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Ex-vivo expansion of red blood cells: How real for transfusion in humans?

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Abstract

Blood transfusion is indispensable for modern medicine. In developed countries, the blood supply is adequate and safe but blood for alloimmunized patients is often unavailable. Concerns are increasing that donations may become inadequate in the future as the population ages prompting a search for alternative transfusion products. Improvements in culture conditions and proof-of-principle studies in animal models have suggested that ex-vivo expanded red cells may represent such a product. Compared to other cell therapies transfusion poses the unique challenge of requiring great cell doses (2.5×10^{12} cells vs 10^7 cells). Although production of such cell numbers is theoretically possible, current technologies generate red cells in numbers sufficient only for safety studies. It is conceived that by the time these studies will be completed, technical barriers to mass cell production will have been eliminated making transfusion with ex-vivo generated red cells a reality.

Keywords

Ex-vivo generated erythroblasts; Red blood cell transfusion; Glucocorticoids; Growth factors

1. Introduction

Blood transfusion is in certain conditions an irreplaceable, life-saving and overall safe treatment.^{1–5} For many years, transfusion-related acute hemolysis and mortality were considered the principal adverse effects of this procedure but are now estimated to occur with a frequency from 1 in 76,000 to 1 in 1.8 million transfusions, respectively.⁶ The evolution of transfusion into a safe routine medical procedure has involved a long and at times difficult path which began with the first proof-of-concept using an animal model by Dr. Richard Lower who reported a successful dog-to-dog blood transfusion in 1665 (For a review of the history of blood transfusion see reference.⁷) This empirical treatment occurred 9 years before the first red cell was observed under a microscope by Antonie van

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Leeuwenhoek. The first successful husband to wife transfusion to treat a post-partum hemorrhage was reported in 1818 by Dr. James Blundel and was followed in 1840 by the report from Samuel Lane, who was aided by Dr. Blundel, of the first successful whole blood transfusion to treat hemophilia. However it was not until 1901, with the recognition of the heterogeneity of the human blood types by Dr. Karl Landsteiner, who provided the first scientific rationale for donor-recipient matching, that transfusion therapy evolved from an empirical practice with an unpredictable success rate into the scientifically based clinical treatment we now know. For the importance of his discovery, Dr. Karl Landsteiner received a Nobel Prize award in 1930.⁷

The blood supply of industrialized countries is adequate.¹⁻⁵ Data from the World Health Organization indicate that over 80 million donations are made every year⁸ and data from the US Department of Health and Human Services indicate that the numbers of transfusions performed in the USA in 2009 exceeded the number of units collected by 13%.¹ Nevertheless, there are chronic shortages of blood for alloimmunized patients and for patients with rare blood types who may become immunized if not transfused with matched blood. Over the years, blood centers have developed frozen blood repositories which contain units from donors with rare alloantigen profiles as well as ORh negative and ORh positive blood that can be stored for more than 10 years.⁹ To meet the growing clinical demands for such units blood centers have established focused recruitment programs to identify rare blood donors and have then typed these selective donors with DNA based and/or serological methods to identify suitable matched units.

In developed countries, there is concern that overall blood donations may become insufficient in the future. This concern increases whenever 1) there is a natural or man-made disaster, 2) new restrictions on donor eligibility are implanted, 3) the number of units discarded because of positivity for transmissible disease rises, 4) an emerging disease (i.e. Dengue) spreads to new geographic areas and 5), more recently, when new scientific information suggests that adverse outcomes are associated with older units of blood.⁹ Based on the projected increase in the median age of the human population in developed countries, it has also been predicted that blood donations will be insufficient by 2050 when the expected number of transfusion recipients (> 60 years old) will become greater than the number of donors (< 60 years of age).²

The transfusion medicine community has addressed these fundamental needs by exploring the development of alternative transfusion products.^{10,11} The alternative transfusion products evaluated for clinical use have included hemoglobin solutions (recombinant or stabilized), perfluorocarbons and red cells modified either by enzymatic cleavage or chemical treatments to mask antigens and produce ORh negative blood. Perfluorocarbons do not transport enough oxygen and are not free from side effects.¹⁰ Hemoglobin solutions, approved for clinical use in South Africa and for veterinarian use in Europe but not approved in the US, have been shown by a recent meta-analysis of 16 clinical trials (using 5 different products) to increase the risk for myocardial infarction and death.¹¹ It was in this context that research on improvements in the formulation of culture conditions for red blood cell (RBC) expansion ex-vivo gained new momentum suggesting the use of these cells as alternative transfusion products. Proof-of-principle in animal models was obtained in 2008 when Nakamura's group demonstrated that transfusion of EBs generated ex-vivo from embryonic stem cells protected mice from lethal hemolytic anemia.¹²

2. The principles of massive red cell amplification ex-vivo

Erythropoiesis is a dynamic and tightly regulated process which begins at the stem cell level.^{13,14} Hematopoietic stem cells first generate committed erythroid progenitors which

then differentiate into morphologically distinct erythroblasts (EBs) which mature into red cells (Fig. 1). This process is regulated by growth factors (GF) and other cues provided by the bone marrow microenvironment. Our understanding of this process has slowly evolved on the basis of experimental observations made during the last 50 years. The milestones of these scientific achievements are summarized in Table 1.

In its early years (1962–1977),^{15–19} research was focused on development of technical tools which allowed reproduction of erythropoiesis ex-vivo. Hematopoietic stem/progenitor cells are present at low frequency in normal tissues.^{20–22} Hence the need for purification methods that, by increasing their frequency, allowed the analyses of their biological properties. Stem/progenitor cells were first isolated in 1968 by Dr. Boyum¹⁶ on the basis of their nuclear/cytoplasmic ratios that confer a uniquely high density ($\rho = 1.0875$ and 1.0770 g/mL per murine and human stem/progenitor cells, respectively). The density separation method developed by Dr. Boyum is still used today to enrich stem/progenitor cells before their purification to homogeneity with other methods based on antigenic surface profiling.^{23,24} The purification of erythropoietin (EPO), a hormone produced by the kidney which is the major regulator of red cell production in vivo, from the sera of polycythemic sheep (step III EPO) in 1962 by Dr. Eugene Goldwasser¹⁵ made possible the development of culture conditions to reproduce erythroid differentiation ex-vivo, first from murine^{17,19} and subsequently human¹⁹ progenitor cells. Also the first biomarker, the benzidine reaction, to recognize EBs at their earliest stage of maturation when they are still morphologically identical to precursor cells of other hematopoietic lineages was discovered in these early years¹⁸ (Table 1).

In the following 10 years, scientific progress was greatly limited by the paucity of EPO available for research. However, investigators started to use in vitro culture techniques to identify the cellular mechanisms underlying abnormalities in hematocrit levels observed in vivo. The clinical observations that Cushing's syndrome²⁵ and estrogen therapy²⁶ are associated with erythrocytosis and anemia, respectively, led to the discovery that nuclear receptors, such as the glucocorticoid and estrogen receptors, as important regulators of erythropoiesis in vitro.^{27,28}

Then from 1985 to 1990, the genes encoding EPO,²⁹ interleukin-3 (IL-3)³⁰ and finally stem cell factor (SCF)^{31–33} were cloned and the proteins produced through recombinant DNA technology. The great amount of GF made available by these discoveries greatly increased the ability to grow EBs ex-vivo leading to the identification of culture conditions for massive expansion.

The era of massive expansion of EBs in liquid culture started with two discoveries: 1) SCF in combination, with EPO, drives unilineage production of EBs in vitro³⁴ and 2) proliferation and maturation of EBs are driven by mutually exclusive culture conditions.³⁵ Therefore, optimal erythroid expansion in vitro is achieved when cells are first cultured in proliferation media and then transferred to media which promote their maturation³⁵ (Fibach's culture, Table 2). In the mean time, genetic studies have shown that the glucocorticoid receptor (GR) is indispensable to activate stress erythropoiesis (an accelerate phase of red cell production observed in the spleen of mice recovering from hemolytic anemia) in mice³⁶ and that under stress conditions, EBs acquire self-renewal ability.^{37,38} EBs acquisition of self-renewal ability is dependent upon the presence of functional EPO and glucocorticoid receptors and of their signaling partner STAT-5.^{37,39} These studies prompted the discovery that liquid cultures stimulated with a combination of glucocorticoids (dexamethasone, DXM, a synthetic GR ligand), estradiol (ES) and GF generate great numbers of EBs.^{39,40} Finally, a seminal paper combined this information establishing Human Erythroid Massive Amplification (HEMA) culture conditions, a two phase liquid

culture system, the first phase of which is designed to promote expansion and is stimulated with SCF, suboptimal concentration of IL-3 (to promote proliferation while limiting myeloid differentiation), EPO, DXM and ES while the second phase is stimulated with EPO and insulin and sustains maturation.⁴¹ It has been calculated that this method allows a theoretical generation of erythroid cell numbers sufficient for 3–50 donations from low-volume cord blood (CB) units⁴² and, if performed utilizing humanized media,⁴³ also from buffy coats from adult blood (AB) donations. Later studies by Dr Douay's group demonstrated that EBs generated under HEMA conditions mature into red cells in vivo, when transfused in immunocompromised mice,⁴⁴ and in vitro, when co-cultured on suitable stromal cell lines.⁴⁵ Since these initial observations, more than 65 publications have described improved methods to generate human red cells ex-vivo for transfusion. These papers have addressed additional important questions such as optimal stem cell source, how to generate more, possibly better, red cells ex-vivo, how to promote enucleation using clinical grade (i.e. stroma-free) conditions, physical and functional properties of EBs generated ex-vivo from different sources, etc. The following sections summarize some of the important achievements obtained to date and provide guidance on how to compare data published by different laboratories and evaluate their relative significance.

3. The biological principles of ex-vivo EB expansion

EB expansion under HEMA conditions is determined by a succession of cellular compartments and molecular events summarized in Fig. 2. In the first 5 days, hematopoietic stem/progenitor cells (i.e., erythroid precursors, EPC) generate immature EBs (iEBs) with a unique CD36^{high}CD235a^{neg/low} phenotype^{41,46} (population A-B of Fig. 1), that is different from that of iEBs generated in vivo under steady state conditions which are instead CD36^{low}/CD235a^{low/neg}.⁴⁷ Ex-vivo generated CD36^{high}CD235a^{neg/low} iEBs may correspond to iEBs generated in vivo under stress conditions since, as the EBs from the spleen of phenyl-hydrazine-treated mice, they are capable of self-replication (Fig. 2).³⁸ From day 6 of HEMA culture, EPC are no longer detectable and iEBs became responsible to generate more iEBs, in addition to mEBs (CD36^{high}/CD235a^{high}). Probably due to the presence of DXM, the autophagic pathway is maximally activated in EBs obtained under HEMA conditions.³⁸ These cells, however, do not use this pathway for maturation and nuclear extrusion but instead undergo autophagic-mediated death unless transferred to maturation media supplemented with EPO and insulin but without DXM.

The relative importance of DXM and ES in the expansion process changes during the culture.⁴³ The presence of DXM is very important at the beginning of the culture while this hormone appears dispensable at later time points (probably for its effect as inducer of autophagy) while ES which is relatively less important at early time points, plays a crucial role in the last days of the culture, probably by preventing telomere exhaustion (Fig. 2).

Calculations of the total numbers of divisions occurring under HEMA conditions indicate that in the case of AB, iEBs undergo between 19 and 20 divisions during a 12 day culture period.⁴⁸ This number is still below the Hayflick limit for normal somatic cells with limited proliferative potential⁴⁹ which is estimated to be 35 mitotic divisions.⁵⁰ England et al.⁵¹ compared the ability of embryonicprimitive (E7.5 yolk sac), embryonic-definitive (E8.5–9.5 yolk sac and E12.5 fetal liver) and adult-definitive murine erythroid progenitors to generate erythroid cells ex-vivo when cultured in the presence of EPO, SCF and DXM. The data presented indicate that embryonic-definitive proerythroblasts, originated from a transient wave of early definitive erythropoiesis, are capable to generate large numbers (10¹⁰- to 10³⁰-fold expansion) of proerythroblasts while adult-definitive proerythroblasts expanded only 10²- to 10⁵-fold. We calculated that 34 divisions were required to obtain 10¹⁰-fold expansion from embryonic-definitive proerythroblasts but that astronomically greater

numbers of divisions were necessary to reach fold increases of 10^{30} (i.e., > 100 mitotic divisions per cell). These data suggest that at least murine EBs may overcome the Hayflick limit, prompting current investigations for the identification of culture conditions which may allow human EBs to overcome this limit as well, generating more, and possibly better, EBs ex-vivo for transfusion. Some of these efforts are summarized below (see also Table 2).

4. Optimization of the expansion process

4.1. Source of stem cells for the production process

For normalization purposes, generation of EBs in culture is expressed as fold increases (FI), i.e. the ratio between number of cells observed at a given day (usually days 10–13) and the one used to start the culture. It is commonly accepted that EPC are present in the CD34^{pos} cell fraction of the MNC of AB and CB. Investigators have indifferently used AB and CB derived CD34^{pos} cells and/or MNC as source of EPC in their EB expansion studies.^{35,39,41,44,45,52–66} FIs observed in cultures with CD34^{pos} cells (range FI: 29– 10^5 ^{41,45,55,57,61–67} and 105– 10^6 ^{44,45,56,60,63} per AB and CB CD34^{pos} cells, respectively) are much higher than those observed with MNC (range FI = 2–150^{35,41,43,52,53,58,64} vs 300–1000^{39,43,54,58} per AB and CB MNC, respectively) (Table 2). However, since CD34^{pos} cells represent a small fraction ($\sim 2.5 \times 10^3$ cells/mL in AB and 2.5×10^4 cells/mL in CB)^{20–22} of MNC, calculations based on FI and number of cells present in the fraction indicate that MNC from both AB and CB generate on average 10 to 100 times more EBs than the corresponding CD34^{pos} cells.⁴⁸ This is due to two factors: 1) cell losses during the purification procedure (average purification procedures recover <30% of CD34^{pos} cells) and 2) presence of CD34^{neg}CD36^{low} EPC in MNC lost during purification.^{46,64} Both MNC and CD34^{pos} cells from AB (FI = 2–150 and 29– 10^5 , respectively) generates lower numbers of EBs than the corresponding cells from CB (FI = 300–1000 and 10^5 – 10^6 , respectively). This difference has been ascribed to a lower proliferation potential of AB cells with respect to those from CB exemplified by the observation that human progenitor cells from CB generate more hematopoietic cells and for longer time than those from AB when engrafted in NOD/SCID mice.⁶⁸ However, it may also be related to different nutrient requirements since under humanized culture conditions, i.e. when culture media is formulated with clinical grade compounds of human origin, the numbers of EBs generated by AB MNC are similar to those generated by CB MNC (FI = 500 in both cases).⁴³

Dr. Hal Broxmeyer, the pioneer of CB transplantation, often mentions that successful translational research should be guided by the overall principle: “the simpler the better” (Dr. H.L Broxmeyer, personal communication, November 2010). This principle supports the use of MNC from either CB or AB as the stem cell of choice for production of transfusion products ex-vivo.

The number of EBs generated by MNC from the same donor in independent cultures is extremely reproducible.⁴¹ By contrast, the number of EBs generated in culture from different donors may vary over 1 log. The heterogeneity of expansion potential observed with individual donors has been ascribed to variability in EPC content in the blood from different donors (which may also span over 1-log).^{22,69–71} However, the biochemical basis for the heterogeneity of the EPC content in the blood is still unknown. *GR* is a highly polymorphic gene containing single nucleotide polymorphisms (SNPs) both in the coding region and in regions associated with alternative splicing and mRNA stabilization.^{72–74} The most studied receptor is the GR isoform which, upon binding to its ligand, migrates to the nucleus where it activates/suppresses expression of target genes.^{72,73,75} An alternative splicing between exons 3 and 4 generates GR_Δ, an isoform containing an additional aa (Arg) in the DNA-binding domain which reduces the transactivation potential by half.⁷⁶ Given the role played by GR in the expansion process, we investigated whether variability in the

expression of GR α and GR β might affect ex-vivo generation of EBs and contribute to the observed heterogeneity in expansion potential among different donors. While GR α was equally expressed in EBs from both AB and CB, GR β was expressed only by EBs obtained from AB donors who generated low numbers of EBs ex-vivo, suggesting that expression of this isoform may predict poor expansion⁷⁷ (Fig. 3). This observation suggests that SNP which favor expression of GR α may prospectively identify poor AB expanders.

4.2. Alternative GF combinations

The first phase of the erythroid expansion system developed by Fibach in 1989³⁵ (step 1) and its modification published in 2000¹¹⁸ does not include EPO and is stimulated with conditioned media (CM) from the 5637-bladder carcinoma cell line. Some investigators still stimulate the step 1 phase of EB expansion cultures with CM, either from the 5637 cell line or from phytohemagglutinin-stimulated leukocytes.^{52,53,78} The majority of the most recent studies, however, replace CM with human SCF and IL-3 produced by recombinant technology.^{34,55,61,66,67,79,80} All of these recent expansion cultures are stimulated also with EPO. There is a widespread consensus to stimulate the maturation phase (step 2) of EB cultures with EPO, either alone or with insulin (or Insulin-like Growth Factor 1 [IGF1], which is 50–70% identical to insulin).^{39,41,43,44,54,64} Both insulin and IGF1 have been reported to synergize the maturation effects of EPO by promoting nuclear condensation and enucleation.⁸¹ In most of these conditions, however, enucleation remains inefficient (see Table 2).

In vivo, EB maturation occurs in highly specialized areas of the microenvironment: the erythroblastic islands, which consist of a central macrophage surrounding with its cytoplasmic protrusions 8–12 EBs at different stages of maturation. Macrophages/EBs establish their interactions through specific adhesion receptors (CXCL12, P-selectin and VCAM1) on the macrophage and counter-receptors on the EBs (CXCR4, P-selectin ligand1 and VLA-4). Macrophage/EB interaction is thought to promote hemoglobinization, by facilitating iron uptake, and enucleation, by providing attachment support facilitating nucleus extrusion. After enucleation, reticulocytes lose their association with the macrophage and egresses into the blood stream⁸² while the pyrenocytes, a cellular element formed by the erythroid nucleus surrounded by a cytoplasmic rim, is phagocytized by the macrophage. These physiological cell interactions have been modeled by including in step 2 cultures either CD34-derived macrophages⁶⁰ or mesenchymal stromal cells, allowing observation of enucleation rates ~ 100%.^{45,63} Satisfactory enucleation rates have been also achieved by inducing a nuclear self-extrusion competent state through stimulation of Step 2 cultures with EPO plus IGF2, VEGF and mifepristone⁵⁶ or thyroid hormone (3,5,3-triiodothyronine [T₃]), human serum and ZK112993.⁵⁴ Both ZK112993 and mifepristone are glucocorticoid antagonists. More recently, Keerthivasan et al.⁸³ have promoted enucleation of ex-vivo generated EBs by treatments with vacuolin-1, an inducer of vesicle trafficking.

In order to potentiate EB expansion, some investigators pre-culture CD34^{pos} cells under culture conditions designed to promote hematopoietic stem/progenitor cell (HSC/HPC) expansion. This preculture or priming phase is EPO-free and contains SCF and Fms-like tyrosine kinase receptor-3 Ligand (Flt-3L) and may or may not contain thrombopoietin (TPO).^{44,57,60,66} The highest proliferation rates (up to FI = 10⁵–10⁶), however, have been so far reported in cultures seeded with CB CD34^{pos} cells^{44,60} pre-cultured with a stromal layer.⁶⁰

Chromatin-modifying agents, such as histone deacetylase inhibitors (HDACIs) and DNA methyltransferase inhibitors (DNMTIs), modulate gene expression through chromatin remodeling. It has been demonstrated that these compounds are capable to enhance the

effects of growth factors on maintenance and expansion of HSC/HPC⁸⁴ and to increase the number of human marrow repopulating cells.^{85,86} Recently, Chaurasia et al.⁸⁷ have demonstrated that the addition of valproic acid to pre-culture of CB CD34^{pos} cells stimulated with SCF, Flt-3L and TPO allows generation of numerous EBs capable to colonize the bone marrow and to mature into circulating red cells when transfused into NOD/SCID/IL2R^{null} mice.

4.3. Additional reagents/nutrients/physical conditions

In agreement with its well established role on erythropoiesis (reviewed in⁸⁸), hypoxia (5–6% O₂) has been reported by Fibach et al.³⁵ and Wada et al.⁵² to improve the numbers of hemoglobinized normoblasts generated in the maturation phase of the culture. More recently, Vlaski et al.⁶³ have demonstrated that hypoxia also favors the proliferation of EBs in Giarratana's cultures⁴⁵ allowing the observation of FIs as high as 3×10^5 .

Based on the positive effect exerted by Insulin-like Growth Factor 2 (IGF2) and Vascular Endothelial Growth Factor (VEGF) on survival, proliferation and/or differentiation of hematopoietic progenitors, Miharada and colleagues⁵⁶ have included these two GF in the expansion medium^{89–91} obtaining greater FI than those observed with the combination of EPO, SCF and IL-3 alone. The EBs generated in the presence of these two GF appeared also competent for nuclear self-extrusion.

Some authors include in the media Cyclosporin A, a lymphotoxic drug, which appears to improve EB growth in cultures containing large numbers of MNC, and are therefore, heavily contaminated with lymphocytes.^{35,61}

4.4. Alternative cell sources under consideration

In addition to hematopoietic stem cells, human embryonic stem cells (hESC)^{92,93} and induced pluripotent stem cells (iPS)^{94–96} are also being actively investigated as a source of EPC for EBs expansion *ex-vivo*. Since red cells do not have a nucleus, safety considerations suggest, in fact, that red cells may represent the first cell therapy product to be generated from hESC and iPS. We believe that the hESC cell lines available up to now are unlikely to advance our ability to generate EBs *ex-vivo* for transfusion because they are available in limited numbers and capable to generate type ORh negative red cells (universal donor blood) but not cells with the complex antigen phenotype necessary for matching of alloimmunized patients.⁹⁷ It is likely that the generation of new hESC lines allowed by the recent lift on hESC generation ban will overcome this barrier.

iPS may be generated from mature somatic cells of any individual (fibroblasts, CD34^{pos} cells and T cells) by forced expression of Oct4, Sox2, Klf4 and cMyc,⁹⁸ and have therefore the potential to generate matched red cells. Chang et al.⁹⁴ have demonstrated that the pluripotent status induced by the reprogramming procedure is not affected by the genetic imprinting (fetal or adult) of the somatic cells utilized (fetal fibroblasts or adult mesenchymal stem cell). However, the process itself is still poorly controlled and generates iPS lines which express great variability in EB expansion at the clonal level. The observation that hESC⁹³- and iPS⁹⁴-derived EBs express embryonic and fetal globin genes (instead of adult globin genes) has raised concerns about their use as transfusion product for the general population. This property, however, may be advantageous under certain conditions. Patients with inherited deficiency to express adult globin genes (such as α -thalassemia and sickle-cell anemia patients) have no or mild clinical phenotype when they co-inherit genetic mutations allowing retention of at least 10–20% of fetal globin gene production in red cells postnatally (hereditary persistence of fetal hemoglobin [Hb] syndrome).⁹⁹ Based on this clinical observation, investigators are attempting to identify

epigenetic drugs that may cure these deficiencies by reactivating expression of fetal Hb in the red cells generated *in vivo*. The observation that EBs expanded *ex-vivo* from iPS derived from these patients express mainly fetal globin genes makes these cells ideal candidates for autologous transfusion. However this interesting possibility remains to be tested because the process of pluripotency induction may also activate expression of antigens capable to induce allo- and/or auto-immune reactions.

5. Comparison of the properties of human EBs generated *ex-vivo* and *in vivo*

5.1. Physical properties

Size and morphological appearance of *ex-vivo* and *in vivo* generated EBs have been compared by various authors.^{39,45,48,54,61,62} The size of *ex-vivo* generated EBs is 3 times greater than normal up to the proerythroblast stage but decreases with maturation. The diameter of *ex-vivo* generated orthochromatic EBs and reticulocytes is normal (~10 µm).⁴⁸

The morphology of the cytoplasm of *ex-vivo* generated EBs is highly vacuolated, a sign of membrane dysfunction. However, the deformability of *ex-vivo* generated reticulocytes is similar to that of native RBC by ektacytometry.⁴⁵

5.2. Metabolic properties

With maturation, *ex-vivo* generated EBs up-regulate the expression of genes related to metabolic pathways important for RBC survival (glycolysis, arginine metabolism, etc.)¹⁰⁰ so that the glucose-6-phosphate dehydrogenase and pyruvate kinase content of *ex-vivo* generated RBC is similar to that of a homogeneous population of young RBC.⁴⁵

5.3. Survival after cryopreservation and chromosome state

Good survival after cryopreservation is an important requisite for cell therapy products. Both Neildez-Nguyen et al.⁴⁴ and Migliaccio et al.⁴⁸ reported that *ex-vivo* expanded EBs survive cryopreservation/thawing procedures without loss of erythroid clonogenic potential. In addition, Migliaccio et al.¹⁰¹ reported that the viability and proliferation/maturation potential of EBs freshly expanded or stored short (1 to 2 months)- and long (8year)-term is comparable. Moreover, long-term stored EBs did not acquire cytogenetic abnormalities upon further extensive expansion *in vitro*.

5.4. Hb content of EBs expanded in different culture systems

The type of Hb (adult or fetal) produced by *ex-vivo* expanded EBs reflects both the cell source from which they are generated (AB, CB, hESC or iPS) and the culture system used for expansion. As mentioned earlier, hESC and iPS derived EBs express mainly HbF but, as expected, AB- and CB-derived EBs^{45,58,90} express predominantly α and β globin chains, respectively. These data were confirmed by gene expression profile studies.^{55,78,102,103} Variations in the levels of α / β ratio expressed by both AB and CB EBs and changes in this ratio with maturation have been reported (Table 2). This variation may reflect the strength of the stress erythropoiesis signal established by the culture components of the individual studies.

Novel insights in the regulation of globin gene expression were provided by Neildez-Nguyen et al.,⁴⁴ who demonstrated that EBs generated *ex-vivo* from CB express mainly HbF (74%) but generate *in vivo* RBC that express primarily HbA (97%). These results suggested that the hemoglobin switch may be accelerated by interactions between CB EBs and the adult microenvironment. This hypothesis is further supported by Fujimi et al.⁶⁰ who

have showed that EBs expanded from CB in the presence of a human adult stromal feeder layer express equal amounts of HbF and HbA.

The efficiency of ex-vivo and in vivo generated RBC as oxygen transporter was compared by Giarratana et al. and Fijimi et al. through carbon monoxide rebinding after flash photolysis, a technique which measures the capability of the Hb contained within a red cell to bind and release oxygen. The slight differences reported in both papers between the oxygen dissociation curves of Hb from ex-vivo expanded RBC and AB RBC may be ascribed to the different type of Hb (HbF or HbA) present in the two cell types.

5.5. Expression profiling

The massive number of EBs that may be generated in vitro with current techniques and the definition of surface markers for the prospective isolation of these cells as they mature allow studies to define the transcriptome during erythroid maturation. These studies, summarized in Table 3,^{55,78,100,102–108} assume that the gene expression profile of cells obtained in vitro may shed light on that of the cells generated in vivo. Proof-of-principle for this assumption is provided by the observations that genes encoding metabolic enzymes (glycolysis or arginine metabolism),¹⁰⁰ surface antigens and structural proteins (tombospondin, glycophorin-A, transferrin receptor, blood group antigens, ankyrin) are upregulated and those encoding proteins present in cellular organelles (ribosome and mitochondria)^{55,78,100,104,106} are down-regulated in expression profiles from EBs as they mature in vitro, that are consistent with the expression patterns of these same genes during EB maturation in vivo. Based on this assumption, it is foreseen that studies on gene profiling of ex-vivo generated cells may provide unique insights on the regulation of genes, such as those involved in chromatin remodeling, DNA repair, transcription, translation and post-translational regulation, cell signaling, cell cycle, apoptosis and autophagy, which are dysregulated in congenital RBC diseases (Table 3).^{55,78,100,104}

However, these studies have the caveat that gene expression is extremely sensitive to culture conditions. In particular, the overall transcriptome of EBs expanded with GF may be different from those obtained under massive amplification conditions (GF plus DXM and ES) since the two cell types may be more similar to cells generated in vivo under steady-state or stress erythropoiesis. As mentioned earlier, this consideration is very important to compare the levels of HbF/HbA expressed by EBs obtained in different culture conditions.¹⁰⁹ Important changes in the EB transcriptome may also be related to the stem cell source (CB, AB, hESC or iPS).

The first erythroid transcriptome was described by Kolbus et al.¹¹⁰ for murine EBs. This paper demonstrated that the EB transcriptome is subjected to modulation by interactions between GF with DXM (see Table 3). EPO and SCF never show opposite effects on the regulation of gene transcription and share common targets but DXM may either synergize or antagonize the regulatory effects of EPO/SCF on gene expression. Genes positively regulated by DXM include CXCR4 (CXC chemokine receptor 4) and MMP2 (matrix metalloproteinase 2), both involved in cell trafficking, VDUP1 (Vitamin D₃ upregulated protein 1), which controls reactive oxygen species, GILZ (glucocortico-idinduced leucine zipper), which interferes with the signaling pathway controlled by NFκB (Nuclear Factor kappa B) and AP-1 (activator protein-1), and CD59 antigen, that inhibits complement-mediated cell lyses and is deficient on red cells from patients affected by paroxysmal nocturnal hemoglobinuria.¹¹⁰ The pivotal role played by culture condition on gene expression was confirmed by Sripichai et al..¹⁰⁸ These authors demonstrated reproducible modulation of the expression of genes encoding nuclear transcription factors which induce HbF production (MAFF, ID2, HHEX, SOX6, EGR1) and γ -globin genes by cytokine combinations (low and high nuclear transcription factors and HbF expression in cultures

stimulated with EPO alone and in those stimulated with EPO, SCF and transforming growth factor- β).

In comparing gene expression profiles reported by different laboratories, it is also important to pay attention to the stage of maturation of the cell population(s) analyzed. Gubin and colleagues¹⁰⁴ analyze the transcriptional profile of CD71⁺⁺⁺⁺ erythroblasts arising after 5 days of stimulation with EPO while Peller et al.,¹⁰⁰ Keller et al.,⁵⁵ Bruchova et al.,¹⁰⁵ Varricchio et al.¹⁰² and Merryweather et al.,⁷⁸ instead, provide “snapshots” of gene expression at discrete phases of maturation defined by the time of the culture in the case of the first three studies and on cells prospectively isolated according to maturation markers (CD36, CD235a and CD71) in the fourth study.⁷⁸ While the approach used by Merryweather et al.⁷⁸ allows for a precise definition of the transcriptome of EBs at specific stages of maturation, it is subject to caveats of interpretations deriving from the poor definition of the GF used to stimulate the cultures (CM from cell lines).

A general guideline when comparing expression profiling published by different authors is to focus attention on the scientific question addressed in study: comparison of EBs versus non-erythroid cells,¹⁰⁴ modulation of gene expression during maturation,^{55,78,100,102,105} changes in expression pattern of genes specifically related to blood group antigens during the maturation of adult blood and cord blood derived EBs,¹⁰⁶ etc. (see Table 3).

6. Prospective time table for clinic application of ex-vivo expanded erythrocytes

Current technologies may easily generate ex-vivo numbers of red cells sufficient for proof-of-principle studies in humans (equivalent to those present in 5–10 mL of blood). Therefore, due to the present excitement for this topic, the first-in-man safety studies are likely underway and will be published soon in peer-review journals (Table 4). The numbers of erythroid cells necessary for transfusion ($> 2.5 \times 10^{12}$), however, cannot be easily produced with current technologies. In addition, in vivo administration of such great numbers of cells poses greater immunological challenges than injection of small blood volumes. The first-in-man transfusion with ex-vivo expanded red cells will likely occur only after considerable improvement of our knowledge on both mass production techniques and immunological properties of these red cells. Safety issues on the use of ex-vivo expanded red cells such as transmission of known and unknown adventitious agents¹¹¹ and neoplastic transformation^{112–114} may be addressed following guidelines already developed for other transfusion products. However, in addition to red blood group antigen related immunization issues that may be addressed with current guidelines, ongoing clinical trials indicate that immunization of recipients to xenogeneic products used for cell production represents a major risk of cell therapy.^{115–117} This risk is higher for products which will be administered multiple times and at great doses, such as ex-vivo generated red cells.

Red cells, however, have additional clinical applications that do not pose a challenge in terms of number of cells required. Clinical applications that are within reach and may be developed in the immediate future are represented by 1) generation of reagent RBC for antibody identification in alloimmunized patients, 2) drug discovery and 3) systemic drug delivery. The use of ex-vivo expanded red cells for systemic drug delivery, by providing information on long-term immunogenicity of these cells, will then pave the way for their use for transfusions of alloimmunized patients and the general population.

6.1. Drug discovery

In vitro tests using red cells from donors with rare blood types are used to predict the suitability of these donors for alloimmunized patients. Since red cells from rare donors are

available in limited numbers, EBs expanded ex-vivo from the buffy coat of these donors discarded at the time of leukoreduction may represent valuable substitutes for in vivo generated reagent RBC in these assays. In addition, to reduce animal experimentation and increase the sensitivity and robustness of pre-clinical toxicology and efficacy studies, future assessment of toxicity and efficacy of new drugs will likely employ in vitro assays using primary cells reflecting age, sex and genetic polymorphism of human populations.¹¹⁸ *Ex-vivo* generated EBs are under consideration to be used for in vitro assays aimed to identify drugs for personalized therapy of Thalassemia and Sickle Cell Anemia (HbF inducers),^{103,119} B19 parvovirus, infection (Inhibitors of the 11 kDa mediated caspase 10 activation),^{118,120} cellular-based malaria vaccines (lentiviral-induced miRNA overexpression strategies to inhibit intracellular proliferation of the parasite),^{121,122} identification of cancer patients that may develop anemia following therapy.

6.2. Drug delivery

The use of red cells for systemic drug delivery has been proposed since some time. The progresses made in recent years in retroviral technology and ex-vivo EBs expansion suggest that ex-vivo generated red cells from molecular engineered CD34^{pos} cells (or iPS) may represent potent vehicles for therapeutic administration of proteins, especially of those with short half lives that require continuous or frequent intravenous administration. Proof-of-principle for this application has been obtained in mice for Hemophilia,¹²³ an X-linked recessive congenital disorder of coagulation due to Factor VIII or IX deficiency affecting 11–13 per 100,000 male births in the US, depending on ethnicity.¹²⁴ The disease is currently treated by prophylactic factor infusion but the treatment is expensive and requires patient compliance.

7. Conclusion

The substantial progress made in expanding EBs ex-vivo suggests that ex-vivo generation of red cell transfusion products may become a reality some time in the future. The possibility of developing hES and iPC that would generate EBs customized for the recipient is an interesting possibility that deserves careful investigation. Development of clinical grade transfusion products, however, must wait for the development of production processes that permit safe ex-vivo expansion of human EBs in a cost-efficient manner.

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References

1. The 2007 National Blood Collection and Utilization Survey Report. Washington, DC: DHHS; 2008.
2. Ali A, Auvinen MK, Rautonen J. The aging population poses a global challenge for blood services. *Transfusion*. 2010; 50:584. [PubMed: 19912582]
3. <http://www.fda.gov/downloads/BiologicsBloodVaccines/SafetyAvailability/ReportalProblem/TransfusionDonationFatalities/UCM254860.pdf>
4. Sullivan MT, Cotten R, Read EJ, Wallace EL. Blood collection and transfusion in the United States in 2001. *Transfusion*. 2007; 47:385. [PubMed: 17319817]
5. Zimrin AB, Hess JR. Current issues relating to the transfusion of stored red blood cells. *Vox Sang*. 2009; 96:93. [PubMed: 19152602]
6. Linden JV, Wagner K, Voytovich AE, Sheehan J. Transfusion errors in New York State: an analysis of 10 years' experience. *Transfusion*. 2000; 40:1207. [PubMed: 11061857]

7. Alter HJ, Klein HG. The hazards of blood transfusion in historical perspective. *Blood*. 2008; 112:2617. [PubMed: 18809775]
8. Glynn SA. Blood supply safety: an NHLBI perspective. *Transfusion*. 2008; 48:1541. [PubMed: 18713102]
9. Hod EA, Zhang N, Sokol SA, Wojczyk BS, Francis RO, Ansaldi D, et al. Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation. *Blood*. 2010; 115:4284. [PubMed: 20299509]
10. Spahn DR. Blood substitutes. Artificial oxygen carriers: perfluorocarbon emulsions *Crit Care*. 1999; 3:R93.
11. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. *JAMA*. 2008; 299:2304. [PubMed: 18443023]
12. Hiroyama T, Miharada K, Sudo K, Danjo I, Aoki N, Nakamura Y. Establishment of mouse embryonic stem cell-derived erythroid progenitor cell lines able to produce functional red blood cells. *PLoS One*. 2008; 3:e1544. [PubMed: 18253492]
13. Papayannopoulou, T.; Migliaccio, A. Biology of Erythropoiesis, Erythroid Differentiation and Maturation. In: Livignstone, C., editor. *Hematology: Basic principle and Practice*. 5. New York: 2009. p. 276
14. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell*. 2008; 132:631. [PubMed: 18295580]
15. Goldwasser E, White WF, Taylor KB. Further purification of sheep plasma erythropoietin. *Biochim Biophys Acta*. 1962; 64:487. [PubMed: 13948830]
16. Boyum A. Collection and handling of bone marrow suspensions for intravenous use. *Scand J Clin Lab Invest*. 1962; 14:419. [PubMed: 13872225]
17. Stephenson JR, Axelrad AA, McLeod DL, Shreeve MM. Induction of colonies of hemoglobin-synthesizing cells by erythropoietin in vitro. *Proc Natl Acad Sci U S A*. 1971; 68:1542. [PubMed: 4104431]
18. Orkin SH, Harosi FI, Leder P. Differentiation in erythroleukemic cells and their somatic hybrids. *Proc Natl Acad Sci U S A*. 1975; 72:98. [PubMed: 164031]
19. Ogawa M, Grush OC, O'Dell RF, Hara H, MacEachern MD. Circulating erythropoietic precursors assessed in culture: characterization in normal men and patients with hemoglobinopathies. *Blood*. 1977; 50:1081. [PubMed: 922159]
20. Bender JG, Unverzagt KL, Walker DE, Lee W, Van Epps DE, Smith DH, et al. Identification and comparison of CD34-positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor flow cytometry. *Blood*. 1991; 77:2591. [PubMed: 1710512]
21. Wong A, Yuen PM, Li K, Yu AL, Tsoi WC. Cord blood collection before and after placental delivery: levels of nucleated cells, haematopoietic progenitor cells, leukocyte subpopulations and macroscopic clots. *Bone Marrow Transplant*. 2001; 27:133. [PubMed: 11281381]
22. Migliaccio AR, Adamson JW, Stevens CE, Dobrila NL, Carrier CM, Rubinstein P. Cell dose and speed of engraftment in placental/umbilical cord blood transplantation: graft progenitor cell content is a better predictor than nucleated cell quantity. *Blood*. 2000; 96:2717. [PubMed: 11023503]
23. Randall TD, Lund FE, Howard MC, Weissman IL. Expression of murine CD38 defines a population of long-term reconstituting hematopoietic stem cells. *Blood*. 1996; 87:4057. [PubMed: 8639761]
24. Gibbs KD Jr, Gilbert PM, Sachs K, Zhao F, Blau HM, Weissman IL, et al. Single-cell phospho-specific flow cytometric analysis demonstrates biochemical and functional heterogeneity in human hematopoietic stem and progenitor compartments. *Blood*. 2011; 117:4226. [PubMed: 21357764]
25. Gursoy A, Dogruk Unal A, Ayturk S, Karakus S, Nur Izol A, Bascil Tutuncu N, et al. Polycythemia as the first manifestation of Cushing's disease. *J Endocrinol Invest*. 2006; 29:742. [PubMed: 17033265]
26. Dukes PP, Goldwasser E. Inhibition of erythropoiesis by estrogens. *Endocrinology*. 1961; 69:21. [PubMed: 13724883]

27. Golde DW, Bersch N, Cline MJ. Potentiation of erythropoiesis in vitro by dexamethasone. *J Clin Invest.* 1976; 57:57. [PubMed: 173742]
28. Singer JW, Adamson JW. Steroids and hematopoiesis. III. The response of granulocytic and erythroid colony-forming cells to steroids of different classes. *Blood.* 1976; 48:855. [PubMed: 1000083]
29. Jacobs K, Shoemaker C, Rudersdorf R, Neill SD, Kaufman RJ, Mufson A, et al. Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature.* 1985; 313:806. [PubMed: 3838366]
30. Yang YC, Ciarletta AB, Temple PA, Chung MP, Kovacic S, Witek-Giannotti JS, et al. Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell.* 1986; 47:3. [PubMed: 3489530]
31. Williams DE, Eisenman J, Baird A, Rauch C, Van Ness K, March CJ, et al. Identification of a ligand for the c-kit proto-oncogene. *Cell.* 1990; 63:167. [PubMed: 1698553]
32. Martin FH, Suggs SV, Langley KE, Lu HS, Ting J, Okino KH, et al. Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell.* 1990; 63:203. [PubMed: 2208279]
33. Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin FH, Atkins HL, et al. Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell.* 1990; 63:213. [PubMed: 1698556]
34. Migliaccio G, Migliaccio AR, Druzin ML, Giardina PJ, Zsebo KM, Adamson JW. 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. [PubMed: 1375106]
35. Fibach E, Manor D, Oppenheim A, Rachmilewitz EA. Proliferation and maturation of human erythroid progenitors in liquid culture. *Blood.* 1989; 73:100. [PubMed: 2910352]
36. Bauer A, Tronche F, Wessely O, Kellendonk C, Reichardt HM, Steinlein P, et al. The glucocorticoid receptor is required for stress erythropoiesis. *Genes Dev.* 1999; 13:2996. [PubMed: 10580006]
37. Dolznig H, Grebien F, Deiner EM, Stangl K, Kolbus A, Habermann B, et al. Erythroid progenitor renewal versus differentiation: genetic evidence for cell autonomous, essential functions of EpoR, Stat5 and the GR. *Oncogene.* 2006; 25:2890. [PubMed: 16407844]
38. Migliaccio G, Masiello F, Tirelli V, Sanchez M, Varricchio L, Whitsett C, et al. Under HEMA conditions, self-replication of human erythroblasts is limited by autophagic death. *Blood Cells Mol Dis.* 2011; 7:182. [PubMed: 21775174]
39. Panzenbock B, Bartunek P, Mapara MY, Zenke M. Growth and differentiation of human stem cell factor/erythropoietin-dependent erythroid progenitor cells in vitro. *Blood.* 1998; 92:3658. [PubMed: 9808559]
40. von Lindern M, Zauner W, Mellitzer G, Steinlein P, Fritsch G, Huber K, 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. [PubMed: 10397722]
41. Migliaccio G, Di Pietro R, di Giacomo V, Di Baldassarre A, Migliaccio AR, Maccioni L, 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. [PubMed: 12064913]
42. Douay L, Lapillonne H, Turhan AG. Stem cells—a source of adult red blood cells for transfusion purposes: present and future. *Crit Care Clin.* 2009; 25:383. [PubMed: 19341915]
43. Migliaccio G, Sanchez M, Masiello F, Tirelli V, Varricchio L, Whitsett C, et al. Humanized culture medium for clinical expansion of human erythroblasts. *Cell Transplant.* 2010; 19:453. [PubMed: 20149301]
44. Neildez-Nguyen TM, Wajcman H, Marden MC, Bensidhoum M, Moncollin V, Giarratana MC, et al. Human erythroid cells produced ex vivo at large scale differentiate into red blood cells in vivo. *Nat Biotechnol.* 2002; 20:467. [PubMed: 11981559]
45. Giarratana MC, Kobari L, Lapillonne H, Chalmers D, Kiger L, Cynober T, et al. Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. *Nat Biotechnol.* 2005; 23:69. [PubMed: 15619619]

46. Tirelli V, Ghinassi B, Migliaccio A, Whitsett C, Masiello F, Sanchez M, et al. Phenotypic definition of the progenitor cells with erythroid differentiation potential present in human adult blood. *Stem Cell Int.* 2011; 2011:602483.
47. Varricchio L, Masselli E, Alfani E, Battistini A, Migliaccio G, Vannucchi AM, et al. The dominant negative {beta} isoform of the glucocorticoid receptor is uniquely expressed in erythroid cells expanded from polycythemia vera patients. *Blood.* 2011; 118:425. [PubMed: 21355091]
48. Migliaccio AR, Whitsett C, Migliaccio G. Erythroid cells in vitro: from developmental biology to blood transfusion products. *Curr Opin Hematol.* 2009; 16:259. [PubMed: 19444099]
49. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res.* 1961; 25:585. [PubMed: 13905658]
50. Hayflick L. The future of ageing. *Nature.* 2000; 408:267. [PubMed: 11089985]
51. England SJ, McGrath KE, Frame JM, Palis J. Immature erythroblasts with extensive ex vivo self-renewal capacity emerge from the early mammalian fetus. *Blood.* 2011; 117:2708. [PubMed: 21127173]
52. Wada H, Suda T, Miura Y, Kajii E, Ikemoto S, Yawata Y. Expression of major blood group antigens on human erythroid cells in a two phase liquid culture system. *Blood.* 1990; 75:505. [PubMed: 2295004]
53. Miller JL, Njoroge JM, Gubin AN, Rodgers GP. Prospective identification of erythroid elements in cultured peripheral blood. *Exp Hematol.* 1999; 27:624. [PubMed: 10210320]
54. Leberbauer C, Boulme F, Unfried G, Huber J, Beug H, Mullner EW. Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors. *Blood.* 2005; 105:85. [PubMed: 15358620]
55. Keller MA, Addya S, Vadigepalli R, Banini B, Delgrosso K, Huang H, et al. Transcriptional regulatory network analysis of developing human erythroid progenitors reveals patterns of coregulation and potential transcriptional regulators. *Physiol Genomics.* 2006; 28:114. [PubMed: 16940433]
56. Miharada K, Hiroyama T, Sudo K, Nagasawa T, Nakamura Y. Efficient enucleation of erythroblasts differentiated in vitro from hematopoietic stem and progenitor cells. *Nat Biotechnol.* 2006; 24:1255. [PubMed: 16980975]
57. Mabaera R, Richardson CA, Johnson K, Hsu M, Fiering S, Lowrey CH. Developmental- and differentiation-specific patterns of human gamma- and beta-globin promoter DNA methylation. *Blood.* 2007; 110:1343. [PubMed: 17456718]
58. Di Baldassarre A, Di Rico M, Di Noia A, Bonfini T, Iacone A, Marchisio M, et al. Protein kinase Calpha is differentially activated during neonatal and adult erythropoiesis and favors expression of a reporter gene under the control of the (A) gamma globin-promoter in cellular models of hemoglobin switching. *J Cell Biochem.* 2007; 101:411. [PubMed: 17212360]
59. Zhang X, Ma YN, Zhang JW. Human erythroid progenitors from adult bone marrow and cord blood in optimized liquid culture systems respectively maintained adult and neonatal characteristics of globin gene expression. *Biol Res.* 2007; 40:41. [PubMed: 17657354]
60. Fujimi A, Matsunaga T, Kobune M, Kawano Y, Nagaya T, Tanaka I, et al. Ex vivo large-scale generation of human red blood cells from cord blood CD34+ cells by co-culturing with macrophages. *Int J Hematol.* 2008; 87:339. [PubMed: 18369691]
61. Ronzoni L, Bonara P, Rusconi D, Frugoni C, Libani I, Cappellini MD. Erythroid differentiation and maturation from peripheral CD34+ cells in liquid culture: cellular and molecular characterization. *Blood Cells Mol Dis.* 2008; 40:148. [PubMed: 17889571]
62. Boehm D, Murphy WG, Al-Rubeai M. The potential of human peripheral blood derived CD34+ cells for ex vivo red blood cell production. *J Biotechnol.* 2009; 144:127. [PubMed: 19735679]
63. Vlaski M, Lafarge X, Chevalyre J, Duchez P, Boiron JM, Ivanovic Z. Low oxygen concentration as a general physiologic regulator of erythropoiesis beyond the EPO-related downstream tuning and a tool for the optimization of red blood cell production ex vivo. *Exp Hematol.* 2009; 37:573. [PubMed: 19375648]
64. van den Akker E, Satchwell TJ, Pellegrin S, Daniels G, Toye AM. The majority of the in vitro erythroid expansion potential resides in CD34(-) cells, outweighing the contribution of CD34(+)

cells and significantly increasing the erythroblast yield from peripheral blood samples. *Haematologica*. 2010; 95:1594. [PubMed: 20378567]

65. Wojda U, Noel P, Miller JL. Fetal and adult hemoglobin production during adult erythropoiesis: coordinate expression correlates with cell proliferation. *Blood*. 2002; 99:3005. [PubMed: 11929793]
66. Carlile GW, Smith DH, Wiedmann M. Caspase-3 has a nonapoptotic function in erythroid maturation. *Blood*. 2004; 103:4310. [PubMed: 14976035]
67. Brugger W, Mocklin W, Heimfeld S, Berenson RJ, Mertelsmann R, Kanz L. Ex vivo expansion of enriched peripheral blood CD34+ progenitor cells by stem cell factor, interleukin-1 beta (IL-1 beta), IL-6, IL-3, interferon-gamma, and erythropoietin. *Blood*. 1993; 81:2579. [PubMed: 7683923]
68. Tanavde VM, Malehorn MT, Lumkul R, Gao Z, Wingard J, Garrett ES, et al. Human stem-progenitor cells from neonatal cord blood have greater hematopoietic expansion capacity than those from mobilized adult blood. *Exp Hematol*. 2002; 30:816. [PubMed: 12135681]
69. Nakagawa R, Watanabe T, Kawano Y, Kanai S, Suzuya H, Kaneko M, et al. Analysis of maternal and neonatal factors that influence the nucleated and CD34+ cell yield for cord blood banking. *Transfusion*. 2004; 44:262. [PubMed: 14962318]
70. Aroviita P, Teramo K, Westman P, Hiilesmaa V, Kekomaki R. Associations among nucleated cell, CD34+ cell and colony-forming cell contents in cord blood units obtained through a standardized banking process. *Vox Sang*. 2003; 84:219. [PubMed: 12670371]
71. Yu J, Leisenring W, Bensinger WI, Holmberg LA, Rowley SD. The predictive value of white cell or CD34+ cell count in the peripheral blood for timing apheresis and maximizing yield. *Transfusion*. 1999; 39:442. [PubMed: 10335991]
72. Zhou J, Cidlowski JA. The human glucocorticoid receptor: one gene, multiple proteins and diverse responses. *Steroids*. 2005; 70:407. [PubMed: 15862824]
73. Nicolaides NC, Galata Z, Kino T, Chrousos GP, Charmandari E. The human glucocorticoid receptor: molecular basis of biologic function. *Steroids*. 2010; 75:1. [PubMed: 19818358]
74. DeRijk R, de Kloet ER. Corticosteroid receptor genetic polymorphisms and stress responsivity. *Endocrine*. 2005; 28:263. [PubMed: 16388115]
75. Hennighausen L, Robinson GW. Interpretation of cytokine signaling through the transcription factors STAT5A and STAT5B. *Genes Dev*. 2008; 22:711. [PubMed: 18347089]
76. Stevens A, Donn R, Ray D. Regulation of glucocorticoid receptor gamma (GRgamma) by glucocorticoid receptor haplotype and glucocorticoid. *Clin Endocrinol (Oxf)*. 2004; 61:327. [PubMed: 15355448]
77. Migliaccio G, Sanchez M, Masiello F, Tirelli V, Varricchio L, Ghinassi B, et al. The Isoform of the Glucocorticoid Receptor Is Ontogenetically Activated and Predicts Poor Ex-Vivo Expansion of Erythroid Cells From Adult Blood. *Blood*. 2009; 114
78. Merryweather-Clarke AT, Atzberger A, Soneji S, Gray N, Clark K, Waugh C, et al. Global gene expression analysis of human erythroid progenitors. *Blood*. 2011; 117:e96. [PubMed: 21270440]
79. Sposi NM, Zon LI, Care A, Valtieri M, Testa U, Gabbianelli M, et al. Cell cycle-dependent initiation and lineage-dependent abrogation of GATA-1 expression in pure differentiating hematopoietic progenitors. *Proc Natl Acad Sci U S A*. 1992; 89:6353. [PubMed: 1631130]
80. Peschle C, Gabbianelli M, Testa U, Pelosi E, Barberi T, Fossati C, et al. c-kit ligand reactivates fetal hemoglobin synthesis in serum-free culture of stringently purified normal adult burst-forming unit-erythroid. *Blood*. 1993; 81:328. [PubMed: 7678510]
81. Muta K, Krantz SB, Bondurant MC, Wickrema A. Distinct roles of erythropoietin, insulin-like growth factor I, and stem cell factor in the development of erythroid progenitor cells. *J Clin Invest*. 1994; 94:34. [PubMed: 7518834]
82. Chasis JA, Mohandas N. Erythroblastic islands: niches for erythropoiesis. *Blood*. 2008; 112:470. [PubMed: 18650462]
83. Keerthivasan G, Small S, Liu H, Wickrema A, Crispino JD. Vesicle trafficking plays a novel role in erythroblast enucleation. *Blood*. 2010; 116:3331. [PubMed: 20644112]

84. De Felice L, Tatarelli C, Mascolo MG, Gregorj C, Agostini F, Fiorini R, et al. Histone deacetylase inhibitor valproic acid enhances the cytokine-induced expansion of human hematopoietic stem cells. *Cancer Res.* 2005; 65:1505. [PubMed: 15735039]
85. Araki H, Mahmud N, Milhem M, Nunez R, Xu M, Beam CA, et al. Expansion of human umbilical cord blood SCID-repopulating cells using chromatin-modifying agents. *Exp Hematol.* 2006; 34:140. [PubMed: 16459182]
86. Araki H, Yoshinaga K, Boccuni P, Zhao Y, Hoffman R, Mahmud N. Chromatin-modifying agents permit human hematopoietic stem cells to undergo multiple cell divisions while retaining their repopulating potential. *Blood.* 2007; 109:3570. [PubMed: 17185465]
87. Chaurasia P, Berenzon D, Hoffman R. Chromatin-modifying agents promote the ex vivo production of functional human erythroid progenitor cells. *Blood.* 2011; 117:4632. [PubMed: 21355088]
88. Prchal JT, Pastore YD. Erythropoietin and erythropoiesis: polycythemia due to disruption of oxygen homeostasis. *Hematol J.* 2004; 5(Suppl. 3):S110. [PubMed: 15190290]
89. Gerber HP, Ferrara N. The role of VEGF in normal and neoplastic hematopoiesis. *J Mol Med.* 2003; 81:20. [PubMed: 12545246]
90. Zhang CC, Lodish HF. Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells. *Blood.* 2004; 103:2513. [PubMed: 14592820]
91. Hiroyama T, Miharada K, Aoki N, Fujioka T, Sudo K, Danjo I, et al. Long-lasting in vitro hematopoiesis derived from primate embryonic stem cells. *Exp Hematol.* 2006; 34:760. [PubMed: 16728281]
92. Olivier EN, Qiu C, Velho M, Hirsch RE, Bouhassira EE. Large-scale production of embryonic red blood cells from human embryonic stem cells. *Exp Hematol.* 2006; 34:1635. [PubMed: 17157159]
93. Chang KH, Nelson AM, Fields PA, Hesson JL, Ulyanova T, Cao H, et al. Diverse hematopoietic potentials of five human embryonic stem cell lines. *Exp Cell Res.* 2008; 314:2930. [PubMed: 18692044]
94. Chang KH, Huang A, Hirata RK, Wang PR, Russell DW, Papayannopoulou T. Globin phenotype of erythroid cells derived from human induced pluripotent stem cells. *Blood.* 2010; 115:2553. [PubMed: 20339107]
95. Ye L, Chang JC, Lin C, Sun X, Yu J, Kan YW. Induced pluripotent stem cells offer new approach to therapy in thalassemia and sickle cell anemia and option in prenatal diagnosis in genetic diseases. *Proc Natl Acad Sci U S A.* 2009; 106:9826. [PubMed: 19482945]
96. Peyrard T, Bardiaux L, Krause C, Kobari L, Lapillonne H, Andreu G, et al. Banking of pluripotent adult stem cells as an unlimited source for red blood cell production: potential applications for alloimmunized patients and rare blood challenges. *Transfus Med Rev.* 2011; 25:206. [PubMed: 21377319]
97. Bonig H, Chang KH, Geisen C, Seifried E, Ware C. Blood types of current embryonic stem cell lines are not conducive to culturing "universal-donor" red blood cells. *Transfusion.* 2008; 48:1039. [PubMed: 18454742]
98. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006; 126:663. [PubMed: 16904174]
99. Olivieri NF. The beta-thalassemias. *N Engl J Med.* 1999; 341:99. [PubMed: 10395635]
100. Peller S, Tabach Y, Rotschild M, Garach-Joshua O, Cohen Y, Goldfinger N, et al. Identification of gene networks associated with erythroid differentiation. *Blood Cells Mol Dis.* 2009; 43:74. [PubMed: 19329339]
101. Migliaccio G, Sanchez M, Leblanc A, Masiello F, Tirelli V, Migliaccio AR, et al. Long-term storage does not alter functionality of in vitro generated human erythroblasts: implications for ex vivo generated erythroid transfusion products. *Transfusion.* 2009; 49:2668. [PubMed: 19659677]
102. Varricchio L, Fabucci ME, Alfani E, Godbold J, Migliaccio AR. Compensated variability in the expression of globin-related genes in erythroblasts generated ex vivo from different donors. *Transfusion.* 2010; 50:672. [PubMed: 19891622]
103. Macari ER, Lowrey CH. Induction of human fetal hemoglobin via the NRF2 antioxidant response signaling pathway. *Blood.* 2011; 117:5987. [PubMed: 21464371]

104. Gubin AN, Njoroge JM, Bouffard GG, Miller JL. Gene expression in proliferating human erythroid cells. *Genomics*. 1999; 59:168. [PubMed: 10409428]
105. Bruchova H, Yoon D, Agarwal AM, Mendell J, Prchal JT. Regulated expression of microRNAs in normal and polycythemia vera erythropoiesis. *Exp Hematol*. 2007; 35:1657. [PubMed: 17976518]
106. Noh SJ, Lee YT, Byrnes C, Miller JL. A transcriptome-based examination of blood group expression. *Transfus Clin Biol*. 2010; 17:120. [PubMed: 20685146]
107. Singleton BK, Burton NM, Green C, Brady RL, Anstee DJ. Mutations in EKLK/KLF1 form the molecular basis of the rare blood group In(Lu) phenotype. *Blood*. 2008; 112:2081. [PubMed: 18487511]
108. Sripichai O, Kiefer CM, Bhanu NV, Tanno T, Noh SJ, Goh SH, et al. Cytokine-mediated increases in fetal hemoglobin are associated with globin gene histone modification and transcription factor reprogramming. *Blood*. 2009; 114:2299. [PubMed: 19597182]
109. Mabaera R, West RJ, Conine SJ, Macari ER, Boyd CD, Engman CA, et al. A cell stress signaling model of fetal hemoglobin induction: what doesn't kill red blood cells may make them stronger. *Exp Hematol*. 2008; 36:1057. [PubMed: 18718415]
110. Kolbus A, Blazquez-Domingo M, Carotta S, Bakker W, Luedemann S, von Lindern M, et al. Cooperative signaling between cytokine receptors and the glucocorticoid receptor in the expansion of erythroid progenitors: molecular analysis by expression profiling. *Blood*. 2003; 102:3136. [PubMed: 12869505]
111. Turner M. The impact of new-variant Creutzfeldt-Jakob disease on blood transfusion practice. *Br J Haematol*. 1999; 106:842. [PubMed: 10519983]
112. Tolar J, Nauta AJ, Osborn MJ, Panoskaltis Mortari A, McElmurry RT, Bell S, et al. Sarcoma derived from cultured mesenchymal stem cells. *Stem Cells*. 2007; 25:371. [PubMed: 17038675]
113. Rubio D, Garcia-Castro J, Martin MC, de la Fuente R, Cigudosa JC, Lloyd AC, et al. Spontaneous human adult stem cell transformation. *Cancer Res*. 2005; 65:3035. [PubMed: 15833829]
114. Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, et al. Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. *Cancer Res*. 2007; 67:9142. [PubMed: 17909019]
115. Selvaggi TA, Walker RE, Fleisher TA. Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. *Blood*. 1997; 89:776. [PubMed: 9028307]
116. Sakamoto N, Tsuji K, Muul LM, Lawler AM, Petricoin EF, Candotti F, et al. Bovine apolipoprotein B-100 is a dominant immunogen in therapeutic cell populations cultured in fetal calf serum in mice and humans. *Blood*. 2007; 110:501. [PubMed: 17395779]
117. <http://www.fda.gov/cber/ind/ind.htm>
118. Migliaccio G, Migliaccio AR. Getting personal with B19 parvovirus. *Blood*. 2010; 115:922. [PubMed: 20133475]
119. Migliaccio AR, Rotili D, Nebbioso A, Atweh G, Mai A. Histone deacetylase inhibitors and hemoglobin F induction in beta-thalassemia. *Int J Biochem Cell Biol*. 2008; 40:2341. [PubMed: 18617435]
120. Chen AY, Zhang EY, Guan W, Cheng F, Kleiboeker S, Yankee TM, et al. The small 11 kDa nonstructural protein of human parvovirus B19 plays a key role in inducing apoptosis during B19 virus infection of primary erythroid progenitor cells. *Blood*. 2010; 115:1070. [PubMed: 19861680]
121. Jiang L, Lopez-Barragan MJ, Jiang H, Mu J, Gaur D, Zhao K, et al. Epigenetic control of the variable expression of a Plasmodium falciparum receptor protein for erythrocyte invasion. *Proc Natl Acad Sci U S A*. 2010; 107:2224. [PubMed: 20080673]
122. Tamez PA, Liu H, Fernandez-Pol S, Haldar K, Wickrema A. Stage-specific susceptibility of human erythroblasts to Plasmodium falciparum malaria infection. *Blood*. 2009; 114:3652. [PubMed: 19706885]

123. Chang AH, Stephan MT, Lisowski L, Sadelain M. Erythroid-specific human factor IX delivery from in vivo selected hematopoietic stem cells following nonmyeloablative conditioning in hemophilia B mice. *Mol Ther.* 2008; 16:1745. [PubMed: 18682698]
124. Ragni, MV.; K, C.; Lozier, JM. Clinical aspects and therapy for hemophilia. In: Livigstone, C., editor. *Hematology: Basic Principles and Practice*. 5th. New York; 2009. p. 1912
125. Leonard M, Brice M, Engel JD, Papayannopoulou T. Dynamics of GATA transcription factor expression during erythroid differentiation. *Blood.* 1993; 82:1071. [PubMed: 8353273]
126. Chang TJ, Scher BM, Waxman S, Scher W. Inhibition of mouse GATA-1 function by the glucocorticoid receptor: possible mechanism of steroid inhibition of erythroleukemia cell differentiation. *Mol Endocrinol.* 1993; 7:528. [PubMed: 8502237]
127. Blobel GA, Orkin SH. Estrogen-induced apoptosis by inhibition of the erythroid transcription factor GATA-1. *Mol Cell Biol.* 1996; 16:1687. [PubMed: 8657144]
128. Blobel GA, Sieff CA, Orkin SH. Ligand-dependent repression of the erythroid transcription factor GATA-1 by the estrogen receptor. *Mol Cell Biol.* 1995; 15:3147. [PubMed: 7760810]
129. Calado RT, Yewdell WT, Wilkerson KL, Regal JA, Kajigaya S, Stratakis CA, et al. Sex hormones, acting on the TERT gene, increase telomerase activity in human primary hematopoietic cells. *Blood.* 2009; 114:2236. [PubMed: 19561322]
130. Schuller CE, Jankowski K, Mackenzie KL. Telomere length of cord blood-derived CD34(+) progenitors predicts erythroid proliferative potential. *Leukemia.* 2007; 21:983. [PubMed: 17344914]
131. Prade-Houdellier N, Frebet E, Demur C, Gautier EF, Delhommeau F, Bennaceur-Griscelli AL, et al. Human telomerase is regulated by erythropoietin and transforming growth factor-beta in human erythroid progenitor cells. *Leukemia.* 2007; 21:2304. [PubMed: 17713555]
132. Fibach E, Rachmilewitz EA. The two-step liquid culture: a novel procedure for studying maturation of human normal and pathological erythroid precursors. *Stem Cells.* 1993; 11(Suppl. 1):36. [PubMed: 8318917]
133. Southcott MJ, Tanner MJ, Anstee DJ. The expression of human blood group antigens during erythropoiesis in a cell culture system. *Blood.* 1999; 93:4425. [PubMed: 10361141]
134. Pope SH, Fibach E, Sun J, Chin K, Rodgers GP. Two-phase liquid culture system models normal human adult erythropoiesis at the molecular level. *Eur J Haematol.* 2000; 64:292. [PubMed: 10863975]
135. Ji P, Yeh V, Ramirez T, Murata-Hori M, Lodish HF. Histone deacetylase 2 is required for chromatin condensation and subsequent enucleation of cultured mouse fetal erythroblasts. *Haematologica.* 2010; 95:2013. [PubMed: 20823130]
136. Giaratana MC, Rouard H, Dumont A, et al. Proof of principle for transfusion of in vitro-generated red blood cells. *Blood.* 2011; 118:5071. [PubMed: 21885599]

Fig. 1.

Antigenic profile and morphology of ex-vivo generated EBs at different stages of maturation. By flow cytometry, ex-vivo generated EBs can be divided into five classes of maturation according to the levels of glycophorin A (CD235a) and thrombospondin receptor (CD36) expression. Representative flow cytometry profiling for CD36/CD235a expression of EBs from days 10 to 12 expansion cultures (immature cultures) and at days 3–4 of maturation cultures are presented on the top. The five maturation classes (populations A to E) are identified by different colors. The maturation state of cells within each gate was confirmed by morphological analyses after cell sorting. Population A contains cells capable to generate colony-forming unit, erythroid (CFU-E)-derived colonies in semisolid assay. CD36 is expressed by cells from the CFU-E up to the basophilic state (population C) and its expression is progressively lost during the transition from polychromatophilic (population D) to orthochromatic (population E) state. CD235a expression is acquired during the transition from CFU-E (population A) to pro-EBs (population B) and progressively increases up to the polychromatophilic-orthochromatic state (population D–E) and declines during the transition from orthochromatic erythroblast to reticulocytes (not shown). Similar classes of EB maturation may be defined by profiling using CD235a in combination with CD71, an antibody that recognize the transferrin type I receptor. Magnification 40× (Modified from⁴⁸).

Fig. 2.

Schematic representation of the cellular compartments responsible for EB expansion in HEMA culture. During the first 5 days, CD34^{pos} and CD36^{pos}/CD34^{neg} erythroid progenitor cells (EPC) present in AB and CB, generate in response to GF, DXM and ES, a population of immature CD36^{high}/CD235a^{pos} cells with the morphology of immature EBs (iEBs).⁴⁶ These cells express CD36 at levels greater than those expressed by any EB found in vivo.⁴⁶ Studies in mice have identified that by contrast with the limited expansion potential of iEBs generated under steady state conditions (<5 divisions), iEBs generated during the recovery from pharmacologically induced hemolytic anemia have extensive self-renewal potential.³⁷ The extensive self-renewal potential of human EBs generated under HEMA conditions³⁸ suggest that these cells may correspond to cells generated in vivo under conditions of stress. EB fate decisions (proliferation vs maturation) are regulated by a switch in the relative expression levels of expression of the two transcription factors GATA2 and GATA1 (the GATA switch).¹⁴ GATA2 controls expression of proliferation related genes while GATA1 suppresses expression of GATA2 and activates expression of maturation specific genes. During their differentiation, EBs first activate GATA2 expression and are capable to proliferate until expression of this gene prevails.¹²⁵ Later on, activation of GATA1 expression suppresses that of GATA2 (preventing further proliferation) and activates maturation. Both DXM and ES affect the GATA switch by decreasing the transcriptional activity of GATA1. DXM induces a caspase-dependent degradation of the GATA1 protein¹²⁶ while ES binds the protein into a transcriptionally inactive complex.^{127,128} ES may also retain EBs in proliferation by upregulating telomerase activity and preventing telomerase loss-dependent cell exhaustion.¹²⁹ The important role exerted by telomerase in EBs expansion is demonstrated by the observations that the telomere length of CD34^{pos} cells may predict the numbers of EBs generated by different CB units in cultures¹³⁰ and that the telomerase expression is low in CD34^{pos} cells but increases by several fold when these cells are induction to mature toward the erythroid lineage.¹³¹

Fig. 3. Semi-quantitative RT-PCR analyses for the levels of α and γ isoforms of GR expressed by EBs generated under HEMA conditions by good (FI>6, 3 AB and 4 CB) and poor (FI<6) expanders. GR α was expressed by EBs obtained from all the 9 donors but the low affinity GR γ isoform was uniquely expressed by EBs generated from poor AB expanders.

Table 1

Milestones of the scientific achievements that have defined the cellular and molecular elements of the erythropoietic process.

The cellular era:	
1962	Purification of erythropoietin from sheep plasma (step III erythropoietin) ¹⁵
1968	Mononuclear cells separation by Ficoll ¹⁶
1971	Identification of murine erythroid colonies in culture ¹⁷
1975	First marker (benzidine staining) for erythroid precursors ¹⁸
1977	Identification of erythroid progenitor cells in human blood ¹⁹
The nuclear receptor era:	
1976	Glucorticoids as erythroid stimulating agents in vitro ²⁷
1976	Estrogens as erythroid stimulating agents in vitro ²⁸
The growth factor era:	
1985	Cloning of human erythropoietin ²⁹
1986	Cloning of human Interleukin-3 ³⁰
1990	Cloning of Human Stem Cell Factor ³¹⁻³³
The mass culture era:	
1989	Development of two step liquid culture ³⁵
1992	SCF plus EPO sustain unilineage erythroid maturation ³⁴
1998	Estrogens increases erythroid expansion ³⁹
1999	Dexamethasone increases erythroid expansion ⁴⁰
2002	Development of HEMA culture ⁴¹
2002-today	>65 publications on ex-vivo expanded human erythroid cells for transfusion

Summary of the stem cell sources and culture methods developed to amplify EB ex vivo from 1989 to date.

Table 2

Study	EB source	Culture conditions	FI	HbF	Enucleation
Fibach et al. 1989 ³⁵	AB MNC	2-phases: <ul style="list-style-type: none"> MEM+10% 5637-CM, 10% FBS w/o EPO (d 0–7) [step 1] Elimination of lymphocytes w anti-lymphocyte antibody (CAMPATH-1) at d 7 of step 1 MEM+30% FBS, 1% BSA, M, Lglut, DXM w EPO at 6% O₂ (d 8–18) [step 2] 	~7.5	ng	Permissive (% unspecified) ³²
Wada et al. 1990 ⁵²	AB MNC	2 phases: <ul style="list-style-type: none"> IMDM+10% FBS, 5% PHA-LCM (or G-CSF+ GM-CSF+IL-3) w/o EPO (d 0–7) [step 1] Elimination of phagocytic cells w carbonyl iron at d 7 of step 1 IMDM+30% FBS, 1% BSA, M, Tf, EPO at 5% O₂ (up to d 11/15) [step 2] 	1.5	ng	18.3%
Migliaccio et al. 1992 ³⁴	CD34 ⁺ from monocyte-deprived CB MNC	1-phase serum-free: IMDM+BSA 2×10 ⁴ mol/L, M, lipids, nucleosides, Tf, inorganic elements, Lglut, C ₃ H ₃ NaO ₃ , EPO, G-CSF, GM-CSF, SCF, IL-3 (d 0–27)	CD34 ⁺ : ~10	ng	ng
Spasi et al. 1992 ⁷⁹	monocyte lymphocyte-deprived AB MNC	1-phase serum-free: IMDM+BSA 10 mg/mL, FeSO ₄ , ins, Lglut, Lipoproteins, Tf, C ₃ H ₃ NaO ₃ , nucleosides, inorganic elements, GM-CSF, EPO, IL-3 (d 0–21)	~500	ng	bd ¹³³
Peschle et al. 1993 ⁸⁰	Monocyte-lymphocyte-deprived AB MNC	1-phase serum-free: IMDM+BSA, FeSO ₄ , ins, Lglut, lipoproteins, Tf, C ₃ H ₃ NaO ₃ , nucleosides, inorganic elements, GM-CSF, EPO, IL-3+SCF (d 0–20)	w/o SCF: ~500 w SCF: ~5×10 ³	By HPLC: w/o SCF: ~5% w SCF: 15%	ng
Brugger et al. 1993 ⁶⁷	LK CD34 ⁺	1-phase: RPMI 1640 medium + 10% FBS, SCF, EPO, IL-1, IL-3, IL-6 (d 0–28)	range: 21–514	ng	ng
Panzenböck et al. 1998 ³⁹	CB MNC	2-phases:	500–1000	ng	9%

Study	EB source	Culture conditions	Fl	HbF	Enucleation
		DMEM + 15% FBS, 1% BSA, 15% dH ₂ O, CHN ₂ O ₃ , M, Tf			
		• EPO, SCF, IGF-1, DXM, ES (d 0–9) [step 1]			
		• EPO, ins (d 10–14) [step 2]			
Miller et al. 1999 ⁵³	AB MNC	2 phases:	ng		ng
		• DMEM + 10% FBS, 10% 5637-CM w/o EPO (d 0–7) [step 1]			
		• MEM + 10% FBS, M, DXM, Tf w EPO (d 8–21) [step 2]			
Pope et al. 2000 ¹³⁴	AB MNC	2-phases:	ng		ng
		• MEM + 10% 5637-CM, 10% FBS, CsA w/o EPO d (0–7) [step 1]			
		• MEM + 30% FBS, 1% BSA, SCF, DXM w EPO (d 8–15) [step 2]			
		Elimination of lymphocytes w Percol stratification at d 11 of step 2			
Neildez-Nguyen et al. 2002 ⁴⁴	CB CD34 ⁺	3-phases serum-free: LCM + 2% BSA, Tf, FeSO ₄ , FeNO ₃ , ins, lipids, hydrocortisone	2×10 ⁵		4%
		• Flt-3 L, TPO, SCF (d 0–7) [step 1a]			
		• SCF, EPO, IGF-1 (d 8–14) [step 1b]			
		• EPO, IGF-1 (up to d 21) [step 2]			
Migliaccio et al. 2002 ⁴¹	AB MNC and CD34 ⁺ (ND and thal)	2-phases (HEMA Culture):	MNC:3.7 CD34 ⁺ :10 ³		ng
		• IMDM, 20% FBS, 4% BSA, Lglut, M, EPO, SCF, IL-3, DXM, ES (up to d 10–16) [step 1]			
		• IMDM, 20% FBS, 4% BSA, Lglut, M, EPO, ins (for additional 5 d) [step 2]			
Wojda et al. 2002 ⁶⁵	LK CD34 ⁺	1-phase: DMEM + 30% FBS, 1% BSA, M, Lglut, Tf, DXM, EPO w/o SCF (d 0–14)	29		Rare
Carlile et al. 2004 ⁶⁶	LK CD34 ⁺	4-phases serum-free: QBSF-60 medium (Quality Biological Inc)	50	ng	30%

Study	EB source	Culture conditions	FI	HbF	Enucleation
Giarratana et al. 2005 ⁴⁵	AB CD34 ⁺ Adult BM CD34 ⁺ LK CD34 ⁺ CB CD34 ⁺	3-phases serum-free: LCM+1% BSA, Tf, FeSO ₄ , FeNO ₃ , ins • IL-3, SCF, EPO, hydrocortisone (d 0–8) [step 1] • Co-culture w stromal layer (MS-5 or MCS) w EPO (d 9–11) [step 2a] • Co-culture w stromal layer (MS-5 or MCS) w/o EPO (up to d 22) [step 2b]	AB, BM: 1.04×10 ⁵ LK: 1.22×10 ⁵ CB: 1.95×10 ⁶	By HPLC: AB/BM: 5% CB: HbF: 64%	100%
Leberbauer et al. 2005 ⁵⁴	Permissive (% unspecified))	CB MNCs	2-phases serum-free: StemSpan medium (STEMCELL Technologies Inc) • SCF, EPO, DXM, lipids, IGF-1 (d 0–14) [step 1]	10 ⁷	ng
Keller et al. 2006 ⁵⁵	AB CD34 ⁺	1-phase: DMEM + serum substitute (STEMCELL Technologies Inc) Lglut, M, Tf, IL-3, SCF, EPO (d 0–14) 4-phases serum-free: • d 0–6 [step 1a]: EDM + SCF, EPO, IL-3 (Protocol A) or EDM + SCF, EPO, IL-3, VEGF, IGF-2 (Protocol B) • d 7–10 [step 2a]: EDM + EPO (6U/mL), SCF • d 11–16 [step 2b]: EDM + EPO (2U/mL), SCF • d 17–20 [step 2c]: IMDM + 0.5% Plasmanate (Bayer Japan), D-mannitol, adenine, H ₂ Na ₂ O ₁₆ P, mifepristone	300	By PCR: / + : <3% at any time point	ng
Miharada et al. 2006 ⁵⁶	CB CD34 ⁺		Protocol B: ~ 1×10 ⁶	ng	100%
Mabaera et al. 2007 ⁵⁷	BM CD34 ⁺	2-phases:	2.5×10 ³	By HPLC: HbF ~ absent	ng

Study	EB source	Culture conditions	FI	HbF	Enucleation
Di Baldassarre et al. 2007⁵⁸	AB and CB MNC	<ul style="list-style-type: none"> IMDM, SCF, Flt-3 L, IL-3 (d 0–7) [step 1] DMEM + 1% BSA, 30% FBS, DXM, M, Tf, EPO (up to d 20–21) [step 2] 	<p>AB: 3.7 ± 2.6 CB: 5.0 ± 5.3</p>	<p>By PCR -globin peaks d13 -globin peaks d16 and is 10-fold higher</p>	ng
Zhang et al. 2007⁵⁹	Adult BM and CB CD34 ⁺	<p>1-phase: IMDM + 1% BSA, DXM, Tf, M, Lglut, EPO, IL-3, GM-CSF</p> <p>For CB CD34⁺: 30% HCS</p> <p>For adult BM: 30% HS, ins, FeSO₄, nucleotides, lipoprotein, inorganic elements (d 0–21)</p>	~ 25	<p>BM EBs: By PCR: / + = 92.4% (d1); 4.3% (d15)</p> <p>HbF^{pos} cells: 9% (d18)</p> <p>CB EBs: By PCR: / + = 61% (d1); 91.3% (d7), 51.6% (d15)</p> <p>HbF^{pos} cells: 93% (d18)</p>	ng
Fujimi et al. 2008⁶⁰	CB CD34 ⁺	<p>4-phases:</p> <ul style="list-style-type: none"> Co-culture w hTERT transduced stroma + SCF, TPO, Flt-3 L in X-VIVO 10 (Biowhitaker™) (d 0–14) [step 1a] 1% BSA, 2% HS, Tf, SCF, IL-3, EPO in X-VIVO 10 (Biowhitaker™) (d 15–28) [step 1b] Co-culture w macrophages + 1% BSA, 2% HS, Tf, EPO in X-VIVO 10 (Biowhitaker™) (d 29–34) [step 2a] Plasmanate (Bayer Japan), mannitol, adenine, H₂₅Na₂O₁₆P w or w/o mifepristone in IMDM (up to d 38) [step 2b] 	<p>After step 1a: 980 After step 1b: 1.17 × 10⁶ After step 2a: 3.63 × 10⁶</p>	HbF slightly > HbA; % unspecified	~ 100%
Ronzoni et al. 2008⁶¹	AB CD34 ⁺	<p>1-phases: MEM + Lglut, hydrocortisone, nucleotides, M, 1% BSA, 30% FBS, IL-3, SCF with EPO and CsA added either at d 0 or d 7 (d 0–21)</p> <ul style="list-style-type: none"> EPO d 0 ± CsA 	<p>EPO alone d0: 68 EPO + CsA d0: 170 EPO ± CsA d7: 20</p>	<p>By PCR: (culture w EPO + CsA added at d0) / + = 0.705 (d 7) / + = 0.125 (d 21)</p>	CsA: Permissive (% unspecified)

Study	EB source	Culture conditions	FI	HbF	Enucleation
Boehm et al. 2009 ⁶²	AB CD34 ⁺	1-phase: IMDM + 1% BSA, Tf, FeSO ₄ , FeNO ₃ , ins, 10% FBS, EPO (until d 15), SCF, IL-3, hydrocortisone (until d 11) (up to d 16–20)	1.5 × 10 ⁶	ng	45%
Viaski et al. 2009 ⁶³	AB and CB CD34 ⁺	Same protocol described by Giarratana et al. at various O ₂ concentrations: • step 1 (d 0–8): 1.5–5% O ₂ (hypoxia) • step 2 (d 9–22): 13–20% O ₂ (normoxia)	AB: 3 × 10 ⁵ CB: 5 × 10 ⁵	By PCR: -globin is 7-fold higher for CB and 2- fold higher for AB in hypoxia vs normoxia	>80% in hypoxic conditions
Migliaccio et al. 2010 ⁴³	AB and CB MNC	• HEMA ^{ser} : IMDM, 20% FBS, 15% BSA, Lglut, M • HEMA ^{def} : IMDM, 10% HSA, M, Tf, lipids, MIX5	• AB HEMA^{ser}: 150 • AB HEMA^{def}: 550 • CB HEMA^{ser}: 300 • CB HEMA^{def}: 500	ng	ng
Van den Akker et al. 2010 ⁶⁴	AB MNC, CD34 ⁺ and CD34 ⁺	3-phases serum-free: StemSpan medium (<i>STEMCELL Technologies Inc</i>) • Lipids, SCF, EPO, DXM, IGF-1, IL-3 (d 0–5) [step 1a] <i>Percoll</i> purification • Lipids, SCF, EPO, DXM, IGF-1 (d 6–17) [step 1b] • EPO, ins, human Plasma, T ₃ , IGF-1, Tf (up to d 24) [step 2]	MNC: 3 CD34⁺: 1 CD34⁺: 150	ng	>80%

Abbreviations:

Cell sources:

AB Adult Blood; **BM:** Bone Marrow; **CB** Cord Blood; **LK** leukafesis product (G-CSF- mobilized).

Culture components:

dH₂O distilled water; **DMEM:** Dulbecco's Modified Eagle's Medium; **EDM:** StemSpanH3000® + 5% Plasmanate®, -tocopherol, linoleic acid, cholesterol, Na₂O₃Se, transferrin, ethanolamine, M; minimal essential medium; **IMDM:** Iscove's modified Dulbecco's medium; **LCM:** long-term serum free culture medium (Iscove's medium, folic acid, L glutamine, heparin, myoinositol, monothioglycerol); **RPMI:** Roswell Park Memorial Institute.

BSA bovine serum albumin; **FBS:** fetal bovine serum; **HCS** human cord serum; **HS** human serum; **HSA** human serum albumin.
Supplements:

M: -mercaptoethanol; **L_{glut}:** L glutamine; **MIX5:** insulin, C₃H₃NaO₃, nucleosides, trace elements, L glutamine; **Tf:** iron saturated human transferrin

Conditioned media and Growth Factors (GF):

5637-CM: conditioned medium collected from the 5637 bladder-carcinoma cell line; **PHA-LCM:** phytohemagglutinin-stimulated leukocyte conditioned medium

BMP-4: Bone Morphogenetic Protein-4; **DXM:** dexamethasone; **EPO:** erythropoietin; **ES:** Estradiol; **Flt-3 L:** Flt-3 Ligand; **G-CSF:** Granulocyte-Colony Stimulating Factor; **GM-CSF:** Granulocyte-Monocyte Colony Stimulating Factor; **IGF-1:** Insulin-like Growth Factor-1; **IGF-2:** Insulin-like Growth Factor-2; **IL-1:** Interleukin-1; **IL-3:** Interleukin-3; **IL-6:** Interleukin-6; **ins:** insulin; **SCF:** Stem Cell Factor; **T3:** thyroid hormone; **TPO:** thrombopoietin; **VEGF:** Vascular Endothelial Growth Factor.

Compounds:

CsA: cyclosporin A.

Cell lines:

hTERT transduced stroma: human telomerase catalytic subunit gene-transduced stromal cells; **FH-B-hTERT cells:** human fetal liver derived human telomerase catalytic subunit gene-transduced cells; **MS-5:** murine stromal cell line; **MSC:** human mesenchymal stromal cells.

General abbreviations:

bd: below detection; **di:** day; **HbF:** fetal hemoglobin; **HPLC:** High-performance liquid chromatography; **ND:** normal donors; **ng:** data not given; **PCR:** Polymerase Chain Reaction; **Thal:** thalassemic patients; **w:** with; **w/o:** without.

Table 3

Expression profile of human erythroid cells generated ex vivo under different culture conditions.

Study	Cells analyzed	Aim	Analysis
Gubin et al. ¹⁰⁴ Culture method: Fibach et al. ³⁵ (see Table 2) GF: 5637-CM + DXM+EPO Cell source: AB	Sorted CD71⁺⁺⁺⁺ (equivalent to B-D[*]) Driver: bulk population obtained with 5637-CM + DXM no EPO	Genes specifically expressed in mature EBs with respect to non-erythroid cells	Up-regulated genes in mature EBs: <ul style="list-style-type: none"> • Membrane proteins and blood group antigens (<i>Thrombospondin</i>, <i>GYPA</i>, <i>Transferrin Receptor</i>, <i>ANK1</i>, <i>Fibronectin Receptor</i>) • Histone proteins (<i>H4</i>, <i>H2A.Z</i>, <i>H3.3</i>, <i>H2A1.2</i>) • High-Mobility Group-proteins (<i>HMG-1</i>, <i>-14</i>, <i>-17</i>) • Transcription factors: <i>NF45</i>, <i>NFIB</i>, <i>dbpB-like protein</i>, <i>SON protein</i> • DNA recombination and repair (<i>topoisomerase I/II</i>, <i>BLM</i>)
Keller et al. ⁵⁵ Culture method: Keller et al. ⁵⁵ (see Table 2) GF: IL-3, SCF, EPO serum-free Cell source: AB	<ul style="list-style-type: none"> • Day 1 • Day 3 • Day 5 • Day 7 • Day 9 • Day 11 	Changes in gene expression with maturation	Progressively up-regulated genes (Day 1 – 11) <ul style="list-style-type: none"> • Blood group antigens (<i>GYPA</i>, <i>KEL</i>, <i>DARC</i>) • Erythroid specific transcription factors (<i>ERAF</i>, <i>BACH1</i>, <i>KLF1</i>, <i>GATA-1</i>), heme biosynthesis enzymes (<i>ALAS2</i>, <i>ferrochelatase</i>), - and -globin genes • Platelet specific genes (<i>selectin P</i>, <i>PF4</i>, <i>proplatelet basic protein</i>) • Other transcription factors: <i>ATF3</i>, <i>CEBP</i>, - zinc-finger proteins Progressively down-regulated genes (Day 1 – 11) <ul style="list-style-type: none"> • Membrane proteins (<i>CD34</i>, <i>CD109</i>, <i>HLA-A</i>, <i>-B</i>, <i>-C</i>) • Transcription factors (<i>ELK3</i>, <i>ETS1</i>, <i>EV12A</i>, <i>GATA-2</i>, <i>KLF12</i>, <i>RUNX3</i>, <i>ZNF512</i>) • Signaling molecules (<i>Flt-3</i>, <i>JAK3</i>, <i>PRKCB1</i>)
Bruchova et al. ¹⁰⁵ Culture method: Neildez-Nguyen et al. ⁴⁴ (see Table 2) GF: TPO, Flt-3L, SCF, EPO, IGF-1 Cell source: AB	<ul style="list-style-type: none"> • Day 1 • Day 7 • Day 9 • Day 11 • Day 14 • Day 16 • Day 19 • Day 21 	Changes in miRNA expression with maturation	Progressively up-regulated miRNAs (Day 1 – 21): miR451, miR16, miR140, miR-149 Progressively down-regulated miRNAs (Day 1 – 21): miR155, miR221, miR222 First up-regulated (Day 9 – 14) then down-regulated (Day 14 – 21) miRNA: miR339 First up-regulated (Day 9 – 14) then down-regulated (Day 14 – 19) then up-regulated (Day 19 – 21): miR378 Not regulated miRNA miR-21, miR26b, miR27a
Singleton et al. ¹⁰⁷ Culture method: Sposi et al. 1989 ⁷⁹ (see Table 2) GF: GM-CSF + EPO + IL-3	<ul style="list-style-type: none"> • Day 6 • Day 11 	Comparison of gene expression profile in rare blood group In(Lu)	Up-regulated genes in In(Lu) vs normal EBs: <ul style="list-style-type: none"> • Antigen processing and presentation (<i>HLA-A</i>, <i>-B</i>, <i>-DPB1</i>, <i>-DRA</i>, <i>-DRB1</i>, <i>-DRB4</i>, <i>-G</i>)

Study	Cells analyzed	Aim	Analysis
Cell source: AB		phenotype and normal donors	<ul style="list-style-type: none"> Defense response (<i>ECRP, ELA2, FCER1A, IL4R, MPO, NCF4, TPSAB1/B2</i>) <p>Down-regulated genes in In(Lu) vs normal EBs:</p> <ul style="list-style-type: none"> Heme/globin synthesis (<i>AHSP, ALAD, ALAS2, globin 1 gene, UROD</i>) Blood group antigens (<i>ANK1, AQP1, BCAM, BSG, CD44, DARC, EPB42, ERMAP, GYPA, SLC4A1</i>) Specific erythroid transcription factors (<i>NFE2</i>) Metabolic enzymes (<i>ARG1, PKLR</i>)
<p>Peller et al.¹⁰⁰</p> <p>Culture method: <i>Modified</i> Fibach et al.³⁵ (see Table 2)</p> <p>GF: SCF + EPO + DXM</p> <p>Cell source: AB</p>	<ul style="list-style-type: none"> S1: Early-stage EBs (day 6–7) (to A-B[*]) S2: Intermediate-stage EBs (days 9–10[*]) (equivalent to C-D) S3: Late stage EBs (day 12–13) (equivalent to E[*]) 	Changes in gene expression with maturation	<p>Progressively up-regulated genes (S1 and S2 S3):</p> <ul style="list-style-type: none"> transcription factors (<i>ZNF354A</i>) signaling molecules (<i>GOLGA3, ACVR2, JAK2</i>) Metabolic enzymes (<i>ARG1</i>) inflammatory response protein folding and post-translational modification (<i>DNAJB9</i>) <p>First up-regulated (S1 S2) then down-regulated (S2 S3) genes:</p> <ul style="list-style-type: none"> cellular components: ribosomes, ribonucleoproteins negative regulators of cell cycle progression <p>Progressively down-regulated genes (S1 S3 and S2 S3):</p> <ul style="list-style-type: none"> cellular components: ribosomes, ribonucleoproteins, mitochondrion, mitochondrial ribosome, T cell receptor complex <p>First down-regulated (S1 S2) then up-regulated (S2 S3) genes</p> <ul style="list-style-type: none"> Defense response (to bacteria, response to virus antigen presentation), antigen processing (via MHC II) Metabolic enzymes (glycolysis)
<p>Sripichai et al.¹⁰⁸</p> <p>Culture method: <i>Modified</i> Wojda et al.⁶⁵ (see Table 2)</p> <p>GF</p>	•Day 7	Gene expression profile in low vs high HbF	<p>Up-regulated genes in high vs low HbF conditions</p> <ul style="list-style-type: none"> Specific erythroid transcription factors (<i>HHEX, ID2, MAFF, EGR1 CBFβ</i>) <p>Not regulated genes in high vs low HbF conditions</p>

Study	Cells analyzed	Aim	Analysis
		culture conditions	<ul style="list-style-type: none"> Specific erythroid transcription factors (<i>GATA1, CREB, HIF1A, ATF2, ATF4</i>)
Low HbF culture: EPO alone			Down-regulated gene in high vs low HbF conditions
High HbF culture: EPO + SCF + TGF			<ul style="list-style-type: none"> Specific erythroid transcription factors (<i>BCL11A, SOX6, KLF1, NFE2</i>)
Cell source: AB			
Varricchio et al. ¹⁰²	Sorted	Changes in erythroid gene expression via qRT-PCR with maturation	Progressively up-regulated genes: , , - <i>globin genes AHSP</i>
Culture method: Migliaccio et al. ⁴¹ (see Table 2)	<ul style="list-style-type: none"> B + C * D + E * 		
GF:			
SCF + EPO + DXM + ES			
Cell source: AB			
Noh et al. ¹⁰⁶	Sorted	Blood group antigen expression with maturation and between AB and CB reticulocytes	Up-regulated genes in immature EBs: <i>GYPC, KEL, GYPA, BSG, RHD, RHCE, CD99</i> Up-regulated genes in mature EBs: <i>AQP1, SLC4A1, DARC, SLC14A1, ERMAP</i> Reticulocytes: No difference between AB and CB, with the exception of the expression of <i>KEL</i> and <i>GYPA</i> , in CB vs AB
Culture method: Wojda et al. ⁶⁵ (see Table 2)	<ul style="list-style-type: none"> CD71⁺GPA⁻ (equivalent to A-B*) CD71⁺GPA⁺ (equivalent to C-D*) Reticulocytes from AB and CB 		
GF DXM+EPO			
Cell source: AB and CB			
Merryweather-Clarke et al. ⁷⁸	Sorted	Changes in gene expression with maturation	Constantly down-regulated genes: Genes related to protein synthesis, RNA post-transcriptional modification, post-translational modification, protein folding Up-regulated genes in immature EBs: Cell growth and proliferation Specific erythroid transcription factors (<i>HHEX, ETV6</i>) Up-regulated genes in mature EBs: Ribosomal and mitochondrial genes, Specific erythroid genes: <i>GATA1, KLF1, -globin, -globin, ALAS (1/2), SMARCA4</i> and blood group antigens Histones, transcription factors involved in chromatin remodeling, high mobility group proteins and DNA repair (topoisomerases) Apoptosis and autophagy (<i>inhibitors: BIRC3; activators: TRIB3, BCL6, HMOX, DNAJB4, GSDMB</i>) Transcription factors: <i>ATF3, ZNF805</i> Additional signaling molecules: JAK/STAT signaling (<i>BRWD3</i>), Ras signaling (<i>RRAD, LIN7B</i>) <i>RFXAP (MHC II activation)</i>
Culture method: Fibach et al. ³⁵ (see Table 2):	<ul style="list-style-type: none"> CFU-E:CD71⁺GPA⁻ (to A*) Pro-Ery: CD71⁺GPA^{-low} (to B*) Int-Ery: CD71⁺GPA⁺ (equivalent to C*) Late-Ery: CD71⁺GPA⁺ (equivalent to D-E*) 		
GF: 5637-CM + DXM + EPO			
Cell source: AB			
Macari et al. ¹⁰³	Day 20	Modulation of globin genes in mature	Up-regulated genes in mature EBs: - <i>globin gene</i>
Culture method:			
<i>Modified</i> Mabaera et al. ⁵⁷ (see Table 2):			
GF: TPO, Flt-3 L, SCF, EPO, DXM			
Additional compounds: tBHQ			
Cell source: LK			

* Refers to EBs populations represented in Fig. 1.

Abbreviations:

Genes:

ACVR2BA: activin A Receptor, type IIb; **AHSP**: alpha Hb Stabilizing Protein; **ALAD**: Aminolevulinate, delta-, dehydratase; **ALAS**: aminolevulinate delta- synthase; **ANK1**: human ankyrin; **AQP1**: Aquaporin 1 (Colton blood group); **ARG1**: Arginase, liver; **ATF2**: activating transcription factor 2; **ATF3**: activating transcription factor 3; **ATF4**: activating transcription factor 4; **BACH1**: BTB and CNC homology 1, basic leucine zipper transcription factor 1; **BCAM**: Basal cell adhesion molecule (Lutheran blood group); **BCL6**: B-cell CLL/lymphoma 6; **BCL11A**: B-cell CLL/lymphoma 11A; **BIRC3**: baculoviral IAP repeat-containing 3; **BLM**: Bloom syndrome protein; **BRWD3**: Bromodomain and WD repeat domain containing 3; **BSG**: Basigin (Ok blood group); **CBFB**: core-binding factor, beta subunit; **CEBP** : CCAAT/enhancer binding protein (C/EBP), ; **CTSL2**: cathepsin L2; **CREB**: cAMP response element-binding; **DARC**: Duffy blood group; **dbpB**: human DNA-binding protein B; **DNAJB4**: DnaJ (Hsp40) homolog, subfamily B, member 4; **DNAJB9**: DnaJ (Hsp40) homolog, subfamily B, member 9; **EGR1**: early growth response 1; **ELK3**: ETS-like protein 3; **EPB42**: Erythrocyte membrane protein band 4.2; **ERAF**: Erythroid associated factor; **ERMAP**: Erythroblast membrane-associated protein (Scianna blood group); **ETS**: erythroblastosis virus E26 oncogene homolog 1; **ETV6**: Ets variant 6; **EVI2A**: ecotropic viral integration site 2a; **Flt-3**: fms-related tyrosine kinase-3; **GOLGA3**: Golgi Autoantigen, golgin subfamily a,3; **GYP A**: glycophorin-A; **GYP C**: glycophorin-C; **GSDMB**: GasderminB; **HHEX**: hematopoieically expressed homeobox; **HIF1A**: hypoxia inducible factor 1, alpha subunit; **HLA**: human leukocyte antigen; **HMOX1**: heme oxygenase1; **JAK2**: Janus Kinase 2; **JAK3**: Janus Kinase 3; **KEL**: Kell blood group; **KLF1**: Kruppel-like factor 1; **KLF12**: Kruppel-like factor 12; **ID2**: inhibitor of DNA binding 2, dominant negative helix-loop-helix protein; **LIN7B**: Lin-7 homolog B (*C elegans*); **MAFF**: v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian); **NF45**: nuclear factor 45; **NFE2**: Nuclear factor (erythroid-derived 2), 45 kDa; **NFIB**: Nuclear factor 1 B-type; **PF4**: platelet factor 4; **PKLR**: Pyruvate kinase, liver and RBC; **PRKCB1**: protein kinase C, beta 1; **RFFL**: Riffylin; **RFXAP**: regulatory factor X-associated protein; **RHCE**: Rh blood group, CcEe antigens; **RHD**: Rh blood group, D antigen; **RRAD**: Ras-related associated with diabetes; **RUNX3**: runt-related transcription factor 3; **SLC4A1**: solute carrier family 4, anion exchanger, member 1 (Diego blood group); **SLC14A1**: solute carrier family 14, anion exchanger, member 1; **Smarca4**: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4; **SOX6**: SRY (sex determining region Y)-box 6; **TRIB3**: tribbles homolog 3; **UROD**: Uroporphyrinogen decarboxylase; **ZNF354A**: Zink Finger Protein 354A; **ZNF512**: Zink Finger Protein 512; **ZNF805**: Zinc finger protein 805.

General:

5637-CM: conditioned medium collected from the 5637 bladder-carcinoma cell line; **AB**: adult blood; **CB**: cord blood; **DXM**: dexamethasone; **EPO**: erythropoietin; **ES**: Estradiol; **Flt-3 L**: Flt-3 Ligand; **GM-CSF**: Granulocyte-Monocyte Colony Stimulating Factor; **HbF**: fetal hemoglobin; **IGF-1**: Insulin-like Growth Factor-1; **IL-3**: Interleukin-3; **LK**: leukapheresis product; **NFR2/ARE**:: erythroid related factor 2/antioxidant responding element; **qRT-PCR**: quantitative Real-Time Polymerase Chain Reaction; **SCF**: Stem Cell Factor; **tBHQ**: Tert-butylhydroquinone; **TGF** : Transforming Growth factor ; **TPO**: thrombopoietin.

Table 4

Time-line for clinical application of ex vivo expanded EBs.

2008	Proof-of-principle in animal models¹²
2011	First in man transfusion¹³⁶
	Drug discovery for personalized medicine:
	<ul style="list-style-type: none"> • Thalassemia and Sickle Cell Anemia^{103,119}
2012	<ul style="list-style-type: none"> • B19 parvovirus infection¹²⁰ • Malaria^{121,122} • Prediction of therapy-related anemia in cancer patients
	Drug delivery:
2015	<ul style="list-style-type: none"> • Factor VIII or factor IX delivery in Hemophilic patients¹²³
2015	Transfusion products for alloimmunized patients and patients with rare blood types
2020	Transfusion products for the general population
2020	Maintenance of a national bank for emergency purposes¹³⁵