Concise Review: Stem Cell-Based Approaches to Red Blood Cell Production for Transfusion

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Key Words. Reticulocytes • Red blood cell transfusion • Pluripotent stem cells • Hematopoietic stem cells • Erythropoiesis

ABSTRACT

Blood transfusion is a common procedure in modern medicine, and it is practiced throughout the world; however, many countries report a less than sufficient blood supply. Even in developed countries where the supply is currently adequate, projected demographics predict an insufficient supply as early as 2050. The blood supply is also strained during occasional widespread disasters and crises. Transfusion of blood components such as red blood cells (RBCs), platelets, or neutrophils is increasingly used from the same blood unit for multiple purposes and to reduce alloimmune responses. Even for RBCs and platelets lacking nuclei and many antigenic cell-surface molecules, alloimmunity could occur, especially in patients with chronic transfusion requirements. Once alloimmunization occurs, such patients require RBCs from donors with a different blood group antigen combination, making it a challenge to find donors after every successive episode of alloimmunization. Alternative blood substitutes such as synthetic oxygen carriers have so far proven unsuccessful. In this review, we focus on current research and technologies that permit RBC production ex vivo from hematopoietic stem cells, pluripotent stem cells, and immortalized erythroid precursors. Stem Cells Translational Medicine 2014;3:346–355

INTRODUCTION

Blood transfusion is a crucial part of modern medicine; supportive care for surgeries and trauma and cancer treatments all involve blood transfusions [1]. A major component of blood is enucleated red blood cells (RBCs), the most abundant cell type in blood or the human body. Whole-blood or RBC transfusion is also important for alleviating symptoms or preventing complications of genetic blood disorders like sickle cell disease [2] and β-thalassemia major [3]. In low-income countries, blood transfusion is used more often for pregnancy-related complications and severe anemia in neonates [1].

Blood available for transfusion is a limited resource and is available only through donations by healthy volunteers. An estimated 103 million blood donations are collected annually worldwide [1]. Approximately 48% of these are donated in high-income countries, which are home to only 15% of the world’s current population. Although blood supply is typically sufficient in these high-income countries, 82 countries of low and middle incomes report having an insufficient supply of less than 10 donations per 1,000 people in the population [1]. Apart from this existing disparity in blood supply and demand, an aging population is set to increase the number of elderly (aged ≥65 years) rapidly in the next five decades, increasing the number of people requiring transfusion [4]. Globally declining fertility rates indicate a further reduction in the donor-eligible population, and blood supply shortages are predicted globally by 2050 [5].

Another challenge in transfusion is blood group compatibility. Including the well-known ABO and Rh antigens that are critical to transfusion compatibility, scientists also know of at least 30 blood group systems that include most of the 308 recognized antigens [6]. The combination of antigens present or absent in an individual is often related to ethnicity, some combinations being present almost exclusively in particular ethnicities [7]. Such “rare” combinations are included in the rare blood groups. If blood is transfused from significantly different donors, recipients may develop antibodies against immunogenic antigens not present in themselves, leading to alloimmunization in recipients. This situation is routinely observed in developed countries in patients with sickle cell anemia, who are predominantly of African ancestry, whereas most donors are white. In cases of chronically transfused patients, antibodies against multiple antigens are gradually augmented [8]. These challenges make it increasingly difficult for finding matched donors for these patients [9]. To solve this problem of blood group matching, technologies such as antigen masking or enzymatic cleavage to generate the “universal” blood type (the O and RhD blood types) were proposed some years ago,
but they are still under development and thus unlikely to be available in the near future [10].

Even with matched groups of blood donors, the risks of infection through transfusion always persist. Regular screening of donated blood for transfusion-transmissible infections, which include HIV and hepatitis B and C viruses, is not a routine process in 39 countries, increasing the risk of such infections. Approximately 33 countries reported irregular supplies of test kits as a factor [1]. In countries where regular screening is carried out, 1.6 million units, or approximately 1.5% of the global donations, are discarded because of the presence of such infectious markers [1]. In comparison, ex vivo production of RBCs in the laboratory can be rigorously monitored and controlled to eliminate infectious risks.

Alternative RBC substitutes have been the focus of research for almost two decades to counter problems with blood transfusion. Hemoglobin-based oxygen carriers and perfluorocarbon-based emulsions are the two prime candidates; however, the development of many such products from both categories has been discontinued in the past because of unsatisfactory results or safety concerns [11, 12].

Any alternative solution would be most beneficial for chronically transfused patients and for patients with rare blood groups. In light of these issues, ex vivo-generated RBCs are becoming increasingly more attractive. Although the idea of producing or manufacturing RBCs in the clinical laboratory from their biological precursors is not new, recent advances in stem cell biology and cell engineering have made it more feasible. This paper reviews the current status of research in ex vivo production of RBCs, as well as the challenges faced, and discusses new possible directions for overcoming those challenges.

**ERYTHROPOIESIS**

Erythropoiesis is a physiological process that generates RBCs, also called erythrocytes (Fig. 1). Developmentally, erythropoiesis in humans occurs in different anatomical locations, and the cells produced are from two distinct phases: primitive and definitive [13]. Primitive erythrocytes are produced in the yolk sac. The first wave of (transient) definitive erythropoiesis also begins in the yolk sac, but the cell maturation occurs in the fetal liver [13, 14]. A second wave of (permanent) definitive erythropoiesis arises from multipotent hematopoietic stem and progenitor cells (HSPCs), expands in the fetal liver, and later homes to the bone marrow. In the postnatal stage, the bone marrow forms the permanent site of erythropoiesis for the rest of adult life [13]. Primitive erythrocytes are large, nucleated cells, whereas definitive erythrocytes are smaller, enucleate RBCs, and express different hemoglobins than the primitive erythroid cells [15]. Briefly, in definitive erythropoiesis, HSPCs differentiate through a series of intermediate cells into the precursor cells of erythropoiesis or erythroblasts. The earliest erythroid-restricted progenitor is the burst-forming unit-erythroid that gives rise to colony-forming unit-erythroid. Both are defined by their ability to give rise to erythroid colonies in methylcellulose colony-forming cultures. The first morphologically distinguishable erythroblast is the proerythroblast, with further cells along this lineage being the basophilic, polychromatophilic, and orthochromatic erythroblasts. The proerythroblast and successive cells differentiate after limited rounds of cell divisions into reticulocytes, going through decreasing cell volume, increased hemoglobin synthesis, and nuclear condensation on the way, and losing the nucleus completely at the reticulocyte stage. This entire process occurs in the bone marrow or the fetal liver, and various cell-cell and cell-extracellular matrix interactions along with different cytokines regulate the process [16]. The reticulocytes, which are slightly larger, mature into erythrocytes within 1–2 days of entering the bloodstream by losing additional plasma membrane and cellular organelles to become smaller and acquire the biconcave disc shape distinctive of the erythrocytes. These cells remain in circulation for approximately 120 days before being cleared by the reticuloendothelial system in the spleen and the liver.

**CULTURED RBCS**

For transfusion, cultured RBCs need to have form and function similar to those of normal RBCs, including hemoglobin type and content, which determines the oxygen carrying capacity of the RBC, and an enucleate, deformable morphology.

**Hemoglobin**

Hemoglobin, the highly abundant protein in RBCs, is a tetramer that imparts the oxygen-carrying capability to erythrocytes. The tetramer is composed of two dimers, each encoded by the α- and β-globin gene clusters that are present on chromosomes 16 and 11, respectively [17]. The former (α) cluster includes the ζ and α1 genes, and the latter has the ε, γα, γβ, δ and β globin genes. The developmental stage of a person determines which chains form the hemoglobin. In early gestation (less than 5 weeks), Gower1 (ζ2γ2) is the predominant form present (primitive erythropoiesis). Gower2 (α2ε2) is also present in smaller quantities. From week 10 to week 34 of gestation (definitive erythropoiesis), fetal hemoglobin (α2γ2) gradually becomes predominant. β-Globin synthesis gradually replaces the γ-globin chain from fetal hemoglobin. By 6 months after birth, adult hemoglobin (α2β2) forms the predominant hemoglobin [17]. However, throughout adult life, small amounts of fetal hemoglobin are always present [18]. Thus, ζ and ε are considered embryonic globin chains, γ as fetal, and β as an adult chain. The regulation of these “switches” in humans from embryonic to fetal and from fetal to adult hemoglobin is still under intensive investigation [19–21].

Fetal hemoglobin (α2γ2) has a higher oxygen affinity than adult hemoglobin (α2β2). This favors easier oxygen uptake from the maternal circulation in the placenta, helping maintain oxygen delivery to fetal tissues. In adult life, however, fetal hemoglobin’s higher oxygen affinity results in lower oxygen delivery to tissues [22]. However, populations with hereditary persistence of fetal hemoglobin that show higher fetal hemoglobin percentages (even as high as 100%) are largely asymptomatic [17, 23, 24]. In fact, the presence of fetal hemoglobin has even been shown to ameliorate the symptoms of sickle cell disease and other hemoglobinopathies [25].

**Enucleation Mechanisms**

The enucleation process involves the division of the orthochromatic erythroblast into two bodies: the nucleus containing pyrenocyte and the enucleated reticulocyte. This process is facilitated in the bone marrow by interactions with macrophages [16] in niches described in the literature as “erythroblast islands.” The maturation process of the erythroblasts also severs the cytoskeletal connections between the plasma and the nucleus. This also makes the process of enucleation dependent on external factors...
such as macrophages for the nucleus extrusion [26]. In ex vivo conditions, in the absence of external forces to provide the right tension, enucleation of mature erythroblasts is a challenge. A detailed discussion of these and some other aspects of erythroblast enucleation has been discussed previously [26]. However, the role of macrophages is debatable because enucleation without exogenous macrophages has also been achieved ex vivo. In the last 3 years, modulators of intracellular vesicle trafficking, Rho-associated protein kinase and nonmuscle myosin IIB have been shown to play essential roles not only in cytokinesis but also in enucleation of human erythroblasts [27, 28].

Other Features
Normal RBCs are small, approximately 8 \( \mu \text{m} \) in diameter [29]. Their membranes are also highly deformable to allow them to squeeze through the tiny capillaries and venules of the human body. The oxygen-carrying capacity of RBCs determines their efficiency, which itself is a product of the nature and quantity of the hemoglobin present in the cells. The Douay group pioneered the testing of cultured RBCs on factors such as deformability and oxygen-carrying capacity [30] that have become some of the benchmark tests carried out to assess the functionality of cultured RBCs.

EX Vivo Production of Human RBCs

In past decades, many groups have successfully generated reticulocytes from fetal and adult HSPCs ex vivo (Table 1) with various protocols following the same set of basic steps. HSPCs are differentiated into the erythroid lineage by following three steps: commitment, expansion, and maturation. To induce erythroid differentiation and maturation, various cytokines (most of them include stem cell factor (SCF) and erythropoietin [EPO]) are added to the culture medium and/or cells are cocultured with feeder cells of murine or human origin. Various groups have used different combinations of cytokines and/or other factors, a summary of which is provided in Figure 2.

A single unit of packed red cells for transfusion contains approximately 2 \( \times 10^{12} \) RBCs. Achieving this large number in culture has so far proven difficult. In adults, self-renewing HSPCs maintain the homeostasis of approximately 2 \( \times 10^{13} \) RBCs. However, to maintain and expand HSPCs ex vivo in their undifferentiated state is a difficult task. Even with improved methods, we can isolate 2.5 \( \times 10^8 \) to 20 \( \times 10^8 \) nucleated cells from 1 ml of marrow harvested [31], of which approximately \( \leq 1.5\% \) are CD34+ HSPCs [32]. A 500-ml bone marrow collection would yield 18 \( \times 10^8 \) to 150 \( \times 10^8 \) CD34+ HSPCs, and possibly much less in some patients who need RBC transfusion. The concentration of CD34+ HSPCs in peripheral blood (PB) is even lower (discussed below). Cord blood (CB) from the umbilical cords of newborns provides an enriched source of CD34+ HSPCs, but total and CD34+ cell number is limited. A single CB unit (80–200 ml of blood) has only approximately 6 to 10 \( \times 10^8 \) nucleated cells, \( \leq 2\% \) of which are CD34+ HSPCs [33]. Clearly, expansion of these cells is necessary. One promising approach is use of the automated fed-batch, scale-up compatible technology, which provided an 80-fold expansion [34]. Addition of an aryl hydrocarbon receptor antagonist, SR1, further increased the expansion of HSPCs [35]. Many other approaches for CB HSPC expansion have been reviewed [36–38].
However, with limited ex vivo proliferation ability of lineage-restricted downstream cells, these approaches are still unable to provide requisite numbers for RBC generation, even for a single transfusion unit. Various approaches have simultaneously tried to increase the proliferation of starting cells used to derive the HSPCs or downstream cells using improved protocols. Ex vivo cultured RBCs can be obtained from various sources (Fig. 3). These sources can be divided into three broad categories: primary HSPCs, pluripotent stem cells, and immortalized erythroid precursors. In addition, a relatively new concept—transdifferentiation—reprogramming human fibroblasts directly to erythroblasts, bypassing the HSPC stage (Fig. 3, approach 5). This approach has been shown to produce reticulocytes, but studies have not yet been conducted on its potential to generate large quantities [39]. The general challenges faced by any of these cell sources are the types of hemoglobin synthesized and the maturation (enucleation) conditions involving animal (murine) feeders and/or animal origin products (serum). Apart from the dependence on animal products, other clinical transfusion problems are blood type matching and inadequate numbers of RBCs generated. These challenges are discussed for each cell type.

Primary HSPCs: Source 1

PB can provide circulating HSPCs in lower numbers, which can be increased by mobilization using granulocyte colony-stimulating factor. CB provides an enriched source of CD34+ HSPCs but the total and CD34+ cell number is limited (Fig. 3, approach 1).

Infrastructure for the routine process of PB and CB collection already exists. The evidence for generating RBCs from HSPCs has been available for more than a decade [40]. The Douay group has demonstrated the functionality of RBCs generated from adult mobilized HSPCs by injecting them into a healthy adult, providing the first proof of principle in human study [41].

The Douay group pioneered the generation of erythrocytes from CB HSPCs, but ex vivo enucleation from the cultured cells appeared low (4%) [42]. Nonetheless, these cells were capable of enucleation in a mouse model. Subsequently, the group achieved near-complete (90%) ex vivo terminal maturation of erythroblasts (derived from CB and PB HSPCs) by coculturing them with mouse MS-5 cells [30]. Little work has been done with PB as a source owing to its low amplification potentials, but the Douay group’s CB protocol, when applied to PB-derived HSPCs, seems to provide similar results (90% enucleation) [30, 41]. The protocol used by Fujimi et al. for RBC generation from CB HSPCs avoids xenogeneic feeders by coculturing with human macrophages derived in culture [43]. The protocol also achieves the highest number of $3.52 \times 10^6$ RBCs per starting cell in a static culture (Table 1). However, it involves a magnetic cell-separation step and the use of human AB blood-type serum for maturation for a total duration of 14 days to derive macrophages. These requirements and the use of an additional CB donor for deriving the macrophages makes this protocol an unlikely candidate for scaling up. Baek et al. developed a protocol using CB or bone marrow-derived mesenchymal stem cells (MSCs) as feeders [44]. Although their protocol allowed for up to 64% enucleation

Figure 2. Summary of current protocols for in vitro RBC generation. The figure provides a brief summary of all factors used for RBC generation by various published protocols. Hematopoietic stem and progenitor cells are expanded and driven to the erythroid lineage in the first step. The second stage involves further expansion of the erythroblast precursor stages and may involve further differentiation down the erythroid lineage. The final step is the maturation phase, in which all cells are driven toward the reticulocyte/erythrocyte (RBC) stage. The various supplementary and growth factors and coculture requirements are shown for each of the three steps. Factors common for all three steps are shown in the lower bottom corner. In the only instance in which IGF-I was used, cells did not mature (enucleate) in vitro. Hydrocortisone/dexamethasone are glucocorticoid receptor agonists. Mifepristone is a glucocorticoid receptor antagonist. Abbreviations: EPO, erythropoietin; hMSC, human mesenchymal stromal cell; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; IL, interleukin; MAP: D-mannitol, adenine, and disodium hydrogen phosphate dodecahydrate; mMS-5, murine marrow stromal cell line MS-5; mOP9, murine marrow stromal cell line OP9; RBC, red blood cell; SCF, stem cell factor.
efficiency, the use of an additional donor for the MSC feeders and inconsistent results with the bone-marrow derived MSC feeders makes this protocol also an unlikely candidate for scale-up and large-scale RBC production. A protocol devoid of adherent feeders is highly desirable to reduce the complexity and expense of using another cell type and is best suited for scale-up using existing bioreactor technology. On the other hand, the Nakamura group provided evidence of high enucleation rates (>70%) in an optimized, feeder-free protocol [45], and Timmins et al. adapted this protocol for use in an agitated bioreactor system, achieving very high amplification on the order of $1.7 \times 10^6$-fold and up to 90% enucleation efficiency [46].

When deciding to use either CB or PB for ex vivo RBC generation, it is worth noting that existing technology for ex vivo RBC generation consistently provides RBCs with predominant fetal hemoglobin from CB-derived cells [30, 42, 43], whereas PB-derived cells provide predominant adult hemoglobin RBCs, which also have elevated fetal hemoglobin levels [30, 41]. However, amplification from PB-derived cells is relatively low (on the order of 120,000-fold) as compared with that from CB-derived cells (>2 million-fold) [30]. Indeed, impressive extrapolated yields of 500 transfusable RBC units from a single donated CB unit can be achieved [46]. Then transusions with CB-derived cells may seem to be the viable option of the two, with better protocols being needed for use of PB as a source. If fetal hemoglobin contained in CB-derived RBCs is acceptable [23, 24], then CB-derived RBCs form an attractive option for transfusion of allo-RBCs.

Based on 2010 estimates, 450,000 CB units are available from 131 CB banks worldwide [47]. Registries maintain information on the blood types and the combinations of the antigens present for each CB unit. However, the percentages of rare blood-type units or of the different antigen combinations have not been reported. This makes it difficult to estimate how many of these units would be beneficial for patients with rare blood groups or for chronically transfused patients who have already developed alloimmunity. Even if special donations were arranged for generating RBCs, these cells are not immortal and cannot form an indefinite source of RBCs that can be provided for patients who need transfusions every 3–4 weeks. However, these protocols may prove useful for RBC generation for patients who cannot benefit from other sources.

**Pluripotent Stem Cells: Source 2**

Human embryonic stem cells (ESCs) [48] and induced pluripotent stem cells (iPSCs) [49] are, by definition, self-renewing and immortal stem cells that can give rise to any cell type. They form another approach to obtaining HSPCs (Fig. 3, approaches 2–4). The mononuclear cell fraction from either CB or adult PB can be separated and reprogrammed to iPSCs. The human iPSCs

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**Figure 3.** Approaches to ex vivo red blood cell generation. Approach 1 involves isolation of HSPCs from umbilical cord blood, whereas approach 2 involves density separation of adult peripheral blood cells to obtain the mononuclear cell fraction. The HSPCs can be isolated from this fraction, or the fraction can be reprogrammed to form induced pluripotent stem cells (iPSCs), forming approach 3. Approach 3, reprogramming to iPSCs, can also use fibroblast skin cells obtained from a donor. These iPSCs can then be differentiated to HSPCs. Approach 4 is differentiating hESCs to HSPCs. hESCs so obtained can be differentiated further to an immature erythroblast stage and then matured further to reticulocytes. Approach 5 uses transdifferentiation of fibroblasts to immature erythroblasts. Immature erythroblasts can also be immortalized, which forms approach 6. **Abbreviations:** hiPSC, human induced pluripotent stem cell; HSC, hematopoietic stem cell; HSPC, hematopoietic stem and progenitor cell; hESC, human embryonic stem cell.
can then be differentiated in vitro to CD45^-CD34^- HSPCs using protocols that were initially developed to differentiate human ESCs [50, 51].

The Nakamura group successfully generated enucleated erythrocytes with mouse ESCs as starting cells [52]. Thereafter, several groups successfully achieved enucleation of erythrocytes starting from human ESCs [53–56]. A comparison of their protocols suggests an important role for EPO and as yet an unidentified factor or factors (provided by feeder lines or by human plasma) that aids enucleation. In the protocols from the Bouhassira group [53] and the Slukvin group [56], without EPO in the final maturation stage, coculture with mouse MS-5 cells leads to <10% enucleation. However, with EPO and either mouse OP9 feeders in the culture or EPO and either mouse OP9 feeders in the final maturation stage, efficient enucleation of human ESCs has been reported with expression of fetal and, later, adult globins. Qiu et al. also provided evidence of increasing β globin expression with increasing time in coculture of human ESCs [53]. This might then suggest that an increased time in cultures may lead to the final hemoglobin switch from fetal to adult hemoglobin. If this is true, better understanding of the underlying regulatory mechanisms governing these switches might provide a way to bypass the increased culture time requirement. Recently, a study used the enforced transgene expression of RUNX1 to enhance hematopoiesis from human ESCs and iPSCs [59] and reported a higher level of β globin expression in differentiated erythrocytes. This study may provide a breakthrough to further improve the synthesis of β globin in human iPSC- or ESC-derived RBCs.

With regard to erythroblast expansion potential, the Slukvin group report no differences between human ESCs and iPSCs [56]. Although the Douay group did not find any differences in the erythroid commitment or hemoglobin types of RBCs generated from human ESCs and iPSCs, the group observed that the human H1 ESC line had seven- to eightfold higher erythroid expansion potential than a human iPSC line (IMR90-16) derived from fetal lung fibroblasts by lentiviral vectors [55]. Feng et al. once reported that human iPSCs derived similarly by integrating viral vectors had significantly decreased hematopoietic and erythroid expansion potential than a human iPSC line (IMR90-16) derived from fetal lung fibroblasts by lentiviral vectors [55].

### Table 1. Chronological history of in vitro RBC generation

| Reference (group, year) | Cell source | Feeder source | Culture length (days) | Hb chains and types | Maturation stage | Numbers of enucleated cells generated ex vivo per starting single cell (enucleation percentage) |
|------------------------|-------------|---------------|----------------------|---------------------|-----------------|----------------------------------------------------------------------------------|
| Neidez-Nguyen et al. [42] (Douay), 2002 | CB CD34^+ | hMSCs/mMS-5 | 21 | HbF | Reticulocyte | 4% in vitro (99% in vivo) |
| Giarratana et al. [30] (Douay), 2005 | CB CD34^+ Adult CD34^+ (mobilized or not) | mMS-5 | 21 | HbF, HbA | Reticulocyte | 1.76 × 10^5 (90%) 1 × 10^5 (90%) |
| Miharada et al. [45] (Nakamura), 2006 | CB CD34^+ | hMSCs/mMSCs | 20 | α, γ (HbF) | Reticulocyte | 55 (6.5%, starting from CD34^+ cells) |
| Qiu et al. [53] (Bouhassira), 2008 | hESCs | hMSCs/mMSCs | 59 | HbF, HbA (α, β, γ) | Reticulocyte | 3.52 × 10^5 |
| Fujimi et al. [43] (Niitsu), 2008 | CB CD34^+ | hMSCs | 38 | ζ, ε, γ | Reticulocyte | 1,000 (10%-30%) 2,500 (30%) 5,000 (65%) |
| Lu et al. [54] (Lanza), 2008 | hESCs | MEFs | 42 | MSC OP9 | Reticulocyte | 64% |
| Baek et al. [44] (Kim), 2008 | CB CD34^+ | MSCs | 21 | Not studied | Reticulocyte | 64% |
| Lapillonne et al. [55] (Douay), 2010 | hiPSCs | hESCs | 46 | HbF, ζ, ε | Reticulocyte | 250–396 (4%-10%) 2,310 (52%-66%) |
| Giarratana et al. [41] (Douay), 2011 | Adult CD34^+ (mobilized) | hMSCs/mMSCs | 18 | 88% HbA, 10.6% HbF | Reticulocyte | 25,000 |
| Chang et al. [57] (Bouhassira), 2011 | hiPSCs | hMSCs/Matrigel | 59 | HbF | Reticulocyte | |
| Dias et al. [56] (Slukvin), 2011 | hESCs | hESC | 60-125 | ε, γ (trace β) | Reticulocyte | 2%–10% |
| Kobari et al. [58] (Douay), 2012 | hiPSCs | MEF/mMSCs | 52 | HbF (predominant); switch to adult in vivo | Reticulocyte | 35 |

Abbreviations: CB, cord blood; Hb, hemoglobin; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; hMSC, human mesenchymal stromal cell; MEF, mouse embryonic fibroblast; mMS-5, murine marrow stromal cell line MS-5; mOP9, murine marrow stromal cell line OP9.
variable erythroid expansion and enucleation efficiencies. It is interesting to note that the hematopoietic potentials of human ESC cell lines vary considerably [61].

In the current list of NIH-approved-for-research lines, a human ESC line with an O and RhD-negative type (a universal blood donor) has not been identified [54]. The minimal set of the 211 currently approved human ESCs may not be able to provide for all blood groups, particularly for rare blood groups. The Douay group postulates that only three selected iPSC lines may be sufficient to provide for RBC-transfusion-dependent patients in France, one of those alone being able to provide for 95.5% of all such patients [62].

Human iPSCs provide the platform for autologous transfusions, especially in the case of alloimmunized patients. It is also possible, using the still-developing genetic correction techniques, to correct genetic defects in iPSCs to produce functional, adult RBCs for patients with hemoglobinopathies such as sickle cell disease [63] and β-thalassemia. This has already been demonstrated in a sickle cell anemia mouse model [64]. Development of better protocols to improve RBC yields from human iPSCs is necessary to bring these technologies to clinical applications.

**Extensively Expanding Erythroid Precursors: Source 3**

Under conditions of stress such as erythrophyly or hypoxia, rapid expansion of erythroid precursors is achieved in vivo by what is defined as “stress erythropoiesis.” Mimicking stress erythropoiesis, erythroid precursors can be expanded in vitro by activation of the glucocorticoid receptor [65, 66]. Along with SCF, EPO, and insulin-like growth factor I, a glucocorticoid receptor agonist such as dexamethasone has been shown to induce proliferation of erythroid precursors on the order of 10^5 to 10^6 [66, 67]. Even with better protocols, the amplification fold of CB-derived erythroid precursors is restricted to <10^10-fold [66]. Using interleukin 3 in this cytokine cocktail, erythroid cells from normal donor PB have also been expanded [68]. However, in the same study, the authors noted that the maximal amplification fold achieved with PB cells lies between values similar to the number of input cells up to only 80-fold higher. A modified approach was not able to significantly improve the expansion, which remained restricted between 6- and 12-fold [69]. These approaches therefore provide yields that are not yet sufficient for producing the large numbers of RBCs required for transfusion.

Many attempts have been made previously to establish immortal human erythroid precursor lines using genetic modifications [70]. However, most of these approaches begin with leukemic origin cells or cells transformed by in vitro genetic manipulations and result in defects in terminal differentiation and maturation [71–73]. However, successful approaches in generating immortalized or extensively expanding erythroblast lines (Fig. 3) that are able to produce enucleated erythrocytes have been demonstrated with mouse erythroblasts differentiated from embryonic stem cells [52, 74]. England et al. recently demonstrated the extensive expansion capability of first-wave definitive erythroid precursors that emerge from the early mouse embryo [75]. The results of these studies suggest that embryonic-stage erythroid cells have a higher proliferation potential than their postnatal counterparts. It is known that the Yamanaka factors are capable of reprogramming adult somatic cells to a different cell state as well as to ESC-like states [49]. If the postnatal erythroid precursors are similarly “reprogrammed” to an epigenetic state similar to the cells described by England et al., they might possess similar proliferation potential [75].

The Cheng laboratory recently reported data testing this hypothesis [76] (Fig. 3, approach 6). By using some of the Yamanaka factors and other factors that are commonly used for reprogramming somatic cells to iPSCs, the group was able to immortalize human CB erythroblasts. These cells were karyotypically normal and stable, maintained cell identity, and preserved ability to differentiate and enucleate into functional RBCs even after 12 months of culture, expanding at least 10^10-fold. The hemoglobin produced in these cells on maturation is of the fetal type, corresponding to the developmental age of the starting CB cell population and also consistent with previous reports that suggest that stress erythropoiesis is accompanied by elevated fetal hemoglobin levels [15].

The enucleation efficiency is approximately 30% when cocultured with irradiated mouse OP9 cells in the presence of serum.

Although such extensively expanding cells are genetically modified and could raise concerns about oncogenicity, the RBCs produced using these approaches could be safely transfused into a patient because these cells lack nuclei. The issue of any remaining nucleated cells could be addressed by leukocyte removal filtration and/or irradiating the cells before transfusion; RBCs remain fully functional even after irradiation. In the future, replacing the retroviral vectors with nonintegrating episomal plasmids, synthetic RNA, or small molecules will further eliminate the oncogenetic concerns, as demonstrated successfully in iPSC reprogramming [77–79]. Recently, another group used a similar approach and derived an immortalized human erythroid progenitor cell line from human iPSCs and CB cells by inducible expression of TAL1 and HPV16-E6/E7 viral genes [80], although the genome stability of these cells was not shown.

If several such cell lines are established from various donors, this approach has the potential to provide for all blood types. In addition, patients with rare blood types and other patients who cannot benefit from the CB-derived cells could potentially benefit by establishing erythroblast lines using adult PB progenitors, which also provide the opportunity for autologous transfusion and the possibility of generating RBCs with adult hemoglobin. The blood group antigen profiling study done by the Migliaccio group on ex vivo expanded erythroblasts provides a starting point in this regard [81].

**Future Directions**

One common problem with any starting cell population, except for adult PB HSPCs, is the absence of adult hemoglobin. Although this may not be an issue, it would be beneficial to have adult hemoglobin expression in adult patients. Recent studies with globin-switching regulatory mechanisms have identified BCL11A and KLF1 as the major players, and a mouse model also confirms these data [82]. Further studies may provide a way to force erythroid cells to express adult hemoglobin.

Another problem involves making culture conditions xenofree for clinical applications. Many protocols use serum or feeder cells of animal origin, especially for increasing enucleation efficiencies [30, 53, 54, 56]. These protocols need to be optimized to avoid the use of animal products. CB feeder-free protocols have already been optimized in this regard [42, 45], and use of alternatives such as human plasma [55] or serum need to be tested. The Migliaccio laboratory has developed a medium that uses human proteins instead of animal products and is equally efficient for erythroblast expansion [69]. Feeder-free protocols are
also easier to scale up because erythroid cells are suspension cells. Enucleation efficiencies of the newly derived immortalized erythroblasts line need to be optimized. Studies aimed at identifying the mechanisms of enucleation and its stimulants would also be greatly helpful.

Protocol optimization for increasing expansion fold to achieve the requisite numbers of RBCs is also necessary. Currently, protocols are better optimized for CB HSPCs than for those from adult PB. It remains to be seen whether the PB HSPCs inherently have lower ex vivo expansion potentials. An approach to expand cells at every stage in differentiation (pluripotent stem cells, HSPCs, and erythroid progenitor populations) might help achieve the requisite numbers of RBCs but at a significant cost for media and cytokines.

One of the most important deciding factors for any cell source and any protocol will be the associated costs, followed by scale-up compatibilities. With the requirement for $2 \times 10^{12}$ RBCs per unit for transfusion and limitations on the cell densities, the amount of medium required in static cultures would be $>1,000$ liters. Assuming feeder-free conditions for clinical applications, even other approaches like bioreactors would require media with cytokines in large quantities. Multiple different approaches are being tried for scaling up, including bioreactors and scaffolds, and have already been reviewed [83–85].

It was recently reported that a significantly simplified medium, E8, which contains eight defined components, including Dulbecco’s modified Eagle’s medium: Nutrient Mixture F-12 basal medium, is sufficient to efficiently expand human iPSCs either by assuming feeder-free conditions for clinical applications, even other approaches like bioreactors would require media with cytokines in large quantities. Multiple different approaches are being tried for scaling up, including bioreactors and scaffolds, and have already been reviewed [83–85].

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