland, Mass, USA) staining. For flow cytometrical characterization, cells were suspended in $Ca^{2+/}Mg^{2+}$-free phosphate-buffered saline, supplemented with 1% BSA, 2 mmol/L ethylenediamine tetraacetate (EDTA), and 0.01% NaN3, stained with either allophycocyanin- (APC-) conjugated CD36 or phycoerythrin- (PE-) conjugated CD235a (antiglycophorin A), or appropriate isotype controls (all from Becton Dickinson Biosciences) and analyzed with the FACS Aria. Dead cells were excluded by SYTOX Blue (0.002 mM, Molecular Probes) staining. Forward and side scatter analyses of cells expressing the mature $CD36^{low}CD235a^{high}$ phenotype and of small size were used for the identification of pyrenocytes [20].

2.6. Statistical Analysis. Results are presented as mean (±SD) of those obtained in at least three experiments per data set. Mean (±SD) were calculated with the computer software Origin 5.0 for Windows (Microcal Software, Inc., Northampton, Mass, USA).

3. Results

3.1. Antigenic Profiling of AB MNC. CD34/CD36 profiling divided AB MNC into 4 populations: $CD34^{pos}CD36^{neg}$ (population A, 0.1 ± 0.05%), $CD34^{pos}CD36^{pos}$ (population B, often present in barely detectable numbers but reaching in some donors a frequency of ~0.1%), $CD34^{neg}CD36^{pos}$ (~23%) and $CD34^{neg}CD36^{neg}$ (population E, ~74%) (Figure 1(a)). $CD34^{neg}CD36^{pos}$ cells could in turn be divided into three populations: the majority of them expressed CD14 and was, therefore, represented by monocytes (monocytes are known to express CD36) [21] (Figure 1(b)). By dot blot distribution and CD42a staining, the remaining could be divided into two additional populations: $CD34^{neg}CD36^{low}$ (population C, ~2%), which does not express CD42a, and $CD34^{neg}CD36^{high}$ (population D, ~1.0), which express high levels of CD42a (mean fluorescence intensity, MFI > 15,000) (Figure 1(b)).

3.2. Prospective Isolation of MNC Populations Based on CD34 and CD36 Expression. AB MNCs were purified on the basis of CD34 and CD36 expression by the combination of magnetic bead enrichment and cell sorting described in Figure 1(b). First, $CD34^{pos}$ cells were enriched by selection with CD34-coated microbeads. The $CD34^{pos}$ fraction (12% pure by reanalyses) was then sorted into $CD34^{pos}$ cells expressing ($CD36^{pos}$, A population) or not ($CD36^{neg}$, B population) CD36. Approximately 75,000 A cells and 10,000 B cells were recovered from the buffy coat of an average donation (Table 1). Population A was >98% pure by reanalyses while the purity of population B was not determined due to low cell recovery.

Reanalyses for CD36 and CD14 expression of the flow-through fraction of the CD34-coated magnetic beads revealed that a great number (~78%) of $CD36^{pos}$ cells expressed also CD14. This flow-through fraction was further divided into CD36-enriched and CD36-deprived fractions by CD36-magnetic bead isolation. The cells adsorbed to the beads which did not express CD14 and CD42a and expressed CD36 at low levels were sorted ($CD34^{pos}CD36^{low}$, population C) (Figure 1(b)). Approximately 7,500 C cells were recovered from the buffy coat of a blood donation (Table 1). This low number prevented reanalyses for purity of this cell population and limited its functional characterization. The $CD14^{neg}CD34^{neg}$ cells which expressed CD36 at high levels

($CD34^{neg}CD36^{high}$, population D) was not sorted because of its high CD42a expression, which suggest that the may have been represented by megakaryocytic precursors [17].

The flow-through fraction of the CD36 magnetic beads was further purified by sorting (population E). A total of 10 million $CD34^{neg}CD36^{neg}$ cells were recovered from an average AB buffy coat (Table 1).

3.3. Cloning Efficiency of AB Populations Prospectively Isolated on the Basis of CD34/CD36 Profiling. The progenitor cell activity in semisolid assays of population A and E is presented in Table 2. AB MNC were analyzed in parallel as control. As expected, population A was greatly enriched for colony forming cells (cloning efficiency 16%) and generated both BFU-E- and CFU-GM-derived colonies (in a 1 : 1 ratio). It also contained few (0.001%) CFU-GEMM. By contrast, population E had a cloning efficiency 40% lower than that of MNC and generated mainly (80%) erythroid bursts. No difference in size and morphology was observed among erythroid bursts originated from population A and E and MNC (insert in Table 1), an indication that the BFU-E present in the different fractions had similar proliferation/maturation potential.

3.4. Expansion Potential under HEMA Conditions of AB Populations Prospectively Isolated on the Basis of CD34/CD36 Profiling. The expansion potential under HEMA conditions of AB populations prospectively isolated on the basis of CD34/CD36 profiling is compared in Figure 2(a) and Table 1. AB MNC were analyzed in parallel as control. As expected, under HEMA conditions, population A had great proliferation potential expressing FIs between 900 (Figure 2(a)) and 24,000 (average FI = 9,000, Table 1) compared to FI < 10 of the corresponding MNC. Significant numbers of cells were also generated by population B and C which expressed FI of 100 and 60 by day 13 (Figure 2(a) and Table 1). By contrast, population E had FI as low as 1–3. However, given the great numbers of cells segregating in this fraction ($>10^7$), population E generated many cells ($\sim 10^7$) under HEMA conditions (FI ~ 1).

3.5. Maturation Potential of AB MNC Populations Prospectively Isolated on the Basis of CD34/CD36 Profiling. The lineage and maturation stage of the progeny of AB MNC and of AB populations prospectively isolated on the basis of CD36/CD34 profiling is presented in Figure 2(b). EBs maturation was defined on the basis of CD36/CD235a profiling which divides EBs into three populations: $CD36^{pos}CD235a^{neg/low}$ (pro-erythroblasts); $CD36^{pos}/CD235a^{med/high}$ (basophilic erythroblasts), $CD36^{low}CD235a^{high}$ (orthochromatic erythroblasts) [5]. A fourth population of $CD36^{low}CD235a^{low}$ cells with low forward and side scatter is composed by pyrenocytes [20].

In cultures of MNC, cells with an immature EBs phenotype ($CD36^{pos}CD235a^{neg}$) became detectable very quickly (2% by day 2) while non-EBs became detectable in modest numbers (6-7%) by day 10. Mature EBs ($CD36^{pos}CD235a^{pos}$) were detected by day 6 (15%) and reached a frequency >70% by day 10. By day 15, immature EBs became barely detectable and numerous cells with $CD36^{low}CD235a^{high}$ phenotype (both larger cells corresponding to orthochromatic EBs, 18%, and smaller cells corresponding to pyrenocytes, 40%) were detected (Figure 2(b)).

In HEMA cultures of population A, immature EBs were also detected very early (10% by day 2) but the frequency of mature EBs reached 10% only by day 8. By day 15, the cultures contained significant numbers (22%) of $CD36^{low}/CD235a^{high}$ orthochromatic EBs but no pyrenocytes (Figure 2(b)).

In HEMA culture of population B, numbers of cells sufficient for antigenic profiling were obtained by day 6. $CD36^{pos}CD235a^{neg}$ cells represented the majority (~83%) of the cells from day 6 to day 8. In these cultures, mature $CD36^{pos}CD235a^{pos}$ EBs were observed at earlier time points with respect to cultures of population A (3% and 10% of $CD36^{pos}CD235a^{pos}$ cells by day 6-7 versus day 7-8 in cultures of population B and A, resp.) (Figure 2(b)). By day 15, the maturation phenotype of the progeny of population B and A was the same.

HEMA cultures of population C were originally seeded with number of cells comparable to those used for population A and B (~7,500 cells with respect to 9,500–10,000 cells used for the two other populations). However, cultures of population C grew very slow (see Figure 2(a)) and the number of cells reached values sufficient for antigenic profiling only by day 8. At day 8, great numbers (39%) of the EBs had already the mature $CD36^{pos}/CD235a^{high}$ phenotype. However, the progeny of population C progressed poorly to the orthochromatic stage and only 6% of them had acquired the $CD36^{low}CD235a^{high}$ phenotype by day 15.

Finally, population E did not generate significant numbers (26%) of $CD36^{pos}CD235a^{neg}$ cells until day 6. The cells progressed then very rapidly to the $CD36^{pos}CD235a^{high}$ stage (10%$CD36^{pos}CD235a^{high}$ cells by day 7) and $CD36^{low}CD235a^{high}$ stage (7.5% by day 13). Pyrenocytes were detectable in these cultures at levels similar to those observed in cultures of MNC (24%) by day 15.

In conclusion, in spite of differences in kinetics, all the populations analyzed in this study generated EBs under HEMA conditions.

4. Discussion

CD36/CD34 profiling identifies at least four populations present in AB MNC capable to generate colonies in semisolid assay and EBs under HEMA conditions.

In semisolid assay, only 9% of the original HPCs activity was recovered among the purified fractions (8.1% in population A and 0.8% in population B). Although the cloning efficiency of population B and C is not known, given the low cell content of these populations (~15,000 cells in total, Table 1), they may contain at most 5% of the MNC HPCs activity. Therefore, >80% of the HPCs activity present in the MNC was lost during the purification procedure. This result suggests the hypothesis that some of the HPCs activity of the MNC is due to pre-HPCs cells which became HPCs in

Table 2: Cloning efficiency of AB MNC and AB cell populations prospectively isolated on the basis of CD34/CD36 expression. Results are presented as mean (±SD) of those observed in three independent experiments. The inserts present the morphology of a representative BFU-E-derived colony obtained in the corresponding semisolid culture (original magnification 10x).

	Cell population		CFC/plate			Total CFC per fraction*	Recovery
		BFU-E		CFU-GM	CFU-GEMM		
	MNC (10^5 cells/plate)		71 ± 24	41 ± 9	1 ± 1	3.1×10^5	100%
A	$CD34^{pos}CD36^{neg}$ (500 cells/plate)		92 ± 5	75 ± 5	0.5 ± 0.5	2.5×10^4	8.1%
E	$CD34^{neg}CD36^{neg}$ (10^5 cells/plate)		34 ± 12	8 ± 3	2 ± 1	2.4×10^3	0.8%

*The total number of CFC per fraction was calculated by multiplying the frequency of CFC (BFU-E + CFU-GM + CFU-GEMM) per the total number of cells in the fraction presented in Table 1.

semisolid assay in response to factors released by accessory cells.

Consistent with the data reported by van den Akker et al. [16], we determined that under HEMA conditions EBs are generated both by $CD34^{pos}$ and $CD34^{neg}$ AB cells (Table 1). Therefore, both populations contain EPC. CD34CD36 profiling identified that in addition to two $CD34^{pos}$ EPC populations ($CD34^{pos}CD36^{neg}$ and $CD44^{pos}CD36^{pos}$), AB MNC contain 2 $CD34^{neg}$ EPC population ($CD34^{neg}CD36^{low}$ and $CD34^{neg}CD36^{neg}$). The antigenic profile which defines the $CD34^{neg}CD36^{neg}$ population is still to be identified, although preliminary results indicate that these cells may express CD44 [22], the receptor for hyaluronic acid which interacts also with osteopontin and collagen [23] (data not shown).

By contrast with the great loss of colony forming cells observed with the purification of AB MNC (Table 2), the purification procedures did not lead to great losses of EPC, as indicated by the observation that the sum of the numbers of EBs generated by the four purified fractions is only modestly (7.5×10^8 versus 2.7×10^9) lower than that generated by MNC (Table 1). Under HEMA conditions, the population which generated the greatest numbers of EBs was population A, only 27% of which had been recovered during the purification procedures (Table 1). Cultivation under HEMA conditions of a population A containing all the $CD34^{pos}CD36^{neg}$ cells present in one donation (100% recovery) would generate as many as 2.3×10^9 EBs, a number very similar to that observed in cultures of MNC. These data indicate cell loss during the purification procedure, rather than great EBs generation by $CD34^{neg}$ HPCs, as the main reason for the overall greater output of EBs from MNC than from $CD34^{pos}$ cells in HEMA culture.

Based on FI and on the time required to mature in culture, the four EPC populations identified in AB were classified according to the hierarchical model presented in Figure 3. $CD34^{pos}CD36^{neg}$ cells may represent earlier cells, probably HPCs (they contain both BFU-E and CFU-GM), while $CD34^{pos}CD36^{pos}$ and $CD34^{neg}CD36^{pos}$ cells may represents early and late erythroid restricted progenitor cells (EPC), respectively. It is possible that these cell populations are linked in a mother-daughter relationship. It is difficult to classify population E in this model. Since the majority of the cells in this population is likely represented by differentiated precursors, it is conceivable that the progenitor cells represent in this fraction are a rare population with such a great proliferation potential to express FI = 1. This hypothesis is also supported by the observation that population E was the slowest population to generate EBs ($CD36^{pos}CD235a^{pos}$ cells were not detected before day 6). It is suggested that this population may contain precursor cells which are capable to generate $CD34^{pos}$ cells. Further studies involving time course analyses of the expression of CD34 among the progeny of $CD34^{neg}CD36^{neg}$ E cells are required to clarify this important point. Since the growth factors used to stimulate HEMA culture were selected for optimal EB, and not CD34 cell, generation [15], it is possible that preculture of $CD34^{neg}CD36^{neg}$ E cells under conditions which promote CD34 cell proliferation (using growth factor combinations including FLT3 ligand or thrombopoietic) [24, 25], will allow generation of greater numbers of EBs when the progeny of their cells will be in turn cultured under HEMA conditions. Also intriguing is the observation that population E is the only purified populations to generate great numbers of pyrenocytes by day 15, an indication that its progeny underwent significant levels of enucleation in HEMA. The presence of macrophages greatly favors the enucleation process [26]. In HEMA culture, macrophages are present as contaminant in cultures of MNC which routinely generate pyrenocytes by day 15 (Figure 1(b)). These cells were removed by the purification process from all the other populations which did not generate pyrenocytes by day 15. Population E, however, although does not contain macrophages ($CD14^{pos}CD36^{pos}$ cells) may contain their precursors, which may maturate in culture, favoring enucleation of EBs. Further studies are required to clarify the role of contaminating macrophages,

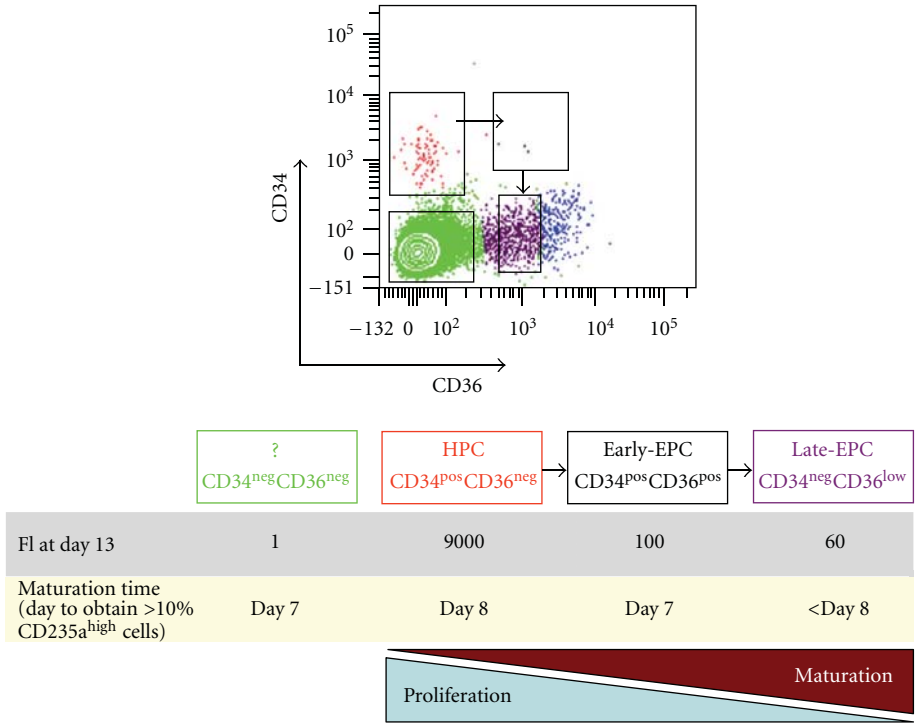


Figure 3: A model for the hierarchical relationship between progenitor cells with erythroid proliferation potential present in AB MNC. This model is based of the proliferation potential (as indicated by the FI) and of the speed of maturation (as indicated by the time required to generate significant numbers, >25%, of mature CD235a^{pos} EBs) of the different populations. See text for further details.

and/or of their precursor cells, in the enucleation of human EBs generated under HEMA conditions.

In conclusion, CD34/CD36 profiling identifies a hierarchy of EPC in AB. Although under HEMA conditions the majority of EBs were generated by CD34^{pos} cells, it is possible that further improvement of the culture system by favoring proliferation of CD34^{neg} cells, may further increase the number of EBs generated by AB.

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Research Article

Human Fetal Liver: An *In Vitro* Model of Erythropoiesis

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We previously described the large-scale production of RBCs from hematopoietic stem cells (HSCs) of diverse sources. Our present efforts are focused to produce RBCs thanks to an unlimited source of stem cells. Human embryonic stem (ES) cells or induced pluripotent stem cell (iPS) are the natural candidates. Even if the proof of RBCs production from these sources has been done, their amplification ability is to date not sufficient for a transfusion application. In this work, our protocol of RBC production was applied to HSC isolated from fetal liver (FL) as an intermediate source between embryonic and adult stem cells. We studied the erythroid potential of FL-derived $CD34^+$ cells. In this *in vitro* model, maturation that is enucleation reaches a lower level compared to adult sources as observed for embryonic or iP, but, interestingly, they (i) displayed a dramatic *in vitro* expansion (100-fold more when compared to CB $CD34^+$) and (ii) 100% cloning efficiency in hematopoietic progenitor assays after 3 days of erythroid induction, as compared to 10–15% cloning efficiency for adult $CD34^+$ cells. This work supports the idea that FL remains a model of study and is not a candidate for *ex vivo* RBCS production for blood transfusion as a direct source of stem cells but could be helpful to understand and enhance proliferation abilities for primitive cells such as ES cells or iPS.

1. Introduction

The cell therapy approach which consists of generating cultured RBC (cRBC) *in vitro* after amplification of stem cells (SC) makes sense in a context of chronic difficulties of obtaining blood supplies. More than blood supplies, hematopoietic differentiation of human stem cells into RBC has important therapeutic implications, including the possibility to produce virus-free units or tailor-designed phenotypes for transfusion purposes. Our team has established an experimental procedure to reproduce *in vitro* terminal erythropoiesis from adult hematopoietic stem cells (HSC) from diverse sources (peripheral blood (PB), bone marrow (BM), and cord blood (CB)) [1, 2]. This protocol, using appropriate cytokines and a specific microenvironment (including stromal murine MS5 or Mesenchymal Stem Cells (MSC)) in a serum-free medium, allows HSC proliferation and terminal differentiation in mature and functional enucleated RBC containing adult haemoglobin [2]. Major advances have allowed towards the *in vitro* production of RBCs from diverse sources in a few years. Nevertheless, their transfusional future will become a reality only if we are able to produce functional transfusable RBCs at a large scale. This will of course require not only the conception of appropriate industrial tools, but also finding the best source of stem cells. To date, the most accessible and proliferative source of HSC in a quantitative aspect is cord blood (CB) [3].

However, this cell source is dependent on donations and *ex vivo* production of RBC is restricted to a system of production in batches, using the finite quantity of HSC available in a CB unit. Such are its limits.

Consequently, our present efforts are focused on establishing conditions to produce RBC using a permanent and inexhaustible source of stem cells. Human embryonic stem cells or recently discovered induced pluripotent stem cells (iPS) [4, 5] are the natural candidates. As other teams, we have shown the possibility to reconstitute *in vitro* erythropoiesis starting from very primitive stem cells [6–13], and we were the first to report the erythroid differentiation and maturation of iPS lines (from fetal and adult fibroblasts) into mature enucleated RBC (4% to 10% compared to 52% to 66% from human ES cell line (H1)), which synthesized functional fetal hemoglobin [9].

The demonstration that primitive cells can be differentiated *in vitro* to cells similar to their natural counterpart was the first step for a potential therapeutic application. But there is still a major problem for a hypothetical application in transfusion: their amplification. Indeed, in our conditions, 1 ES or 1 iPS cell can generate 5000 or 1000 RBC, respectively, [9] whereas 1 $CD34^+$ from CB can give up to 2.5×10^5 RBCs [2]. This crucial difficulty of amplification of ES or iPS has not yet been solved by any team.

Rollini et al. have demonstrated that fetal liver (FL) could be an alternative source of HSC despite a limited total cell number per tissue, due to their high proliferative capacity [14]. Indeed, the site of erythropoiesis migrates during development. It takes place first in the yolk sac and the para-aortic region. Erythropoiesis then migrates to the fetal liver between the 8th and the 22nd week of gestation to finally take place in the BM [15]. In the present work we investigated the capacity of $CD34^+$ cells isolated from human FL, an ontogenic source intermediate between very primitive cells (ES and iPS) and adult stem cells, to expand and differentiate into erythroid lineage cells.

We cocultured FL-derived $CD34^+$ cells over an *in vitro* reconstituted bone marrow (BM) microenvironment. As observed for hES or iPS, only a fraction of erythroid cells reached terminal differentiation into mature RBC (15%). These few RBCs contained nearly exclusively fetal hemoglobin at the protein level. However, as compared to adult HSC, we could observe a dramatic expansion of FL-derived erythroid cells, 100-fold higher when compared to CB $CD34^+$.

FL is an alternative model that might be relevant to understanding mechanisms both implied in amplification and differentiation of HSC in erythropoiesis and could be used as a model for other differentiation pathways.

2. Materials and Methods

2.1. Fetal Liver. Fetal livers were surgically obtained from 6 aborted fetuses (after 12–14 weeks of amenorrhoea) after informed consent of patients was given. All experiments and procedures were in agreement with the guidelines from the French Health ministry and the Agence de la Biomédecine. FL was dissociated within less than 4 hours after collection and flushed through a 70 μm filter and preserved in a PBS/BSA 10% (v/v) (Sigma Aldrich, Lyon, France) solution until CD34 cell immunomagnetic separation, which was performed within a maximal 1 hour delay.

2.2. CD34 Cell Isolation. FL $CD34^+$ cells were isolated by immunomagnetic separation according to the manufacturer's guidelines (Miltenyi Biotech, Bergisch Gladbach, Germany). $CD34^+$ purity was evaluated by flow cytometry.

2.3. FL-Derived Mesenchymal Stem Cells (MSCs). The CD34 negative fractions were used for MSC generation according to the technique described by Doucet et al. [16]. Briefly, cells were plated at a density of 2×10^5 cells/cm^2 and cultured in alpha-MEM medium (Biological Industries, ATGC Biotechnologies, Noisy le Grand, France) supplemented with 5% (v/v) platelet-enriched plasma lysate (PL) and 2 IU/mL heparin (Roche Diagnostics, Basel, Switzerland). Cultures were fed every 3-4 days with fresh medium. At confluence cells were replated at 6×10^3/cm^2. The grown cells had a fibroblast-like morphology. After 2 passages, cells were assessed for immunophenotyping, and their phenotype corresponded to MSCs, that is, $CD73^+$, $CD105^+$, $CD90^+$, $CD45^-$.

2.4. Generation of Red Blood Cells Ex Vivo. $CD34^+$ cells were cultured in a three-step procedure in a serum-free medium as previously described [2] with some modifications such as the source of serum or the use of FL mesenchymal stem cells (MSCs) as stroma. 2×10^4 $CD34^+$ cells/mL were first cultured in IMDM medium (Biological Industries, ATGC Biotechnologies) supplemented either with 1% BSA or 1% human AB plasma, 10 μg/mL insulin (Sigma), and 120 μg/mL iron-saturated human transferrin (Sigma). In the first step (days 0–8), 2×10^4/mL $CD34^+$ cells were cultured in the presence of 10^{-6} M hydrocortisone (Sigma), 100 ng/mL stem cell factor (SCF, Peprotech, Neuilly sur Seine, France), 5 ng/mL IL-3 (R&D Systems), and 3 IU/mL erythropoietin (Eprex, kindly provided by Janssen-Cilag). On day 4, one volume of cell culture was diluted in four volumes of fresh medium containing hydrocortisone, SCF, IL-3, and erythropoietin. In the second step (days 8–10), the cells were replated at 5×10^4/mL and cocultured on an adherent stromal layer in fresh medium supplemented with erythropoietin. In the third step (days 10–18), cells were cultured on an adherent stromal layer in fresh medium without cytokines. The cultures were maintained at 37°C in 5% CO_2. The adherent cell layer consisted of either the MS-5 stromal cell line (provided by K. Mori) or fetal mesenchymal stromal cells (MSCs) established from the CD34 negative fraction of FL liver samples.

2.5. Colony Assay. Colony-Forming Unit Granulocyte Macrophage (CFU-GM), Colony-Forming Unit Erythroid cells (CFU-E), Burst-Forming Unit Erythroid cells (BFU-E) and Colony-Forming Unit Mix (CFU-Mix) were assayed in a semisolid methylcellulose medium as previously described [17].

2.6. Flow Cytometry Analyses. All immunophenotyping analyses were performed using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest Pro software. Immunophenotyping of fetal

MSCs was carried out with the following antibodies: FITC-conjugated anti-CD45 (Beckman Coulter, Imunotech, Roissy en France, France), anti-CD105 (Serotec, Cergy Saint Christophe, France), PE-conjugated anti-CD90, and anti-CD73 (Pharmingen-Becton Dickinson Franklin Lakes, USA). Immunophenotyping of fetal RBCs was carried out with the following antibodies: FITC-conjugated anti-CD36 and PE-conjugated anti-CD71 (Beckman Coulter, Imunotech). Immunophenotyping of fetal mononuclear cells was carried out with the following antibodies: FITC-conjugated anti-CD38 (DAKO, Glostrup, Denmark), anti-Glycophorin A, anti-CD36, anti-CD71, anti-CD45, PE-conjugated anti-CD117, anti-CD33, anti-CD34, anti-CD2, anti-CD3, anti-CD8, (Beckman Coulter-Imunotech), anti-CD19, anti-CD4, anti-CD14, and anti-CD56 (Becton Dickinson). Appropriate negative controls (mouse anti-human irrelevant control) were performed for each FACS analysis.

2.7. Cells and Hemoglobin Characterisation

Hematological Staining. Cell differentiation was monitored throughout culture by morphological analysis of the cells after cytocentrifugation and May-Grünwald-Giemsa (MGG) staining.

Hemoglobin Analysis. The pattern of hemoglobin (Hb) synthesis was analyzed by high-performance liquid chromatography (HPLC) (Biorad variant II, BioRad Laboratories, Munich, Germany) with a high-resolution betathalassemia program.

2.8. Semiquantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Using the protocol of erythroid differentiation described above, we generated RBCs from $CD34^+$ cells isolated from different ontogenic sources (i.e., from CB, BM, and FL) in order to compare the kinetics of the transcriptomic profile of genes known to be involved in erythroid differentiation (GATA-1, FOG 1, SOX6, GFI 1b, STAT5a, FOXO3a, and $\alpha, \beta, \gamma, \varepsilon, \zeta$ globin chains).

Total RNA was prepared from cells collected at different times of RBCs culture (days 8, 11, 15, 18). RNA was extracted using the Trizol method according to the manufacturer's instructions (Invitrogen, Paisley, Scotland). 1 μg of DNase-treated RNA was transcribed into cDNA using 200 units of SuperScript II reverse transcriptase (Invitrogen) and 150 ng of random primers (Invitrogen). The resulting cDNA was aliquoted to avoid repeat freeze/thaw cycles. Real-time PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif, USA). The 18S gene was used as an internal reference. All primers and probes were from Applied Biosystems.

Other genes were amplified using SYBRgreen chemistry, and the GAPDH gene was used as an internal reference. All primers were from Eurogentech (Liege, Belgium).

For both chemistries, PCR were performed in duplicate using either the TaqMan Master Mix or SYBR green Master Mix with 10 ng of cDNA and 300 nM of primers in a final volume of 25 μL. After 2 minutes incubation at 50°C, the AmpliTaq Gold was activated by 10-minute incubation at 95°C. A total of 40 amplification cycles were run with an annealing temperature of 60°C. Calibration curves were established to check that PCR efficiency was similar for all target genes and references. The relative expression of a given target gene was given by the $2^{-\Delta\Delta Ct}$ method [18], where $\Delta\Delta Ct$ represents the difference of an unknown sample (days 8, 11, 15, 18 of culture) versus a control (day 0 of culture) for a target gene normalized to a control gene (18S or GAPDH). This calculation method is suitable for investigating physiological changes in gene expression levels.

Table 1: Flow cytometric analysis of fetal liver cells before $CD34^+$ immunomagnetic separation. Fetal liver was dissociated, and the mononuclear cells were isolated by density-gradient centrifugation and analyzed by flow cytometry for different cell surface markers. Results are presented as the percentage of positive cells. CD: cluster of differentiation.

Phenotypical analysis of mononuclear cells	
CD45	29%
CD117	20%
CD38	15%
CD14	2.2%
CD13	14.5%
CD33	11%
CD2, CD3, CD19, CD56	Negative
CD36	72%
GlycoA	68%
CD71	78%
CD34	**22**%

3. Results

Starting from FL-derived $CD34^+$ cells, we used a three-step stimulation protocol. Firstly, cell proliferation and erythroid differentiation were induced with stem cell factor (SCF), interleukin-3 (IL3), and erythropoietin (Epo). Secondly, the cells were cocultured with additional Epo alone on an *in vitro* reconstituted BM microenvironment (human fetal MSC or murine MS5 stromal cell line). In the third step, all exogenous factors were withdrawn, and the cells were incubated on the stroma alone.

3.1. Massive Expansion and Commitment of Human Erythroid Cells from FL-Derived $CD34^+$ Cells. In the starting FL population, smear examination showed that a majority of cells belonged to the erythroid lineage, more than 50% being proerythroblasts. As analyzed by flow cytometry, most cells expressed markers of the erythroid lineage (69% double positive for CD235a/CD36 and 74% for CD235a/CD71), and $22 \pm 2.8\%$ were $CD34^+/CD38^-$ HSC. Other cells belonged to the myeloid lineage while no lymphoid B or T cells were detected (Table 1). After $CD34^+$ immunomagnetic separation, we obtained a purity of over 90%. The three-step protocol resulted in a dramatic amplification of $CD34^+$

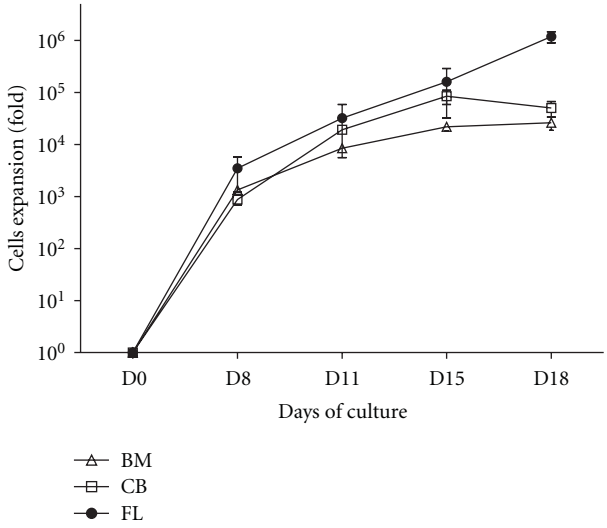

Figure 1: Amplification of CD34$^+$ FL cells during erythroid differentiation. CD34$^+$ hematopoietic stem cells from fetal liver (FL) were grown in liquid culture according to the three-step protocol described in Section 2, and total cells were counted at the indicated times. Mean values with the standard deviation for three experiments are shown, and the results are compared to those for amplification of cells from cord blood (CB) and bone marrow (BM).

	Day of culture		
Type of colony	D3	D8	D10
BFU-E	228	100	20
CFU-E	32	18	31
CFU-GM	167	22	3
Mix (CFU-GM + BFU-E)	91	14	2
Total	518	154	56

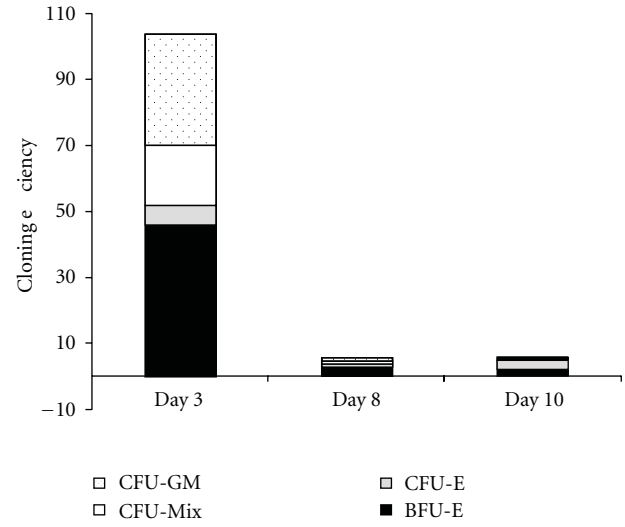

Figure 2: Colony formation of FL-derived erythroid cells. The colony forming ability of FL-derived erythroid cells was measured on days 3, 8, and 10 of liquid culture. Aliquots of nonadherent cells were grown in methylcellulose medium in the presence of SCF, GM-CSF, IL3, and erythropoietin. CFU-E colonies were counted on day 7 and BFU-E, CFU-GM, and CFU-mix colonies on day 14 of semisolid culture.

stem cells by $1.2 \pm 0.3 \times 10^6$-fold (mean of six different liver samples) by day 18. This amplification represents 1 or 2 logs higher expansion as compared to CB ($8 \times 10^4 \pm 2 \times 10^4$-fold, $n = 3$) and BM ($2.6 \times 10^4 \pm 0.8 \times 10^4$-fold, $n = 3$) cells, respectively, (Figure 1).

The development of a large and almost pure erythroid cell population can probably be attributed to the preferential proliferation of the generated erythroid progenitors and their subsequent differentiation and maturation. On day 3, 100% of the cells were clonogenic, most belonging to the erythroid lineage (44% BFU-E, 7% CFU-E, 33% CFU-GM and 16% CFU-mix). On day 8, 5% of the cells were clonogenic, 90% of these being BFU-Es. Late erythroid CFU-E progenitors proliferated extensively and constituted 57% of the clonogenic cell population by day 10 (Figure 2). Cells in culture lost their immaturity as early as day 8 as assessed by CD34, CD117, and CD33 negativity, while their erythroid commitment was confirmed by CD235a, CD71, and CD36 positivity. Day 18 cultured RBCs were CD235a$^+$. These cells still expressed CD36 and CD71 markers, as was also observed in our model using CB and BM sources (Figure 3(a)).

On an MS5 stromal layer, FL CD34$^+$ cells displayed the same erythroid maturation kinetics as CD34$^+$ cells from other sources (CB, BM, or peripheral blood, data not shown) up to day 15. However, their erythroid maturation was blocked by day 18 at the acidophilic stage, and very little nucleus expulsion was observed (3 to 15%) (Figure 3(b)). On the hypothesis that a fetal microenvironment would provide a more suitable niche to achieve complete maturation, we established cocultures over autologous or allogeneic MSCs grown from the FL CD34 negative fractions, but with no improvement in the enucleation efficiency even by extending culture up to day 24.

3.2. Hemoglobin Analysis of FL-Derived Erythroid Cells. Quantitative real-time PCR analysis of total RNA at different times of culture (days 8, 11, 15, and 18) showed that, except for the β globin gene whose expression is constant regardless of the stage of the culture, the expression level of mRNA coding for the other globin genes is upregulated during the erythroid differentiation process.

More particularly, the globin gene expression of the FL-derived erythroid cells showed to be mainly embryonic (ζ and ε globin chains) and fetal (γ globin chains). No adult β globin gene expression could be detected. BM-derived erythroid cells showed an adult globin profile as they mainly expressed β globin chains and to lesser extent fetal (α, γ) globin chains. No embryonic genes (ζ- and ε-globin chains) could be detected. CB-derived erythroid cells showed a similar gene profile but had a stronger expression of the fetal γ-globin chains; embryonic genes (ζ- and ε-globin chains) could be detected but with a variable and low expression (Figure 4(a)).

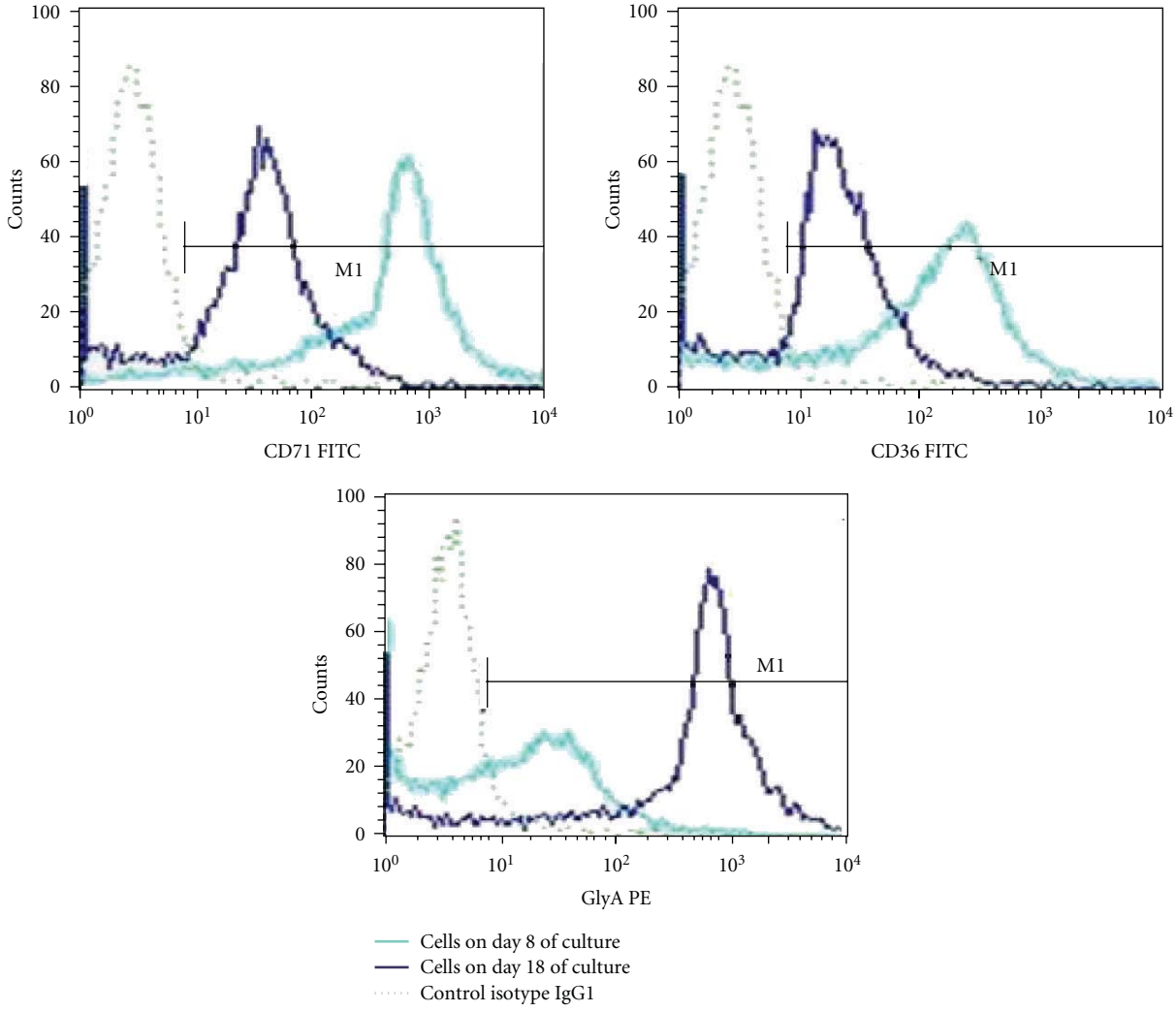

Figure 3: Maturation of FL-derived erythroid cells. (a) FACS analyses of transferrin receptor (CD71), CD36 and glycophorin A (CD235a) expression on days 8 and 18 of liquid culture. Data are from one representative experiment and the green, light blue and dark blue, tracings represent the negative control and cells from days 8 and 18 of culture, respectively. (b) Photographs of the cells on days 8, 10, 15, and 18 of liquid culture after May-Grünwald-Giemsa staining. Every stage of erythroid maturation is represented: proerythroblast, basophilic erythroblast, polychromatophilic erythroblast, orthochromatic erythroblast, and RBCs (magnification ×50).

HPLC analysis ($n = 4$) performed on erythroid cells derived from FL revealed mainly fetal hemoglobin (Hb) regardless of the time of culture. On day 8, the cells contained 68% (59–77%) fetal hemoglobin (HbF) including an acetylated fraction. A very early migrating subfraction was detected and could be identified as Bart Hb, which corresponds to the association of four γ globin chains. At the end of culture (days 19–24), 86% (80–89%) of the cells contained HbF and less than 2% (1.2–1.8%) adult HbA (Figure 4(b)).

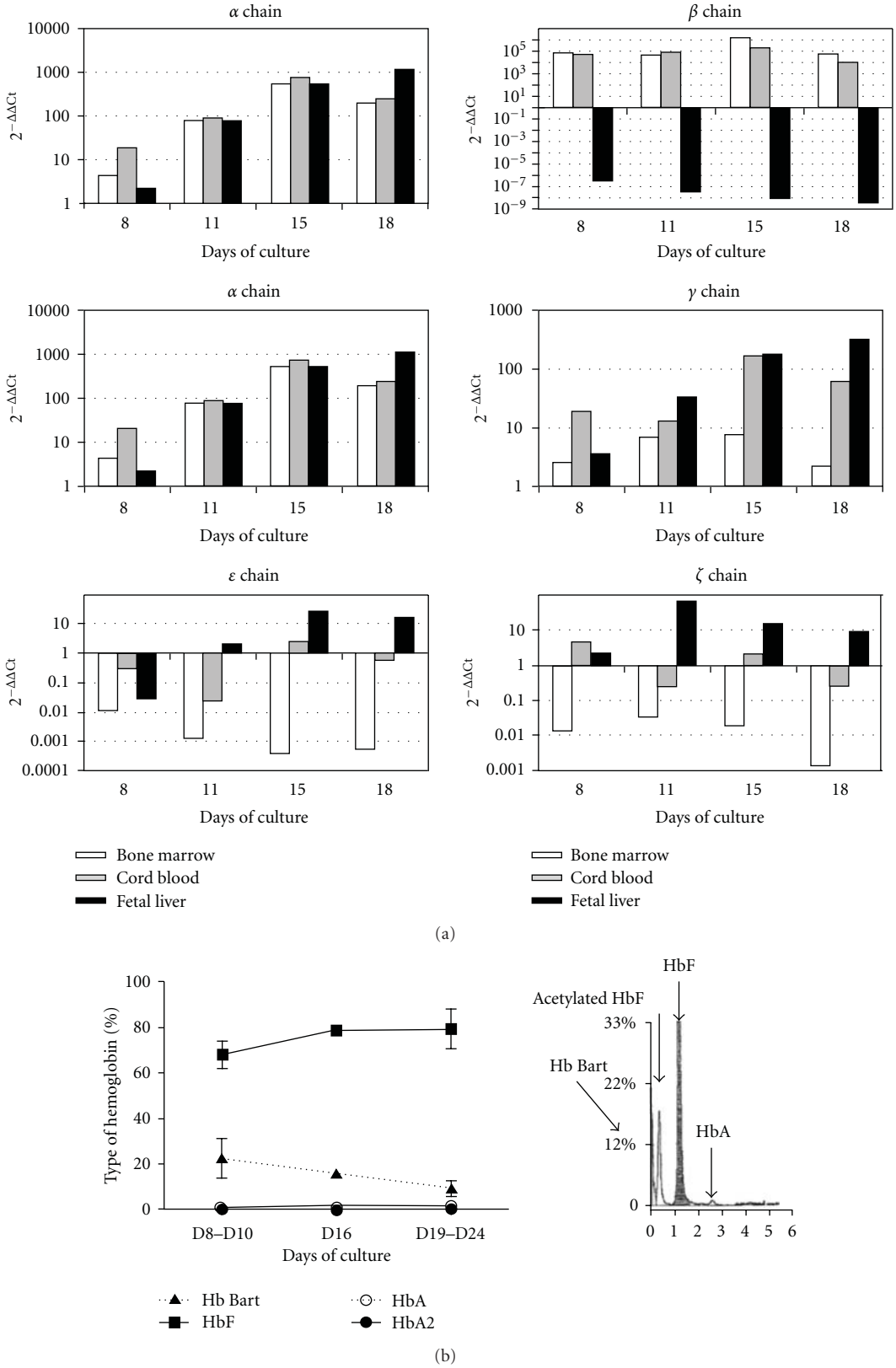

Figure 4: Analysis of the hemoglobin produced by FL-derived erythroid cells. (a) Quantitative real-time PCR analysis of the globin chains at different times of culture (days 8, 11, 15, and 18). Relative gene expression is given by the $2^{-\Delta\Delta Ct}$ method where $\Delta\Delta Ct$ represents the difference of an unknown sample (days 8, 11, 15, and 18 of culture) versus a control (day 0 of culture) for a target gene (globin genes) normalized to a control gene (18S). (b) HPLC analysis of the hemoglobin (Hb) produced on days 8 ($n = 6$), 16 ($n = 2$), and 19–24 ($n = 5$) of erythroid differentiation. Graph and profile show mainly fetal hemoglobin (HbF) including an acetylated fraction.

3.3. Gene Expression of Factors Involved in Definitive Erythropoiesis. In an attempt to explain the blockage at the acidophilic stage of the FL-derived erythroid cells, we analyzed the expression pattern of factors known to be involved in definitive erythropoiesis. We compared PCR results with those obtained for BM and CB samples taken at the same time of erythroid culture (days 8, 11, 15, 18). The $2^{-\Delta\Delta Ct}$ method was applied to establish relative expression of genes (Figure 5).

As compared to BM or CB samples, RT-PCR revealed a strong downregulation of all genes for FL-derived cells regardless of the stage of the culture. BM and CB had the same expression pattern of factors except for STAT5a factor (for which CB showed the same profile as FL). We also observed that the levels of gene expression were higher for BM than for CB.

4. Discussion

A large variety of ontogenic sources of HSC have been explored for cellular therapies. Because of constantly increasing demand in blood supplies, one particular field of interest is transfusion. Thus, we and others have described experimental procedures which allow the massive expansion of $CD34^+$ cells from various sources and the generation of mature RBCs [1, 2, 19–22]. To date, the most proliferative and accessible source of stem cells for generating cultured RBCs (cRBCs) is cord blood. It presents, nevertheless, the drawbacks of being restricted to voluntary donations and is not unlimited; 1 CB unit gives one batch of cRBC production.

Because they are an unlimited source of stem cells, many studies are being carried out with ES cells and more recently with iPS. Critical advances have allowed progress towards the *in vitro* production of functional RBC from these sources in a few years. However their limited ability to expand *in vitro* is a limitation to large-scale production.

In human development, FL is a rich source of hematopoietic progenitors between the 8th and the 22nd week of gestation as it is the exclusive site of erythropoiesis. Although "conventional" sources of HSC for generating cRBC include both adult (mobilized peripheral blood or CB) and primitive stem cells (ES or iPS cells), evidence suggests that FL may represent a rich alternative source of "early" HSCs, displaying multipotency, possessing high proliferative and repopulating potential [23–26]. Indeed, it has been proven to be efficient in transplantation [27–29].

In the present work, we addressed the question of the possibility of generating *in vitro* mature cells from HSC of such an intermediate ontogenic source (FL cells) between ES cells and adult cells. In our experimental conditions, FL cell maturation is mainly blocked at the acidophilic stage. Indeed, only few cells (3–15%) achieved terminal differentiation. We made the hypothesis that the blockade of maturation observed *in vitro* was the consequence of an inappropriate microenvironment. On the assumption that a microenvironment from the same ontogenic stage would better support erythroid differentiation, we cocultured FL-derived $CD34^+$ cells on FL-derived MSC instead of the murine MS5 stromal cell line. However, no difference was observed in terms of terminal maturation, and the produced cells were mostly nucleated, whereas in the same culture conditions adult stem cells could generate fully enucleated cRBC. When we analyzed by RT-PCR globin transcripts of FL-derived erythroid cells, the transcriptomic profiles were mainly fetal (γ-globin chains) and embryonic (ζ- and ε-globin chains). In the same way, BM-derived erythroid cells showed an adult globin profile as they mainly expressed β-globin chain and to lesser extent fetal chains (α-, γ-globin chains). No embryonic genes (ζ- and ε-globin chains) could be detected. In comparison, CB-derived erythroid cells showed a similar gene profile but had a stronger expression of the fetal γ-globin chains and embryonic genes (ζ- and ε-globin chains) could be detected but with a variable and low expression. By analyzing the globin genes expression, we could observe that our system of culture mimics what happens during development and leads to globin gene expression patterns in accordance with the cells' ontogeny.

The precise molecular mechanisms that regulate the erythroid differentiation process remain to be fully elucidated despite many genes and pathways have been described to be essential in erythropoiesis. In order to understand the reason for the enucleation deficiency observed with FL cells, we analyzed by RT-PCR the expression of some genes known to be involved in definitive erythropoiesis (i.e., GATA-1, FOG 1, SOX6, GFI 1b, STAT5a, FOXO3a) upon the following rationale. GATA-1 is found exclusively in hematopoietic cells and is particularly important in erythroid and megakaryocytic lineages. *In vitro*, in the absence of GATA-1, a maturation deficit can be observed, indicating its crucial role in terminal erythroid differentiation [30]. FOG-1-null embryos present a defect both in primitive and definitive erythropoiesis with a blockage of maturation at the stage of proerythroblast [31].

The signal transducer and activator of transcription STAT5 is responsible for the control of proliferation, differentiation, and apoptosis, via its effect on gene expression. STAT5 proteins [32] are suggested to play an important role in hematopoiesis, but there are numerous contradictory reports on the role of STAT5 in normal hematopoietic cell development. A recent study [33] shows that overexpression of an activated mutant of (STAT5) induces erythropoiesis and increases long-term proliferation of human hematopoietic stem/progenitor cells. siRNA silencing of FOXO3a as well as the inactivation of GFI 1b led to erythroid differentiation blockage [34, 35]. Finally, it has been reported that, in $CD34^+$ from CB, SOX6 accelerated the kinetics of erythroid maturation and increased the number of cells that reached the final enucleation step [36].

Our results clearly show that, in our culture conditions, all the genes we studied are strongly downregulated in FL cells, whereas they are upregulated both in BM and CB cells, notably in BM cells. The pattern of gene expression that we observed was in concordance with the enucleation defect observed in FL but could not fully explain it. Indeed this molecular analysis is qualitative and not functional. Nevertheless, it suggests that genes involved in terminal differentiation are differently regulated in the function of the cell ontogeny and might at least in part explain the

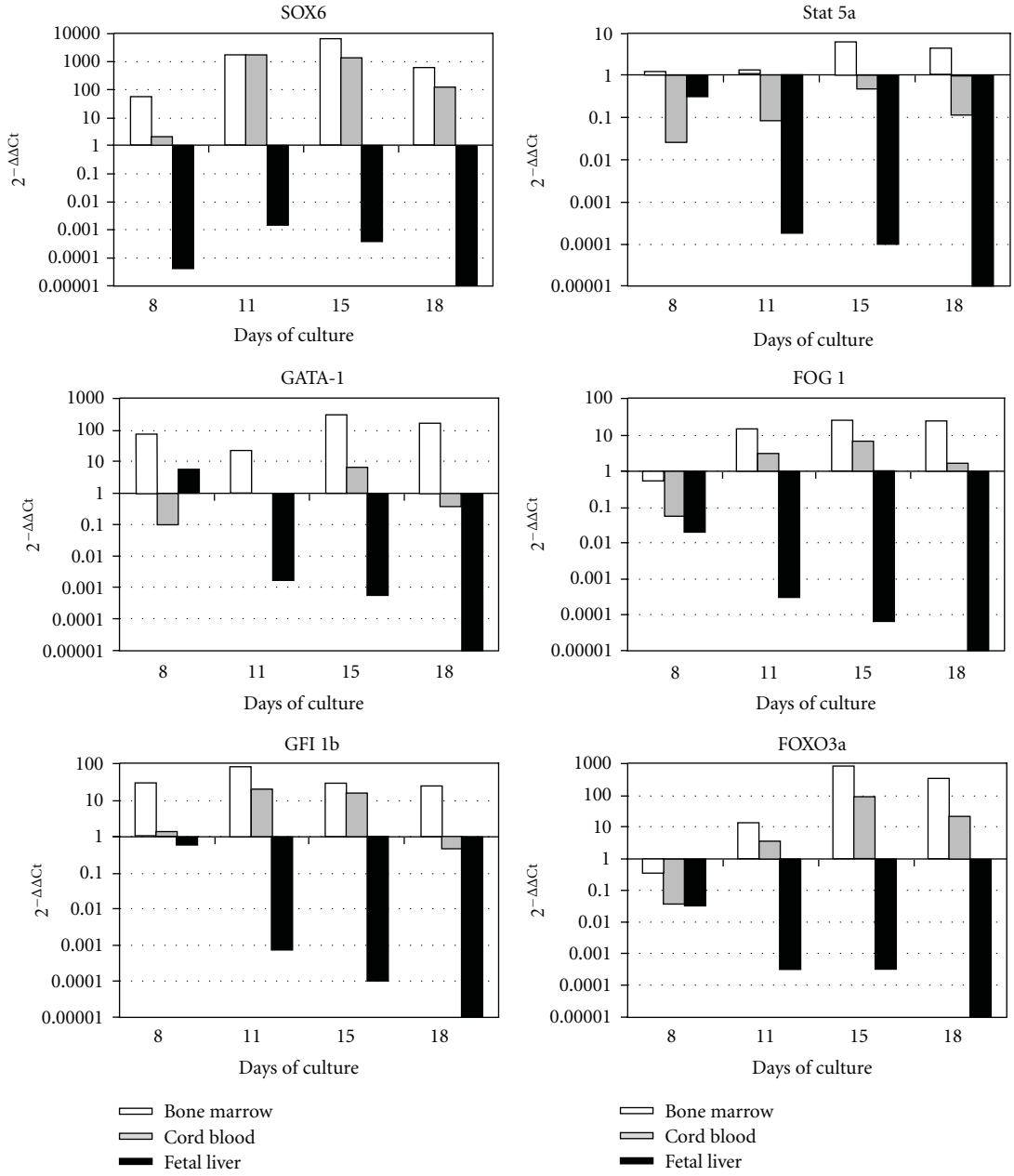

Figure 5: Gene expression of factors involved in definitive erythropoiesis. Quantitative real-time PCR analysis of genes known to be implied in definitive erythropoiesis at different times of culture (days 8, 11, 15, and 18). Relative gene expression is given by the $2^{-\Delta\Delta Ct}$ method where $\Delta\Delta Ct$ represents the difference of an unknown sample (days 8, 11, 15, and 18 of culture) versus a control (day 0 of culture) for a target gene normalized to a control gene (GAPDH).

differences observed in the ability to generate RBC *in vitro*. For many reasons, ES or iPS cells would be the natural candidates for the regenerative medicine of tomorrow. Through this model of erythropoiesis, one can understand how different cells behave according to their ontogeny. As it is titled in the publication of Williams et al. "Children are not little adults: just ask their hematopoietic stem cells" [37]. Extensive analysis on models explaining these differences would be of great interest to fully control the differentiation pathway of stem cells and would be helpful for the next generation of stem cell therapy.

Even if the generation of RBCs from ES cells or iPS has been quite routinely achieved by several teams [6–13], none succeeded in a 100% enucleated population whatever the culture conditions. Primitive cells seem to underperform in terms of production yield of fully mature cRBCs *in vitro*. The question whether this is due to their ontogeny or related to inappropriate culture conditions remains open.

If FL has no advantage in terms of final maturation over pluripotent stem cells, we observed an interest in terms of amplification. As far as the hematopoietic pathway is concerned, FL is known to contain a compartment of

colony-forming cells with a high proliferative potential [14, 23–25]. These observations were confirmed by our findings of (i) a strong expansion of $CD34^+$ FL cells and (ii) 100% cloning efficiency in hematopoietic progenitor assays after 3 days of erythroid induction, as compared to 10–15% cloning efficiency for adult $CD34^+$ cells [17]. The proliferation capacity of stem cells from various sources is partly related to their ontogenic origin. ontogeny-related changes in proliferative potential of human hematopoietic cells *in vitro* have already been suggested in the literature, and analyses have shown, by comparing bone marrow, peripheral blood, umbilical cord blood, and fetal liver, that FL appears to represent a good target for *ex vivo* stem cell expansion [38, 39].

We reproduced this observation in our culture conditions. Indeed, we observed a 100,000- and 25,000-fold expansion for cells from CB, BM, respectively. Strikingly, for FL-derived $CD34^+$ cells, in the same culture conditions, the expansion reached more than 1.2×10^6-fold.

FL remains a model of study and is not a candidate as a direct source of stem cells for *ex vivo* RBCs production for blood transfusion. On the contrary, because iPS cells can proliferate indefinitely, they are obviously the best candidate to set up complementary sources of RBCs for transfusion. But their clinical application will need a drastic increase of their ability to proliferate *in vitro*. The present observations relating to erythropoiesis from FL could pave the way for the design of new strategies to generate induced pluripotent stem cells. This idea is supported by the work of Kim et al. [40]. In a murine model, these authors observed that iPS harbor residual methylation signatures of their somatic tissue of origin, which favors their differentiation into lineages related to the donor cells while restricting alternative cell fates. According to this logic, we could imagine that a cell with a high proliferative capacity thereby would be optimal for the production of RBC.

Acknowledgments

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Review Article

Optimization Manufacture of Virus- and Tumor-Specific T Cells

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Although *ex vivo* expanded T cells are currently widely used in pre-clinical and clinical trials, the complexity of manufacture remains a major impediment for broader application. In this review we discuss current protocols for the *ex vivo* expansion of virus- and tumor-specific T cells and describe our experience in manufacture optimization using a gas-permeable static culture flask (G-Rex). This innovative device has revolutionized the manufacture process by allowing us to increase cell yields while decreasing the frequency of cell manipulation and *in vitro* culture time. It is now being used in good manufacturing practice (GMP) facilities for clinical cell production in our institution as well as many others in the US and worldwide.

1. Introduction—T Cell Transfer

Cell therapy is a new but rapidly expanding field in biotechnology which involves the administration of autologous or allogeneic cells that carry out a therapeutic effect *in vivo*. The first adoptive T cell transfer protocols in the allogeneic hematopoietic stem cell transplant (HSCT) setting were based on the premise that donor peripheral blood contained T cells able to mediate antitumor and/or antiviral activity in the HSCT recipient. Accordingly, donor lymphocyte infusions (DLIs) have been extensively used to provide both antitumor and antiviral immunity. However, the relatively high frequency of alloreactive cells compared with virus- and/or tumor-specific T cells results in a significant incidence of graft-versus-host disease (GvHD), thereby limiting the applicability of this approach. Infusion of enriched antigen-specific T cells with reactivity against a particular antigen potentially increases therapeutic potency while decreasing undesired "off-target" effects or GvHD, and this field has grown over the past two decades. This paper focuses on the production of *in vitro* expanded antigen-specific T cells, discusses conventional and current technologies for T cell generation, and outlines recent advances in cell production techniques which may ultimately move this therapeutic modality from a boutique application towards a "standard of care."

2. Infusion of *Ex Vivo* Expanded CTL

The infusion of *in vitro* expanded donor-derived virus-directed cytotoxic T lymphocytes (CTLs) targeting one (Epstein-Barr virus (EBV)), two (EBV and Adenovirus (Adv)), or three viruses (EBV, Adv, cytomegalovirus (CMV)) has proven to be safe, effective, and protective *in vivo* [1–4]. The adoptive transfer of tumor antigen-directed T cells has also induced objective tumor responses and complete remissions in patients with advanced lymphoma, melanoma, and nasopharyngeal carcinoma [5–10]. Recent advances in molecular biology techniques have increased the enthusiasm for this therapeutic modality by (1) allowing the genetic modification of T cells with a wide range of genes which confer new antigen specificity by transferring T cell receptors (TCRs) or chimeric antigen receptors (CARs) [11–14], (2) improving the homing and proliferative properties of effector cells [15, 16], and (3) controlling unwanted T cell proliferation or *in vivo* activity [12, 17–20].

Although the administration of *in vitro* expanded antigen-specific CTLs has produced promising clinical results, there are several factors limiting the extension of this approach beyond the research arena. A major practical constraint is the current complexity associated with production of large number of cells using traditional manufacture

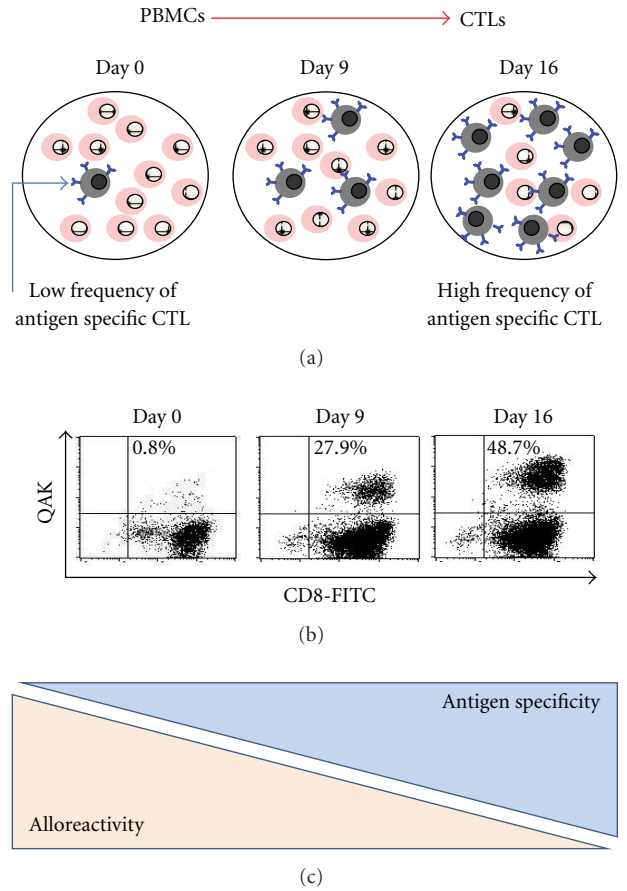


FIGURE 1: Increased frequency of antigen-specific CTLs after *in vitro* stimulation. (a) illustrates the low frequency of antigen-specific CTLs present in peripheral blood and the subsequent enrichment after antigen stimulation. (b) shows the enrichment of QAKWRLQTL- (HLA-B8-restricted EBV epitope-) specific T cells in a seropositive donor as evaluated by tetramer analysis. (c) illustrates the inverse correlation between the frequency of antigen-specific and alloreactive T cells in peripheral blood (left) and in vitro expanded CTLs (right).

protocols. However, some recent advancements streamlined the production process.

3. *Ex vivo* Expansion of Antigen-Specific T Cells

The *ex vivo* generation of antigen-specific T cells is conventionally accomplished by repeat in vitro stimulation with professional or artificial antigen presenting cells (APCs) which express the protein or peptide of interest and culture in the presence of cytokines which promote T cell proliferation, such as interleukin- (IL-) 2 [1, 21, 22]. This process results in the amplification and enrichment of T cells directed against the stimulating antigen/peptide with a corresponding decrease in the frequency of cells with undesired specificities such as alloreactive T cells (Figure 1). Once sufficient cells (required for adoptive transfer) are generated, these are then tested for potency, purity, identity, and sterility prior to infusion.

For example, EBV-specific CTLs can be expanded *ex vivo* from EBV-specific T cell precursors generally present at a frequency of up to 1% in the peripheral blood of most seropositive individuals. Traditionally, enriched T cell lines are prepared by coculturing 1×10^6 peripheral blood mononuclear cells (PBMCs) per cm^2 with gamma-irradiated (40 Gy) autologous EBV-transformed lymphoblastoid cell lines (EBV-LCLs) at a 40 : 1 ratio (PBMC : LCLs) in a total volume/well (of a tissue culture treated 24-well plate) of 2 mL CTL growth media (RPMI 1640 supplemented with 45% Click medium (Irvine Scientific, Santa Ana, Calif), 2 mM GlutaMAX-I, and 10% FBS). Between days 9 and 12 CTLs are harvested, counted, resuspended in fresh media, re-seeded at 5×10^6 per cm^2 in a total volume of 2 mL of CTL media, and then fed with recombinant IL-2 (50 U/mL) 4 days later. This initial 13–16-day culture period in the absence of exogenous cytokines gives a proliferative/survival advantage to the small population of EBV-specific T cells present in PBMCs, which both produce and use IL-2 in an autocrine manner upon stimulation with EBV-LCL. However, at later time points, when cultures are exclusively EBV specific the level of available cytokine becomes limiting and thus cultures must be supplemented to ensure that CTL proliferation is adequately supported [23]. Subsequent stimulations are performed every 7 days using a 4 : 1 CTL:EBV-LCL ratio with twice weekly addition of IL-2 (50 U/mL). This *ex vivo* propagation of EBV-specific T cells continues until sufficient cells are generated for cryopreservation and quality control analysis including HLA typing to confirm identity, purity, and safety testing. All products must meet the specified release criteria before they are released for infusion. Additional analysis on specific products such as assessment of transgene expression may also be performed. For example, one of the release criteria for chimeric-antigen-receptor- (CAR-) modified EBV-CTLs is that at least 15% of cells must express the transgene. Though there are different CTL generation protocols used by different groups, even for the generation of the "same" product, the component parts/core requirements (antigen, APC, and cytokine) are essentially the same.

4. Traditional *in vitro* Culture of Antigen-Specific T cells

A large variety of manufacturing protocols have been described for the *in vitro* expansion of T cells. Small numbers of suspension cells ($<5 \times 10^7$) can be relatively easily propagated using conventional multiwell tissue culture treated plates or flasks. However, when the number of cells required exceeds the maximum capacity of a single plate or flask (e.g., $>5 \times 10^7$) this platform becomes time consuming and cumbersome to manipulate.

Cell propagation *in vitro* is limited by requirements for nutrients and oxygen (O_2) and by the accumulation of metabolic waste such as lactic acid and carbon dioxide (CO_2). Cell culture in conventional cultureware is restricted to the use of specific media volumes per surface area unit, that is, a maximum of 1 mL media should be added per cm^2 since this is permissive to gas diffusion. However, this shallow

media volume limits both the available nutrients and the buffering capacity of the media. In addition, as cell numbers increase, O_2 and nutrient requirements progressively increase, so that cultures must be fed and re-seeded regularly. These frequent medium changes and cell manipulations are time consuming and expensive, reduce the reproducibility of cell production, and increase the risk of contamination.

5. Alternative Vessels for T Cell Expansion

One way to overcome the limitations associated with scale-up using conventional cultureware is to instead utilize a cell bioreactor that provides mechanical rocking or stirring to perfuse media with gas. The use of such bioreactors augments cell expansion, resulting in higher cell densities beyond that attained using conventional plasticware.

A large number of bioreactors (hollow fiber bioreactors, stirred tank bioreactors, and WAVE bioreactors) have been explored for the expansion of suspension cells such as activated T cells, genetically modified T cells, or antigen-specific CTL [23–27]. In these bioreactors oxygen is provided by mechanical rocking or stirring or by pumping gas through the culture while medium can be exchanged by perfusion. Stirred bioreactors allow excellent gas exchange and can be scaled up relatively easy. However, shear stress associated with the stirring rate adversely affects cell viability and thus it has not been broadly adapted [28]. In contrast, hollow fiber bioreactors allow a constant perfusion of the culture, thus diluting metabolites without shear stress. However, accessibility to this device makes it difficult to efficiently recover the expanded cells [24]. Static culture bags limit the achieved cell densities (per input media volume). Thus, the generation of large cell numbers requires the use of large media volumes with a resultant increase in the frequency of manipulations required to obtain the final product [29]. Although the WAVE Bioreactor has been effectively adapted for the expansion of primary T cells, resulting in the generation of large numbers of cells (10^{15}), the culture bag cannot be accommodated in a standard incubator and must be heated and rocked in an expensive, custom-made device [30, 31]. In addition, optimal cell growth is maintained by regular measurement of oxygen and lactic acid and a peristaltic pump is needed to move medium in and out of the bag, necessitating the incorporation of special filters to prevent cells being damaged by the pump. Further, gas is propelled through the culture using a control flow meter which ensures that culture osmolarity is maintained.

Although antigen nonspecific T cell cultures have been grown with some success in these various bioreactors, antigen-specific T cells have strict requirements for cell-to-cell contact and have proven difficult to consistently adapt to moving cultures. Therefore many groups, including our own, have found it difficult to improve upon results achieved using the 2 cm^2 wells of standard tissue culture-treated 24-well plates, which are ideal for the expansion of small numbers of cells required for preclinical and proof of concept studies but limit the translation of antigen-specific T-cell-based therapies beyond the academic level (Figure 2). Table 1 shows the relative advantages and disadvantages associated with each of the culture vessels which have been used to produce T cell products for clinical applications.

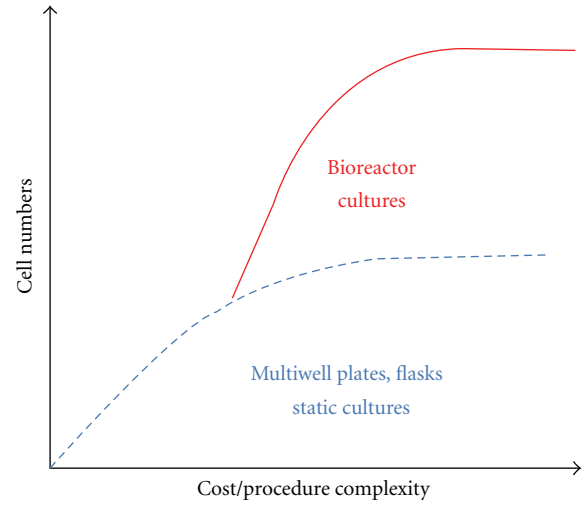


Figure 2: Increased cost and procedure complexity with large-scale cell requirements. As illustrated multiwell plates or flasks are ideal for the expansion of small numbers of antigen-specific CTLs ($<5 \times 10^7$). However, this system becomes ineffective for the expansion of large numbers of cells. In contrast cell bioreactors are ideal for the production of large cell numbers, but this platform is difficult to adapt and requires specialized equipment.

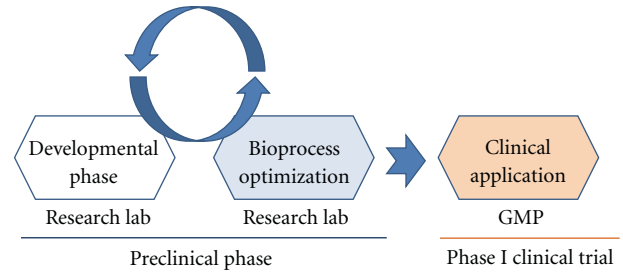


Figure 3: Dynamic bioprocess optimization. This dynamic interaction between the optimization and the preclinical phase allows for easy transition of a cell product into the cGMP.

6. Dynamic Bioprocess Optimization

The problem with most manufacturing processes is the misconception that a product can be produced on a large scale by simply using a linear scale-up model. In most cases this is simply not feasible given that the production protocols are, for the most part, specialized, highly complicated, and convoluted. One way to overcome this scale-up problem, which is a bottleneck in conventional cellular therapies, is to incorporate bioprocess optimization in the manufacturing process. That will ultimately pave the way for an easy transition into the GMP and will almost guarantee manufacturing success, thus positively impacting the outcome of a clinical study. This bioprocess optimization (as illustrated in Figure 3) should not be considered

Table 1: Suitability and properties of different culture vessels for T cell expansion.

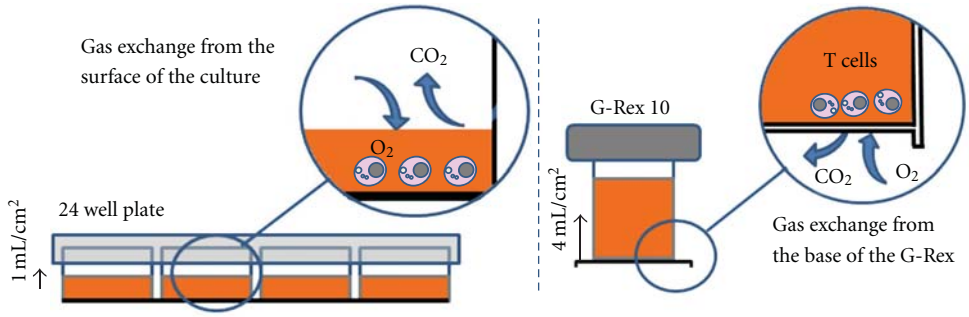
Cell culture vessels	Gas exchange	Volume of media	Cell concentration	Disadvantages	Advantages
Multiwell plates/flasks (static cultures)	Limited	Limited: low ratio of medium to surface area	Low	High risk of contamination Extensive processing time Frequent interventions Not scalable	Suitable for small-scale cell production
Gas-permeable bags (static cultures)	Good	Limited: low ratio of medium to surface area	Medium	Low output per bag requires constant culture maintenance Limited microscopic cell examination Not linearly scalable from research to production	Sterility of closed system
G-Rex (gas-permeable static cultures)	Excellent	Unrestricted: high ratio of medium to surface area	High	Limited microscopic cell examination	Excellent O_2 exchange Linearly scalable from research to large-scale production Significantly reduced culture manipulation Compatible with closed system
Wave action bioreactors with CO_2/O_2 aeration & pH controllers (dynamic cultures)	Good	Unrestricted: high medium capacity in each bag	High	Complex, costly, requires special equipment. Not well suited to coculture stage of CTL production Requires constant culture maintenance. Limited microscopic cell examination Not linearly scalable from research to large-scale production	Excellent O_2 exchange yields large cell numbers Closed system

a "validation stage" but instead a dynamic interaction between the preclinical phase and manufacturing optimization that seeks to simplify the product generation, while ensuring that the cell product maintains the biological properties achieved in small scale manufacture.

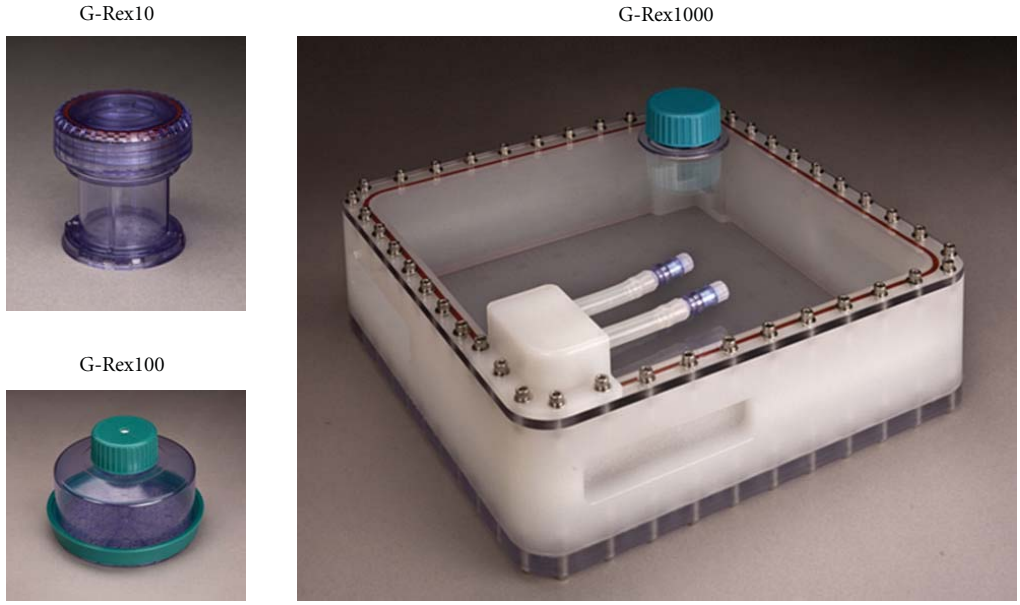
7. Our Experience

One example of manufacture optimization that we have undertaken over the past 4 years at the Center for Cell and Gene Therapy (CAGT) at Baylor College of Medicine and supported by Production Assistance for *Cellular Therapies* (PACT) surrounds our search for simpler and more rapid strategies to expand antigen-specific T cells for adoptive transfer. Traditionally our group and others have cultured virus- and tumor-directed T cells in 2 cm^2 wells of tissue culture treated 24-well plates. These T cells are often propagated for 8 weeks or longer to achieve the cell numbers required for clinical application. However, the restricted media ratio (1 mL/cm^2) associated with gas diffusion limits the supply of nutrients, which are rapidly consumed by proliferating T cells. Consequent acidic pH and waste build-up rapidly impedes cell growth and survival. Therefore, the only alternative for cell propagation is frequent reseeding and medium exchange which increases the frequency of manipulation required with a concomitant increase in the risk of contamination. Thus, we sought to optimize our antigen-specific T cell culture process which led us to evaluate a novel cell culture device (gas-permeable cultureware (G-Rex)), developed by *Wilson Wolf Manufacturing*, and in which O_2 and CO_2 are exchanged across a silicone membrane at the base of the flask. Because gas exchange occurs from below this allows an increased depth of medium above, which provides more nutrients required by the cells while waste products are diluted, thus not adversely affecting cell growth (Figure 4).

These optimal culture conditions provided by the G-Rex result in improved cell viability and increased final cell numbers without increasing the number of cell doublings, and decreasing the feeding frequency and the number of manipulations required [32]. For example, for the expansion of EBV-CTLs using the G-Rex we co-culture 1×10^6 PBMCs per cm^2 using a G-Rex10 (surface area of 10 cm^2—total



(a)



(b)

Figure 4: G-Rex culture device. (a) shows the limited gas exchange that occurs in conventional cultureware, which limits the volume of media and consequently the available nutrients. In contrast the G-Rex provides gas exchange from the base of the flask which allows cells to be cultured with a superior ratio of media per surface area. (b) shows the G-Rex10 with a surface area of 10 cm^2 and a volume capacity of 40 mLs, the G-Rex100 with a surface area of 100 cm^2 and a volume capacity of 500 mLs, and the G-Rex1000 with a surface area of 1000 cm^2 and a volume capacity of 5000 mLs.

of 1×10^7 PBMCs) with gamma-irradiated (40 Gy) EBV-LCLs at a 40 : 1 ratio in a final volume of 40 mL of CTL medium. On days 9–12 the second stimulation is performed by removing 20 mL of media (aspirated from the top) and adding 20 mLs of fresh CTL medium containing irradiated EBV-LCLs, resuspended at a cell density appropriate to stimulate T cells at a ratio 4 : 1. Four days after the second stimulation 50 U/mL of IL-2 is added directly to the culture. Once the cells have expanded to a density of $>5 \times 10^6$ per cm^2 the cells are transferred to a G-Rex100 (surface area 100 cm^2) and stimulated with irradiated EBV-LCL (4 : 1) in a final volume of 500 mLs of media. These culture conditions have allowed us to decrease the frequency of culture manipulation while increasing the cell output (3–20-fold) and shortening the time of culture [32] (Figure 5). We demonstrated that this novel culture system supports the expansion of almost any type of suspension cell, is GMP-compliant, and reduces the number of technician interventions approximately 4-fold [32].

This manufacture optimization has been validated, transferred to our GMP facility in 2009 and is now used for all of our CTL production processes. Since that time we have allowed other centers, including the NCI, to cross-reference our IND to enable the use of this cell culture technology in other GMP facilities both within the US and beyond, and this platform is currently used for production of numerous cellular products including activated T cells, antigen-specific CTLs, NK cells, regulatory T cells, and feeder cells including EBV-LCLs and aK562 [32]. Importantly, cell culture in the G-Rex can also be linearly scaled which allows an easy transition of protocols from small to large scale. We recently demonstrated this using the new G-Rex600 and G-Rex1000 (surface area of 600 and 1000 cm^2, resp.), which can generate up to 6×10^9–1×10^{10} cells, respectively, in a single device.

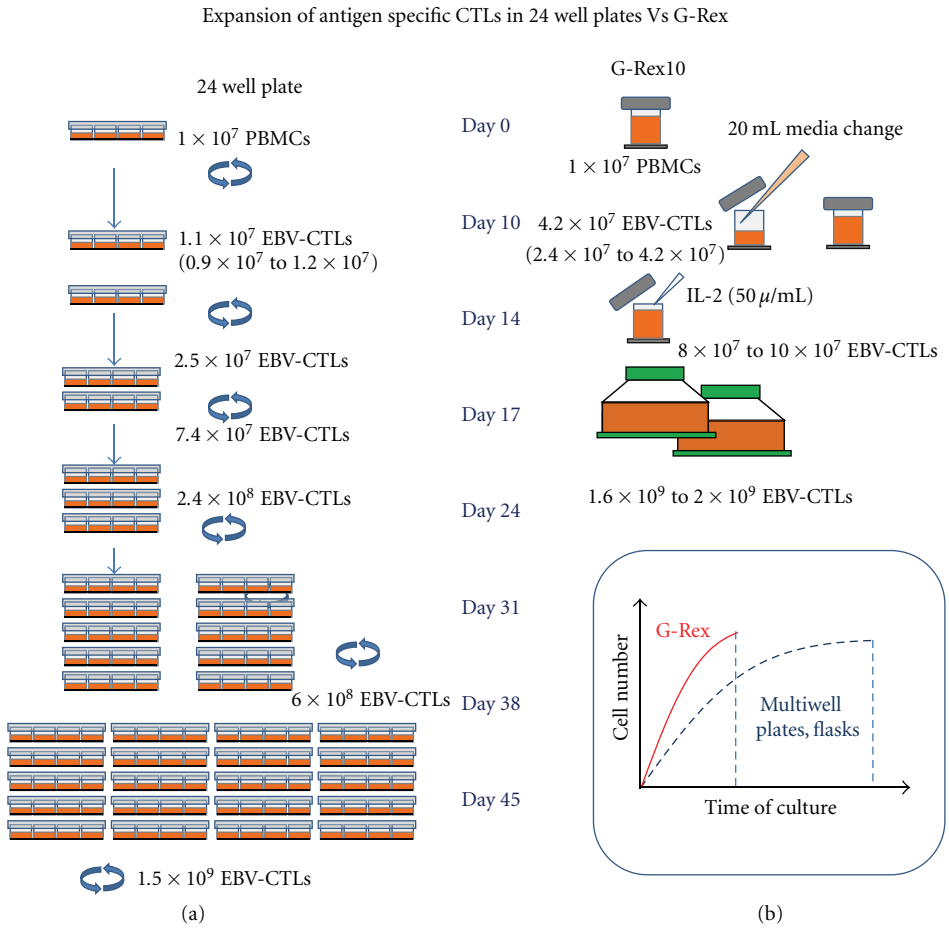


Figure 5: Optimization of antigen-specific CTL manufacture decreases the number of interventions while increasing the cell output. (a) illustrates the level of complexity associated with the generation of antigen-specific CTLs using conventional 24-well plates and the reduced number of interventions required when reproducing the same protocol using the G-Rex. (b) shows how the implementation of the G-Rex device decrease the *in vitro* culture time when compared with the conventional method.

8. Third-Party CTLs

These manufacturing improvements have allowed us to consider the use of virus-specific CTLs in the 3rd-party setting and recently we have developed a cell bank to facilitate this endeavor. Administration of this "off-the-shelf" product raises two potential concerns: (i) the risk of inducing GvHD by administering a partially HLA-mismatched CTL product and (ii) limited *in vivo* persistence, due to recipient alloreactivity directed against nonshared HLA antigens. Nevertheless a number of small studies have demonstrated the feasibility of this approach in the patients with EBV lymphoma arising after HSCT or solid organ transplant. Haque and colleagues used 3rd-party EBV-specific CTLs to treat PTLD after solid organ transplant or SCT and showed an encouraging response rate of 64% and 52% at 5 weeks and 6 months, respectively [33]. In this study the CTLs were selected by low-resolution typing and screened for high-level killing of donor EBV-LCLs and low-level killing of patient PHA blasts. The level of HLA matching ranged from 2/6 to 5/6 antigens, and there was a statistically significant trend towards a better outcome with closer matching at 6 months. Importantly, no patient developed GVHD after CTL administration. In another report two cord recipients with EBV lymphoma received closely matched EBV-specific T cells resulting in complete resolution of their lesions [34].

Currently we are evaluating the safety and potency of using "off-the-shelf" trivirus CTL for the treatment of CMV, adenovirus, or EBV infections in patients after HSCT with active infection and that do not respond to conventional therapy. Preliminary results in >35 recipients, most of whom had received alternative donor transplants, are encouraging, with minimal toxicity and >80% achieving complete or partial responses. If this trend continues, we will generate a larger CTL bank to cover as many racial groups as possible and progress to a phase II clinical trial where we can ask more specific questions regarding the persistence and function of the CTL *in vivo*. Such a study is dependent on the ability to produce large numbers of CTLs that maintain their specificity and functional activity and are not "exhausted" by excessive *in vitro* passaging, and this has become possible only recently with the advent of optimized culture protocols in the G-Rex cultureware that effectively supports CTL expansion.

9. Future Prospects

Manufacture optimization arises from constant and critical reflection on the different processes involved in the generation of a cellular product. The G-Rex culture device is just one example of manufacture optimization taking place at the CAGT. We have also recently simplified the process of virus-specific CTL generation by replacing viral vectors and live virus (previously used as antigen sources) with clinical grade plasmids and overlapping peptide libraries [35]. We have also discovered that certain combinations of enhancing and stimulatory cytokines support the efficient activation and expansion of both virus- and tumor-reactive CTLs, leading to the new GMP-compliant protocols that enable the rapid generation of high-quality cellular products. Although the manufacture optimization is a research phase that requires time, money, and effort, this is an investment and a prerequisite for the manufacturing success of a cell product. Ultimately, the final "value" of a cell product depends on the *in vivo* therapeutic efficacy; however, it is the manufacture process that either facilitates or restrains the evolution of such products from the boutique to the mainstream.

Abbreviations

Adv: Adenovirus
APC: Antigen presenting cells
CAR: Chimeric antigen receptor
CMV: Cytomegalovirus
CTL: Cytotoxic T lymphocytes
DLI: Donor lymphocyte infusions
EBV: Epstein-Barr virus
FBS: Fetal bovine serum
GVHD: Graft-versus-host disease
HSCT: Hematopoietic stem cell transplant
IL: Interleukin
LCL: Lymphoblastoid cell line
PACT: Production assistance for *cellular therapies*
PBMC: Peripheral blood mononuclear cells
TCR: T cell receptor.

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Research Article

Recovery and Biodistribution of *Ex Vivo* Expanded Human Erythroblasts Injected into NOD/SCID/IL2Rγ^{null} mice

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Ex vivo expanded erythroblasts (EBs) may serve as advanced transfusion products provided that lodgment occurs in the macrophage-niche of the marrow permitting maturation. EBs expanded from adult and cord blood expressed the receptors (CXCR4, VLA-4, and P-selectin ligand 1) necessary for interaction with macrophages. However, 4-days following transfusion to intact NOD/SCID/IL2Rγ^{null} mice, CD235a^{pos} EBs were observed inside CD235a^{neg} splenic cells suggesting that they underwent phagocytosis. When splenectomized and intact NOD/SCID/IL2Rγ^{null} mice were transfused using retrovirally labeled human EBs, human cells were visualized by bioluminescence imaging only in splenectomized animals. Four days after injection, human CD235a^{pos} cells were detected in marrow and liver of splenectomized mice but only in spleen of controls. Human CD235a^{pos} erythrocytes in blood remained low in all cases. These studies establish splenectomized NOD/SCID/IL2Rγ^{null} mice as a suitable model for tracking and quantification of human EBs *in vivo*.

1. Introduction

Culture conditions capable of generating *ex vivo* human erythroblasts (EBs) in numbers sufficient for transfusion have been established by several investigators (reviewed in [1]) increasing the likelihood that these cells could serve as alternative transfusion products [2]. The preclinical evaluation of these products includes testing the *in vivo* potency of *ex vivo* generated cells in animal models [3]. The development of animal models to assess the potency of human EBs generated *ex vivo* requires a better understanding of the interactions between human EBs and the murine microenvironment and identification of animal manipulations which may favor lodgment of these cells in the marrow.

In vivo, EBs mature in specialized areas of the marrow in close proximity to macrophages which favors both hemoglobinization, by facilitating iron uptake, and enucleation [4, 5]. Following enucleation, reticulocytes lose their association with the macrophage and egress into the blood stream. EBs interact with the macrophages through the surface adhesion receptors CXCR4 (CD184), P-selectin ligand1 (PSGL1, CD162), and VLA-4 (CD49d, α4 integrin) which recognize CXCL12 (also known as SDF1), P-selectin and VCAM1, respectively, on the surface of the macrophage [4]. Under steady-state conditions, few EBs egress from the marrow and these are usually cleared by macrophages in the spleen [6]. Smears of cultured human EBs often contain macrophages surrounded by clusters of EBs indicating that cultured EBs are capable of interacting with human macrophages derived from blood. However, the adhesion receptor profile of *ex vivo* generated EBs and the efficiency

of the interaction of these cells with murine macrophages are presently unknown.

Neildez-Nguyen et al. have reported that human carboxyfluorescein diacetate succinimidyl ester- (CFSE-) labeled EBs expanded *ex vivo* from $CD34^{pos}$ cells isolated from cord blood differentiate into red blood cells when transfused to NOD/SCID mice [7]. The transfusion protocol included coadministration of exogenous human erythropoietin (EPO) and intraperitoneal administration of packed human red cells to block the reticuloendothelial system (leading to transient functional splenectomy). Initially, human $CFSE^{pos}$ cells were detected in bone marrow, liver, spleen and lung of the transfused animals and, beginning at day 4, also in peripheral blood. However, these experiments have been viewed with skepticism because human $CD34^{pos}$ cells engrafted in immunodeficient mice typically generate robust levels of lymphoid and myeloid cells but barely detectable numbers of erythroid cells [8]. These data have been interpreted as evidence that the murine microenvironment is not permissive for maturation of EBs. Although the molecular structures of human and murine EPO are very similar, it has been suggested that murine EPO may fail to support optimal maturation of human EBs because it does not induce dimerization after binding to the human receptor [9]. This hypothesis was indirectly supported by Nicolini et al who reported that treatment with human EPO after transplantation greatly increases the generation of human erythroid cells when human $CD34^{pos}$ cells are injected into immunodeficient mice [10]. However, the relative contribution of administration of human EPO and packed human red cells to the success of the animal model for human transfusion developed by Neildez-Nguyen et al. [7] has not been clarified as yet.

The aim of our study was to test whether *ex vivo* generated EBs express the adhesion receptor profile necessary to complete their maturation *in vivo* and whether inhibition of the interaction between *ex vivo* generated EBs and splenic macrophages will permit establishment of an *in vivo* model for functional evaluation of *ex vivo* expanded human EBs.

2. Materials and Methods

2.1. Human Subjects. Low volume cord blood units (CB) were obtained from the New York Blood Center (New York, NY, USA). Peripheral blood (PB) was collected from normal adult donors at the transfusion center of "La Sapienza" University (Rome, Italy). Both specimens were collected according to guidelines established by institutional ethical committees and provided as deidentified samples.

2.2. Mice. 12-week-old female NOD/SCID/IL2Rγ^{null} mice were purchased from Jackson laboratory and housed in the animal facility of Memorial Sloan-Kettering Cancer Center. All murine studies were performed according to institutional animal care and use committee approved protocols.

2.3. Cell Preparation. Mononuclear cells (MNCs) from CB and AB were obtained by centrifugation over Ficoll-Hypaque (Amersham Pharmacia Biotec, Uppsala, Sweden). $CD34^{pos}$ cells from CB were separated using the Human Cord Blood CD34 Selection Kit (STEMCELL Technologies Inc, Vancouver, BC, Canada), as described by the manufacturer.

2.4. Expansion of Human Erythroblasts. $CD34^{pos}$ cells and MNC (5×10^4 and 10^6 cells/mL, resp.) were cultured under human erythroid massive amplification (HEMA) conditions in a proliferative phase stimulated with stem cell factor (SCF, 10 ng/mL, R&D System, Minneapolis, MN, USA), erythropoietin (EPO, 5 U/mL, Epogen, Amgen, Thousand Oaks, CA, USA) and interleukin 3 (IL-3, 1 ng/mL, Biosource, San Jose, CA, USA), dexamethasone and estradiol (both at 10^{-6} M, Sigma, St. Louis, MO, USA) up to 10 days and subsequently in a differentiative phase stimulated with EPO (5 U/mL), insulin (10 ng/mL, Calbiochem, La Jolla, CA, USA), and $FeSO_4$ (10^{-6} M, Sigma) up to 4 days, as previously described [11, 12].

2.5. Colony Forming Assay. The colony forming ability of sorted cells was evaluated in standard semisolid methylcellulose cultures (40%, Fluka Biochemika) stimulated with SCF (10 ng/mL), IL-3 (10 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF, 10 ng/mL), granulocyte colony-stimulating factor (G-CSF, 100 ng/mL) and EPO (5 U/mL). The cultures were incubated at 37°C in a fully humidified 5% pCO_2 atmosphere and scored after 2 days for colony forming unit erythroid (CFU-E) and after 8 days for burst forming unit erythroid (BFU-E) and colony forming unit granulocyte-macrophages (CFU-GM) colonies.

2.6. Cell Viability and Phenotypic Analysis. Cell numbers and viability were assessed by microscopic evaluation after trypan blue (Boston Bioproducts, Ashland, MA, USA) staining. Erythroid cells were identified using standard morphological criteria by visual examination of cytocentrifuged cell preparations (Cytospin 3, Shandon, Astmoor, England) stained with May-Grünwald-Giemsa (Fisher Scientific, Pittsburg, PA, USA) using the Axioscope light microscope equipped with a Coolsnap video camera (Zeiss, Oberkochen, Germany). For flow cytometric analyses, cells were suspended in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline, supplemented with 1% BSA, stained with either phycoerythrin- (PE-) conjugated CD36 (antithrombospondin receptor) [13], -CD184 (CXCR4), -CD162 (PSLG1), -CD49d (VLA-4), or allophycocyanin- (APC-) conjugated CD235a (anti-glycophorin A), pan hematopoietic human- (FITC-) conjugated CD45 or appropriate isotype controls (all from Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). For GFP determinations, autofluorescence of untreated cells was analysed as negative control. Fluorescence was analyzed with the FACS Canto (Becton Dickinson Biosciences) equipped with three air-cooled and solid-state lasers (488 nm, 633 nm and 407 nm). Dead cells were excluded by propidium iodide (PI, 5 μg/mL, Sigma) staining.

2.7. Fluorescence Labelling of Human EBs. CFSE Labeling. EBs obtained at day 11 of HEMA cultures were stained with

CFSE (10 μM, Invitrogen, Carlsbad, CA, USA) as described [14] and cultured for 24 additional hrs under HEMA conditions. *Retrovirally mediated labeling*. CB CD34^{pos} cells were cultured for 3 days in X-VIVO 10 media (Lonza Walkersville, Walkersville, MD, USA) containing thrombopoietin (TPO, 100 ng/mL), SCF (100 ng/mL), Flt3 Ligand (Flt3L, 100 ng/mL), IL-3 (20 ng/mL), and the PG-13 retroviral producer supernatant [15, 16] which contains the membrane anchored form of the Gaussia luciferase enzyme (extGLuc) [16] was added twice 24 hrs apart. The transfected CD34^{pos} cells were then cultured under HEMA conditions for 6 additional days. An aliquot of nontransduced CD34^{pos} cells from the same isolation was cultured in parallel under HEMA condition as control.

2.8. Transfusion Protocol. Two protocols were investigated. In the first experiment (See Supplementary Figure 1 in Supplementary Material available online at doi: 10.4061/2011/673752), 2 NOD/SCID/IL2Rγ^{null} mice were injected via the tail vein with 25×10^6 CFSE-labeled EBs. Twenty-four hrs prior to injection, mice were bled (500 μL) to increase endogenous EPO levels. In the second experiment (Supplementalry Figure 2), intact and splenectomized NOD/SCID/IL2Rγ^{null} mice (4 mice per group) were used. NOD/SCID/IL2Rγ^{null} mice were anaesthetized with isoflurane (Baxter, Deerfield, USA) and splenectomized after double ligation of the splenic artery and vein [17]. The muscle, peritoneum, and skin were closed in separate layers using sterile 5–0 absorbable suture. Intact and splenectomised mice were transfused via the tail vein with 25×10^6 retrovirally labeled EBs together with human EPO (20 U/mouse). Twenty four hours earlier, mice had been bled (500 μL). In both experiments, mice receiving culture media only served as negative controls and all the mice were sacrificed 4-5 days following transfusion for further analyses.

2.9. Immunocytochemistry. Smears of single cell suspensions from femur, spleen, and liver and cultured EBs prepared by cytocentrifugation (Shandon, Astmoor, England) were fixed with paraformaldehyde (3.7%, Electron Microscopy ScienceHatfield, PA, USA) for 30 min and rinsed 2 times with double distilled water. Smears were incubated with an antihuman glycophorin A antibody (CD235a, Abcam, Cambridge, MA, USA) and the immunoreaction detected with the avidin-biotin immunoperoxidase system (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) on slides counterstained with hematoxylin-eosin, as described by the manufacturer. Aliquots of injected EBs and smears from media-treated mice were used as positive and negative controls, respectively. Histological observations were carried out using a ZEISS AXIOSKOPE light microscope (Jena, Germany) equipped with a Coolsnap Videocamera. Enumeration of human CD235a^{pos} cells was performed by two separate investigators in a blinded fashion.

2.10. Bioluminescence Imaging. Bioluminescence was detected using a Xenogen IVIS Imaging System (Xenogen) as previously described [18]. Imaging was performed 24–48 hrs following intravenous injection of coelenterazine (250 μg) (Nanolight Technology) via the tail vein. Dorsal and ventral images of the animals were acquired with acquisition times in the range of 1 to 3 min. Field of view of 15, 20, or 25 cm with low, medium, or high binning in an open filter was utilized to maximize signal intensity and sensitivity. We obtained acquisition of image datasets and measurements of signal intensity through region of interest (ROI) analysis using Living Image software (Xenogen). Normalized images are displayed on each dataset according to color intensity.

2.11. Statistical Analyses. Results are expressed as mean ($\pm$SD) of at least three replicate experiments unless stated otherwise. Statistical analysis was performed by Anova using the computer software Origin 5.0 for Windows (Microcal Software, Inc., Northampton, MA, USA).

3. Results

3.1. Cord Blood MNC Generate Greater Numbers of Less Mature Erythroid Cells Than Adult Blood MNC under HEMA Conditions. Our group has previously identified a culture strategy for massive production of EBs *ex vivo* from both CB and AB MNC defined as human erythroid massive amplification (HEMA) culture [11]. Under these culture conditions, in the experiments included in this paper, AB MNC generated EBs with an FI = 15 while the number of EBs generated by CB MNC was 3-times greater (FI = 40 Figure 1).

By CD36/CD235a profiling, EBs generated under HEMA conditions may be separated into 4 classes of progressively more mature cells represented by CD36^{high}/CD235a^{neg} (class I, gate R1) and CD36^{high}CD235a^{low} (class II, gate R2) cells, which contain colony-forming unit-erythroid (CFU-E) and pro-EBs, and CD36^{high}CD235a^{high} (class III, gate R3) and CD36^{low}CD235a^{high} cells (class IV, gate R4), which contain basophilic-polychromatic and orthochromatic EBs, respectively (Supplemental Figure 3). BFU-E- and CFU-GM-derived colonies are not detectable in cells generated in HEMA culture from day 8 on (data not shown). At day 10 of HEMA culture, a significant proportion of EBs generated in cultures of AB and CB MNC are represented by immature class I and class II EBs (38 and 78%, resp.) (Figure 1 and Table 1). Exposure of Day 10 EBs derived from both AB and CB to EPO alone for 4 days increased the proportion of mature class III and IV EBs up to 52–70%. CB-derived EBs contained a lower fraction of mature cells than AB-derived EBs both in HEMA culture and when cultured with EPO alone (Figure 1 and Table 1).

Macrophage islands surrounded by 6–9 EBs were commonly detected in smears of both AB and CB derived EBs beginning at day 7 and up to day 10 of HEMA culture (Figure 1(c)). The frequency of these islands on the smears was correlated with the frequency of nonerythroid cells which had survived in culture (data not shown). The random nature the macrophages in HEMA culture prevented quantification of this phenomenon. Further studies in which cultured EBs will be exposed to monocytes purified from

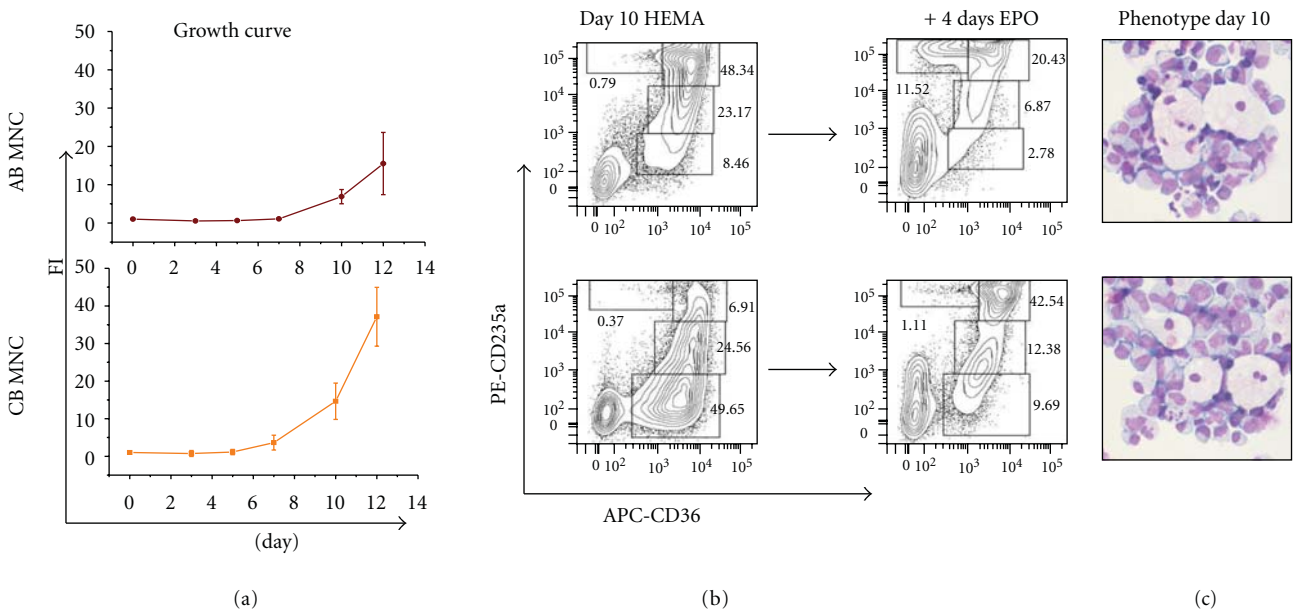


Figure 1: MNC from AB and CB generate great numbers of EBs under HEMA conditions. (a) Cell number (as Fold Increase, FI, with respect to day 0), (b) maturation profile (cytofluorimetric analysis on the basis of the expression of CD36 and CD235a), and (c) representative morphology (by May-Grunwald staining) of EBs generated in HEMA culture seeded with MNC from either CB or AB. The ability of CB and AB EBs obtained at day 10 to proceed along the maturation pathway after 4 days of culture in the presence of EPO only is also compared (b). The flow cytometric profile used to define EBs maturation is presented in Supplemental Figure 3. The flow charts are representative of those obtained in at least three independent experiments with MNC from different CB or AB donors. The numbers in the quadrants present the frequency of EBs in the gates R1 to R4. Frequencies obtained in multiple experiments are presented as mean (±SD) in Table 1.

Table 1: Frequency of EBs divided according to their maturation profile at day 10 of HEMA culture seeded with AB and CB MNC and after 4 days of additional culture in EPO alone to induce maturation. The gates used to define the 4 maturation classes used in this study are described in Supplemental Figure 3.

Maturation classes	AB MNC		CB MNC	
	Day 10 (%)*	Day 4 + EPO (%)	Day 10 (%)	Day 4 + EPO (%)
Class I ($CD36^{high}CD235a^{neg}$)	17.0 ± 6.2	1.6 ± 0.6	53.8 ± 8.9	11.9 ± 4.8
Class II ($CD36^{high}CD235a^{low}$)	21.7 ± 4.1	9.7 ± 1.1	21.5 ± 6.5	10.6 ± 4.5
Class III ($CD36^{high}CD235a^{high}$)	49.2 ± 2.4	55.1 ± 7.4	9.1 ± 4.6	49.5 ± 6.4
Class IV ($CD36^{low}CD235a^{high}$)	1.2 ± 1.1	15.8 ± 4.4	0.2 ± 0.6	1.7 ± 1.1

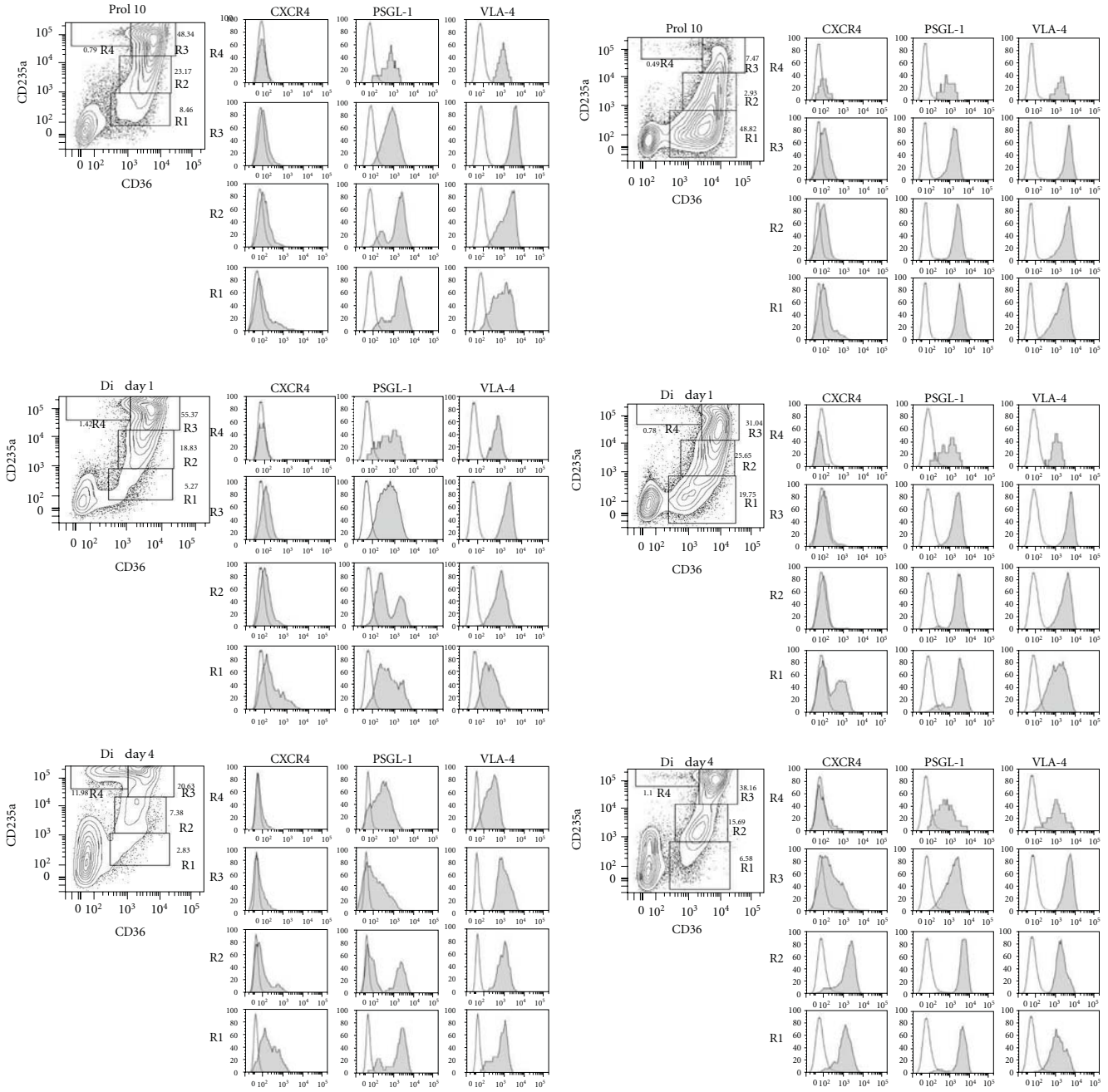
* Percentages were calculated with respect to the total cell population present in culture (EBs and non EBs).

human blood, marrow, and spleen are required to define the interaction between these cell populations *in vitro*.

3.2. Dynamic Pattern of Adhesion Receptor Profiling during the Maturation of AB- and CB-Derived EBs in Culture. The expression patterns of CXCR4, PSLG1, and VLA-4 on EBs obtained from AB and CB at day 10 of HEMA and induced to mature for 4 days in cultures containing EPO alone is presented in Figures 2, 3, and Supplemental Figure 4.

Expression of CXCR4 was barely detectable either in AB- or CB-derived EBs at day 10 of culture, irrespective of the stage of maturation (Figures 2 and 3). Exposure of these cells to EPO alone rapidly (within 1 day) activated CXCR4 expression first on immature cells (Class 1, R1) and then by day 4 also on cells expressing a more mature phenotype (Class 2 for AB EBs and Class 2 + 3 for CB EBs). The most mature Class 4 EBs (both from AB and CB) never expressed CXCR4 (Figures 2 and 3). The dynamic pattern of CXCR4 expression is visualized by the direct comparison of CD184 (CXCR4) and CD235a (glycophorin A) expression as AB and CB EBs matured in EPO alone culture presented in Supplemental Figure 4. Although similar patterns of CXCR4 expression were observed in EBs derived from AB and CB, a greater percentage (30–80% versus 15–40%) of CB-derived EBs expressed CXCR4 than AB EBs and the mean fluorescence intensity (MFI) was also greater for CB (range 1000–2500 versus 500–1000).

At day 10, both AB and CB derived EBs expressed high levels of PSLG1 regardless of their maturation stage (80–90% $PSLG1^{pos}$ cells with MFI in the range of 1000–4000) (Figures 2 and 3). The expression of PSLG1 was conserved when EBs were induced to mature with EPO alone for 4 days (Figures 2 and 3) although direct comparison of PSLG1 expression against CD235a indicated an association



(a) Adult Blood (b) Cord Blood

Figure 2: Adhesion receptor profiling during *in vitro* maturation of EBs expanded from AB (a) and CB (b). Three color flow cytometry analysis for CD36 and CD235a in combination with either CXCR4 (CD184), PSGL-1 (CD162), or VLA-4 (CD49d) of EBs obtained at day 10 of HEMA culture from AB and CB MNC (Prol 10) and at day 1 (Diff Day 1) and 4 (Diff Day 4) of maturation with EPO alone, as indicated. EBs were divided into 4 maturation classes defined by the levels of CD36 and CD235a expression identified by the gates R1 to R4 and corresponding to Class 1 to 4 (see Supplemental Figure 3 for further details).

between reduced expression of this receptor and maturation which was particularly evident in the case of AB-derived EBs (Supplemental Figure 4).

A great percentage (60–100%) of day 10 EBs expressed robust levels of VLA-4 (MFI in the range of 1000–3000 for AB EBs and 2000–6000 for CB EBs) regardless of their maturation status. The frequency of VLA4^{pos} EBs remained high when the cells were induced to mature with EPO for 4 days, although in the case of AB EBs the levels of VLA4 expression per cell decreased with time in culture when stimulated with TPO alone (Figures 2 and 4). The apparently high maturation-independent pattern of VLA4 expression in AB and CB EB was also observed by direct analyses of VLA4/CD235a expression patterns (Supplemental Figure 4).

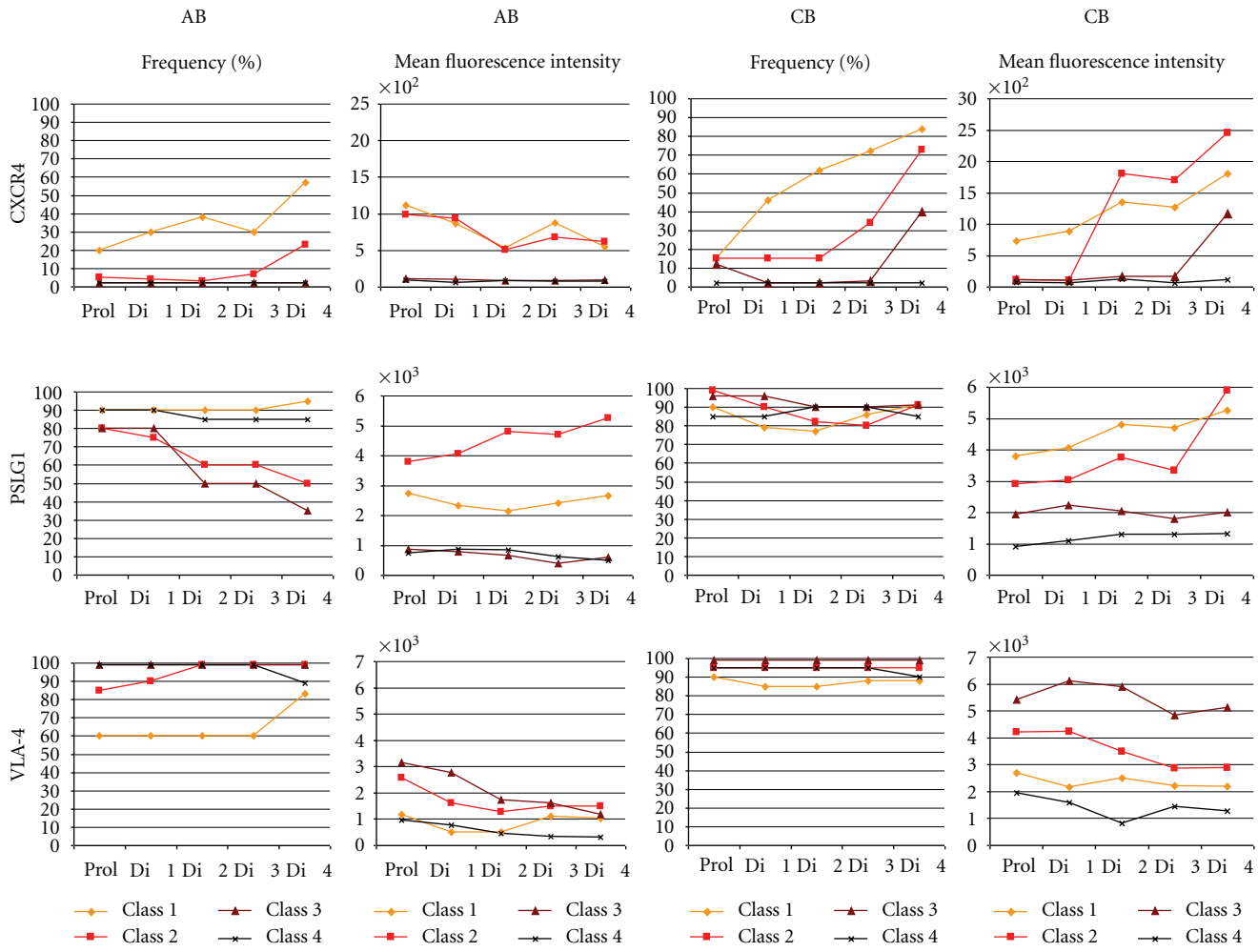


Figure 3: Time course of the frequency and MFI of CXCR4, PSL1, and VLA4 expression during *in vitro* maturation of EBs expanded from adult and cord blood, as indicated. Values observed with EBs at different stages of maturation are color coded: Class 1 (gate R1): light orange, Class 2 (gate R2): red, Class 3 (gate R3): brown, and Class 4 (gate R4): black (see also Supplemental Figure 1). The results correspond to the flow charts presented in Figure 2.

In conclusion day 10 EBs express low levels of CXCR4 but robust levels of PSLG1 and VLA4. Induction of these cells to mature by exposure to EPO alone rapidly activates CXCR4 expression but has modest or no effect on the expression of PSLG1 and VLA4, with the exception of a reduction in the number of PSLG1^{pos} class 2 and class 3 EBs observed in culture of AB-derived cells.

3.3. Cell Fate of Ex Vivo Expanded CB EBs in NOD/SCID/IL2Rγ^{null} Mice. The greater levels of adhesion receptor expression of CB EBs suggested that these cells might be more able than AB EBs to establish the cellular interactions necessary for maturation *in vivo* and therefore more suitable for use in animal models to analyze cell fate following transfusion. We chose the NOD/SCID/IL2Rγ^{null} mouse as the animal model for these experiments because it has been shown to readily support engraftment of human CD34^{pos} hematopoietic stem cells and represents a superior, long-lived model suitable for studies employing xenotransplantation strategies [20, 21].

In preliminary experiments, the fate of murine RFP-labeled EBs derived from liver and transfused into syngeneic thalassemic mice was determined. Murine RFP-labeled EBs ($30–150 \times 10^6$) transfused in unbled thalassemic mice generated undetectable or barely detectable levels of red cells. By contrast, when mice were bled 150 to 600 μL 24 hrs before transfusion, the same numbers of murine RFP-labeled EBs generated as many as 5×10^9-5×10^{10} red cells (A.R. Migliaccio and P. Frenette, unpublished observations). These results indicate that bleeding facilitates *in vivo* maturation of transfused murine EBs, probably by increasing endogenous EPO levels. On the basis of these data we decided to bleed animals 24 hr prior to transfusion with human EBs.

In the initial three experiments, CFSE-labeled EBs derived from CD34^{pos} CB cells after 11 days in HEMA culture were transfused via the tail vein (25×10^6 EBs per mouse) into NOD/SCID/IL2Rγ^{null} mice that were bled (500 μL) the day before (Supplemental Figure 3). Mice were sacrificed at day 4 after transfusion for detection of human CD235a^{pos} EBs and

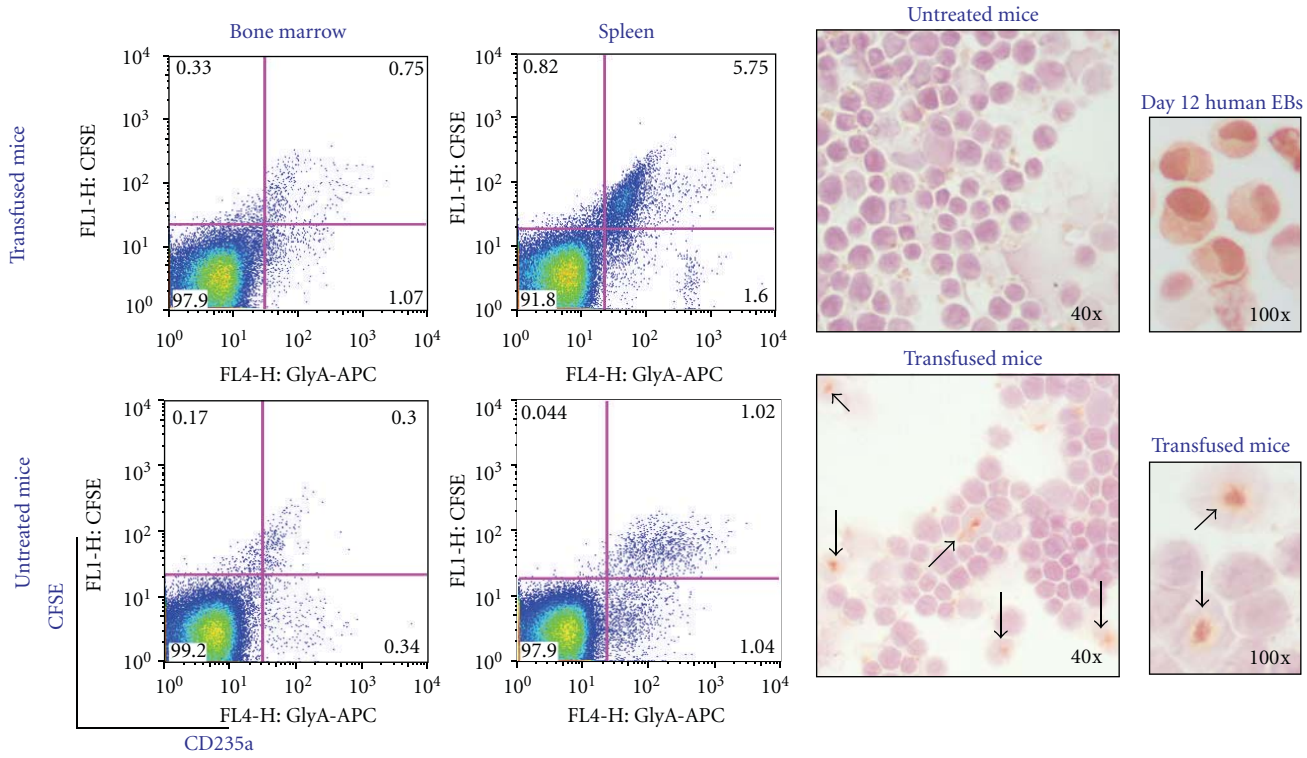


Figure 4: At day 4 after transfusion, human EBs are mostly detectable in spleen of NOD/SCID/IL2Rγ^{null} mice. (a) Flow cytometric analysis for human CD235a and CFSE expression of bone marrow and spleen cells from transfused and nontransfused (negative control) NOD/SCID/IL2Rγ^{null} mice. The results are representative of those obtained with 2 mice per group (see Table 2 for further details) and are representative of those observed in three separate experiments. (b) Immunocytochemistry for human CD235a of cells from the spleen of untransfused (top left panel) and transfused (lower panels) mice sacrificed 4 days after the human EBs transfusion, as indicated. Cells from control mice (top left panel) are negative while cultured human EBs (top right panel) are all positive. In transfused mice, human CD235a^{pos} cells (arrows) represented up to 18.2 ± 5.6% of total splenic cells. However, they were trapped inside larger CD235a^{neg} cells, probably macrophages of murine origin. Magnifications 40x and 100x, as indicated.

Table 2: Frequency and total number of cells expressing the human CD235a in bone marrow and spleen of NOD/SCID/IL2Rγ^{null} mice transfused 4 days earlier with CFSE-labeled EBs. Both CFSE-positive and CFSE-negative human CD235a^{pos} cells were detected in the organs of the animals after transfusion, an indication that some of the human Ebs had proliferated *in vivo*.

	CSFE$^+$CD235a$^+$ (%)	CSFE$^-$CD235a$^+$ (%)	CSFE$^+$+CSFE$^-$CD235a$^+$−control (%)	Total no. of CD235$^+$ cells/organ*
BM				
Control	0.3	0.34	0	
Transfused mice (2 mice)	0.53–0.75	1.1–1.07	0.99–1.18	$3.0–3.5 \times 10^6$
Spleen				
Control	1.02	1.04	0	
Transfused mice (2 mice)	3.34–5.75	1.3–1.6	2.57–5.29	$2.6–5.3 \times 10^6$

The frequency of human CD235a$^+$ cells was obtained after subtracting the background signal observed in untreated controls.
*The total number of cells per organ was calculated assuming that a mouse contain 300×10^6 BM and 100×10^6 spleen cells [19].

at day 21 for the presence of human hematopoietic cells of all lineages (CD45^{pos}).

At day 4, FACS analyses revealed the presence of 0.99–1.18% and 2.57–5.29% of CD235a^{pos} cells (both CFSE^{pos} and CFSE^{neg}) in bone marrow and spleen, respectively, of the transfused mice (Figure 4(a) and Table 2). The presence of CFSE^{neg}CD235a^{pos} cells indicated that some of the human EBs had undergone proliferation *in vivo* during the 4 days after transfusion. Assuming that the total number of cells in bone marrow and spleen of a mouse is 300×10^6 cells and $\sim 10^8$ cells, respectively [19], it is calculated that the bone marrow and spleen of the transfused animals contained

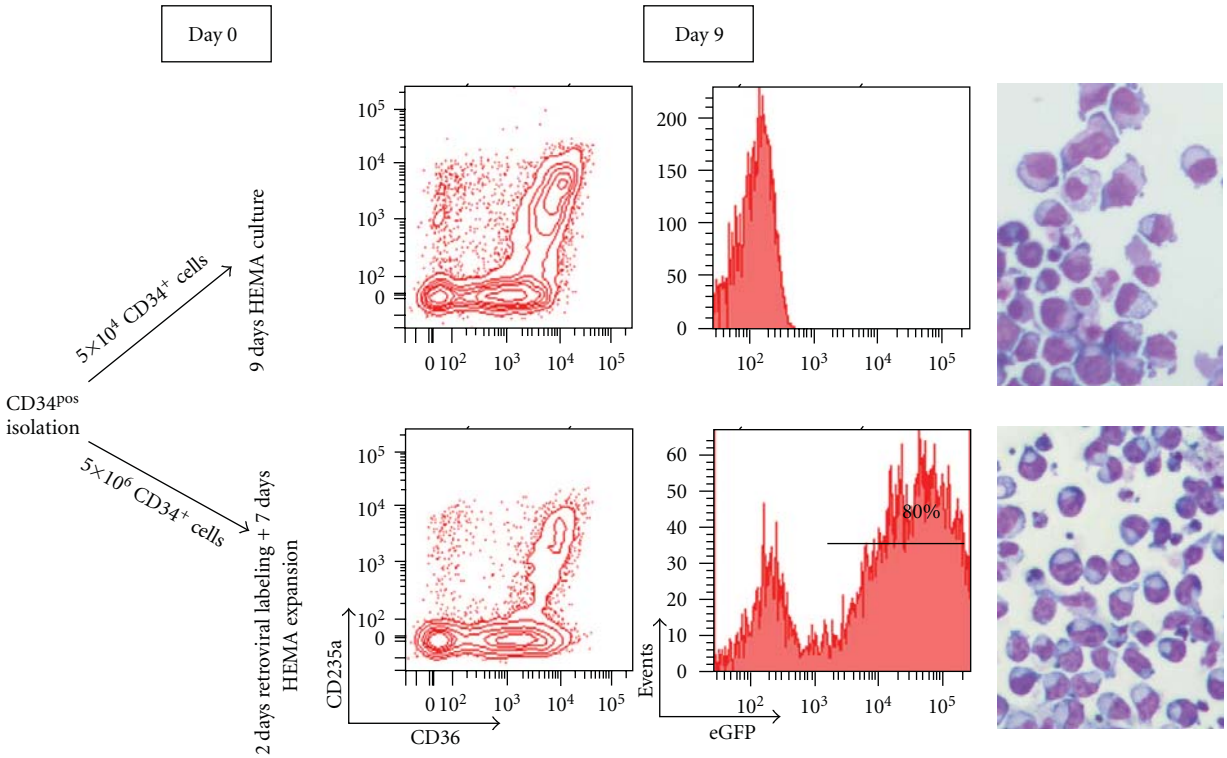


Figure 5: *Ex vivo* expanded extGLuc$^+$ EBs express high level of eGFP by FACS analyses. Maturation profile and transfection efficiency of nontransfected (as control, top panels) and transfected (bottom panels) EBs at day 9 of HEMA culture identified on the basis of CD36/CD235a and eGFP expression (on the left) and on the basis of morphology (May-Grunwald staining, on the right), magnification 40x. The transfected EBs express great levels of eGFP. The transfection did not alter the morphology of the cells but slightly reduced the expression of the CD235a probably due to interference with the GFP signal. At day 9, in transfected and control cultures the fold increase was 48-fold and 325-fold, respectively, and the viability was 98% in both cases.

$\sim 3.5 \times 10^6$ (1.7% of 3×10^8 cells) and 7×10^6 (6.8% of 10^8 cells) human EBs, respectively (Table 2). Therefore, the majority of human EBs lodged in the spleen. Immunocytochemical analyses for the expression of the human erythroid CD235a marker confirmed the presence of numerous (18% of the total cell population) human CD235a^{pos} cells in the spleen of the transfused animals (Figure 4(b)). However, the CD235a^{pos} cells were trapped inside larger CD235a^{neg} cells, probably macrophages of murine origin. By day 18, very few (0.01–0.06%) human CD235a^{pos} cells were detectable in the blood. By day 21, human CD45^{pos} cells were undetectable in marrow, spleen, and liver of the transfused NOD/SCID/IL2Rγ^{null} mice, confirming the colony forming data, which indicated that CB EBs expanded for 10 days under HEMA conditions no longer contain hematopoietic progenitor cells. These results suggest that human EBs lodged preferentially in the spleen where they were engulfed and probably destroyed by macrophages.

To determine if splenectomy would facilitate lodgment in the marrow, the fate of *ex vivo* expanded EBs transfused to splenectomized and intact NOD/SCID/IL2Rγ^{null} mice was compared. Cell biodistribution was analyzed by retroviral-mediated labeling with the external Gaussia luciferase genes (extGLuc), using bioluminescence imaging, and e green fluorescent protein (eGFP) was used for flow cytometry determinations of CD34^{pos} CB cells which were then expanded under HEMA conditions (see [16] and Supplemental Figure 2). The extGLuc enzyme is a newly engineered Gaussia luciferase which contains the CD8 transmembrane domain at its carboxy terminus. The presence of this domain allows membrane anchoring and cell surface retention of extGLuc, significantly enhancing the bioluminescent signal [16].

The expansion potential of retrovirally transduced and control CD34^{pos} CB cells is compared in Figure 5. By day 9, 5×10^4 unmanipulated CD34^{pos} CB cells generated 16×10^6 EBs (FI = 325-fold) while 5×10^6 CD34^{pos} CB cells which had been cultured for 3 days under conditions permissive for retroviral transduction (see Supplemental Figure 2) generated 240×10^6 EBs (FI = 48), the great majority of which (80%) expressed robust levels of eGFP (MFI > 5×10^4). The difference in FI between HEMA culture of unmanipulated and retrovirally transduced CD34^{pos} CB cells was not associated with changes in death rates, as evaluated by propidium iodide staining (data not shown) and may be related to the likelihood of underestimations of the low number of cells (5×10^4) used to initiate the cultures of unmanipulated cells. By FACS analysis, the majority of CD36^{pos} EBs derived from retrovirally transduced CD34^{pos} CB cells did not express CD235a. However, by morphological analysis the maturation stage of these cells was comparable to that of EBs derived from un-manipulated CB cells (Figure 5).

We believe that lower expression of CD235a by the retrovirally transduced EBs was a consequence of interference between the fluorescence signal of this antibody and that of eGFP.

Aliquots (25×10^6) of retrovirally transduced EBs were injected via the tail vein together with 20 U of human EPO in splenectomized and control NOD/SCID/IL2Rγ^{null} mice which had been bled (500 μL) the day before. Bioluminescent imaging was performed 24 and 48 hrs after transfusion. Twenty-four hrs after transfusion, the extGLuc bioluminescent signal was virtually undetectable, other than at the site of injection in intact mice (Figure 6). In contrast, significant levels of extGLuc signal were observed in legs and skull of splenectomized mice, an indication that human EBs may have homed to the BM. In these mice, significant levels of bioluminescent signal were also detected in the abdomen (Figure 6). These data demonstrate that in the absence of the spleen CB EBs lodge in marrow and liver.

Four days after transfusion, mice were sacrificed and the presence of human CD235a^{pos} cells in bone marrow and liver of the transfused NOD/SCID/IL2Rγ^{null} mice was analyzed by FACS. In the case of intact mice, great numbers (~15% of the total cell population) of human CD235a^{pos} cells were detected in spleen while human CD235^{pos} cells in marrow and liver were barely detectable. By contrast, in splenectomized animals, great numbers of human CD235a^{pos} cells were detectable in bone marrow (25%) and liver (7%) (Figure 7). Interestingly these cells did not express CD36, an indication that they had matured *in vivo*. However, red cells in the blood remained low both in intact and splenectomized mice (data not shown).

These results indicate that the removal of the spleen favors homing of human EBs to the bone marrow of NOD/SCID/IL2Rγ^{null} mice.

4. Discussion

An important issue for the quality and safety of cellular products lies in the development of a surrogate assay for a cell product that is as close as possible to the final product intended for therapeutic use. Short of a phase I clinical study, the optimal evaluation of human cell products is represented by preclinical studies performed in immunodeficient mouse models. In our experience, such an approach can not only help validation studies and the establishment of a potency assay, but also help guide the development of the production process itself by defining optimal cell purification procedures, culture conditions, and scale-up systems. This study focused on establishing a xenogeneic model to test EBs expanded *ex vivo* under HEMA conditions as potential transfusion products. The availability of such a model may advance other areas of research by providing a single method to compare the functional status of EBs derived from primary hematopoietic stem/progenitor cells with those derived from other stem cells sources such as human embryonic stem cells and induced pluripotent stem cells.

This study demonstrates that the maturation of *ex vivo* generated CB- and AB-derived EBs is associated with a dynamic pattern of adhesion receptor expression. At day 10–11 of HEMA culture, the majority of immature AB- and CB-derived EBs expressed low levels of CXCR4 and high levels of both VLA-4 and P-selectin ligand 1 (Figures 2 and 3). Once induced to mature by exposure to EPO alone, these EBs rapidly (within 24 hrs) activated expression of CXCR4 while retaining expression of VLA-4 and down-modulating expression of PSGL-1. By day 4 of maturation culture, the majority of EBs had progressed to the mature CD235a^{high}CD36^{high} phenotype (Figure 1 and Table 1) and had reduced expression of all the adhesion receptors. Minor ontogenetic differences where observed since CB EBs expressed greater levels of CXCR4, PSGL-1, and VLA4 and activated CXCR4 expression upon EPO exposure more readily than AB EBs. While the adhesion receptor pattern expressed by human erythroblasts maturing *in vivo* is not known, these data are available in mice. Immature murine EBs obtained from both fetal liver and adult bone marrow express robust levels of CXCR4, PSGL-1 and α4 integrins. These cells lose PSGL-1 expression and retain CXCR4 and VLA-4 expression with maturation [22, 23]. These high levels of CXCR4 represent the major difference between the pattern of adhesion receptors expressed by murine cells and those expressed by *ex vivo* expanded human EBs. The low levels of CXCR4 expression in human EBs generated in HEMA and the fact that these cells rapidly, within 24 hrs, acquire CXCR4 expression upon exposure to EPO alone are consistent with reports that CXCR4 expression is suppressed by dexamethasone [24], one of the components of HEMA culture [11]. On the other hand, the low levels of CXCR4 expression observed when human EBs mature (Class 4) is expected since these cells are supposed to downregulate expression of CXCR4, the major receptor involved in cell retention in the marrow [25], as part of the process which allows them to egress from the marrow [6].

A separate set of experiments evaluated the fate of CFSE- or retrovirally-labeled CB-derived human EBs when transfused in NOD/SCID/IL2Rγ^{null} mice. Bleeding and/or infusing the animals with human EPO to increase EPO levels greatly increased the detection of human EBs in the animals. By day 4 after transfusion, 1.5–5% of the cells in BM and spleen, respectively, of the animals were positive for human CD235^{pos} by flow cytometry (Figure 4(a)). However, by immunohistochemistry, high numbers (18%) of cells positive for human CD235a by immunohistochemistry were detectable in the spleen (Figure 4(b)). The human CD235a^{pos} cells, however, appeared trapped inside larger CD235a cells suggesting that they had probably undergone phagocytosis by murine macrophages. These results indicated that preferential splenic lodgment and clearance of human EBs may represent a barrier to the use of mouse models for functional evaluation of human transfusion products.

CXCR4 plays a major role in cell lodgment and retention in bone marrow [25] but is dispensable for cell homing in spleen [17, 19]. We hypothesized that failure of human CB EBs to lodge in bone marrow may be determined by unbalanced expression of CXCR4 with respect to the expression of other adhesion receptors, such as PSGL1, VLA-4, and CD36 (CD36 specifically guide cell interaction with the endothelial system [26]). Therefore, removal of

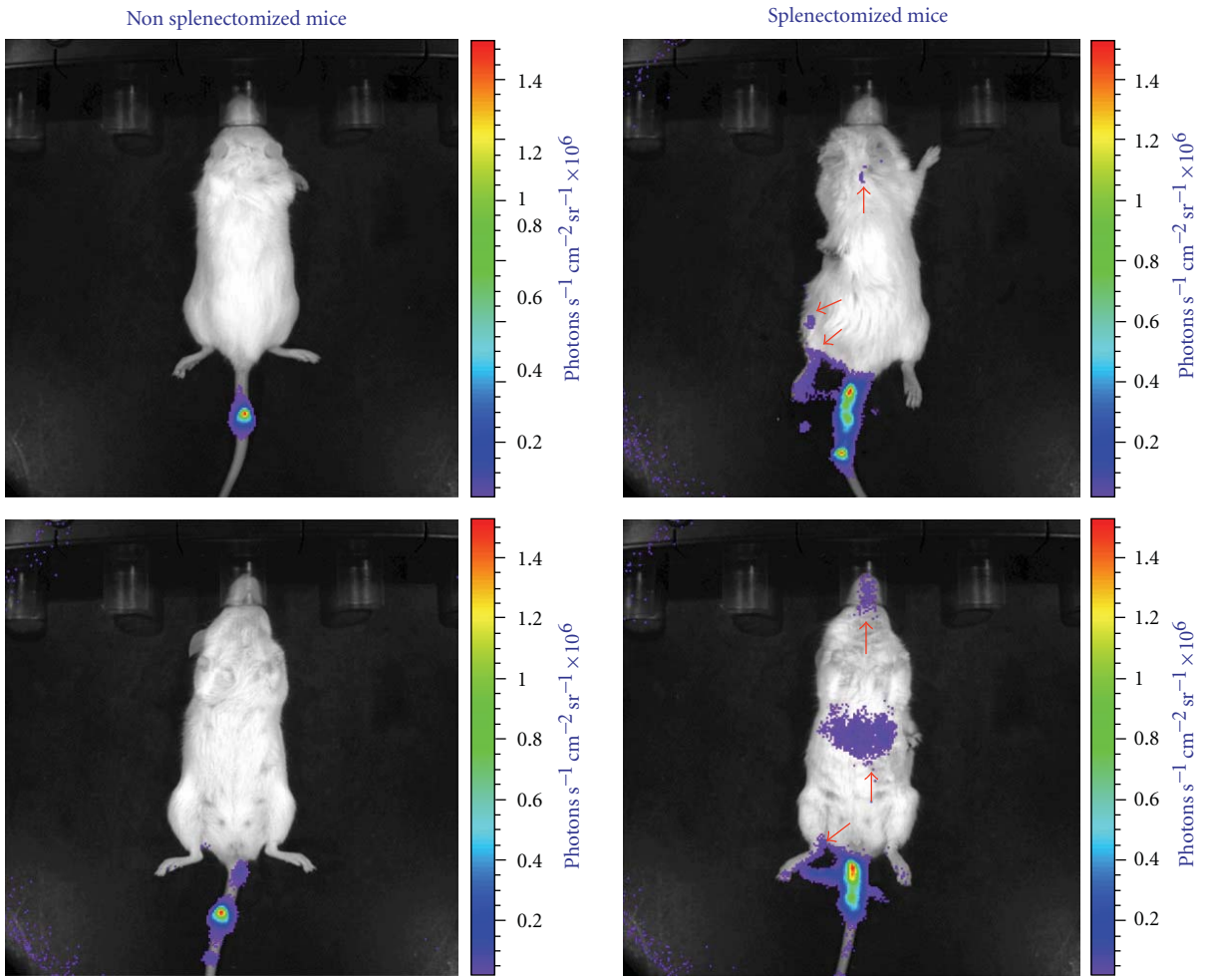


Figure 6: Significant level of extGLuc signal is detectable in splenectomized mice 24 hrs after transfusion. Bioluminescent imaging of nonsplenectomized (left panels) and splenectomized (right panels) mice 24 hrs after injection with extGLuc$^+$EBs, as indicated. Dorsal and ventral imaging is presented in the top and bottom panels, respectively. In intact mice, the bioluminescent signal was virtually undetectable other than at the site of injection (the tail). In the splenectomized mice, significant signal levels were observed in limbs (lower extremity), skull (indicating marrow localization), and abdomen (indicating liver localization because the spleen had been removed). Arrows indicate the positive signal. Data are representative of those observed with 4 mice per experimental point.

the spleen, by reducing cell interactions mediated by these receptors, may favor lodgment in the marrow of EBs expressing low levels of CXCR4. This hypothesis was tested by comparing lodgment of human EBs in the bone marrow of intact and splenectomized NOD/SCID/IL2Rγ^{null} mice. Bioimaging, followed by flow cytometry analyses, indicated that in the absence of the spleen retrovirally-labeled human EBs lodge in greater numbers in the marrow of the transfused animals (Figure 6). These results indicate that removal of the spleen greatly improves the survival of human EBs in mice. However, although signs of *in vivo* maturation were detected (detection in the marrow of cells positive for human CD235a but negative for CD36), the numbers of human erythrocyte detected in the blood of the transfused animals remained low.

Since CXCR4 expression was likely upregulation when human EBs were transfused *in vivo* (the cells were no longer exposed to DXM but exposed to human EPO), it is possible that, in addition to splenectomy, lodgment of human EBs in the marrow of the transfused animals may be improved by experimental approaches designed to increase CXCR4 expression, such as short pretransfusion exposure to EPO *in vitro* (Figures 2 and 3). Since cycling cells are known to have inferior homing [27, 28], in addition to increasing CXCR4 expression, these pretreatments may also improve homing by reducing the number of proliferating EBs. Indirect proof that CXCR4 over-expression may facilitate homing of human EBs in the marrow was recently obtained. Although treatments with many chromatin modifying agents decreases CXCR4 expression [29] the HDAC inhibitor valproic acid (VPA) is well known for its ability to upregulate CXCR4 expression while downmodulating expression of α-integrins and other adhesion receptors in several cell types [30, 31]. Recently Chaurasia et al. [32] have demonstrated that *ex vivo* expanded EBs obtained from VPA-treated CB CD34^{pos} cells colonize, in addition to the spleen, the bone marrow when transfused into NOD/SCID and NOD/SCID/IL2Rγ^{null} mice which had been functionally splenectomized by blocking the reticuloendothelium system with intraperitoneal injection

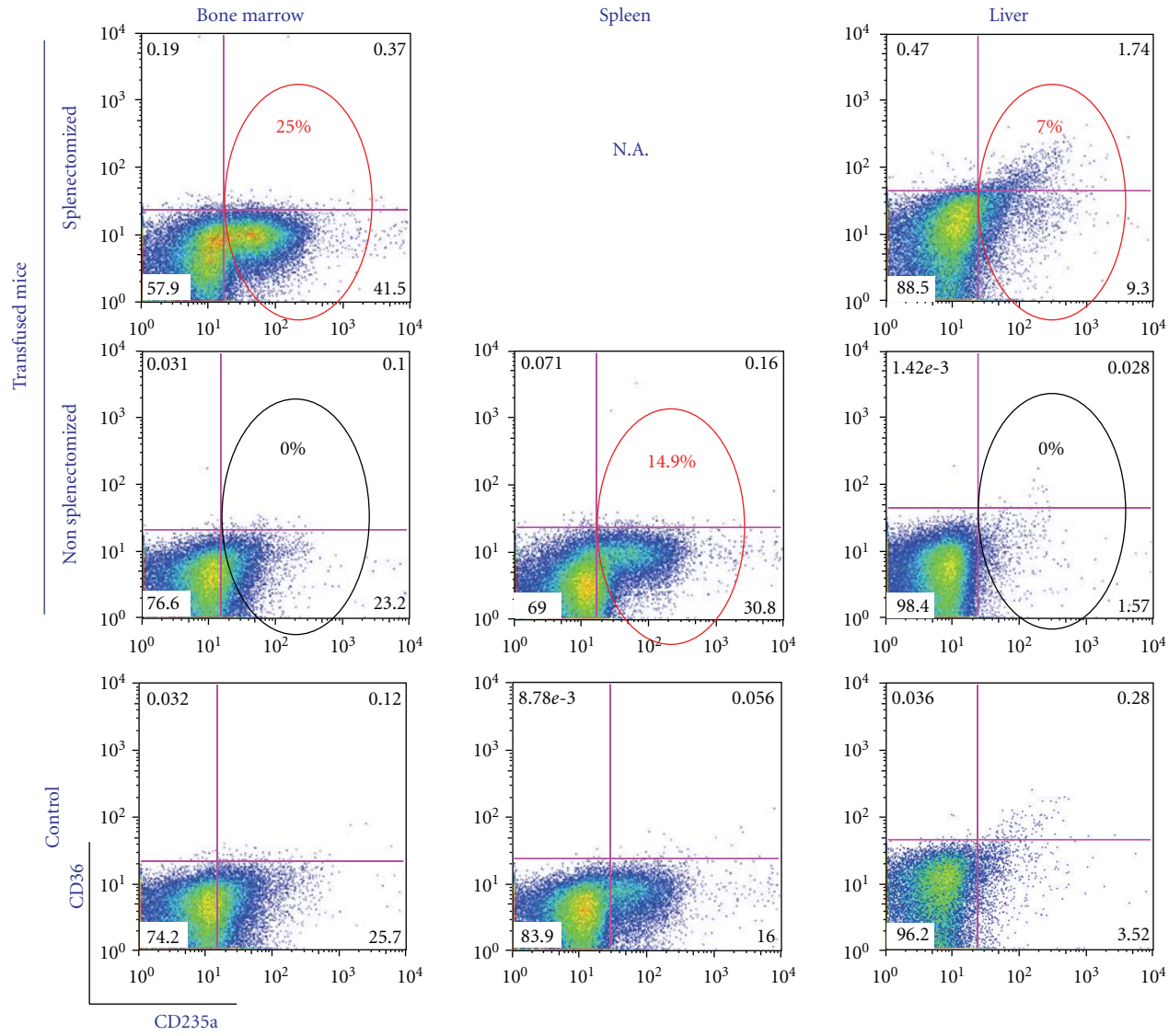


FIGURE 7: By flow cytometry of human CD36/CD235a expression, human CD235a^{pos} cells were detectable in marrow and liver of splenectomized mice 4 days after transfusion. Flow cytometry analysis of bone marrow (left panels), spleen (middle panels) and liver (right panels) from splenectomized (top panels), non splenectomized (middle panels), and nontransfused controls (bottom panels) of representative mice are shown. The small numbers indicate the frequency of the cells detected within the respective quadrant. Values within circles correspond to the percentage of human cells calculated by subtracting the frequency detected in the negative controls in the corresponding bottom panels. The red circles indicate the frequency of human cells. Of note, the majority of the human CD235a positive cells were negative for CD36, an indication that the human EBs had matured *in vivo*. Similar results were obtained by analyzing the cells for eGFP expression. Data are representative of those obtained with 3 mice per experimental point.

of human type O+ red cells, as first reported by Neildez-Nguyen et al. [7]. However, since Chaurasia et al. [32] did not measure the levels of adhesion receptors expressed by VPA-treated EBs, it is not possible to assess the relative contribution of functional splenectomy and/or upregulation of CXCR4 (and/or down regulation of VLA-4 and PSGL-1 expression) to the improved lodgment of these cells in the marrow. Further studies are necessary to clarify this issue in order to establish the optimal pre-treatment (splenectomy versus functional reticulo-endotheium blockade) of the mouse model for functional evaluation of transfusion products generated by different stem cell sources.

A major difference between this study and the report by Chaurasia et al. [32] is represented by the levels of human erythrocytes detected in the blood of the transfused animals. In our study, human erythrocytes were barely detectable in the blood at any time point after transfusion while in the study of Chaurasia et al. discrete numbers of mature erythrocytes were detectable in the circulation starting from day 7 after transfusion (15% in NOD/SCID mice and 30% in the improved NOD/SCID/IL2Rγ^{null} model). However, progenitor cells were no longer detectable among the human EBs expanded under HEMA conditions in this study (CD45^{pos} cells were undetected in the transfused mice)

while representing a significant proportion (7%) of the VPA-treated cells transfused by Chaurasia et al. [32]. It is therefore possible that in this case release of human erythrocytes in the blood was favored by the presence of human macrophages generated by human hematopoietic progenitors in the animal model. Another possibility, however, is that in our experiments, the erythrocytes released in the blood were cleared by the immune system.

Immunological responses have represented a barrier to the development of mouse models for functional assessment of human red cell products [33]. These barriers include cell-mediated and humoral immune responses triggered by antigens specifically expressed on red cells. Since some of the epitopes present on red cell antigens are expressed also by proteins on the membrane of bacteria presents in gut flora [34], red blood cells may trigger immune reactions leading to red cell lyses even in the absence of any prior sensitization. NOD/SCID, and the improved NOD/SCID/IL2Rγ^{null} mice, lack cell mediated and immunoglobulin-mediated humoral responses (these mice do not have B cells). In addition, a great proportion of the immune-response against red blood cell antigens are often initiated in the spleen [35] which was transiently inactivated in [7, 32] and permanently removed in our study. The complement system plays a significant role in the humoral mediated red cell clearance in vivo. Since NOD/SCID/IL2Rγ^{null} mice lack C5 [21], they have reduced activation levels of the alternative complement pathway. However, these mice express C3 [21] and may activate the classical complement pathway responsible for lysis of the human CD55 and CD59 deficient red cells observed in paroxysmal nocturnal hemoglobinuria (PNH) [36], preventing the use of NOD/SCID/IL2Rγ^{null} mice for development of animal models for PNH studies. Human CB EBs expanded *ex vivo* under HEMA conditions express CD55 (53–87% positive EBs, MFI = 110–330) and CD49 (54–70%, MFI = 1,900–2500). However, the expression of these antigens greatly decreased when the cells were induced to mature with EPO for 5 days *ex vivo* (CD55: 17–27% EBs, MFI = 30–32; CD59: 28–56%, MFI = 20–76, data from 2 separate cultures) (A.R.Migliaccio and C.Whitsett, unpublished data). It is therefore possible that *ex vivo* expanded human EBs also lost CD55/CD59 expression during their maturation in the marrow of NOD/SCID/IL2Rγ^{null} mice making their progeny susceptible to C3-mediated lysis. Since C3 deficient mice have been recently developed [37], we predict that C3-deficient NOD/SCID/IL2Rγ^{null} mice will represent a better read-out animal model for the presence of human erythrocytes in the blood.

In conclusion, we describe data indicating that splenectomized NOD/SCID/IL2Rγ^{null} mice represent a surrogate *in vivo* model to assess the potency of transfusion products generated *ex vivo* from different stem cell sources.

Disclosure

All the authors have read the paper, concur with its content, and state that its content has not been submitted elsewhere. The authors have no conflict of interest to disclose.

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Review Article

Plasticity of Cells and *Ex Vivo* Production of Red Blood Cells

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The supply of transfusable red blood cells (RBCs) is not sufficient in many countries. If transfusable RBCs could be produced abundantly from certain resources, it would be very useful. Our group has developed a method to produce enucleated RBCs efficiently from hematopoietic stem/progenitor cells present in umbilical cord blood. More recently, it was reported that enucleated RBCs could be abundantly produced from human embryonic stem (ES) cells. The common obstacle for application of these methods is that they require very high cost to produce sufficient number of RBCs that are applicable in the clinic. If erythroid cell lines (immortalized cell lines) able to produce transfusable RBCs *ex vivo* were established, they would be valuable resources. Our group developed a robust method to obtain immortalized erythroid cell lines able to produce mature RBCs. To the best of our knowledge, this was the first paper to show the feasibility of establishing immortalized erythroid progenitor cell lines able to produce enucleated RBCs *ex vivo*. This result strongly suggests that immortalized human erythroid progenitor cell lines able to produce mature RBCs *ex vivo* can also be established.

1. Introduction

Transfusion therapies involving RBCs, platelets, and neutrophils depend on the donation of these cells from healthy volunteers. However, unpredictable adverse results can ensue from transfusion therapies because of the donation of cells from a very large number of anonymous volunteers. For example, transfusion of blood products that include hazardous viruses or prions is difficult to prevent completely, because, occasionally, tests to detect them yield pseudo-negative results. There is little doubt that RBCs, platelets, and neutrophils produced *ex vivo* would be candidate materials to replace cells donated from such a large group of anonymous individuals.

The development of technologies such as PCR and gene knockout that enable the manipulation of an organism's genetic material contributed tremendously to progress in the life sciences in the final decades of the last century. This century looks to continue this progress through the development of further new technologies relating to cell manipulation.

2. Discovery of Plasticity in Terminally Differentiated Cells

It was believed for a long time that epigenetic modifications in differentiated somatic cells were irreversible. This meant that terminally differentiated cells could never return to being immature cells. However, in 1962, it was reported that the nuclei of somatic cells of an amphibian (frog) were reprogrammed following transfer into enucleated unfertilized eggs [1]. Following transfer of a somatic cell nucleus, the egg could undergo cell division and differentiate to produce an adult frog. This result clearly indicated that epigenetic modifications in terminally differentiated somatic cells were reversible. Dr. John Gurdon, who performed this groundbreaking study, received the Albert Lasker Basic Medical Research Award in 2009.

Initially, many biologists believed that this reversibility of epigenetic modifications in terminally differentiated cells was restricted to amphibian somatic cells and did not occur in mammalian somatic cells. However, in 1997, a nuclear transfer experiment in sheep in which somatic nuclei were

transferred into unfertilized eggs showed that epigenetic modifications in terminally differentiated mammalian somatic cells were also reversible [2]. This experiment famously resulted in the birth of the first live cloned sheep, named "Dolly".

3. Immortalization of ES Cells

The methodology for isolating and culturing mouse ES cells was first developed in 1981 [3] and has aided research in a wide range of biological studies. Dr. Martin Evans, who developed the technology for establishing mouse ES cell lines, was awarded a Nobel Prize in 2007 together with Dr. Mario Capecchi and Dr. Oliver Smithies, who developed homologous recombination technology in mouse ES cells. As a result of these technical advances, functional analysis of genes has progressed considerably using mice with gene knockouts or other genetic modifications.

It is well known that mouse cells can be immortalized simply by continuous *in vitro* culture, for example, using the so-called "3T3 protocol". One widely exploited example of an immortalized cell line is NIH3T3, which continues to be used in a wide range of experiments. In contrast, it is not possible to immortalize human somatic cells in a similar manner and this difficulty gave rise to the widespread assumption that it would not be possible to establish human ES cell lines. However, in 1998, 17 years after the first establishment of mouse ES cell lines, it was reported that human ES cell lines could also be produced by continuous *in vitro* culture [4].

4. Therapeutic Cloning

The ability to reprogram mammalian somatic cells by nuclear transfer and to establish human ES cell lines stimulated medical scientists to investigate the creation of ES cell lines using nuclear transfer as a potential means of achieving "therapeutic cloning". If this technology could be established as a viable therapy, then patients who would benefit from somatic cell transplantation could be treated with nuclear-transferred ES cells produced using their own somatic cells, which would avoid the possibility of transplant rejection as the cells possess the same major histocompatibility (MHC) antigens as host tissue.

Although an earlier report of successful therapeutic cloning by a group in Korea proved false, it was recently reported that primate ES cell lines have been established by nuclear transfer technology [5]. Since unfertilized primate eggs are much more fragile than those of rodents, it may still take some time to establish the technology for use in human therapeutic cloning. However, the prospect of using such therapy no longer seems to be so distant.

5. A Search for Alternative Technologies to Therapeutic Cloning

An important limitation to the use of therapeutic cloning is that it requires unfertilized eggs. Human eggs are very difficult to obtain, and, moreover, their use for this purpose also raises serious ethical issues. For these reasons, a search has been initiated for alternative methodologies that avoid nuclear transfer. One approach has been to search for factors in unfertilized eggs that may be required for the reprogramming of transferred somatic nuclei. Another avenue of research has been to elucidate which genes specifically function in ES cells, since these genes may maintain the undifferentiated state of ES cells, and thus might be able to induce reprogramming of nuclei in terminally differentiated somatic cells.

The research group led by Dr. Shinya Yamanaka reported the first success in the latter approach. They were able to induce differentiated mouse somatic cells to become pluripotent stem cells by the application of four defined factors [6]. The enforced expression of the transcription factors Oct3/4, Sox2, Klf4, and c-Myc in terminally differentiated somatic cells induced cellular reprogramming and changed the cells into ES-like pluripotent stem cells. These reprogrammed cells were named "induced pluripotent stem (iPS) cells". Subsequently, in the year after establishment of human iPS cell lines was first reported, several other groups also succeeded with this methodology [7–10]. Dr. Shinya Yamanaka, who developed the method, was given the Albert Lasker Basic Medical Research Award in 2009 together with Dr. John Gurdon.

The mechanisms underlying the reprogramming of terminally differentiated somatic cells following the enforced expression of the four factors remain to be elucidated. It is now known that expression of these factors after exogenous introduction is completely suppressed in established iPS cells. Thus, the factors seem to be required only for the reprogramming process but not for maintenance of pluripotency. Regardless of the mechanisms involved, this discovery clearly indicated that terminally differentiated somatic cells could be reprogrammed without nuclear transfer into unfertilized eggs and opened a new dawn for therapeutic cloning [11–13].

6. *Ex Vivo* RBC Production from Hematopoietic Stem/Progenitor Cells

The rapid progress relating to cell manipulation technology described above prompted many scientists in various fields to consider cell therapy using the cells produced and/or manipulated *ex vivo*. The scientists in the field of hematology are naturally aiming to produce the terminally differentiated blood cells able to use in the clinic. RBC transfusion was the first transplantation procedure to be established and is now routine and indispensable for many clinical purposes. However, in many countries, the supply of transfusable materials is not always sufficient. In Japan, for example, the supply of RBCs with an AB/RhD(−) phenotype is always lacking, because individuals with this RBC phenotype are rare. This problem of inequalities in the supply and demand for RBCs has stimulated interest in the development of *ex vivo* procedures for the generation of functional RBCs from hematopoietic stem cells or progenitor cells.

The hematopoietic stem cells that are present in bone marrow and umbilical cord blood are promising materials

for *ex vivo* production of RBCs. In particular, umbilical cord blood cells are readily available, as they are usually discarded. Provided the mother of a neonate consents to use of the umbilical cord, this material can provide a useful resource without any further complicating critical or ethical concerns.

Neildez-Nguyen et al. reported that human erythroid cells (nucleated cells) produced on a large scale *ex vivo* could differentiate *in vivo* into enucleated RBCs [14]. They developed a culture protocol to expand $CD34^+$ erythroid progenitor cells based on a 3-step expansion of cells by sequential supply of specific combinations of cytokines to the culture medium [14]. This study demonstrated that erythroid progenitor cells produced *ex vivo* from hematopoietic stem and/or progenitor cells could have a clinical application as an alternative method for transfusing terminally differentiated RBCs. Later, the same group described an *ex vivo* methodology for producing fully mature human RBCs from hematopoietic stem/progenitor cells [15]. The enucleated RBCs produced by this approach are potentially even more valuable, as they should be functional immediately after transfusion without requiring time for enucleation as is necessary with the erythroid cells.

7. Enucleation of Erythroid Progenitor Cells

The mechanism of erythroblast enucleation, a critical step in RBC production, has not yet been fully elucidated [16, 17]. The role of the interaction of erythroblasts with other cells, such as macrophages, is a controversial topic in this process [18–22]. Macrophages in retinoblastoma gene- (*Rb*-) deficient embryos are unable to physically interact with erythroblasts, and RBC production is impaired in these embryos [21]. In addition, *in vitro* production of enucleated RBCs from immature hematopoietic progenitor cells proceeds efficiently in the presence [15] but not in the absence [14] of feeder cells.

However, enucleation can apparently be initiated *ex vivo* in erythroblasts that have been induced to differentiate *in vivo* to a developmental stage that is competent for nuclear self-extrusion [22, 23]. Consistent with these findings, our group discovered a method to produce enucleated RBCs efficiently *ex vivo* without use of feeder cells [24]. Our system for expanding erythroid progenitor cells and inducing efficient enucleation of those progenitor cells is shown in Figure 1.

The method we developed included VEGF and IGF-II in the culture medium [24]. These two factors have been reported to promote the survival, proliferation, and/or differentiation of hematopoietic progenitors [25–27]. Consistent with these findings, these factors promoted the expansion of erythroid progenitors [24]. However, a much more important feature of our culture system is that it allowed erythroid cells to differentiate to a developmental stage competent for nuclear self-extrusion [24]. It has generally been thought that efficient enucleation of erythroblasts is largely dependent on signals mediated by cells in their local environment [18–21]. However, the data we reported demonstrate that the interaction of erythroblasts with other cells is not necessary for efficient erythroblast enucleation [24]. Signals mediated by humoral factors appear to be sufficient for the efficient autonomous completion of erythroblast enucleation. In addition, since culture without the use of feeder cells is technically easier and less expensive, the method we developed has the potential to be a cost-effective means of producing transfusable RBCs on a large scale from immature hematopoietic stem/progenitor cells.

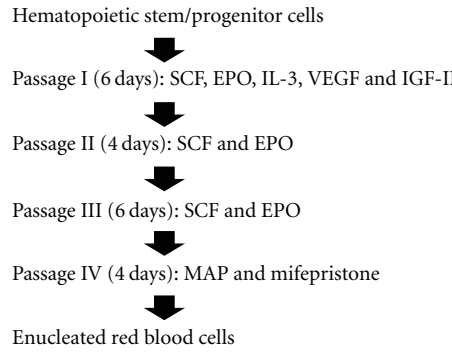

Figure 1: Culture protocol for the efficient production of enucleated red blood cells without feeder cells from hematopoietic stem/progenitor cells. Passage I∼III are the steps to expand erythroid progenitor cells. Passage IV is the step to induce enucleation of progenitor cells. SCF, stem cell factor; EPO, erythropoietin; IL-3, interleukin-3. VEGF, vascular endothelial growth factor; IGF-II, insulin-like growth factor-II; MAP, mixture of D-mannitol, adenine, and disodium hydrogen phosphate dodecahydrate.

8. RBC Production from ES/iPS Cells

ES/iPS cells possess the potential to produce various differentiated cells able to function *in vivo,* and thus represent another promising resource for RBC production *ex vivo*. Furthermore, since ES/iPS cell lines are immortalized, they can be used repeatedly and have potential to produce abundant differentiated cells in the quantities required for clinical use. However, it will be important to carry out routing screening of the ES/iPS cell lines for *de novo* chromosomal aberrations and/or genetic mutations that may arise during culture, before these long-term cell cultures are applied in the clinic. Unsurprisingly, there is now a widespread and enthusiastic debate on standardization of the characteristics of ES/iPS cells for regenerative medicine protocols that exploit these cell lines. In our opinion, since chromosomal aberrations and genetic mutations are inevitable in long-term cell cultures, only ES/iPS cell lines that have been cultured for a limited period, for example, less than 30 passages, should be selected for clinical use.

Hematopoietic cells, including those in the erythroid lineage, have been generated from mouse ES cells [28–31], nonhuman primate ES cells [32–34], and human ES cells [35–41]. Our group has also established a long term *in vitro* method for culturing hematopoietic cells derived from ES cells of the nonhuman primate, the cynomolgus monkey [27]. Recently, abundant productions of mature RBCs from human ES cells [42] and human iPS cells [43] were also reported.

9. Establishment of Immortalized Erythroid Progenitor Cell Lines Able to Produce Enucleated RBCs

As described above, we can now produce mature RBCs by *in vitro* culture of ES/iPS cells or the hematopoietic stem/progenitor cells present in umbilical cord blood. In practice, however, the efficiency of RBC generation varies with the quality of the ES/iPS cell line, or the umbilical cord blood sample. Since ES/iPS cell lines can be utilized repeatedly, derivation of RBCs from ES/iPS cells appears to be more practical. However, even with optimal experimental procedures and the most appropriate ES/iPS cell line the generation of abundant RBCs directly from ES/iPS cells is a costly and time-consuming process. If immortalized human erythroid progenitor cell lines can be established that have efficient production of mature RBCs, they would provide a much more useful resource than ES/iPS cell lines.

Several mouse and human erythroid cell lines have been established. However, to the best of our knowledge, there is no cell line that can efficiently differentiate into enucleated RBCs. It is generally difficult to establish hematopoietic cell lines from adult hematopoietic stem and progenitor cells, as both are sensitive to DNA damage and are unable to maintain the lengths of telomere repeats on serial passage [43]. In contrast, ES cells are relatively resistant to DNA damage and maintain telomere lengths on serial passage [44]. Therefore, these characteristics of ES/iPS cells may be advantageous for the establishment of cell lines, since differentiated cells derived from ES/iPS cells may retain them. In fact, an erythroid cell line has been established from *in vitro*-differentiated GATA-1-deficient mouse ES cells [45].

Recently, we developed a robust method to obtain differentiated cell lines following the induction of hematopoietic differentiation of mouse ES cells (Figure 2) and established five independent hematopoietic cell lines using this method [46]. Three of these lines exhibited characteristics of erythroid cells, and they were designated **m**ouse **E**S cell-**d**erived **e**rythroid **p**rogenitor (MEDEP) cell lines. Although their precise characteristics varied, each of the MEDEP lines could differentiate *in vitro* into more mature erythroid cells, including enucleated RBCs. Following transplantation into mice suffering from acute anemia, MEDEP cells proliferated transiently and subsequently differentiated into functional RBCs. Treated mice showed a significant amelioration of acute anemia. In addition, MEDEP cells did not form tumors following transplantation into mice. This paper was the first to demonstrate the feasibility of establishing immortalized erythroid cell lines able to produce mature RBCs.

After the work above, we have continuously cultured the established MEDEP cell lines so as to observe whether the characteristics of them were stable. After long-term cultures for more than one and a half year, all MEDEP cell lines maintained the characteristics able to differentiate into mature erythroid cells producing hemoglobin abundantly (Figure 3). Of note, the characteristics of one of the MEDEP cell lines, MEDEP-BRC5, have changed to that able to produce enucleated RBCs very efficiently; that is, more

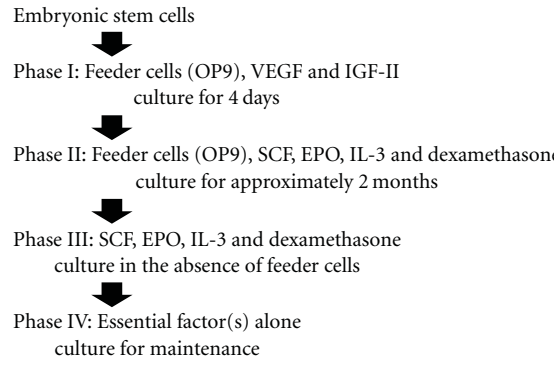

Figure 2: Culture protocol to establish erythroid progenitor cell lines from embryonic stem cells. In most cases, the cells failed to proliferate within two months of the initial induction of differentiation from ES cells. Induced cells that could proliferate continuously for approximately two months (60 days) were subsequently cultured in the absence of OP9 cells and in the presence of hematopoietic humoral factors. Cells that could proliferate in the absence of OP9 cells were cultured further. Approximately four months after the initial induction of differentiation of the cells, we evaluated the factors that were essential for the proliferation of each cell line. VEGF, vascular endothelial growth factor; IGF-II, insulin-like growth factor-II; SCF, stem cell factor; EPO, erythropoietin; IL-3, interleukin-3.

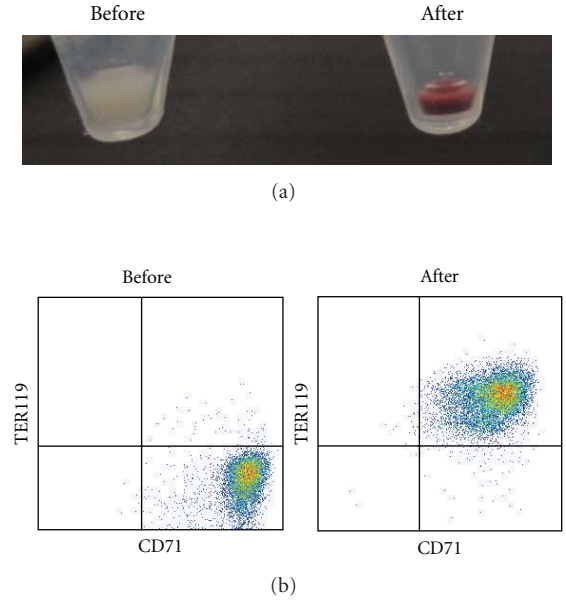

Figure 3: *In vitro* differentiation of a MEDEP cell line, MEDEP-BRC5. MEDEP-BRC5 cells cultured continuously for more than one and a half year was analyzed. The *in vitro* differentiation of MEDEP-BRC5 was performed by culture for four days after deprivation of stem cell factor and addition of erythropoietin. (a) Cell pellets before and after *in vitro* differentiation. Red cell pellet indicates abundant hemoglobin production in the cells. (b) Flow cytometric analyses. Before and After, the cells before and after *in vitro* differentiation; CD71, transferrin receptor; TER119, a cell surface antigen specific for mature erythroid cells.

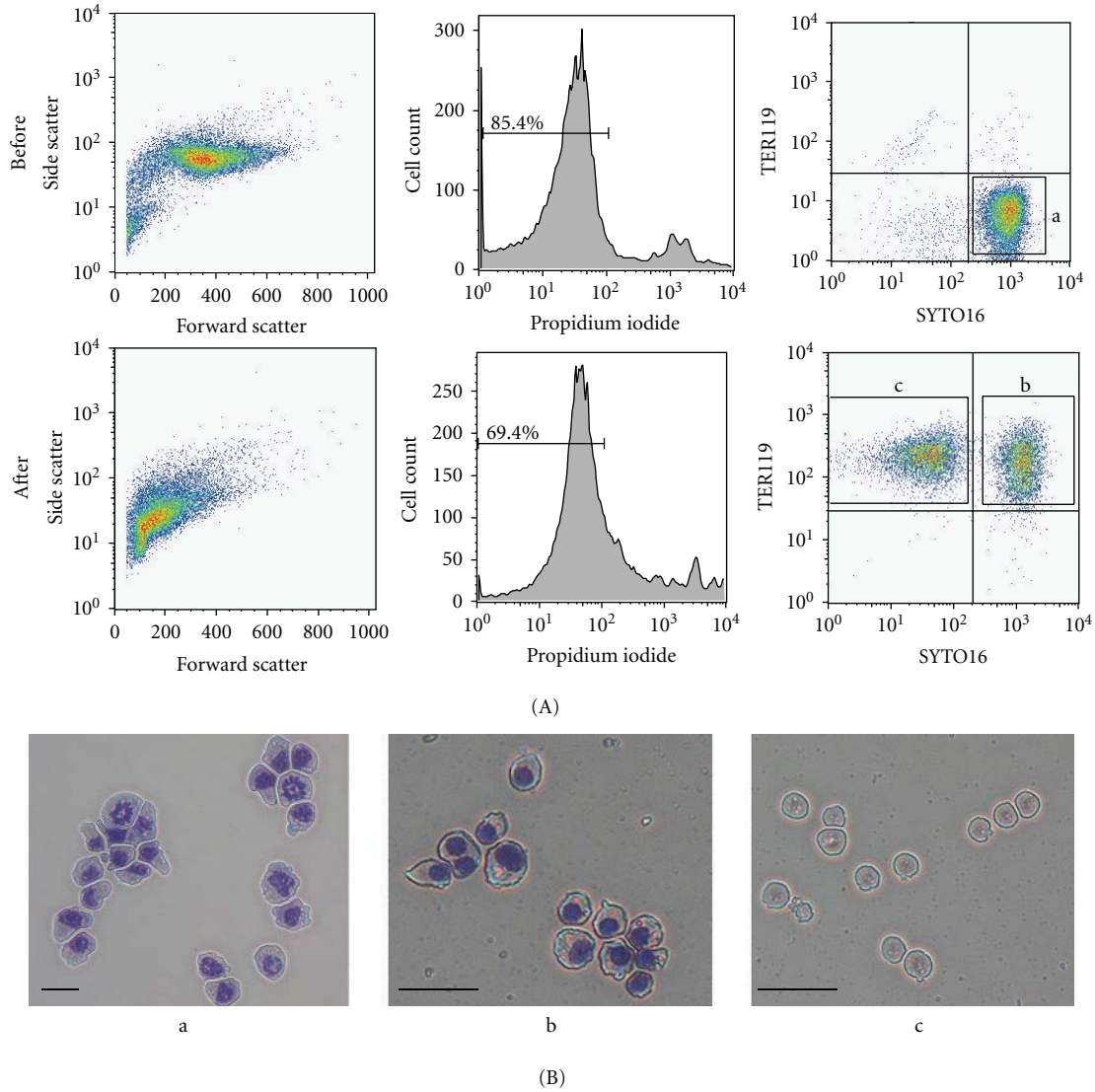

Figure 4: *In vitro* enucleation of a MEDEP cell line, MEDEP-BRC5. The *in vitro* differentiation of MEDEP-BRC5 was performed as described in Figure 3. (A) Flow cytometric analyses. Before and After, the cells before and after *in vitro* differentiation. Percentages of propidium iodide-negative viable cells are shown. TER119, see Figure 3. SYTO16, a cell membrane-permeable fluorochrome dye to stain nucleic acids. Following *in vitro* differentiation, 52% of the cells were TER119-positive and SYTO16-negative cells, that is, the cells lacking nuclei. (B) Morphology of cells collected from the a, b, and c fractions shown in (A). Scale bars indicate 20 μm.

than 50% of the cells were the enucleated RBCs following the induction of differentiation into mature erythroid cells (Figure 4). This result demonstrates that the interaction of erythroid progenitor cells with other cells is not necessary for efficient enucleation.

At present, the mechanism underlying the establishment of differentiated cell lines from ES cells has not been elucidated. Nevertheless, the data we reported clearly indicate that useful erythroid cell lines can be reproducibly obtained from mouse ES cells. Given that differentiation strategies developed for mouse ES cells often differ from those applied to human ES cells [47], the method we developed [46] may not be directly applicable to human ES cells and will require some modification.

10. iPS Cells as a Source for Establishing Immortalized Erythroid Progenitor Cell Lines

To establish the MEDEP cell lines, we screened eight types of mouse ES cell line and succeeded in establishing MEDEP cell lines from three of these [46]. By extrapolation from this result, it may be that many more human ES cell lines than currently available worldwide will be necessary to establish usable erythroid cell lines. In this context, the establishment of human iPS cell lines [7–10] should help to solve the problem of a potential shortfall, since human iPS cells have very similar characteristics as human ES cells.

Therefore, we attempted to establish human iPS cell lines and were able to establish a number of human iPS cell lines using fibroblast-like cells derived from neonatal tissues [48]. Fortunately, we were able to induce abundant numbers of hematopoietic cells from some of these iPS cell lines and also to establish immortalized hematopoietic cell lines from the induced hematopoietic cells (unpublished results). Currently, we are investigating the characteristics of these immortalized hematopoietic cell lines. Some seem to be erythroid cell lines.

11. Clinical Application of Erythroid Progenitor Cell Lines

We reported that MEDEP cells did not exhibit tumorigenicity *in vivo* [46]. Nevertheless, the tumorigenic potential of any human erythroid cell line will need to be thoroughly analyzed prior to clinical use [49, 50]. In addition, it may be advisable to engineer these cells in such a way that they are eliminated if a malignant phenotype arises for any reason [51].

Alternatively, the use of terminally differentiated cells that no longer have the capability of proliferating should allow clinical applications of ES/iPS cell derivatives without the associated risk of tumorigenicity. Thus, for example, RBCs lack nuclei following terminal differentiation and are highly unlikely to exhibit tumorigenicity *in vivo*. As such, even if the original ES/iPS cells and/or their derivatives possessed abnormal karyotypes and/or genetic mutations, they might, nonetheless, be useful for clinical applications, provided that they can produce enucleated RBCs. Indeed, the MEDEP lines included many cells possessing abnormal karyotypes; however, the vast majority of the cells in each cell line, nevertheless, differentiated into mature erythroid cells and transplantation of these cells significantly ameliorated anemia [46].

As described in this paper, various methods have been developed that enable the *ex vivo* production of enucleated RBCs from human hematopoietic stem/progenitor cells [14, 15, 24] and ES/iPS cells [42, 43]. Therefore, once appropriate erythroid progenitor cell lines have been established, it should be possible to apply these methods for producing enucleated RBCs *ex vivo*. Since RBCs are much smaller than normal nucleated cells, RBCs produced *ex vivo* could be selected by size prior to use in the clinic so as to exclude nucleated cells, for example, by filtration. In addition, X-ray irradiation might be useful for eradicating any contaminating nucleated cells without affecting the RBCs.

Another potential obstacle to the clinical use of ES/iPS cell derivatives is that of immunogenicity [52, 53]. Transplanted MEDEP cells could not ameliorate acute anemia in mouse strains other than those from which each individual cell line was derived or in immunodeficient mice [46], suggesting immunological rejection in heterologous strains. Hence, the clinical application of erythroid cell lines will require use of many cell lines that express different major histocompatibility (MHC) antigens. However, *ex vivo*-generated RBCs need to be compatible with ABO and RhD antigens alone. Furthermore, the establishment of an immortalized human erythroid cell line lacking the genes to produce A, B, and RhD antigens would be a very useful resource for clinical application, since such a cell line would produce O/RhD(−) RBCs, which would, in theory, be transfusable into all individuals.

12. Conclusions

It is now highly likely that immortalized human erythroid progenitor cell lines able to produce enucleated RBCs can be established in the near future. We believe that the transfusion of RBCs produced *ex vivo* from such cell lines will become a standard procedure in the clinic.

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