



Novel, High-Yield Red Blood Cell Production Methods from CD34-Positive Cells Derived from Human Embryonic Stem, Yolk Sac, Fetal Liver, Cord Blood, and Peripheral Blood

EMMANUEL OLIVIER, CAIHONG QIU, ERIC E. BOUHASSIRA

Key Words. Erythroid • Adult stem cells • Fetal human liver • Embryonic stem cells • Hematopoiesis

Departments of Medicine and Cell Biology, Albert Einstein College of Medicine, Bronx, New York, USA

Correspondence: Eric Bouhassira, Ph.D., 1300 Morris Park Avenue, Bronx, New York 10461, USA. Telephone: 718-430-2188; Fax: 718-824-3153; e-mail: eric.bouhassira@einstein.yu.edu

Received May 22, 2012; accepted for publication July 10, 2012; first published online in *SCTM EXPRESS* ____.

©AlphaMed Press 1066-5099/2012/\$20.00/0

<http://dx.doi.org/10.5966/sctm.2012-0059>

ABSTRACT

The current supply of red blood cells expressing rare blood groups is not sufficient to cover all the existing transfusion needs for chronically transfused patients, such as sickle cell disease homozygous carriers, because of alloimmunization. In vitro production of cultured red blood cells is slowly emerging as a possible complement to the existing collection-based red blood cell procurement system. The yield of cultured red blood cells can theoretically be maximized by amplifying the stem, progenitor, or precursor compartment. Here, we combined methods designed to expand these three compartments to optimize the yield of cultured red blood cells and found that exposing CD34⁺ cells to a short pulse of cytokines favorable for erythroid differentiation prior to stem cell expansion followed by progenitor expansion produced the highest yield of erythroid cells. This novel serum-free red blood cell production protocol was efficient on CD34⁺ cells derived from human embryonic stem cells, 6–8-week yolk sacs, 16–18-week fetal livers, cord blood, and peripheral blood. The yields of cells obtained with these new protocols were larger by an order of magnitude than the yields observed previously. Globin expression analysis by high-performance liquid chromatography revealed that these expansion protocols generally yielded red blood cells that expressed a globin profile similar to that expected for the developmental age of the CD34⁺ cells. *STEM CELLS TRANSLATIONAL MEDICINE* 2012;1:000–000

INTRODUCTION

The in vitro production of cultured red blood cells (cRBCs) has recently emerged as a potential long-term alternative to the current donation-based red blood cell (RBC) procurement system. The current RBC collection system is expensive to maintain, is vulnerable to major disruption, and does not adequately serve the needs of chronically transfused, alloimmunized individuals, such as sickle cell disease patients, who often require RBCs expressing rare blood groups. Production of cRBCs from stem cells holds the promise of revolutionizing transfusion medicine and overcoming dependence on the existing RBC supply system by eliminating the current sporadic shortages, securing the supply lines, and providing back-up capability. In 2011, Giarratana et al. provided a proof of principle for this strategy by successfully testing autologous cRBCs in one human patient [1].

Source of Cells

Many of the methods developed to produce cRBCs are based on the expansion of progenitors

obtained from peripheral blood (PB) or cord blood (CB). These methods can potentially increase the blood supply because expansion of the progenitors from one unit of blood can yield multiple units of cRBCs. An alternative solution to improving yields is the development of a permanent source of cells that could be used for cRBC production. The isolation of human embryonic stem cells (hESCs) by the Thomson laboratory [2] and the development of methods to produce induced pluripotent stem cells (iPSCs) by the Yamanaka laboratory [3] have created the opportunity to develop such a permanent cell source because pluripotent cells are immortal.

Kaufman et al. reported in 2001 that hESCs could be differentiated into erythroid cells by coculturing hESCs on a feeder layer of S17 cells [4]. The Bouhassira laboratory expanded on these studies [5–8] by showing that hESC and iPSC differentiation closely parallels normal human development since these cells can be induced to sequentially produce cRBCs containing hemoglobin (Hb) Gower 1, Hb Gower 2, and Hb F [5]. Several other laboratories have reported similar findings using a variety of

methods to increase the yield of RBCs from hESCs [9–16]. In contrast to cRBCs derived from pluripotent cells, cRBCs produced from PB and CB express predominantly adult and fetal Hb, respectively.

The hemoglobin content is an important characteristic of cRBCs because hemoglobins have different oxygen affinities that affect their oxygen transport capacity. It is generally believed that whereas a high adult hemoglobin (Hb A) content is preferable for transfusion product, high Hb F cells are likely to be adequate because individuals carrying hereditary persistence of fetal hemoglobin in which the Hb F to Hb A switch occurs partially or not at all are asymptomatic [17].

Stem and Progenitor Expansion Strategy

Production of cRBCs can theoretically be achieved by stimulating the growth of the stem, progenitor, or precursor compartment. Fibach et al. were the first to publish a two-step liquid culture method to produce RBC *in vitro* on the basis of the expansion of progenitors [18]. Other authors have reported methods to amplify hematopoietic progenitors using defined cytokine cocktails [19, 20]. Over the last few years, the Douay laboratory has published several reports describing serum-free methods based on progenitor expansion to produce large numbers of enucleated red blood cells in serum-free conditions [21–23]. A major innovation was the use of a feeder layer of mouse bone marrow stromal cells (MS-5) in the last phase of the culture system that greatly facilitated cRBC final maturation and resulted in nearly 100% enucleation. Subsequently, Miharada et al. reported that a high rate of enucleation could be obtained without the use of feeder layers [24].

Beug and coworkers observed, first in chicken and then in mammals, that high levels of steroids such as dexamethasone could be used to induce extensive proliferation of proerythroblasts, providing a way to specifically amplify early erythroid progenitors [25–28]. Building on this work, the Migliaccio laboratory has developed the human-erythroid massive-amplification procedure to expand human adult peripheral blood cells and has shown, using a variety of *in vitro* assays, that human cRBCs produced in the presence of high levels of steroids were highly similar to their *in vivo*-produced counterparts [29–34]. One of the goals of this report was to determine whether culture medium containing high concentrations of steroid could be used to amplify erythroblasts derived from human pluripotent stem cells.

Because CB units generally do not contain sufficient numbers of hematopoietic stem cells (HSCs) to transfuse adults, several authors have developed methods to amplify repopulating hematopoietic stem cells [35–37]. Zhang et al. [38] have developed a procedure based on the STIF cytokine cocktail (stem cell factor [SCF], thrombopoietin, insulin-like growth factor 2 [IGF2], fibroblast growth factor 2) and angiopoietin-like proteins to amplify HSCs approximately 20-fold. More recently, Boitano et al. [39] demonstrated a 50-fold HSC expansion using SR-1, an aryl hydrocarbon receptor antagonist. Another goal of our study was to determine whether the addition of an HSC-expansion step could be used to increase the yield of cRBCs.

MATERIALS AND METHODS

Tissue Procurement

Human yolk sacs (6–8 weeks) and fetal livers (16–18 weeks) were obtained from the Einstein Fetal Tissue repository under an

institutional review board (IRB)-approved protocol. Cord blood was obtained from the Obstetric Department at the Weiler Hospital of the Albert Einstein College of Medicine (Bronx, NY) under an IRB-approved protocol. Peripheral blood was obtained from adult volunteers under an IRB-approved protocol. H1 hESCs were obtained from WiCell Research Institute (Madison, WI), <http://www.wicell.org>.

Human Embryonic Stem Cells

H1 hESCs were cultured and differentiated into CD34⁺ cells by coculture for 35 days on FHB-hTERT, a human fetal liver-derived cell line, as described previously [5].

Expansion of Progenitors in Erythroid Expansion and HSC-Expansion Conditions

Cells were grown at 37°C and 100% humidity in StemSpan basal medium (StemCell Technologies, Vancouver, BC, Canada, <http://www.stemcell.com>) plus the cytokines described in supplemental online Tables 1–3. All cell counts were performed in duplicate using trypan blue and a hemacytometer. Cytokines and tissue culture reagents are described in the supplemental online Methods.

Methylcellulose and Fluorescence-Activated Cell Sorting Assays

Colony forming unit-erythroid (CFU-E) and fluorescence-activated cell sorting (FACS) assays were performed as described previously [5–7].

Expansion of Progenitors Under High Steroid Concentration

Sorted CD34⁺ cells derived from hESCs differentiated by coculture on FHB-hTERT feeder stroma were placed in StemSpan basal medium (StemCell Technologies) supplemented with 1 μM synthetic glucocorticoid dexamethasone (Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>), 2 U/ml erythropoietin (Epo), 40 ng/ml IGF1, 100 ng/ml SCF, and 40 μg/ml cholesterol-rich lipid mix (Sigma-Aldrich). After a few days, the cultures developed into a homogeneous cell suspension (see Results section). The cells were then fed every other day with cell density adjustment to 1 million cells per milliliter. A month after the culture, a Ficoll gradient centrifugation was performed to eliminate dead and matured cells. The remaining isolated progenitors were placed back in the same culture medium. This procedure was repeated every 2 weeks.

RESULTS

To determine whether HSC expansion could be used to improve the yield of cRBCs, we have compared the cRBC yields and the types of hemoglobin produced when we combined HSC-expansion methods derived from the protocols of the Lodish laboratory [38] with an erythroid expansion (E-expansion) protocol that we developed based, in part, on the reports of Giarratana et al. [22] and Freyssonier et al. [20]. Twenty different cytokine combinations were tested on sorted CD34⁺ cells derived from either 18-week fetal liver (FL) or CB. FL-derived cells were included in the study because we have shown that they are much closer to embryonic stem cell (ESC)-derived cells than adult cells and because a major goal of the study was to develop methods that

would be effective on cells that have the potential of becoming a permanent cell source of cRBCs.

Several HSC-expansion protocols in which CD34⁺ cells are incubated with various cytokine cocktails prior to incubation in E-expansion conditions were compared with a basic E-expansion protocol, termed protocol O1. Protocol O1 involves a 1-week progenitor expansion step in the presence of low-dose steroids, SCF, Flt-3-L, interleukin 3 (IL3), bone morphogenetic protein-4 (BMP-4), and Epo, followed by a second week of incubation with low-dose steroids SCF, IGF1, IL3, BMP-4, and Epo. These E-expansion culture conditions yield a population of cells composed almost entirely of erythroid cells at the pro- and basophilic stages of differentiation. This population of cells can be induced to mature into enucleated cRBCs by coculture for 7–10 days with a feeder layer of MS-5 cells as described earlier [22].

The basic HSC-expansion medium tested was the STIF medium described by Zhang et al. [38, 40]. This medium can be used to expand CB-derived CD34⁺ cells and increase the number of repopulating HSCs in a xenotransplant model up to 20-fold. However, it had never been tested for its capacity to increase the yield of cRBCs. In addition to testing the basic STIF cocktail (protocol L1), we also tested whether the proteins angiopoietin-like 3 and 4, individually or in the presence of either platelet-derived growth factor α (PDGF α) or vascular endothelial growth factor (VEGF), could improve the yield of this HSC-expansion step (protocols L2–L5). The angiopoietin-like 3 and 4 proteins were tested because Zhang et al. have reported that these two cytokines increase the level of HSC expansion by severalfold [40]. PDGF α and VEGF were tested because it was reported that they can increase the proliferation of cells of mesodermal origin. Sorted FL-derived or CB-derived CD34⁺ cells were incubated in these various cytokine cocktails for either 1 or 2 weeks prior to being placed into our E-expansion conditions. Figure 1A and 1B and supplemental online Figure 1A and 1C summarize the yield of cells and the fold increase over the O1 E-expansion protocol obtained when a 1-week HSC-expansion step was added. Preincubation of FL-derived and CB-derived CD34⁺ cells for 1 week in HSC-expansion conditions led to cell yield increases of 6–8-fold and 10–20-fold, respectively. All of the variations of the HSC-expansion protocol tested (L1–L5) had similar effects on the yield of cRBCs compared with the basic E-expansion protocol. We therefore conclude that an HSC-expansion step might be useful to improve the yield of cRBCs but that the STIF cocktail was sufficient since adding angiopoietin-like 3 and 4, VEGF, and PDGF α only had a marginal effect. Additional experiments will be necessary to rule out small effects on yield of RBCs associated with the addition of angiopoietin-like 3 and 4, VEGF, and PDGF α during the HSC-expansion step. However, for the rest of the discussion, we averaged the L1–L5 experiments since all five experiments gave similar results. To determine whether a longer HSC-expansion step could further increase the yield of cRBCs, we set up cultures in which the cells were grown in all of the above cocktails for 2 weeks (protocols L12–L53). These experiments revealed that a 2-week HSC-expansion step did not significantly improve the yield of erythroid cells over the 1-week HSC-expansion step (Fig. 1A, 1C; supplemental online Fig. 1B, 1D). We therefore conclude that a 1-week HSC-expansion step is optimal to increase the yield of erythroid cells from FL-derived and CB-derived CD34⁺ cells. As in the experiments described above, the additional cytokines in the STIF medium (angiopoietin-like 3 and

4, VEGF, and PDGF α) had little or no effect. We therefore averaged the L12–L53 experiments for the rest of the discussion.

To determine the mechanism of the increase in cRBCs observed after culture in HSC-expansion conditions, we performed FACS analysis using a panel of antibodies. We first compared the persistence of stem and progenitor cells using CD34 and CD133 antibodies when an HSC-expansion step was included in the protocol. Because the cells were sorted for expression of CD34, the percentage of expression was close to 100% the day the cultures were started. At the start of the experiment, the percentage of CD133 expression was also close to 100%, which is not surprising because CD133 and CD34 have similar expression patterns in stem and progenitor cells. After 1 week in E-expansion protocol O1 culture, the percentages of FL-derived and CB-derived CD34⁺ and CD133⁺ cells dropped to less than 5%, and after the second week of culture, they dropped to less than 1% (Fig. 1E; supplemental online Figure 2C, 2D). Importantly, when the cells were grown in the HSC-expansion medium for 1 week, the percentage of CD34⁺ cells ranged between 18.5% and 32.5% (mean, 27.7 \pm 4.8%) for FL-derived cells and between 29.6% and 50.8% (mean, 41.0 \pm 7.1%) for CB-derived cells (Fig. 1D; supplemental online Figure 2A–2D). In both cases, the percentage of CD34⁺ cells dropped to less than 2% when the cells were switched to the E-expansion week 1 culture conditions. These data suggest that the HSC-expansion step leads, as expected, to an expansion of the stem and progenitor cells and to a delay in their differentiation.

In the case of FL-derived cells, the number of cells plated in conditions L1–L5 had increased an average of 38.1 \pm 5.9-fold after the week in HSC-expansion conditions (supplemental online Table 4); since an average of approximately 28% of these cells had retained CD34 expression, the number of CD34⁺ cells present at the end of the HSC expansion step had increased by 10.8-fold, a number that is quite close to the 7.5 \pm 0.8-fold increase in the yield of basophilic erythroblasts obtained at the end of the culture. This suggests that the 1-week HSC-expansion step did not decrease the erythroid potential of the CD34⁺ cells. Similar calculations for the CB-derived CD34⁺ cells revealed that the total number of cells after the week in HSC-expansion conditions had increased an average of 10.3-fold and therefore that the number of CD34⁺ cells had increased by 4.1-fold.

We then examined the expression of CD235a, a marker of late erythroid differentiation (Fig. 1F; supplemental online Figure 2E, 2F). These experiments revealed that CD235a remained low during the HSC-expansion step and therefore confirmed the lack of differentiation during the week of HSC expansion. Analysis of the cells at the end of the E-expansion period demonstrated that the vast majority of the cells obtained at the end of the experiments were erythroid cells whether or not the cells had been preincubated for a week in HSC conditions. No major differences were observed when protocols L1–L5 were compared.

To functionally characterize the cells grown with or without the HSC-expansion step, we then compared the number and types of colonies obtained in methylcellulose assays. After a week of culture in E-expansion medium, FL-derived and CB-derived CD34⁺ cells yielded approximately 75% CFU-E and 25% myeloid colonies (colony forming unit-macrophage and colony forming unit-granulocyte/macrophage colonies; Fig. 2). These

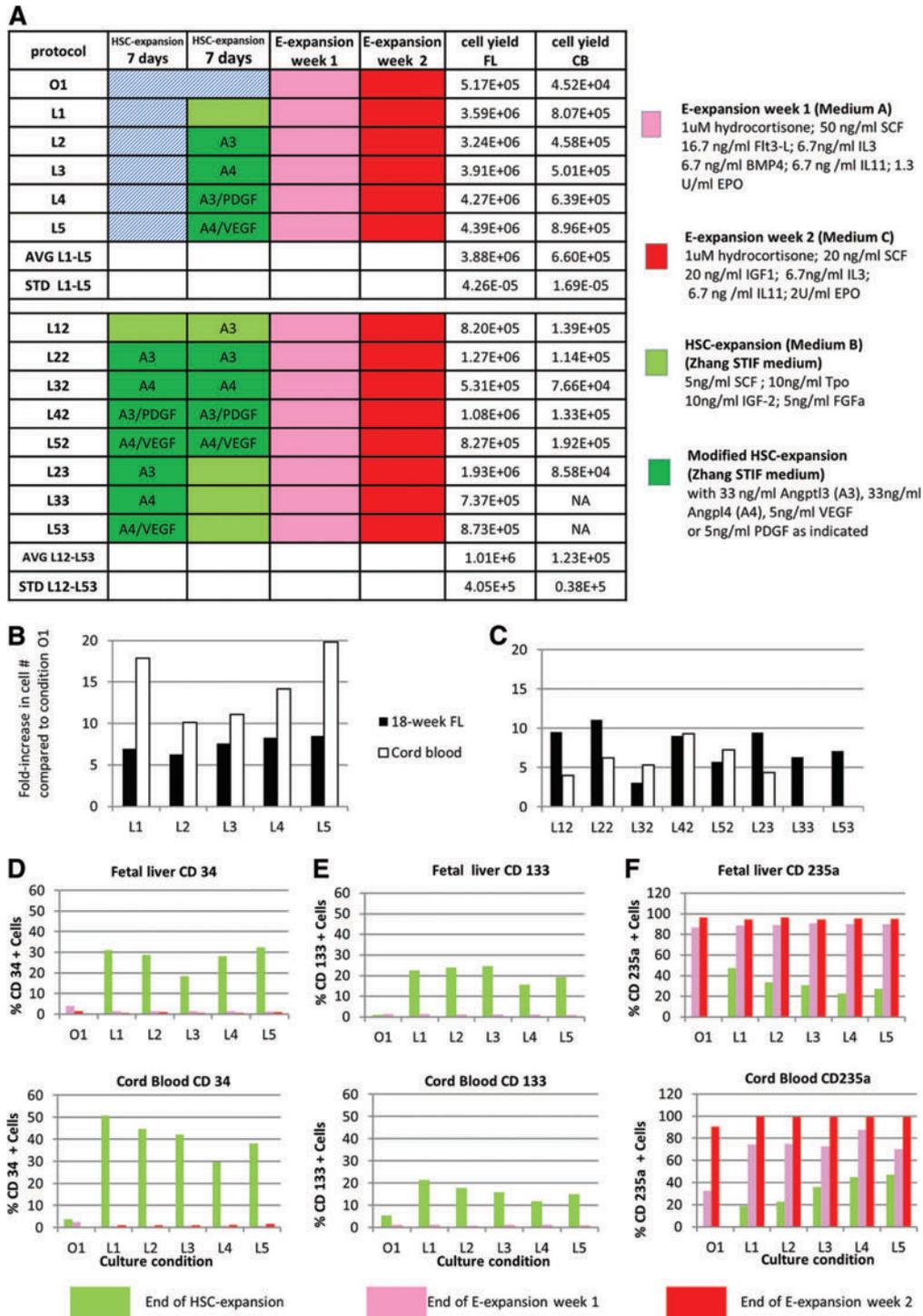


Figure 1. Incubation of CD34⁺ cells for 1 week in HSC-expansion conditions increases the yield of cultured red blood cells. **(A):** Table illustrating the fold expansion of CD34⁺ cells grown in various conditions. Incubation conditions are color-coded (composition of the medium is indicated on the left). **(B, C):** Fold increase in the number of cells obtained at the end of the experiments as compared with condition O1. Incubation for 1 week in HSC-expansion conditions prior to incubation in E-expansion conditions increased cell yield 6–20 times. **(D–F):** Fluorescence-activated cell sorting analysis of CD34⁺ incubated for 1, 2, and 3 weeks in conditions O1, L1–L5, and M3. Dot-plots illustrating the results are presented in supplemental online Figure 2. Analysis with the CD34 and CD133 antibodies revealed that CD34 and CD133 were almost completely silenced at the end of the first week of culture in condition O1 but remained expressed in a significant portion of the cells after a week in HSC-expansion conditions. Cells incubated for 1 week in HSC-expansion conditions and then in E-expansion conditions (condition O1) silenced CD34 and CD133 expression with a kinetics similar to that of cells that were placed directly in condition O1. Analysis of cells with CD235a antibodies revealed that these antigens exhibited a delayed activation when they were incubated in HSC-expansion conditions. Abbreviations: AVG, average; BMP, bone morphogenetic protein; CB, cord blood; E-expansion, erythroid expansion; EPO, erythropoietin; FGFa, fibroblast growth factor a; FL, fetal liver; HSC, hematopoietic stem cell; IGF, insulin-like growth factor; IL, interleukin; NA, not applicable; PDGF, platelet-derived growth factor; SCF, stem cell factor; STD, standard deviation; STIF, cytokine cocktail (stem cell factor, thrombopoietin, insulin-like growth factor 2, fibroblast growth factor 2); Tpo, thrombopoietin; VEGF, vascular endothelial growth factor.

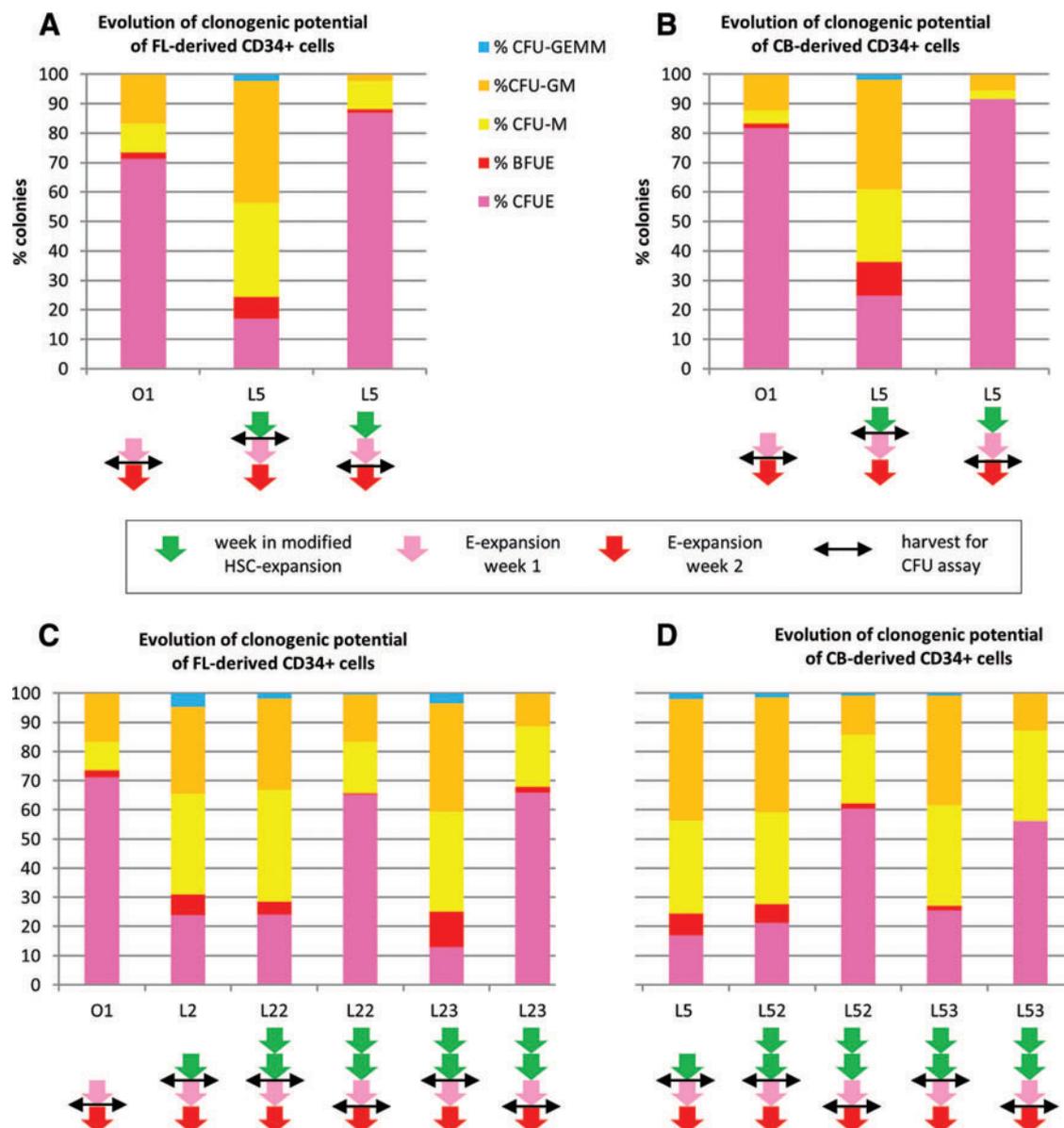


Figure 2. Incubation of CD34⁺ cells for 1 week in hematopoietic stem cell (HSC) expansion preserves their clonogenic potential. **(A, B):** Histograms summarizing the results of clonogenic assays performed on CD34⁺ cells from FL **(A)** or CB **(B)** incubated in condition O1 for 1 week or in condition L5 (HSC expansion for 1 week followed by O1 conditions for 1 week, as shown in Fig. 1). The vertical arrows below the histograms represent the incubation conditions; horizontal double-sided arrows represent the time of harvest for the colony forming unit assays. After a week of incubation in condition O1, the progenitor content of the culture consisted predominantly of CFU-E with a few BFUE and some myeloid progenitors. By contrast, incubation of CD34⁺ cells in HSC-expansion conditions preserved a more primitive progenitor content consisting of smaller amounts of CFU-E and larger amounts of CFU-GEMM, BFUE, and myeloid precursors. **(C, D):** The experiment was performed as above, but the cells were incubated in HSC-expansion conditions for 2 weeks. Incubation in HSC-expansion conditions for 2 weeks (L22 and L23 for FL and L52 and L53 for CB) led to colony profiles similar to those observed after 1 week in HSC-expansion conditions (compare L2 and L5 with L22, L23, L52, and L53). Abbreviations: BFUE, blast forming unit-erythroid; CB, cord blood; CFUE, colony forming unit-erythroid; CFU-GEMM, colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte; CFU-GM, colony forming unit-granulocyte/macrophage; CFU-M, colony forming unit-macrophage; E-expansion, erythroid expansion; FL, fetal liver.

results are similar to a previous report and were expected because these conditions are designed to amplify erythroid progenitors [41]. By contrast, for both the FL-derived and CB-derived cells, incubation for 1 week in the HSC-expansion conditions yielded a broader and more immature profile of colonies, including a lower overall erythroid contribution but a higher number of blast forming unit-erythroid (BFU-E) and colony forming unit-granulocyte, erythrocyte, monocyte, mega-

karyocyte colonies. Importantly, cells that had been cultured for a week using the HSC-expansion protocol and then for a week using the E-expansion protocol gave profiles of colonies very similar to the profiles obtained from cells grown only in the E-expansion protocol for 1 week. Together, these data strongly support the conclusion that incubation in HSC-expansion conditions leads to proliferation of early progenitors without causing their differentiation, since very similar colony profiles were obtained

after a week in E-expansion conditions whether or not a week of HSC expansion was included. As predicted from the FACS data, these results were true for all the HSC-expansion protocols tested in the methylcellulose assays (L1, L2, and L5).

To understand why incubation for 2 weeks of culture in HSC-expansion media did not increase the yield of erythroid cells above that observed after 1 week of HSC expansion, we also performed colony assays on cells obtained in these conditions. As shown in Figure 2, the cells cultured for 2 weeks in HSC-expansion conditions had not lost their differentiation potential since they had the same colony profiles as the cells that had been in HSC-expansion conditions for only 1 week. Furthermore, when cells grown for 2 weeks in HSC-expansion conditions were incubated in E-expansion conditions for 1 week, they differentiated into cells with the same colony assay profile as cells that had not been cultured in HSC-expansion conditions. Therefore, 2 weeks of HSC-expansion culture did not lead to the differentiation of the CD34⁺ cells.

Supplemental online Table 4 summarizes the weekly expansion rate of cells grown using protocols O1, L1–L5, and L12–L53. Analysis of these data combined with the above colony assay results suggests that the lack of an overall increase in yield of erythroid cells observed after the second week in HSC conditions is not caused by differentiation of the cells but by the combination of a low proliferation rate during the second week of HSC expansion as compared with the first week of HSC expansion (average expansion of 4.1 vs. 38.1 for FL and 2.3 vs. 10.3 for CB), and by a loss of proliferation capacity of the CD34⁺ cells incubated for 2 weeks in HSC-expansion conditions (average fold increase of 63.0 vs. 240.9 for FL and 64.0 vs. 189.9 for CB during the second week of E-expansion).

Serendipitous preliminary observations indicated that it might be possible to further increase the yield of cRBCs by including a short pulse of growth in erythroid conditions prior to expansion in HSC-expansion conditions (data not shown). To test this hypothesis, we incubated FL- and CB-derived CD34⁺ cells using novel expansion protocols O2, O3, O22, O32, and O33 in which the cells were pulsed for 2 days in E-expansion conditions prior to incubation in HSC-expansion conditions for 5 days (protocols O2 and O3) or 12 days (protocols O22, O32, and O33). As shown in Figure 3A and 3B, incorporation of a 2-day pulse prior to incubation in HSC-expansion conditions led to a considerable increase in erythroid cell yield as compared with the previous experiments (61.1 vs. 7.5 for FL and 110.1 vs. 14.6 for CB). Protocols O2 and O3, which differed by the presence or absence of PDGF α in the STIF medium during HSC amplification, yielded very similar results, confirming the results described in Figure 1, which had shown that adding PDGF α to the STIF medium did not increase the yield of erythroid cells. Conditions O22, O32, and O33, which differ from conditions O2 and O3 by a week-long prolongation of the HSC-expansion step, yielded lower numbers of cells, confirming that a 1-week HSC-amplification step is optimal to maximize cell yield.

FACS analysis revealed that the short pulse in E-expansion conditions led to partial differentiation of the cells during the first week of culture when compared with cells grown for a week without the short pulse (Fig. 3C, 3D). After 1 week of culture using protocols L1–L5, 30%–50% of the cells had retained CD34 expression (Fig. 1D), whereas after 1 week in conditions O2 and O3, only 5%–8% had retained CD34 expression (Fig. 3C). Colony assay analysis revealed that despite having partially differenti-

ated after 1 week in culture using HSC-expansion protocol O2 or O3 (which included the 2-day pulse), the cells had retained significant multilineage differentiation potential (Fig. 3E).

To extend these results to CD34⁺ cells at other developmental ages, we repeated the above experiments using protocols O1 (basic E-expansion protocol), L1 (basic HSC-expansion protocol for 1 week followed by protocol O1), and O2 (2-day pulse in E-expansion conditions followed by 5 days in HSC-expansion condition and by protocol O1) on CD34⁺ cells derived from two 8-week-old yolk sacs and two PB samples. Because pluripotent stem cells are some of the most promising source of cells for ex vivo production of cRBCs, we also determined whether these growth conditions could be used to expand CD34⁺ cells derived from H1 hESCs. The latter cells were produced by coculture of embryonic stem (ES) cells for 5 weeks with a feeder layer of FHB-hTERT in conditions that give rise to progenitor cells that differentiate in RBCs expressing globins similar to what would be found in a late yolk sac or early fetal liver. Additionally, we repeated the FL and CB experiments to reproduce our results in donors with different genetic backgrounds.

The three protocols again produced robust numbers of basophilic erythroblasts expanded from CD34⁺ cells regardless of the developmental ages of the starting tissues (Fig. 4A–4C; supplemental online Table 5). Importantly, the results confirmed that protocol L1 increases the yield of erythroblasts approximately 10-fold over protocol O1 and that protocol O2 further improves the yield by an order of magnitude or more.

To determine whether any of the protocols tested altered hemoglobin expression, we quantified globin expression patterns by high-performance liquid chromatography (HPLC) after an additional week of culture in maturation conditions (Fig. 4D, 4E). As expected, this analysis revealed that cRBCs express globin chains similar to the pattern found at the time of development at which the stem and progenitor cells were harvested: cRBCs produced from CD34⁺ cells harvested from yolk sac (YS) or FL expressed mostly Hb F and small amounts of embryonic globins. cRBCs produced from CD34⁺ cells harvested from CB expressed a mixture of fetal and adult hemoglobins, and CD34⁺ cells harvested from PB expressed mostly adult Hb. CD34⁺ cells produced from hESCs were very similar to cRBCs produced from yolk sac or fetal liver. Minor differences between samples from the same developmental stage were observed: the two FLs tested differed slightly in the amount of Hb A expressed, whereas the two CB and PB samples tested differed in the amount of Hb F expressed. This was expected and likely reflects either gestational age or genetic differences.

Importantly, the patterns of globin expression were not dramatically changed by the three protocols tested. Generally, cells grown in condition L1 or O2 expressed slightly more immature hemoglobin profiles than cells cultured in O1 conditions, but the effect was minor: the ES- and YS-derived cells expressed slightly more ϵ -globin, whereas the CB and PB-derived cells expressed slightly more Hb F. Expression of ζ -globin was low in all conditions and slightly more unpredictable, maybe because this gene was in the process of shutting down at the time of the harvest of the cRBCs [5].

As discussed in the Introduction, it has previously been reported that it is possible to expand late human erythroid progenitors for long periods of time by culturing them in a culture medium supplemented with cytokines and with a high concentration of dexamethasone (equivalent to 100-fold more steroids

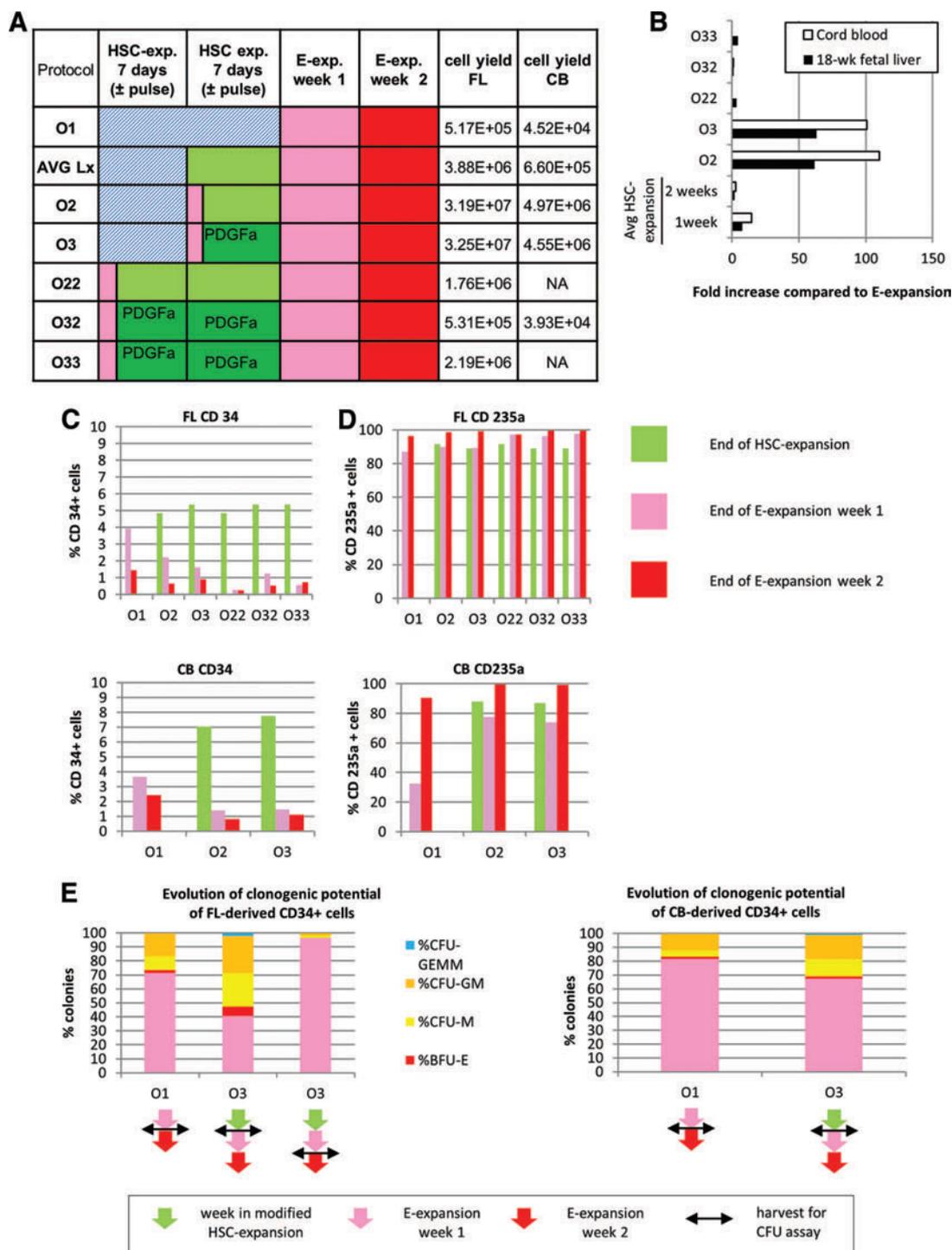
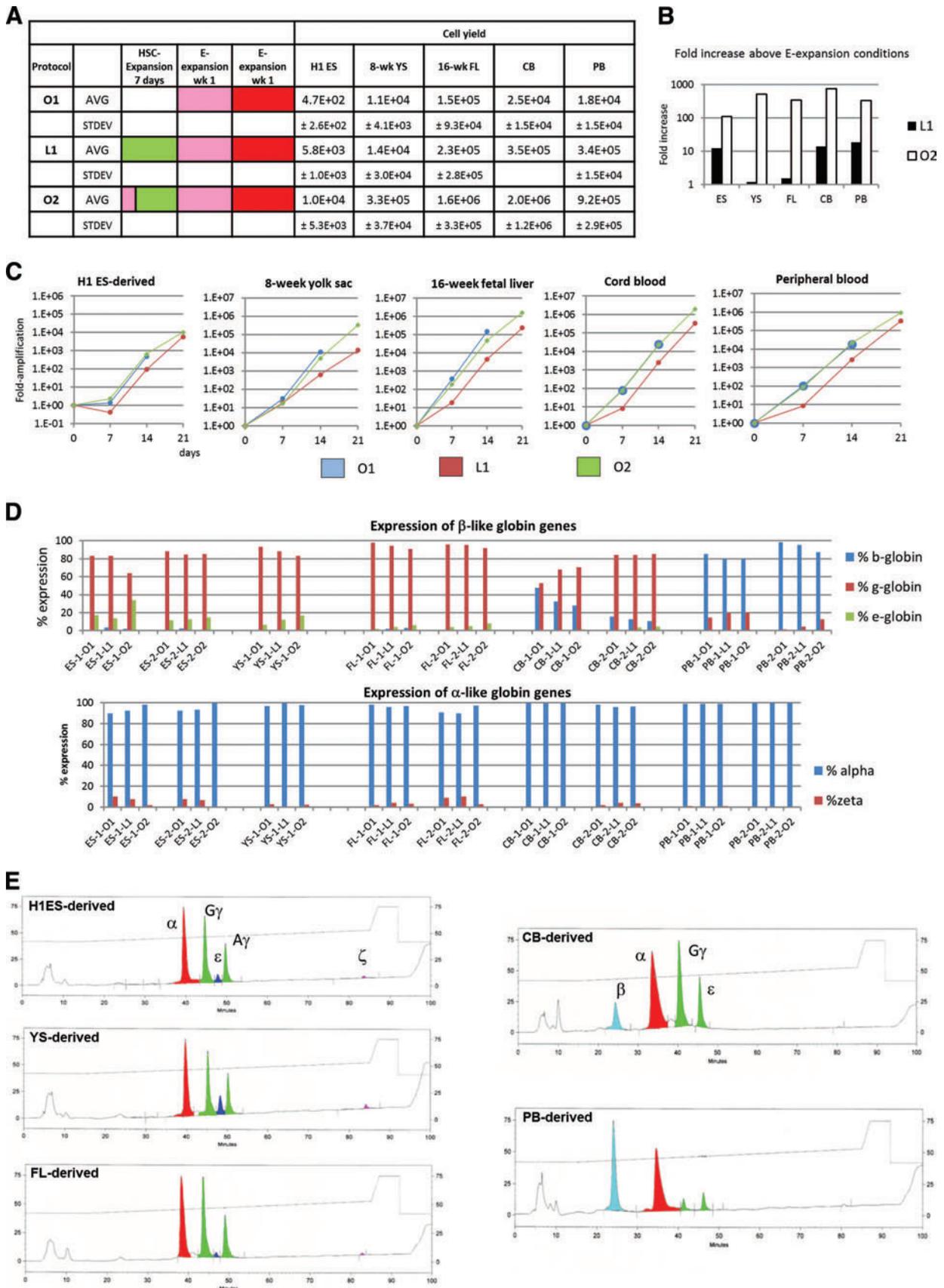


Figure 3. A short pulse in E-expansion conditions prior to incubation in HSC-expansion conditions increased the yield of cultured red blood cells. **(A):** Table illustrating the fold expansion of CD34⁺ cells grown in various conditions. Incubation conditions are color-coded (composition of the medium is indicated on the left). **(B):** Fold increase in the number of cells obtained at the end of the experiments as compared with condition O1. Conditions O2 and O3 yielded much higher numbers of cells than condition O1. As earlier, growth for 2 weeks in HSC-expansion conditions (O22, O32, and O33) did not improve the yield compared with a single week in HSC-expansion conditions. **(C, D):** Histograms summarizing a fluorescence-activated cell sorting analysis of cells observed after 1–3 weeks of culture in conditions O1 to O33 with CD34 and 235a antibodies. Cells grown in conditions O2 to O33 maintained CD34 expression longer than cells in O1 conditions but to a much smaller degree that cells grown in conditions L1–L5 (Fig. 1). CD235a analysis reveals that after a week in conditions O2–O33, most of the cells already expressed CD235a, suggesting that they were committed to the erythroid lineage. This contrasts with the results after a week in conditions L1 to L5 (Fig. 1F). **(E):** Histograms illustrating clonogenic assays performed on cells grown for 1–2 weeks. Cultures of cells grown in condition O3 for 1 week retained a greater differentiation potential than cells grown in condition O1. Abbreviations: Avg, average; BFU-E, blast forming unit-erythroid; CB, cord blood; CFU, colony forming unit; CFU-GEMM, colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte; CFU-GM, colony forming unit-granulocyte/macrophage; CFU-M, colony forming unit-macrophage; E-expansion, erythroid expansion; FL, fetal liver; HSC-exp., hematopoietic stem cell-expansion; NA, not applicable; PDGFa, platelet-derived growth factor α ; wk, week.



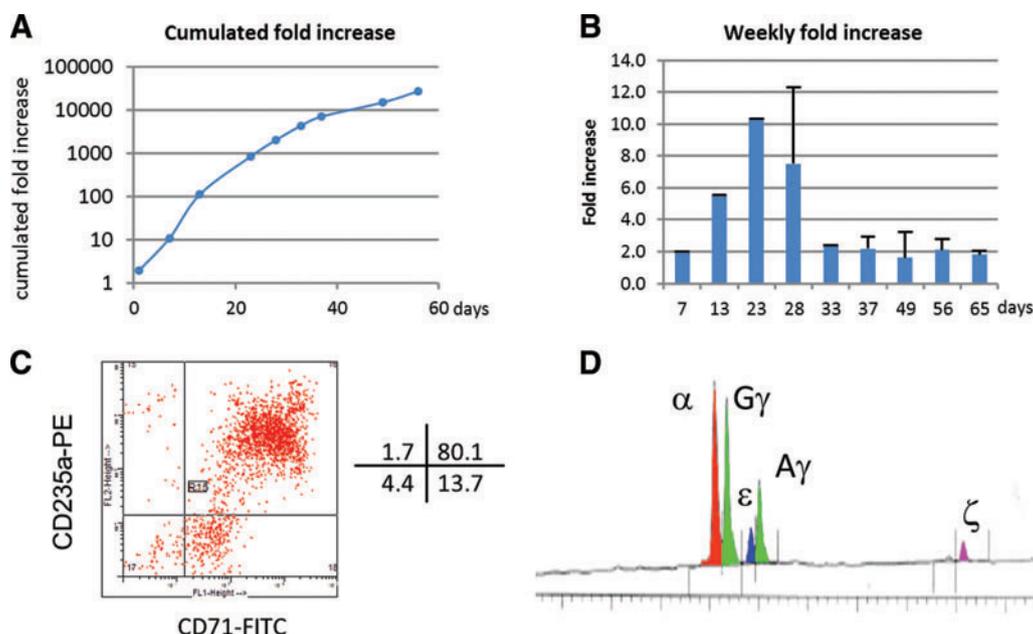


Figure 5. Expansion of human embryonic stem cell (hESC)-derived CD34⁺ cells in high steroid conditions. **(A):** H1 hESCs were differentiated for 35 days on FHB-hTERT. CD34⁺ cells were isolated and placed in culture under high steroid conditions. x-y-scatter plot represents the cumulative fold increase in cell number. **(B):** Histogram representing the weekly fold increase, which reached 10-fold on the third week and then slowed to approximately 2-fold per week. **(C):** Histogram illustrating CD235a and CD71 expression. After approximately 4 weeks, the vast majority of cells present in the culture were erythroid. **(D):** Chromatogram illustrating globin expression at day 28 of the culture. Cells expressed mostly fetal globin and a small amount of embryonic globins. Similar results were observed after 35, 42, 49, and 56 days of culture. Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin.

than the concentration of hydroxycortisone used in the E-expansion conditions). To determine whether it was possible to expand H1 hESC-derived erythroid progenitors in the presence of high steroid concentration, we incubated hESC-derived CD34⁺ cells in serum-free medium supplemented with Epo, SCF, and 1 μ M dexamethasone, as described earlier [42]. As shown in Figure 5A, these experiments revealed that hESC-derived erythroblasts could be cultured and expanded for at least 9 weeks in these conditions. The average total expansion reached 5×10^4 -fold, and the cells were still growing when the experiments were terminated. The rate of expansion was quite rapid during the first 4 weeks, reaching a maximum of approximately 10-fold, equivalent to 3–4 divisions per week, but then decreased to approximately 2-fold per week and remained at that rate (Fig. 5B). To characterize the cells present in the culture, we performed FACS analysis using CD71 and CD235a at different time points. As shown in Figure 5C, the vast majority of the cells present in the culture starting at day 30 were CD71⁺CD235⁺ erythroid cells.

To determine the type of hemoglobin expressed by these cells, HPLC analysis was performed on a weekly basis from week 2 to week 7 of culture (Fig. 5D). These experiments revealed that

globin expression was constant over time and consisted mostly of α and γ globin, the constituents of Hb F, and of small amounts of ϵ and ζ globin, the constituents of Hb Gower I.

DISCUSSION

We have tested CD34⁺ cells derived from H1 hESCs and from two YS, three FL, three CB, and two PB samples using a variety of cRBC production protocols. We found that although CD34⁺ cells from various developmental stages have different proliferation potentials, the same in vitro conditions can be used to expand all of these cells. Overall, fetal liver-derived cells had the highest proliferation potential (fold expansion in O2 conditions of 3×10^7 , 2.5×10^6 , and 0.6×10^6 for the three livers tested), followed by cord blood (fold expansion using protocol O2 of 5.0×10^6 , 1.5×10^6 , and 2.4×10^6), peripheral blood (fold expansion using protocol O2 of 1.1×10^6 and 1.1×10^6), and yolk sac (fold expansion using protocol O2 of 0.6×10^6 and 0.3×10^6). The fold expansion for hESC-derived CD34⁺ cells was lower (approximately 10^4 using protocol O2), but this number does not reflect the true potential of these cells because we have

Figure 4. HSC expansion can be used to increase the cultured red blood cell yield from CD34⁺ cells obtained from human embryonic stem cells, YS, FL, CB, and PB. **(A):** Table illustrating the fold expansion of CD34⁺ cells grown in various conditions. Incubation conditions are color-coded (composition of the medium is indicated on the left). SDs calculated from two embryonic stem cell (ESC), two YS, two FL, two CB, and two PB experiments are indicated below the averages. **(B):** Fold increase in the number of cells obtained at the end of the experiments as compared with condition O1. The results confirm that condition L1 yielded more cells than condition O1 and that condition O2 yielded more cells than condition L1. These conclusions are true regardless of the origin of the CD34⁺ cells, although the magnitude of the differences differs. **(C):** x-y-scatter plot illustrating the evolution of cell numbers for CD34⁺ cells of different origins grown in conditions O1, L2, and O2. **(D):** Histogram illustrating globin expression at the end of the culture period. ES-1 and ES-2 represent the results of two independent experiments starting from H1 human ESCs. FL-1, FL-2, CB-1, CB-2, PB-1, and PB-2 represent the results obtained from CD34⁺ cells harvested from different FL, CB, or PB samples. **(E):** Chromatograms illustrating the high-performance liquid chromatography analysis. Abbreviations: AVG, average; CB, cord blood; E-expansion, erythroid expansion; ES, embryonic stem; FL, fetal liver; HSC, hematopoietic stem cell; PB, peripheral blood; STDEV, standard deviation; wk, week; YS, yolk sac.

previously reported that the vast majority of the CD34⁺ cells produced from hESCs are not hematopoietic and die rapidly in culture. We have estimated that only 1%–5% of the cells placed in culture are still alive after 2 days of culture. The true proliferation potential of hESC-derived CD34⁺ cells is therefore 20–100 times higher than 10⁴ and is likely similar to the proliferation potential of YS or maybe even FL CD34⁺ cells. There was significant variation in proliferation potential and to a much lesser degree in globin expression when different samples from the same source were tested. This variation is likely due to a combination of the effect of gestational age and genetics.

Our most important conclusion is that combining HSC-expansion and E-expansion protocols can dramatically increase the yield of cRBCs. Using the basic HSC expansion for 1 week increased cell yield by approximately 10-fold. Adding a 48-hour cytokine pulse to promote the erythroid lineage prior to culture in HSC-expansion conditions improved the yield of cells by at least another order of magnitude. We tested several variations of the basic HSC-expansion protocol, but overall, addition of angiopoietin-like 3, angiopoietin-like 4, VEGF, or PDGF α did not have any reproducible measurable effects on the yield of cRBCs. A week of culture in HSC-expansion conditions seemed optimal since 2 weeks of HSC expansion did not lead to any significant increase in yield.

It is important to note that differentiation of hESCs yields CD34⁺ cells that are embryonic in nature and that are different from adult HSCs. Most importantly, hESC-derived CD34⁺ cells are unable to repopulate lethally irradiated immunodeficient mice and therefore do not fulfill the most important criterion used to characterize adult HSCs. In the case of this cell source, the HSC expansion cocktail probably acts by increasing the proliferation of earlier progenitors rather than of stem cells.

Analysis of progenitor levels using clonogenic assays and flow cytometry suggests that exposure to HSC culture conditions, without the 48-hour pulse, leads to expansion of the progenitors and retention of the CD34 and CD133 antigens that is roughly proportional to the number of cRBC produced. Pulsing the cells with our erythroid differentiation cocktail, prior to expansion in HSC conditions, increases the yield even further. This result was very robustly reproduced with cells at all stages of differentiation and is more difficult to explain. Our current hypothesis is that the pulse in erythroid differentiation conditions primes the multipotent progenitors to commit to the erythroid differentiation pathway without causing their immediate differentiation while potentiating the amplification potential of early erythroid progenitor more efficiently than constant culture conditions inducing continuous differentiation of these cells. These primed multipotent progenitors proliferate during the 5 days of culture in HSC conditions and partly differentiate into committed

mature erythroid progenitors. These committed progenitors retain a very strong proliferation potential when they are reexposed to the erythroid conditions. Additional experiments will be necessary to test this hypothesis. Importantly, we have also found that hESC-derived CD34⁺ could be amplified using the high steroid approach. With this method, expansion levels close to 5 \times 10⁴ were obtained after 9 weeks of culture. England et al. have shown in mice that very long lived progenitors, termed extensively self-renewing erythroblasts (ESREs), can be isolated from similar cultures using mouse ESCs as the source of cells [43]. It will be interesting to determine whether ESREs can be isolated from hESC-derived cultures.

CONCLUSION

We previously reported [7] that it is possible to routinely generate at least 3 \times 10⁵ CD34⁺ cells per six-well plate of ESCs (approximately 5 \times 10⁶ cells). This suggests that more than 10¹⁰ cRBCs can be generated using the procedures described above using a single plate of ESCs. Combining HSC expansion with E-expansion and expansion in high steroid conditions might lead to an additional 1–2 order of magnitude increase in the number of cells that can be produced from a single plate of hESCs. This suggests that although large-scale production of cRBCs will require the use of large bioreactors to expand and differentiate the CD34⁺ cells into cRBCs, production of sufficient numbers of hESCs or iPSCs to support large culture operations capable of producing hundreds of units of cRBCs per week could be done using relatively low numbers of pluripotent cells.

ACKNOWLEDGMENTS

E.E.B. is supported in part by Grants N08T-006 (C024405) and N08S-001 (C024172) from NYSTEM, the funding agency of New York State Empire Stem Cell Board. E.O. is currently affiliated with the Institute of Cardiovascular and Medical Sciences, University of Glasgow, Glasgow, U.K.; C.Q. is currently affiliated with the Stem Cell Institute, Yale University, New Haven, CT.

AUTHOR CONTRIBUTIONS

E.O. and C.Q.: performance of experiments, contributions to experimental design, data interpretation, manuscript preparation; E.E.B.: contributions to experimental design, data interpretation, manuscript preparation.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

- 1 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.
- 2 Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;282:1145–1147.
- 3 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006;126:663–676.
- 4 Kaufman DS, Hanson ET, Lewis RL et al. Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA* 2001;98:10716–10721.
- 5 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.
- 6 Olivier EN, Qiu C, Velho M et al. Large-scale production of embryonic red blood cells from human embryonic stem cells. *Exp Hematol* 2006;34:1635–1642.
- 7 Qiu C, Hanson E, Olivier E et al. Differentiation of human embryonic stem cells into hematopoietic cells by coculture with human fetal liver cells recapitulates the globin switch that occurs early in development. *Exp Hematol* 2005;33:1450–1458.
- 8 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.
- 9 Zambidis ET, Peault B, Park TS et al. Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive

stages resembling human yolk sac development. *Blood* 2005;106:860–870.

10 Chadwick K, Wang L, Li L et al. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood* 2003;102:906–915.

11 Choi KD, Vodyanik MA, Slukvin II. Generation of mature human myelomonocytic cells through expansion and differentiation of pluripotent stem cell-derived lin-CD34+CD43+CD45+ progenitors. *J Clin Invest* 2009;119:2818–2829.

12 Vodyanik MA, Bork JA, Thomson JA et al. Human embryonic stem cell-derived CD34+ cells: Efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood* 2005;105:617–626.

13 Dias J, Gumenyuk M, Kang H et al. Generation of red blood cells from human induced pluripotent stem cells. *Stem Cells Dev* 2011;20:1639–1647.

14 Lu SJ, Feng Q, Park JS et al. Biologic properties and enucleation of red blood cells from human embryonic stem cells. *Blood* 2008;112:4475–4484.

15 Ma F, Ebihara Y, Umeda K et al. Generation of functional erythrocytes from human embryonic stem cell-derived definitive hematopoiesis. *Proc Natl Acad Sci USA* 2008;105:13087–13092.

16 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.

17 Forget BG. Molecular basis of hereditary persistence of fetal hemoglobin. *Ann NY Acad Sci* 1998;850:38–44.

18 Fibach E, Manor D, Oppenheim A et al. Proliferation and maturation of human erythroid progenitors in liquid culture. *Blood* 1989;73:100–103.

19 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.

20 Freyssonier JM, Lecoq-Lafon C, Amsellem S et al. Purification, amplification and characterization of a population of human erythroid progenitors. *Br J Haematol* 1999;106:912–922.

21 Lapillonne H, Kobari L, Mazurier C et al. Red blood cell generation from human induced pluripotent stem cells: Perspectives for transfusion medicine. *Haematologica* 2010;95:1651–1659.

22 Giarratana MC, Kobari 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.

23 Neildez-Nguyen TM, Wajzman H, Marden MC et al. Human erythroid cells produced ex vivo at large scale differentiate into red blood cells in vivo. *Nat Biotechnol* 2002;20:467–472.

24 Miharada K, Hiroyama T, Sudo K et al. Efficient enucleation of erythroblasts differentiated in vitro from hematopoietic stem and progenitor cells. *Nat Biotechnol* 2006;24:1255–1256.

25 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.

26 Carotta S, Pilat S, Mairhofer A et al. Directed differentiation and mass cultivation of pure erythroid progenitors from mouse embryonic stem cells. *Blood* 2004;104:1873–1880.

27 von Lindern M, Zauner W, Mellitzer G et al. The glucocorticoid receptor cooperates with the erythropoietin receptor and c-Kit to enhance and sustain proliferation of erythroid progenitors in vitro. *Blood* 1999;94:550–559.

28 Wessely O, Deiner EM, Beug H et al. The glucocorticoid receptor is a key regulator of the decision between self-renewal and differentiation in erythroid progenitors. *EMBO J* 1997;16:267–280.

29 Migliaccio AR, Whitsett C, Papayannopoulou T et al. The potential of stem cells as an in vitro source of red blood cells for transfusion. *Cell Stem Cell* 2012;10:115–119.

30 Ghinassi BTM, Chang K-H, Halverson G et al. Comparative blood group profiling of human erythroid cells (EBs) generated from adult blood (AB), cord blood (CB), human embryonic stem cells (hESC) and induced pluripotent stem cells (iPS). Abstract presented at: 53rd American Society of Hematology Meeting; December 10, 2011; San Diego, CA.

31 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.

32 Migliaccio G, Sanchez M, Masiello F et al. Humanized culture medium for clinical expansion of human erythroblasts. *Cell Transplant* 2010;19:453–469.

33 Migliaccio AR, Whitsett C, Migliaccio G. Erythroid cells in vitro: From developmental biology to blood transfusion products. *Curr Opin Hematol* 2009;16:259–268.

34 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 thalassemic patients. *Blood Cells Mol Dis* 2002;28:169–180.

35 Cellot S, Sauvageau G. In vitro stem cell expansion: Stepping closer towards self-renewal. *Gene Therapy* 2006;13:1617–1618.

36 Sauvageau G, Iscove NN, Humphries RK. In vitro and in vivo expansion of hematopoietic stem cells. *Oncogene* 2004;23:7223–7232.

37 Sorrentino BP. Clinical strategies for expansion of hematopoietic stem cells. *Nat Rev Immunol* 2004;4:878–888.

38 Zhang CC, Kaba M, Iizuka S et al. Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. *Blood* 2008;111:3415–3423.

39 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.

40 Zhang CC, Kaba M, Ge G et al. Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. *Nat Med* 2006;12:240–245.

41 Palii CG, Pasha R, Brand M. Lentiviral-mediated knockdown during ex vivo erythropoiesis of human hematopoietic stem cells. *J Vis Exp* 2011;53:2813.

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

43 England 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.



See www.StemCellsTM.com for supporting information available online.

**Novel, High-Yield Red Blood Cell Production Methods from CD34-Positive Cells
Derived from Human Embryonic Stem, Yolk Sac, Fetal Liver, Cord Blood, and
Peripheral Blood**

Emmanuel Olivier, Caihong Qiu and Eric E. Bouhassira
Stem Cells Trans Med published online August 2, 2012

This information is current as of August 20, 2012

**Updated Information
& Services**

including high-resolution figures, can be found at:
<http://stemcellstm.alphaamedpress.org/content/early/2012/08/01/sctm.2012-0059>

Supplementary Material

Supplementary material can be found at:
<http://stemcellstm.alphaamedpress.org/content/suppl/2012/08/01/sctm.2012-0059.DC1.html>