Concise Review: Production of Cultured Red Blood Cells from Stem Cells

ERIC E. BOUHASSIRA

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Einstein Pluripotent Stem Cell Center, Departments of Cell Biology and Medicine, Albert Einstein College of Medicine, New York, New York, USA

Correspondence: Eric E. Bouhassira, Ph.D., Einstein Pluripotent Stem Cell Center, Departments of Cell Biology and Medicine, Ullman Building 903, 1300 Morris Park Avenue, Bronx, New York 10461, USA.

Telephone: 718-430-2188; Fax: 718-824-3153; E-Mail: eric.bouhassira@einstein.yu.edu

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ABSTRACT

In the Western world, the volunteer-based collection system covers most transfusion needs, but transient shortages regularly develop and blood supplies are vulnerable to potentially major disruptions. The production of cultured red blood cells from stem cells is slowly emerging as a potential alternative. The various cell sources, the niche applications most likely to reach the clinic first, and some of the remaining technical issues are reviewed here.

INTRODUCTION

Red blood cell (RBC) transfusions, the first successful and most commonly used cell-based therapy, were developed more than 80 years ago, before all other cell therapies. In developed countries, 1 unit of blood is used on average per 20 people per year. In 2009, more than 17.3 million units of RBCs were collected in the U.S. [1]. Currently, RBC transfusions are used extensively in emergency medicine, serve as an essential component of major surgical procedures and chemotherapy, and are one of the major treatment options for individuals with hereditary anemias, including β-thalassemia major and sickle cell disease (SCD). The number of patients with SCD managed with chronic RBC transfusions is rising because prophylactic transfusions prevent stroke in some children with SCD [2, 3].

WHY PRODUCE RED BLOOD CELLS IN VITRO?

In the Western world, the volunteer-based collection system covers most transfusion needs, but in many parts of the developing world, shortages of RBCs are endemic because the blood collection system is not sufficiently developed. Even in collection systems that currently post an overall surplus of cells, as is the case in several countries today, shortages commonly develop either because of local conditions that transiently affect collection or distribution or because of locally high numbers of individuals who are particularly difficult to transfuse. The most underserved population is probably chronically transfused patients because of their specific needs: acute transfusion therapy involves matching the patient with the donor for ABO and RhD blood types, but chronic transfusion therapy in patients with SCD requires more precise matching because chronically transfused patients often generate allo-antibodies against normally nonreactive blood group antigens. Once they have developed antibodies, these patients can only be transfused with cells with very rare blood groups and RBC shortages regularly develop for this particular population, leading to patients going untreated or treated with less effective therapies. The shortages occur in part because of antigen mismatch between the mostly white RBC donor population and the black sickle cell patient population, and in part because of the large antigen diversity in blacks [4].

The existing transfusion system is expensive to maintain and vulnerable to potentially major disruptions that could be caused by the emergence of novel pathogens or social upheaval. Historical analysis of emergency responses after a major natural or man-made disasters suggests that short-term blood needs in such circumstances are relatively small and can be fulfilled by locally available supplies [5]. Rather, the most threatening scenarios involve long-term disruption of the supply chain caused, for instance, by a major pandemic that would decrease the ability of the population to donate blood for an extended period of time [6].

Generally, supply problems and transient shortages are expected to worsen over the next 20 to 30 years because of current demographic trends in the Western world, with an increasing proportion of older people needing transfusion therapy and much smaller proportion of younger donors [7].

Thus, whereas the current system covers most needs, it does not adequately address the needs of patients requiring chronic transfusions.
and is potentially vulnerable. Creation of alternate sources of RBCs, particularly rare cells, would greatly benefit the system by reducing shortages and could provide a critical backup capability in case of major emergencies.

The in vitro differentiation of RBCs from stem and progenitor cells has recently developed as a potential alternative to the current procurement system. Production of cultured RBCs (cRBCs) from stem cells holds the promise of revolutionizing transfusion medicine and the existing RBC supply system. Several groups of researchers are developing procedures to generate clinically useful cRBCs. We briefly review below the various strategies that are being explored to produce cRBCs.

### Erythropoiesis

Human RBCs have a limited life span and are the progeny of immortal self-renewing hematopoietic stem cells (HSCs) [8]. Since approximately 1% of the RBCs are eliminated from the circulation every day, maintenance of the average adult RBC mass of $2.5 \times 10^{13}$ requires the daily production of more than 200 billion RBCs. The differentiation of HSCs into RBCs is well understood and involves the generation of a series of progenitors with increasingly restricted differentiation potential: HSCs sequentially differentiate into common myeloid progenitors and megakaryocyte-erythroid progenitors and then into unipotent progenitors restricted to the erythroid lineage. These unipotent erythroid progenitors include burst-forming unit erythroid, colony-forming unit erythroid, and the morphologically recognizable erythroblast series, which ultimately terminally differentiate into orthochromatic erythroblasts that enucleate into reticulocytes and mature into RBCs [9].

### Sources of Cells for the Production of Cultured RBCs

Production of cRBCs for clinical purposes will require the identification of cell sources that can reliably be tapped and used to develop a highly scalable production procedure that recapitulates the erythropoiesis process briefly outlined above. Three major sources of cells are under consideration: circulating stem and progenitor cells from adults or from cord blood, immortalized progenitors, and pluripotent stem cells.

#### Circulating Stem and Progenitor Cells

Laboratory scale methods to produce cRBCs from circulating stem and progenitor cells are already well developed. Indeed, a clinical trial involving a single patient demonstrated recently that autologous cRBCs were functional, although their half-life was shorter than expected, suggesting that the culture conditions are not yet optimal [10].

With the best available methods, stem and progenitor cells found in 1 unit of cord blood can theoretically be expanded into more than 500 units of RBCs [11–14]. This level of amplification is sufficient to significantly expand the blood supply of rare blood groups, which would be an important milestone for the field, and justifies investment in this technology.

The main drawback of the use of circulating stem and progenitor cells is their limited proliferation potential. Since these cells are not immortal, production of cRBCs remains dependent on the donation-based collection and testing system. This increases cost and does not eliminate the risks to the blood supply associated with potential emerging pathogens or with social upheaval. Another problem is the genetic variability of primary cells, which can affect growth conditions and therefore complicate the production process and increase cost. These drawbacks are the major impetus for efforts to find a permanent source of cells that could be used to produce RBCs in a more cost-efficient manner.

#### Immortalized Progenitors

One potentially attractive source of cells would be the generation of immortalized progenitors that could be grown in large amounts in simple medium but would retain their capacity to differentiate into cRBCs upon induction. In general, cellular immortalization requires simultaneous stimulation of proliferation and inhibition of terminal differentiation and cell death [15–17].

Studies of murine erythroid precursors and murine erythroleukemia cells have led to the identification of several factors that control these processes in erythroid cells. These factors include GATA-1, which promotes erythroid development [18], and PU.1, which binds to GATA-1 and inhibits erythroid terminal differentiation [19, 20]. PU.1 is also required for proliferation and survival of very early erythroid progenitors [21, 22]. Other factors that are important for erythroid progenitor survival include the anti-apoptotic proteins BCL-2 and BCL-XL [23], the proapoptotic p53 [24], and c-Kit, which also promotes erythroid progenitor proliferation [25]. In addition, the well-known immortalizing transcription factor c-Myc has been shown to block erythroid differentiation [26–31].

Methods to immortalize hematopoietic cells with erythroid potential have been developed in chicken and mouse over the last 20 years. These extensive studies have shown that immortalization of erythroblasts can be achieved by manipulating the expression of transcription factors, tumor suppressors, and nuclear receptors. In addition to the factors mentioned above, v-Erb-a (THRA), v-Erb-b, and Raf, as well as several other genes, have been shown to have the potential to contribute to immortalization of erythroid precursors [32–36]. Some of the cell lines created with these factors have retained the capacity to differentiate and enucleate.

These animal studies provide a strong theoretical basis for the engineering of human erythroid cell lines that could be developed into highly scalable precursors for cRBC production. Importantly, this approach might be safer because cRBCs are not oncogenic since they are enucleated and remain fully functional after irradiation. The use of transformed cells to produce cRBCs is therefore not a safety issue since contaminating precursors could be eliminated. However, whether fully functional cRBCs could be derived in large quantities from a transformed cell line remains to be demonstrated.

#### Pluripotent Stem Cells

The third potential permanent source of cells for the production of cRBCs is pluripotent stem cells, such as human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). The main advantages of these cells are that they are immortal and...
karyotypically stable and that they can be reproducibly generated from any individual with a variety of well-developed methods [39]. The capacity of human pluripotent cells to differentiate into the erythroid lineage was demonstrated in 2007 by Kaufman et al., who used coculture with S17 cells, a mouse bone marrow cell line, to induce hESC differentiation toward the hematopoietic lineage [40]. Since this initial report, a number of methods based on other mouse (OP-9) [41, 42] or human feeder layers (FHB-hTERT) [43] or on the generation of embryoid bodies have been developed [44 – 46]. Methods that do not use any xenobiotic components and that might therefore be suitable for clinical production have also been developed [47]. Genetic engineering of pluripotent stem cells, which is technically possible, further expands the potential use of these cells as a source of cRBCs.

**DEVELOPMENTAL AGE AND HEMOGLOBIN CONTENT OF cRBCS**

The developmental age and the hemoglobin content are important characteristics of transfused RBCs. Adult and fetal cells differ by many characteristics, including the longer half-life of adult cells as compared with fetal cells [48]; the higher oxygen affinity of fetal hemoglobin (Hb F) as compared with adult hemoglobin (Hb A), which is caused by a lack of binding of Hb F to 2,3-diphosphoglycerate; and the differential antigen expression and altered membrane characteristics between the two cell types [49, 50].

Hemoglobin expression in humans is complex but well understood [51]. Briefly, early human erythropoiesis is characterized by several hemoglobin switches, as well as major morphological changes. The first morphologically recognizable erythroid cells are produced at week 3 of gestation in the yolk sac. These cells are primitive macrocytic pro- and basophilic erythroblasts that predominantly express hemoglobin Gower I. These primitive erythroblasts differentiate in the embryonic circulation into orthochromatic erythroblasts that predominantly express hemoglobin Gower II. Starting at weeks 6 – 7 of gestation, enucleated erythroid cells that express mostly Hb F and that likely originate from erythroid myeloid progenitors (EMPs) can be detected [52]. EMPs are a transient population of hematopoietic cells with differentiation potential greater than the primitive lineage but that do not self-renew. EMP-derived RBCs are then progressively replaced by definitive RBCs that originate from self-renewing HSCs. These definitive RBCs express mostly Hb F throughout gestation with a slow progressive increase in Hb A expression. Therefore, two types of RBCs predominantly express Hb F: the RBCs that are derived from the EMP wave of hematopoiesis and the RBCs derived from self-renewing definitive hematopoietic cells. Whether these two types of RBCs are identical is not known at this time. Around birth the switch to adult hemoglobin production accelerates dramatically, and Hb A becomes the predominant hemoglobin. Hb A represents more than 97% of all hemoglobins in most adults.

Currently, RBCs transfused in adults and neonates express almost exclusively adult hemoglobins because they are collected from adults. In the case of adults, there are no obvious indications against using RBCs containing high levels of Hb F since individuals with hereditary persistence of Hb F are asymptomatic [53] and since transfusion of umbilical cord blood cells in adults has been reported to be safe and did not result in any unusual immunological or nonimmunological transfusion reaction [54, 55]. Nevertheless, the longer half-life of adult cells might be preferable in some cases. In the case of neonates, Hb F-containing cells might actually be preferable because increased oxygen transport by Hb A might be associated with retinopathy in premature neonates [56].

The level of Hb F in RBCs that are collected for transfusion from adult individuals is generally lower than 1% of total Hb produced. The hemoglobin content of cRBCs varies widely. Depending on the expansion method used, cRBCs derived from adult stem and progenitor cells express between 1% and 10% Hb F. Cocktails of factors that favor the proliferation of adult stress progenitors that are used in the most efficient expansion protocols (see below) can lead to higher levels of Hb F, up to 50% of total Hb produced. Cord blood-derived cRBCs express mostly Hb F and a smaller amount of Hb A [57].

Collectively, this raised an important question regarding the type of hemoglobin that would be produced by cRBCs derived from hESCs. Since these cells are embryonic in nature, it was expected that they would express mostly embryonic hemoglobins.

The Bouhassira laboratory has developed a method to differentiate hESCs and iPSCs into RBCs that successively express embryonic hemoglobins Gower I and Gower II, and it has also developed conditions that yield RBCs expressing mostly Hb F [43, 57, 58]. Other investigators have also reported that hESC-derived RBCs can express both embryonic and fetal hemoglobins [59, 60]. Differentiation of pluripotent stem cells toward the erythroid lineage can be induced using a variety of methods, including embryoid body formation and coculture with feeder layers, but regardless of the method used, RBCs expressing embryonic hemoglobins are always obtained before RBCs expressing fetal hemoglobins. Therefore, differentiation of pluripotent cells toward the erythroid lineage closely recapitulates normal human development.

Whether the Hb F-producing RBCs obtained from pluripotent stem cells are equivalent to EMP- or HSC-produced RBCs is unclear, although the former possibility is much more likely because production of self-renewing HSCs from pluripotent stem cells has been very difficult to achieve. In a recent study, Chang et al. have shown that iPSC differentiation into RBCs is very similar to hESC differentiation since they produce cells that exhibit the same embryonic globin switches [60]. Therefore, iPSCs, even when produced from adult cells, do not retain any epigenetic memory that would alter the type of hemoglobin produced upon induction of their differentiation.

In conclusion, hemoglobin expression in cRBCs generally corresponds to the developmental age of the source of cells that were used to produce them. Both Hb A-containing cells and Hb F-containing cells are likely to be appropriate for most transfusions. No data are available about the potential effects of the presence of embryonic hemoglobin in cRBCs derived from pluripotent stem cells. Detailed studies comparing cRBCs with different developmental ages and different hemoglobin content are necessary. This is particularly true for cRBCs derived from pluripotent stem cells because they express embryonic Hb and because they might carry antigens not usually present in adult cells.

Another important parameter is the shelf life of the cRBCs. Shelf life is a critical determinant of cost and of the overall resilience of any supply system because inventory management can be used to minimize waste and alleviate temporary decreases in supply.

Currently, RBCs are generally stored up to 42 days in the U.S. and up to 35 days in the U.K. The shelf life of placental (cord) RBCs,
which contain mostly Hb F, is reported to be up to 35 days [61]. This suggests that cells with a young developmental age can be stored for approximately the same amount of time as adult cells. The shelf life of cRBCs is currently unknown and will have to be assessed.

**Liquid Culture Methods to Produce CRBCs**

The first method to produce CRBCs in liquid culture was developed by Fibach et al., who described a two-step procedure designed to first amplify and then favor the maturation of erythroid progenitors present in umbilical cord and adult peripheral blood [62]. Whereas this method was effective for the production of erythroid precursors, it did not yield large numbers of enucleated CRBCs and required serum and conditioned medium. Subsequently a variety of methods were developed that improved on this protocol [63, 64]. The Douay group made significant contributions by developing methods to amplify cord blood stem and progenitor cells using completely defined conditions [13, 65, 66]. Other important contributions were made to the field over the last 20 years by the Beug group and others who have developed SED, a cocktail of factors containing stem cell factor (SCF), erythropoietin, and dexamethasone, that can be used to induce extensive proliferation of a subset of progenitors that differentiate into stress erythroblasts. These stress erythroblasts are believed to be the first line of defense of the organism in case of major blood loss [12, 67, 68]. Additionally, using a variation of the SED cocktail, the Migliaccio laboratory has developed a procedure termed human erythroid massive amplification culture that yields very high numbers of human CRBCs that are antigenically very similar to RBCs produced in vivo [69–74].

Recently, England et al. have discovered the existence of primary erythroid progenitors in early mouse embryos that are capable of extensive (10^6- to 10^8-fold) ex vivo proliferation when exposed to the SED cocktail [75]. These extensively self-renewing erythroblasts, if they exist in humans, might represent a semi-permanent source of cells for CRBC production.

Human CRBCs produced using the SED cocktail express high levels of Hb F (10%–60%), which is consistent with the idea that they are stress RBCs since an elevated level of Hb F is a hallmark of stress erythropoiesis in humans. Olivier et al. recently presented evidence that the SED cocktail could be used to induce proliferation of CD34^+ cells derived from hESCs [76].

In parallel to the development of CRBC production methods, many laboratories have developed procedures to specifically amplify in culture long-term, repopulating HSCs because of their importance for transplantation applications [77]. In particular, Zhang et al. have developed a procedure based on the STIF cytokine cocktail (stem cell factor, thrombopoietin, insulin-like growth factor-2, fibroblast growth factor-2) and angiopoietin-like proteins to amplify HSCs approximately 20-fold [78, 79]. More recently, Boitano et al. demonstrated a 50-fold HSC expansion using SR-1, an aryl hydrocarbon receptor antagonist [80].

Recently, Olivier et al. combined the STIF cocktail designed to specifically amplify the stem cells compartment with cytokine cocktails designed to amplify the progenitor compartment and developed a CRBC production protocol that is much more efficient than the protocols focused on a single compartment [76]. The method reported by these authors can be used to expand and differentiate human CD34^+ cells purified from tissues at developmental ages ranging from 6-week yolk sac to adult. The procedure is also efficient for CD34^+ cells derived from hESCs. This study suggests that the yield of CRBCs can be increased by specifically focusing on amplification of each of the stem, progenitor, and precursor compartments separately, and that amplification of the cells in one compartment does not decrease their proliferation potential once they reach the next compartment. This compartment-specific approach might be useful to further increase yield, maybe by combining the SED and STIF cocktails.

**Enucleation**

In vitro enucleation and separation of extruded nuclei from CRBCs are the most challenging and least understood steps of CRBC production protocols. The Douay laboratory was the first to obtain nearly complete enucleation of CRBCs [13]. This was achieved by coculturing maturing CRBCs with specific stromal cell lines, such as mouse MS-5, and human mesenchymal stem cells (hMSCs). Subsequently, Miharada et al. reported that high rate of enucleation of cord blood-derived CRBCs could be obtained without the use of feeder layers by optimization of the culture conditions [81]. This was an important report because the coculture method is not easily scalable. Building on this work, Timmins et al. recently obtained 90% enucleation and very high yields corresponding to 500 units of CRBCs per initial unit of cord blood using a stirred bioreactor, a simple dilution feeding protocol, and a humanized medium containing SCF, Epo, and interleukin 3 [14].

Together these results show that enucleation can be achieved without a feeder layer, at least for low-density cultures and cord blood-derived CRBCs. High percentages of enucleation without feeder cells in high-density cultures have not yet been demonstrated.

Enucleation is more difficult to achieve with fetal liver-, hESC-, and hiPSC-derived CRBCs. The Bouhassira laboratory has reported 6% enucleation of hESC-derived CRBCs after a maturation step with cocultivation with MS-5 cells [57]. Lu et al. have reported 40%–60% enucleation of hESC-derived CRBCs when cocultured with hMSCs or mouse OP9 stromal cells [82]. The Bouhassira laboratory has also observed that fetal liver-derived CRBCs do not enucleate well in vitro (E. Bouhassira, personal observation). Therefore enucleation rates with current protocols seem to be highly dependent on the tissue of origin of the CRBCs. The causes of the difficulties in obtaining enucleation of CRBCs derived from CD34^+ cells at early developmental ages (fetal liver and earlier) are unclear since RBCs produced in the fetal liver in vivo are all enucleated.

**Scaling Up**

As reviewed by Timmins and Nielsen, the number of RBCs transduced every year is astronomical [83, 84]. Producing a sufficient amount of CRBCs to contribute to the existing transfusion system even a few percent of the total RBCs transfused is a significant technical challenge. A major issue is the maximal concentration of cells that can be achieved in a bioreactor. Since a unit of blood contains approximately 1–2 × 10^12 cells, more than 1,000 liters of medium would be necessary to produce that many cells in static flask culture, which allows a maximal density of approximately 2 × 10^9 cells per milliliter.

However, significant progress is being made in increasing the density of CRBCs that can be attained in bioreactors. Recently, Housler et al. have used a three-dimensional (3D) reactor based on a four-compartment hollow fiber culture platform to grow
cRBCs [85]. The bioreactor used contained two bundles of hydrophilic hollow fiber microfiltration membranes for transport of culture medium (forming two medium compartments), interwoven with one bundle of hydrophobic hollow fiber oxygenation membranes for transport of oxygen and carbon dioxide (forming a gas compartment), to closely mimic the continuous perfusion conditions in the bone marrow. Using this setup, cord blood CD34+ cells inoculated at an initial seeding density of 800,000 cells per milliliter could be amplified more than 14,000-fold and reach a density of 2–4 × 10^9 per milliliter [85]. This density is approximately 10% of the cell density in human bone marrow. These 3D bioreactors can be scaled up with the current design to approximately 10 liters, which would be sufficient to produce 1–2 units of cRBCs per reactor in a 3–4-week period of time and might therefore be suitable for small-scale niche applications.

One such niche application might be the production of reagent RBCs. Reagent RBCs are panels of cells with known antigen profiles that are used prior to transfusion to test the serum of the patients for the presence of antibodies that might react with the transfused cells. Current panels of reagent RBCs represent antigen profiles found primarily in Caucasian populations. Rare or uncommon African phenotypes are essential for testing serum from patients with SCD to help identify the great diversity of antibodies that develop when they become allo-immunized. Such cells are very difficult to procure and are not available on the market. In addition, many transfusion services rely on automated procedures that are based on the availability of reagent red cells and shortage of these critical reagents might develop in case of emergencies [6]. Worldwide needs for such reagent cells for both SCD patients and the general population could be fulfilled with existing bioreactor technology.

Another niche application that is more technically demanding than reagent RBCs but of critical importance would be supplying antigen-negative cRBCs for sickle cell anemia or other allo-immunized poly-transfused patients. As discussed in Peyrard et al. [86], only a few types of cells would be sufficient to cover all needs and even relatively small numbers of units would alleviate current shortages. Once the technology for these niche applications has been developed, scaling up to tackle the larger number of units necessary to supplement the donor based system, for instance in group O negative cells, will likely look less daunting than it does today.

**CONCLUSION**

Dramatic progress has been made toward the production of cRBCs for clinical use. The basic methods to produce such cells have been developed and there are many avenues of research that can be explored to decrease cost. Each cell source has advantages and disadvantages. The major advantage of primary cells is that the cRBCs are most similar to in vivo-produced RBCs since the cells are minimally manipulated and since the hemoglobins produced are mostly adult. The major drawbacks are that it might be difficult to procure enough cells for large-scale production, particularly for rare blood groups, and that the supply system would still be vulnerable to emerging pathogens. Expansion of primary cells will be most useful if the yield of the culture can be further increased by maximizing the expansion of each compartment as shown recently by Olivier et al. [76] and if it can be shown that the same culture conditions can be used to expand many genetically different donors. The second promising approach is the development of permanent cell lines from either primary cells or from iPSCs that could be grown in extremely large amounts in relatively simple media and that could be induced to terminal differentiation by addition or withdrawal of a small molecule. Considerable preliminary work has been performed on immortalization of chicken and mouse erythroblasts, but little has been published on the production of human cell lines capable of full enucleation. The development of protocols to reliably produce karyotypically normal stable cell lines that yield transfusible cRBCs in large quantities using simple growth media would be an extremely important step for the field, since simple cell culture conditions are key to scaling up to industrial production. The third potential source of cells is pluripotent hESCs and iPSCs. The major advantages of these cells are that they are immortal and karyotypically stable and that they can be reliably produced from practically any individuals. The drawbacks are that the cRBC production protocols from pluripotent cells are complex and the yield of enucleated cRBCs is relatively low when compared with primary cells. To overcome these drawbacks, the focus should be placed on simplifying the existing differentiation protocols, increasing the yield, and developing new protocols that produce adult-like RBCs. The fact that pluripotent cells are amenable to precise genetic manipulation using zinc-finger or transcription activator-like effector (TALE) nucleases should speed up progress with these cells.

A progressive increase in the clinical use of cRBCs seems inevitable in the long term if clinical trials do not reveal any unexpected problems, but significant engineering and economic challenges will have to be overcome before the technology can supplement even a fraction of the cells currently obtained from donation. All of the cell sources discussed above have the potential to eventually reach the clinic, although it is likely that a single technology will eventually dominate the market. The race to develop the winning technology will be of great scientific and technological interest and will certainly lead to the development of methods that will have broad applications in the stem cell and cell engineering fields.

**AUTHOR CONTRIBUTIONS**

E.E.B.: manuscript writing.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The author indicates no potential conflicts of interest.

**REFERENCES**

1. Whitaker BI, Schlumpf K, Schulman J et al. The 2009 National Blood Collection and Utilization Survey Report. Washington, D.C.: U.S. Department of Health and Human Services, Office of the Assistant Secretary for Health, 2011.

2. Adams RJ, Brambilla D. Discontinuing prophylactic transfusions used to prevent stroke in sickle cell disease. N Engl J Med 2005;353: 2769–2778.

3. Adams RJ, McKie VC, Hsu L et al. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial Doppler ultrasonography. N Engl J Med 1998;339:5–11.

4. Westhoff CM, Reid M. Transfusion medicine: Human blood group antigens and antibodies. In: Hoffman R, EB, Shattil S, Furie B et al., eds. Hematology Basic Principles and Practice. 5th ed. 2010.

www.StemCellsTM.com
5 Hess JR, Thomas MJ. Blood use in war and disaster: Lessons from the past century. Transfusion 2003;43:1622–1633.

6 Zimrin AB, Hess JR. Planning for pandemic influenza: Effect of a pandemic on the supply and demand for blood products in the United States. Transfusion 2007;47:1071–1079.

7 Ali A, Auvinen MK, Rautonen J. The aging population poses a global challenge for blood services. Transfusion 2010;50:584–588.

8 Orkin SH, Zon Li. Hematopoiesis: An evolving paradigm for stem cell biology. Cell 2008;132:631–644.

9 Hattangadi SM, Wong P, Zhang L et al. From stem cell to red cell: Regulation of erythropoiesis at multiple levels by multiple proteins, RNAs, and chromatin modifications. Blood 2011;118:6258–6268.

10 Giarratana MC, Rouard H, Dumont A et al. Proof of principle for transfusion of in vitro-generated red blood cells. Blood 2011;118:5071–5079.

11 Fujimi A, Matsunaga T, Kobune M et al. Ex vivo large-scale generation of human red blood cells from cord blood CD34+ cells by coculturing with macrophages. Int J Hematol 2008;87:339–350.

12 Leberbauer C, Boulme F, Unfried G et al. Different steroid co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors. Blood 2005;105:85–94.

13 Giarratana MC, Kobiari L, Lapillonne H et al. Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. Nat Biotechnol 2005;23:69–74.

14 Lachman HM, Cheng GH, Skoulitchi AI. Transfection of mouse erythroblast leukemia cells with myc sequences changes the rate of induced commitment to differentiation. Proc Natl Acad Sci USA 1988;83:6480–6484.

15 Lachman HM, Skoulitchi AI. Expression of c-myc changes during differentiation of mouse erythroblast leukemia cells. Nature 1986;340:319–324.

16 Prochownik EV, Kukowska J. Dereguated expression of c-myc by murine erythroblast leukemia cells prevents differentiation. Nature 1986;322:848–850.

17 Skoda RC, Tsai SF, Orkin SH et al. Expression of c-MYC under the control of GATA-1 regulatory sequences causes erythroblast leukemia in transgenic mice. J Exp Med 1995;181:1603–1613.

18 Ghysdael J, Tran Quang C, Deiner EM et al. Erythroid cell development and leukemic transformation: Interplay between signal transduction, cell cycle control and oncogenes. Pathol Biol (Paris) 2000;48:2011.

19 Schuetze S, Henegar DJ, Kebat D. The Ets-related transcription factor PU.1 immortalizes erythroblasts. Mol Cell Biol 1993;13:5670–5678.

20 Weiss MJ, Yu C, Orkin SH. Erythroid-cell-specific properties of transcription factor GATA-1 revealed by phenotypic rescue of a gene-targeted cell line. Mol Cell Biol 1997;17:1642–1651.

21 Metz T. Oncogenes and erythroid differentiation. Semin Cancer Biol 1994;5:125–135.

22 Metz T, Harris AW, Adams JM. Absence of p53 allows direct immortalization of hematopoietic cells by the myc andraf oncogenes. Cell 1995;82:29–36.

23 Nakamura Y, Hiroyama T, Mihara K et al. Red blood cell production from immortalized progenitor cell line. Int J Hematol 2011;93:5–9.

24 Hiroyama T, Mihara K, Sudo K et al. Establishment of mouse embryonic stem cell-derived erythroid progenitor cell lines able to produce functional red blood cells. Plos One 2008;3:e1544.

25 Okita K, Yamanaka S. Induced pluripotent stem cells: Opportunities and challenges. Philos Trans R Soc Lond B Biol Sci 2011;366:2198–2207.

26 Kaufman DS, Hanson ET, Lewis RL et al. Hematopoietic potential of c-MYC under the control of GATA-1 regulates the self-renewal capacity of erythroid progenitors. Int J Hematol 2011;93:1398–1411.

27 Chang KH, Nelson AM, Cao H et al. Definitive-like erythroid cells derived from human embryonic stem cells coexpress high levels of embryonic and fetal globins with little or no adult globin. Blood 2006;108:1515–1523.

28 Mazerur C, Douay L, Lapillonne H. Red blood cells from induced pluripotent stem cells: Hurdles and developments. Curr Opin Hematol 2011;18:249–253.

29 Pearson HA. Life-span of the fetal red blood cell. J Pediatr 1967;70:166–171.

30 Bunn HF, Biehl RW. The interaction of 2,3-diphosphoglycerate with various human hemoglobins. J Clin Invest 1970;49:1088–1095.

31 Mohandas N, Gallagher PG. Red cell membrane: Past, present, and future. Blood 2008;112:3939–3948.

32 Maniatis T, Fritsch EF, Lauer J et al. The molecular genetics of human hemoglobins. Ann N Y Acad Sci 1980;14:145–178.

33 Chen MJ, Li Y, De Obaldia ME et al. Erythroid/myeloid progenitors and hematopoietic stem cells originate from distinct populations of endothelial cells. Cell Stem Cell 2011;9:541–552.

34 Forger BG. Molecular basis of hereditary persistence of fetal hemoglobin. Ann N Y Acad Sci 1998;850:38–44.

35 Ziyzursky A. The erythrocytes of the newborn infant. Semin Hematol 1965;2:167–203.

36 Bhattacharya N. Placental umbilical cord whole blood transfusion: A safe and genuine whole blood source at emergency. J Am Coll Surg 2005;33:557–563.

37 Bianchi M, Landini A, Giannatonio C et al. Allogeneic cord blood red cells for transfusion. Transfus Med Rev 2012;26:90–91, author reply 91–92.

38 Qiu C, Olivier EN, Velho M et al. Globin switches in yolk sac-like primitive and fetal-like definitive red blood cells produced from human embryonic stem cells. Blood 2008;111:2400–2408.

39 Olivier EN, Qiu C, Velho M et al. Large-scale production of embryonic red blood cells...
from human embryonic stem cells. Exp Hema-
tol 2006;34:1635–1642.
59 Vodyanik MA, Slukvin, II. Hematoendo-
thelial differentiation of human embryonic stem cells. Curr Protoc Cell Biol 2007;Chapter 23:Unit 23.6.
60 Chang CJ, Mitra K, Koya M et al. Production of embryonic and fetal-like red blood cells from human induced pluripotent stem cells. PLoS One 2011;6:e25761.
61 Garratsen HS, Brune T, Louwen F et al. Autologous red cells derived from cord blood: Collection, preparation, storage and quality controls with optimal additive storage medium (Sac-mannitol). Transfus Med 2003;13:303–310.
62 Fibach E, Manor D, Oppenheim A et al. Proliferation and maturation of human erythroid progenitors in liquid culture. Blood 1989;73:100–103.
63 Freysinier JM, Lecoq-Lafon C, Amsellem S et al. Purification, amplification and charac-
terization of a population of human erythroid progenitors. Br J Haematol 1999;106:912–922.
64 Panzenböck B, Bartunek P, Mapara MF et al. Growth and differentiation of human stem cell factor/erythropoietin-dependent erythroid progenitor cells in vitro. Blood 1998;92:3658–3668.
65 Douay L, Giarratana MC. Ex vivo genera-
tion of human red blood cells: A new advance in stem cell engineering. Methods Mol Biol 2009;482:127–140.
66 Neilléz-Nguyen TM, Wajcman H, Marden MC et al. Human erythroid cells pro-
duced ex vivo at large scale differentiate into red blood cells in vivo. Nat Biotechnol 2002;20:467–472.
67 Dolznig H, Kolbus A, Leberbauer C et al. Expansion and differentiation of immature mouse and human hematopoietic progenitors. Methods Mol Med 2005;105:323–344.
68 Carotta S, Pilat S, Mairhofer A et al. Di-
rected differentiation and mass cultivation of pure erythroid progenitors from mouse embry-
yonic stem cells. Blood 2004;104:1873– 1880.
69 Migliaccio G, Masiello F, Tirelli V et al. Under HEMA conditions, self-replication of hu-
man erythroblasts is limited by autophagic death. Blood Cells Mol Dis 2011;47:182–197.
70 Ghinassi BTM, Chang K-H, Halverson G et al. Comparative blood group profiling of hu-
man erythrocytes (E6) generated from adult blood (AB), cord blood (CB), human embryonic stem cells (hESC) and induced pluripotent stem cells (iPS). Blood (ASH Annual Meeting Ab-
stracts) 2011;118:1027.
71 Migliaccio G, Sanchez M, Masiello F et al. Humanized culture medium for clinical expan-
sion of human erythroblasts. Cell Transplanta-
tion 2010;19:453–469.
72 Migliaccio AR, Whitsett C, Migliaccio G. Erythroid cells in vitro: From developmental bi-
ology to blood transfusion products. Curr Opin Hematol 2009;16:259–268.
73 Migliaccio G, Di Pietro R, di Giacomo V et al. In vitro mass production of human erythroid cells from the blood of normal donors and of tha-
lassemic patients. Blood Cells Mol Dis 2002;28:169–180.
74 Migliaccio G, Migliaccio AR, Druzin ML et al. Long-term generation of colony-forming cells in liquid culture of CD34+ cord blood cells in the presence of recombinant human stem cell factor. Blood 1992;79:2620–2627.
75 Englund SJ, McGrath KE, Frame JM et al. Immature erythroblasts with extensive ex vivo self-renewal capacity emerge from the early mammalian fetus. Blood 2011;117:2708–2717.
76 Olivier E, Qiu C, Bouhassira, EE. Novel, high-yield red blood cell production methods from cells derived from human embryonic stem, yolk sac, fetal liver, cord blood, and peri-
pheral blood. Stem Cells Translational Medicine 2012;1:604–614.
77 Sorrentino BP. Clinical strategies for ex-
pansion of haematopoietic stem cells. Nat Rev Immunol 2004;4:878–888.
78 Zhang CC, Kaba M, Iizuka S et al. Angio-
poietin-like 5 and IGFBP2 stimulate ex vivo ex-
pansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplanta-
tion. Blood 2008;111:3415–3423.
79 Zhang CC, Kaba M, Ge G et al. Angiopoi-
etin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. Nat Med 2006;12:240–245.
80 Boitano AE, Wang J, Romeo R et al. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. Science 2010;329:1345–1348.
81 Miharada K, Hiroyama T, Sudo K et al. Efficient enucleation of erythroblasts differen-
tiated in vitro from hematopoietic stem and progenitor cells. Nat Biotechnol 2006;24:1255–1256.
82 Lu SJ, Feng Q, Park JS et al. Biologic prop-
erties and enucleation of red blood cells from human embryonic stem cells. Blood 2008;112:4475–4484.
83 Timmins NE, Nielsen LK. Manufactured RBC: Rivers of blood, or an oasis in the desert? Biotechnol Adv 2011;29:661–666.
84 Timmins NE, Nielsen LK. Blood cell manu-
ufacture: Current methods and future chal-
lenges. Trends Biotechnol 2009;27:415–422.
85 Housler GJ, Miki T, Schmelzer E et al. Compartmen
tal hollow fiber capillary mem-
brane-based bioreactor technology for in vitro studies on red blood cell lineage direction of hematopoietic stem cells. Tissue Eng Part C Methods 2012;18:133–142.
86 Peyrard T, Bardiaux L, Krause C et al. Banking of pluripotent adult stem cells as an unlimited source for red blood cell production: Potential applications for alloimmunized pa-
tients and rare blood challenges. Transfus Med Rev 2011;25:206–216.