

Cover Page



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Title: Anemia of prematurity : time for a change in transfusion management?

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**Exploring the use of expanded erythroid cells for
autologous transfusion for anemia of prematurity**

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Abstract

Background: Autologous cord blood (CB) red blood cells (RBCs) can partly substitute transfusion needs in premature infants suffering from anemia. To explore whether expanded CB cells could provide additional autologous cells suitable for transfusion, we set up a simple one-step protocol to expand premature CB cells.

Study design and methods: CB buffy coat cells and isolated CD34 positive (CD34^{pos}) cells from premature and full-term CB and adult blood were tested with several combinations of growth factors while omitting xenogeneic proteins from the culture medium. Cell differentiation was analyzed serially during 21 days using flow cytometry, progenitor assays, and high performance liquid chromatography.

Results: Expanded CB buffy coat cells resulted in a threefold higher number of erythroblasts than the isolated CD34^{pos} cells. However, the RBCs contaminating the buffy coat remained present during the culture with uncertain quality. Premature and full-term CB CD34^{pos} cells had similar fold expansion capacity and erythroid differentiation. With the use of interleukin-3, stem cell factor, and erythropoietin, the fold increases of all CD34^{pos} cell sources were similar: CB 3942 ± 1554 , adult peripheral mobilized blood 4702 ± 1826 , and bone marrow (BM) 4143 ± 1908 . The proportion of CD235a expression indicating erythroblast presence on Day 21 was slightly higher in the adult CD34^{pos} cell sources: peripheral blood stem cells ($96.7 \pm 0.8\%$) and BM ($98.9 \pm 0.5\%$) compared to CB ($87.7 \pm 2.7\%$; $p = 0.002$). We were not able to induce further erythroid maturation *in vitro*.

Conclusion: This explorative study showed that fairly pure autologous erythroid expanded cell populations could be obtained by a simple culture method, which should be optimized. Future challenges comprise obtaining *ex vivo* enucleation of RBCs with the use of a minimal manipulating approach, which can add up to autologous RBCs derived from CB in the treatment of anemia of prematurity.

Introduction

Potentially deleterious effects of allogeneic red blood cell (RBC) transfusions have led to the development of alternatives for RBC transfusion. Explored alternatives are the use of hemoglobin (Hb)-based oxygen carriers, recombinant human erythropoietin (EPO), and intra- or postoperative RBC salvage. For the treatment of anemia of prematurity these options are however no suitable alternatives.¹⁻⁵ Currently, *ex vivo* expansion of CD34positive (CD34^{pos}) hematopoietic stem cells derived from adult blood or cord blood (CB) embodies the center of research on transfusion alternatives. Clinical-grade RBCs have been generated *ex vivo*, and also the use of expanded erythroblasts as an alternative transfusion product has been suggested.⁶⁻¹² Clinical application of these expanded cells is, however, still held back due to the high costs, the complex production process, and the presence of xenogeneic material in the culture medium.¹² Alongside the potential use of *ex vivo* expanded RBCs for allogeneic transfusion purposes, these cells could also serve for autologous transfusions for patients with a high probability of transfusion needs, that is, premature infants suffering from anemia of prematurity.^{13,14} A therapeutic allogeneic RBC transfusion for adults contains approximately 2 ± 10^{12} RBCs preserved in 350 mL of storage medium. Premature infants receive, depending on the hospital transfusion practice, 10 to 20 mL of RBC product per kg of body weight per transfusion. Extremely low birth weight infants weighing less than 1000 g are most often in need of transfusions. For these infants approximately 5×10^{10} to 10×10^{10} RBCs would be enough for a transfusion. We previously showed that autologous RBCs could be harvested from CB collected after premature birth and, if successful, could cover up to 58% of the transfusion needs.¹⁵ These premature CB-derived RBC transfusion products had a shorter shelf life (maximally 21 days) compared to adult RBCs, which can be stored for 35 days.¹⁶ To obtain RBCs, the premature CB was buffy coat depleted by centrifugation and the removed buffy coats contained approximately 40% of the white blood cells (WBCs). We evaluated whether this WBC fraction or the isolated CD34^{pos} cells could provide additional expanded autologous RBCs. We aimed to set up a simple one-step culture protocol using only human proteins and recombinant human cytokines in the culture medium. In view of the practical transfusion needs of premature infants, we focused on the cultured cells that could be obtained within 21 days of expansion, which could theoretically supplement autologous CB RBCs, which have a shelf life up to 21 days.¹⁶ To test the efficiency and efficacy of this culture protocol, we compared both premature and full-term CB and cells derived from adult blood and bone marrow (BM).

Material and methods

Cell sources

Premature CB units were obtained after parental consent as part of our prospective clinical study.¹⁵ CB units from full-term deliveries were obtained from the Umbilical Cord Blood Bank Leiden from donors who consented for experimental use of the CB. Mobilized peripheral blood stem cell (PBSC) samples were obtained from healthy adult donors. Before donation, patient consent was obtained for experimental use of a small part of the collection for research (approx. 2%). Adult BM samples were obtained from Stem Cell Technologies, Grenoble, France.

Cell isolation and culture medium

Buffy coat preparation

To produce a transfusion product from premature CB, the autologous RBCs and buffy coat were separated by centrifugation.¹⁶ The recovery of autologous CB RBCs was $75 \pm 8\%$. Consequently, the premature CB buffy coats contained a relatively large RBC fraction. Further RBC depletion from the buffy coat by hydroxyethyl starch (HES) sedimentation (ratio 1:4, HES:blood) and centrifugation at $50 \pm g$ for 10 minutes at room temperature resulted in a further RBC reduction of $45 \pm 18\%$. The remaining buffy coat fraction after HES sedimentation was washed with phosphate-buffered saline (PBS; Hospital Pharmacy LUMC, Leiden, the Netherlands) and 500,000 total nucleated cells (TNCs) per well were seeded on 24-well culture plates.

CD34^{pos} cell isolation

Mononuclear cells (MNCs) were separated by a Ficoll-Isopaque gradient (1.079 g/cm³; Hospital Pharmacy LUMC) centrifuged and enriched in CD34^{pos} cells by using columns (MiniMACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the enriched cells was 77 to at least 95%; 50,000 CD34^{pos} cells were seeded per well.

Culture medium and growth factors

The HES buffy coat fraction and the isolated CD34^{pos} cells were incubated at 37°C and 5% CO₂ humidified atmosphere for 21 days. The culture medium consisted of Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA), supplemented with 20% human AB-citrated plasma (Sanquin Blood Bank South West, Rotterdam, the Netherlands), 0.165 mg/mL CaCl₂ (Sigma, Steinheim, Germany), 1% penicillin-streptomycin (Invitrogen), 0.34% human serum albumin (Sanquin Blood Bank, Amsterdam, the Netherlands), 10 U/mL heparin (Hospital Pharmacy LUMC), 0.5 mg/mL human transferrin (Sigma), 0.05 mmol/L 2b-mercaptoethanol (Sigma), and 0.001 mol/L dexamethasone (Hospital Pharmacy LUMC). Cells were cultured in presence of several combinations of recombinant human 3 ng/mL thrombopoietin (TPO; KIRIN Brewery Co. Ltd, Yokohama, Japan), 50 ng/mL recombinant human stem cell factor (SCF; Endogen, Rockford,

IL), or/and 10 ng/mL recombinant human interleukin-3 (IL-3; Peprotech, London, UK), as early acting growth factors, in combination with 4 IU/mL human recombinant EPO (Eprex, Janssen-Cilag, Tilburg, the Netherlands). Half of the medium was refreshed at least once weekly or more often if cell proliferation increased. Both TPO and IL-3 were removed by washing after 6 days of culture. SCF and EPO were present during the entire 21-day culture period. The cultured cells were harvested on Days 4, 7, 10, 14, and 21. TNCs were counted on an automated blood cell counter (AcT 10 cell analyzer, Beckman Coulter, Woerden, the Netherlands). Expansion rate was calculated as follows: harvested TNCs, divided by the original number of seeded cells per well and corrected for the dilution factor for well transfer.

Induction of enucleation

To induce further differentiation and maturation of the expanded erythroblasts, we added thyroid hormone and insulin in the last week of culture.¹⁷⁻¹⁹ CD34^{pos} CB cells were cultured during 10 days in the standard medium with IL-3, SCF, and EPO to induce erythroid expansion. On Day 10 the expanded cells were washed with PBS and reseeded in the standard medium without dexamethasone and additionally supplemented with 10 mg/mL insulin (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 1 mmol/L thyroid hormone (T3; Sigma-Aldrich). The culture media of these cells were refreshed every other day. On Day 17 all wells were harvested for counting, phenotypic analysis, and cell smear preparation.

Phenotypic analysis

After being harvested and counted, the expanded cells were both washed and resuspended in PBS. The cells were incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies for 20 minutes at room temperature and resuspended in PBS before measurement. Anti-CD45-FITC, anti-CD34-PE, anti-CD14-FITC, anti-CD15-PE, anti-CD62p-FITC, anti-CD41-PE, anti-CD34-FITC, anti-CD71-PE, anti-CD36-FITC, 7-aminoactinomycin D (Beckman Coulter), and CD235a (glycophorin A)-PE (Dako, Glostrup, Denmark) were used for analysis. Controls were isotype FITC and PE antibodies.

Nucleated RBCs (NRBCs) were enumerated by flow cytometry using propidium iodide and CD45-FITC identified cells with a low forward and side scatter.²⁰ All analyses were performed on a flow cytometer (FC 500, Beckman Coulter).

Colony-forming assay

Colony-forming unit (CFU) and erythroid burst-forming unit (BFU-E) progenitors were assayed in methylcellulose cultures (Methocult GF H4434, Stem Cell Technologies) on Day 0 and after 7, 10, 14, and 21 days of *ex vivo* expansion. Cell concentrations of 0.32×10^4 /mL on Day 0; 0.32×10^5 /mL on Day 7; and 0.32×10^6 /mL on Days 10, 14, and 21 were seeded. The cultures were incubated at 37°C and 5% CO₂ in humidified atmosphere. Colonies were counted after 14 days.

Unless otherwise specified, all erythroid colonies (BFU-E; CFU-granulocyte, erythrocyte; CFU-megakaryocyte, erythrocyte; and CFU-granulocyte, erythrocyte, monocyte, megakaryocyte) were combined and counted as total erythroid colony formation expressed per 10^3 plated (expanded) cells.

Cell morphology

Cell morphology was analyzed on Days 17 and 21 on smears of the expanded cells. The smears were stained with May-Grunwald-Giemsa. Photographs were made with a digital camera (AxioCam MRc5, Zeiss, Gottingen, Germany), enlargement 100 ± 1.30 oil, $\infty/0.17$.

Hb analysis

Harvested cells were washed with NaCl 0.9% and kept at minus 80°C until processing. The separation by reversed phase high-performance liquid chromatography (HPLC) of the globin chains was performed with HPLC (AKTA Purifier 100, GE Healthcare Europe, Diegem, Belgium) with a sample injector and a variable UV detector operating at 215 nm. A LiChrospher 100 RP-8 column (VWR, Amsterdam, the Netherlands) was used and elution was obtained in a linear gradient of 25 column volumes of acetonitrile, methanol, NaCl in ultrapure water (milliQ, Millipore Corp., Billerica, MA) at a flow rate of 0.8 mL/min. The gradient started with 30% Solvent B (acetonitrile, methanol, 0.155 mol/L NaCl 68:4:28) and 70% Solvent A (acetonitrile, methanol, 0.077 mol/L NaCl 26:33:41) and ended with 55% Solvent B.

Statistical analysis

Data are presented as mean $\pm$ SD from generally three separate experiments unless indicated otherwise. Computer software (SPSS for Windows, Version 18, Chicago, IL) was used for data analysis. Continuous variables were analyzed using independent t tests or one-way analysis of variance (ANOVA) for differences between more than two groups. Bonferroni correction was used to correct for multiple testing if applicable. A *p*-value of less than 0.05 was considered significant.

Results

Premature CB cells after fractionation

After processing of the autologous CB products as described in our earlier clinical study, approximately 40% of the WBCs were removed by buffy coat depletion. The premature CB RBCs had a mean cell volume of 117 ± 5 fL. The products had hematocrit levels of 0.60 ± 0.05 L/L and contained a median of 5.1 ± 10^9 CB RBCs/mL (range, 3.6×10^9 - 6.6×10^9 /mL).¹⁶ The absolute RBC number per product was (median) 10.8×10^{10} (range, 2.8×10^{10} - 3.5×10^{11}). The residual buffy coats (*n* = 10) contained a median of 1.9×10^8 TNCs (range, 2.4×10^7 - 6.8×10^8) and a median of 2.5×10^6 CD34^{pos} cells (range, 1.2×10^5 - 1.1×10^7).

Expansion of premature CB buffy coats

Expansion of the premature CB buffy coat HES fraction was performed with the use of SCF and EPO ($n = 3$). Cells expressing both CD36 and CD235a could be distinguished from the native RBCs that were present at the start of the culture. After 21 days, the mean fold TNC increase was 48.3 ± 16.8 . Total CD235a^{high} expression on Day 21 was $91.4 \pm 9.6\%$, of which a minor proportion of $5.4 \pm 7\%$ coexpressed CD36^{high} (indicative of basophilic or polychromatic erythroblasts). The major proportion of these cells were CD36^{neg}CD235a^{high} (indicative of polychromatic or orthochromatic erythroblasts). CD71 (transferrin receptor) expression was $80.7 \pm 16\%$. The fold increase of CD34^{pos} fractions from the premature CB buffy coats with SCF and EPO was 1324 ± 1295 , with total CD235a^{high} expression of $92.7 \pm 5.6\%$ on Day 21 of which $13 \pm 2.1\%$ coexpressed CD36^{high}. Total CD71 expression was $96.7 \pm 1.5\%$. Thus the buffy coat HES fractions yielded approximately three times more erythroblasts than the CD34^{pos} fractions without obvious differences in purity and erythroblast maturation.

Premature and full-term CD34^{pos} CB cells

We then compared CD34^{pos} fractions from premature and full-term CB. Addition of SCF and EPO to the culture medium resulted in a higher expansion rate compared to TPO and EPO ($p < 0.001$). Premature and full-term CB had a similar fold increase. Also the appearance of erythroid cell markers was similar (Fig. 1). SCF-EPO cultures had a higher fold increase with similar proportions of CD36^{high}CD235a^{high} cells and CD36^{low/neg}CD235a^{high} cells, respectively, $19 \pm 5.6\%$ in full-term CB and $13 \pm 2.0\%$ in premature CB, and $75.7 \pm 7.2\%$ in full-term CB and $80 \pm 6\%$ in premature CB, respectively ($n = 6$ full-term CB vs. $n = 3$ premature CB). In the premature CB cultures expanded with SCF and EPO, we counted a similar amount of NRBCs per harvested well, respectively, $1.5 \times 10^6 \pm 0.9 \times 10^6$ NRBCs in premature CB and $1.3 \times 10^6 \pm 1.2 \times 10^6$ NRBCs in full-term CB. Given the similar fold expansion rate and generation of erythroid cells in both premature and full-term CB cultures, full-term CB was used for subsequent experiments.

Growth factor combinations: erythroid cell expansion and differentiation

Addition of TPO to SCF and EPO did not result in a substantial synergistic effect, in contrast to supplementation of IL-3, which resulted in a higher expansion rate of full-term CB compared to the other combinations (one-way ANOVA for differences between the groups $p = 0.04$; Fig. 1). Erythroid marker expression in the tested growth factor combinations were almost similar. The TPO-EPO combination resulted in a higher percentage of CD41 positive cells ($10.2 \pm 2.6\%$) as well as in residual CD34^{pos} cells after 21 days of culture compared to the other combinations. There was no difference in lineage negative cells (defined as CD235a/CD45-negative cells; Table 1). Although statistically not different, the proportion of CD36^{high}CD235a^{high} cells in the IL-3-SCF-EPO combination was higher, suggestive for less advanced erythroblast maturation. Expanded NRBCs on Day 21 were similar in SCF-EPO ($1.3 \times 10^6 \pm 1.2 \times 10^6$ /well), TPO-SCF-EPO (2.1×10^6

$\pm 3.2 \times 10^6$ /well), and IL-3-SCF-EPO ($5.9 \times 10^5 \pm 4.5 \times 10^5$ /well; one-way ANOVA for differences between the groups, $p = 0.779$; after correction for fold expansion, $p = 0.545$). Because of the optimal expansion rate of the TNCs, the combination IL-3-SCF-EPO was used for subsequent experiments to compare CB and adult CD34^{pos} cell sources. Removal of dexamethasone and addition of insulin and thyroid hormone to the culture medium was compared to the standard medium. Fold expansion of the TNCs was similar, respectively, 192 ± 210 for standard and 188 ± 257 for the two-step method. NRBCs were respectively $7 \times 10^5 \pm 2.4 \times 10^5$ /well for the standard medium and $6.6 \times 10^5 \pm 2.2 \times 10^5$ /well for the two-step method. After correction for well passaging the NRBC yield was also similar. We observed small differences in erythroid surface marker expression. Total CD235a expression was, respectively, $97.7 \pm 1.2\%$ for standard and $99.4 \pm 0.3\%$ for two-step method ($p = 0.067$). CD36^{high} coexpression was, respectively, $11.7 \pm 6\%$ and $3.8 \pm 2.4\%$ ($p = 0.052$), indicating more mature erythroid cells in the two-step method. Total CD71 expression was similar; $98.2 \pm 0.6\%$ in standard and $97.3 \pm 1.7\%$ in the two-step method. However, cell smears showed similar erythroid morphology without reticulocytes or RBCs in either of the cultures (not shown).

Table 1: Cell surface marker expression of expanded full term cord blood CD34^{pos} at day 21

	TPO-EPO (n=6)	SCF-EPO (n=6)	TPO-SCF-EPO (n=3)	IL3-SCF-EPO (n=3)
Fold expansion	47 ± 41	988 ± 842	802 ± 364	3942 ± 1554
CD36 ^{pos} -CD235a ^{neg}	11.4 ± 3.5 % *	4.4 ± 2 %	6.0 ± 1.7 %	9.4 ± 1.2 %
CD36 ^{pos} -CD235a ^{pos}	8.4 ± 6.3 %	17.6 ± 7.8 %	17 ± 10.4 %	37.2 ± 2.4 %
CD36 ^{neg} -CD235a ^{pos}	75.2 ± 8 %	76.2 ± 9.2 %	75.7 ± 11.5 %	50.6 ± 0.2 %
CD36 ^{neg} -CD235a ^{neg}	5.1 ± 5.9 %	1.8 ± 1.3 %	1.3 ± 1.2 %	2.8 ± 1.5 %
CD235a ^{neg} -CD45 ^{neg}	5 ± 3.9 %	2.9 ± 1.1 %	2.7 ± 0.6 %	5.1 ± 0.8 %
CD71 ^{pos}	86.7 ± 4.8 %	92 ± 5.3 %	92.3 ± 4.0 %	93 ± 0.9 %
CD41 ^{pos}	10.2 ± 2.6 % †	0%	0.7 ± 1.1 %	0.2 ± 0.2 %
CD34 ^{pos}	2.8 ± 1.6 %	0.3 ± 0.4 %	0.3 ± 0.6 %	0.6 ± 0.3 %

*: TPO-EPO compared SCF-EPO and SCF-TPO-EPO $p=0.01$ after correction for multiple testing. †: TPO-EPO compared to all other combinations $p<0.001$ after correction for multiple testing.

Abbreviations: TPO: Thrombopoietin, EPO: erythropoietin, SCF: Stem Cell Factor; IL-3: interleukin 3; CB: cord blood

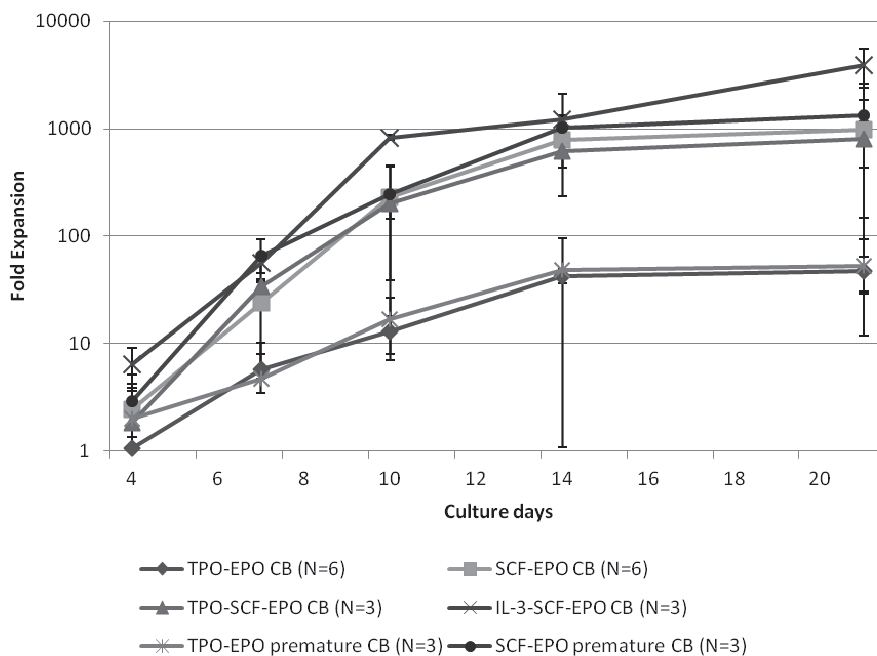


Figure 1. Fold expansion of premature and term cord blood

Abbreviations: TPO: Thrombopoietin, EPO: erythropoietin, SCF: Stem Cell Factor; CB: cord blood, IL3: interleukin 3

Erythroblast formation from adult PBSC-, BM-, and CB-derived CD34^{pos} cells

After 21 days of expansion with IL-3-SCF-EPO we observed similar fold expansion rates between the three CD34^{pos} cell sources: 3942 ± 1554 fold for full-term CB, 4702 ± 1826 fold for mobilized PBSCs, and 4143 ± 1908 for BM (one-way ANOVA for differences between the groups, $p = 0.82$; Fig. 2). Erythroid total CD235a expression on Day 21 is depicted in Table 2. Adult CD34^{pos} cell cultures were more purely erythroid compared to CB; CD235a expression was, respectively, $87.7 \pm 2.7\%$ in CB, $96.7 \pm 0.8\%$ in mobilized PBSCs, and $98.9 \pm 0.5\%$ in BM ($p = 0.002$ after correction for multiple testing). In the CB cultures there were more lineage-negative cells, depicted as both CD235a/CD45 negative, instead of remaining CD34^{pos} cells. CD71 expression, indicative of proliferating cells, was higher in the adult cell cultures compared to the CB cultures ($p = 0.032$ after correction for multiple testing; Table 2). The development of CD235a^{pos} expression was plotted against CD36^{pos} cell expression. In adult BM and PBSC cultures we observed earlier presence of CD36^{high}CD235a^{high} cells, indicative of basophilic and/or polychromatic erythroblast formation (Fig. 3). NRBC counts on Day 21 were $5.9 \times 10^5 \pm 4.5 \times 10^5$ /well in CB, $9.4 \times 10^5 \pm 0.6 \times 10^5$ /well in mobilized PBSCs, and $2.6 \times 10^6 \pm 1.3 \times 10^6$ /well in BM. After correction for the number of cell passages and fold expansion, the absolute NRBC numbers in the BM cultures were statistically higher compared to CB ($p = 0.022$) and PB ($p = 0.027$). Myeloid cell presence at 21 days estimated

by CD14 and CD15 expression was similar: $2 \pm 0.9\%$ in CB, $0.7 \pm 0.5\%$ in mobilized PBSCs, and $1.4 \pm 1.7\%$ in BM (one-way ANOVA difference between the groups, $p = 0.43$).

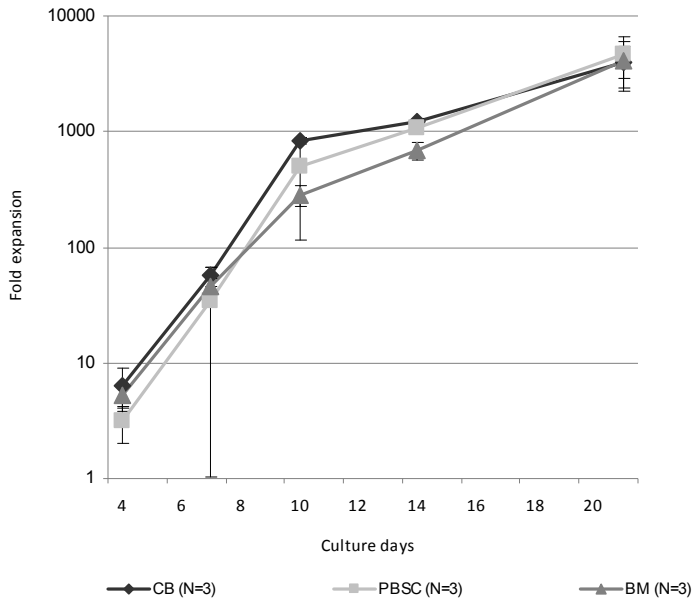


Figure 2. Comparison of fold expansion of cord blood and adult blood

Abbreviations: EPO: erythropoietin, IL3: interleukin 3, SCF: Stem Cell Factor; CB: cord blood, PBSC: mobilized peripheral blood; BM: bone marrow

Table 2: Cell surface marker expression after 21 days of expansion with IL3, SCF and EPO, in relation to adult (PBSC/BM) or CB derived CD34^{pos} cell source

	CB	PBSC	BM	<i>p</i> -value
Fold expansion	3942 ± 1554	4702 ± 1826	4143 ± 1908	ns
CD36 ^{pos} -CD235a ^{neg}	9.4 ± 1.2 %*	2.7 ± 0.7 %	0.9 ± 0.5 %	<0.001
CD36 ^{pos} -CD235a ^{pos}	37.2 ± 2.4 %	54.4 ± 11.2 %	48.2 ± 9.3 %	ns
CD36 ^{neg} -CD235a ^{pos}	50.6 ± 0.2 %	42.4 ± 11.9 %	50.4 ± 9.4%	ns
CD36 ^{neg} -CD235a ^{neg}	2.8 ± 1.5 %†	0.6 ± 0.1 %	0.3 ± 0.1 %	0.04
CD235a ^{neg} -CD45 ^{neg}	5.1 ± 0.8 %‡	1.0 ± 0.2 %	0.4 ± 0.1 %	0.002
CD71 ^{pos}	93 ± 0.9 %§	97.9 ± 1.5 %	96.8 ± 1.5 %	0.032
CD41 ^{pos}	0.2 ± 0.2 %	0.1 ± 0.1 %	0.4 ± 0.2 %	ns
CD34 ^{pos}	0.6 ± 0.3 %	5.1 ± 4.2 %	3.3 ± 2.5 %	ns

*: CB statistically significant different compared to both PBSC and BM after correction for multiple testing; †: CB statistically significant compared to BM after correction for multiple testing. ‡: CB statistically significant different compared to both PBSC and BM after correction for multiple testing; §: CB statistically significant different compared to both PBSC and BM after correction for multiple testing

Abbreviations: CB: cord blood, PBSC: mobilized peripheral blood; BM: bone marrow; EPO: erythropoietin, SCF: Stem Cell Factor; IL3: interleukin 3

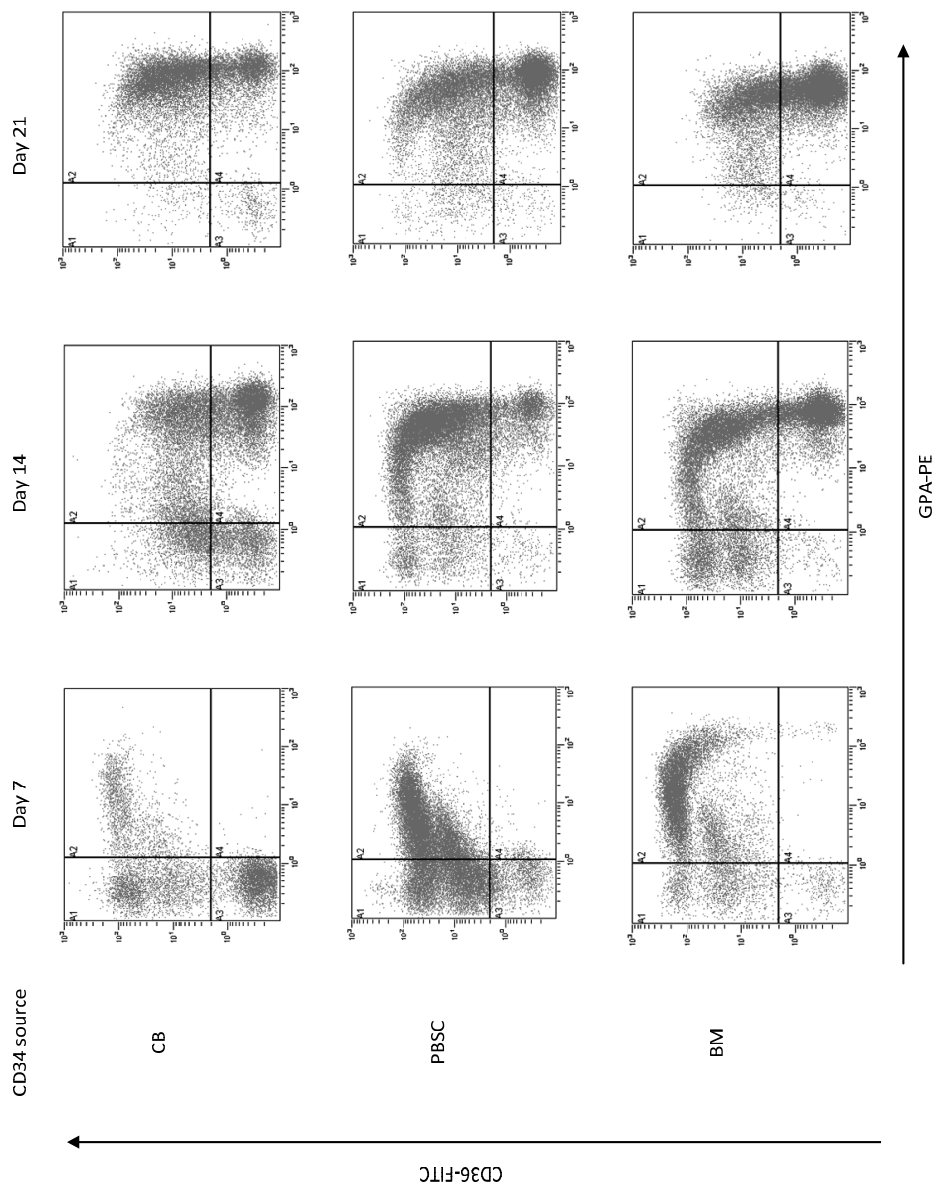


Figure 3. CD235a (GPA) cell expression during expansion plotted against CD36 expression. (All pictures are representative of three separate experiments.)

Colony-forming capacity of expanded cells

The colony-forming capacity of the expanded adult and CB cells were measured on Days 7, 10, 14, and 21 of culture. After 10 days of expansion only few CD34^{pos} cells could be detected in the CB cultures ($0.4 \pm 0.4\%$), compared to PBSCs ($8.2 \pm 2\%$) and BM ($21.5 \pm 3\%$). Nevertheless, after 7 and 10 days of expansion, both the expanded adult and the CB cells were still able to form BFU-E, although between Day 7 and Day 10 the colony-forming capacity sharply declined. After Day 10 there were almost no colonies formed. The expanded BM cells were less able to form BFU-E colonies after 7 days of expansion compared to CB and PBSCs (Table 3).

Table 3: Colony forming capacity of expanded adult and cord blood cells

Material	Colonies per 1000 seeded cells	Days of culture		
		Day 0	Day 7	Day 10
CB	Total HPC colonies	$438 \pm 13^*$	152 ± 4	$13 \pm 3^*$
	Total Erythroid colonies	$169 \pm 18^*$	104 ± 31	4 ± 4
	BFU-E colonies	59 ± 22	91 ± 39	4 ± 3
PBSC	Total HPC colonies	118 ± 119	120 ± 53	4 ± 3
	Total Erythroid colonies	27 ± 33	79 ± 25	2 ± 2
	BFU-E colonies	25 ± 30	79 ± 25	2 ± 2
BM	Total HPC colonies	138 ± 77	$17 \pm 4^\dagger$	3 ± 4
	Total Erythroid colonies	72 ± 46	$11 \pm 2^\dagger$	3 ± 3
	BFU-E colonies	62 ± 38	$11 \pm 1^\ddagger$	3 ± 3

*CB significantly different compared to PBSC and BM ($p < 0.04$) after correction for multiple testing; † BM significantly different compared to PBSC and CB ($p < 0.03$) after correction for multiple testing; ‡ BM significantly different compared to CB ($p < 0.03$) after correction for multiple testing.

Abbreviations and symbols: CB: cord blood; PBSC: mobilized peripheral blood; BM: bone marrow; HPC: hematopoietic progenitor cell colonies; BFU-E: erythroid burst forming unit; Total erythroid: BFU-E, CFU-GE, CFU-ME and CFU-GEMM

Hb analysis

On Days 11, 14, and 21, the globin chain expression was examined by reversed-phase HPLC. The HbA:HbF expression rates in the CB cultures were, respectively, 49:51 on Day 11, 37:63 on Day 14, and 24:76 on Day 21. In the adult CD34^{pos} cell cultures the HbA:HbF expression rates on Day 21 were 82:18 for BM and 84:16 for PB.

Cell morphology

We observed several stages of erythropoiesis. The majority of the cells were polychromatic erythroblasts, but also basophilic and orthochromatic erythroblasts were present. We also detected a few proerythroblasts. Reticulocytes and mature RBCs were not detected. Besides the expected stages of erythropoiesis we also detected a trinuclear cell in one of the smears, which can be a sign of either dyserythropoiesis or stress erythropoiesis. The cell membrane of this last cell was not intact and was possibly in degradation (Fig. 4).

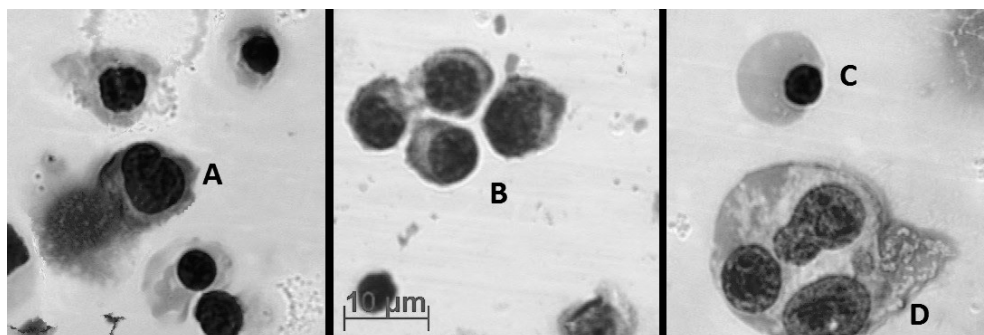


Figure 4. Erythroid progenitor cells after 21 days of expansion

A: pro-erythroblast

B: several basophilic erythroblasts

C: orthochromatic erythroblast

D: trinuclear cell

Potential quantitative erythroblast formation after ex vivo expansion

The buffy coats contained an absolute number of 2.4 ± 10^7 to 6.8 ± 10^8 nucleated cells and contained an absolute range of 1.2 ± 10^5 to 1.1 ± 10^7 CD34^{pos} cells ($n = 10$). With the use of the optimal combination of IL-3, SCF, and EPO, this would lead to a range of 4.8 ± 10^8 to 4.4 ± 10^{10} expanded erythroblasts when isolated CD34^{pos} cells from the premature buffy coats are cultured. Based on the threefold higher yield of erythroblasts when the buffy coat was used instead of CD34^{pos} cells, this could imply approximately 5 ± 10^{10} cells if the premature buffy coats could have been cultured without RBC contamination.

Discussion

Current research on *in vitro* production of RBCs provides methods to expand adult or CB MNCs and CD34^{pos} cells into mature RBCs suitable for transfusion.⁶⁻¹² The use of expanded erythroblasts has also been mentioned as an alternative transfusion product, as these cells seem to mature *in vivo* when a suitable mouse model was used.^{21,22} In an earlier study we showed that autologous RBCs derived from premature CB could be used for transfusion purposes. This amount of harvested autologous RBCs is limited. The premature CB buffy coat that was removed during autologous RBC production contained CD34^{pos} and other progenitor cells. We aimed for a simple one-step culture intended to obtain additional RBCs. Ideally these expanded autologous cells would be available within 14 to 21 days when the shelf life of CB RBCs had expired.¹⁶ Expansion of the premature CB buffy coat yielded three times more erythroblasts than CD34^{pos} cells, confirming the results of van den Akker and colleagues.¹⁷ However, the CB buffy coats contained a relatively large amount of CB RBCs. During 21 days of culture these RBCs may have acquired “storage” lesions posing a risk factor for transfusion. We further explored the erythroid differentiation characteristics in

our one-step protocol with CD34^{pos} cells. In line with previous studies, the combination of IL-3, SCF, and EPO was most effective.^{11,23} Premature and full-term CB had similar expansion potential and erythroid marker appearance. The premature CB buffy coats contained a median of 2.5 ± 10^6 CD34^{pos} cells (range, 1.2×10^5 - 1.1×10^7). With the observed mean fold expansion rate of 3942 ± 1554 , a range of 4.8×10^8 to 4.4×10^{10} (median, 1.2×10^{10}) erythroblasts could be generated in theory. Only the maximum of this range would provide just enough RBCs for one neonatal transfusion for very low birth weight neonates. We were unable to induce further differentiation and enucleation of the CD36^{neg}CD71^{high}CD235a^{high} polychromatic erythroblasts by adjustment of the culture medium with insulin and thyroid hormones and removal of dexamethasone. It is possible either that a density purification step is essential for further differentiation of the erythroblasts or that our supplemented growth factor combination was still too simple.^{17,18} Adult PBSC-, BM-, and CB-derived CD34^{pos} cells all had a similar fold increase. The adult cells had a more accelerated erythroid differentiation compared to CB (Fig. 3). Moreover, a higher residual lineage negative fraction remained in the CB cultures, whereas more CD34^{pos} cells remained in adult cultures. Loss of CD34 expression, with maintenance of expansion depending on growth factors toward hematopoietic particular lineages, has been previously observed to be typical for CB.²⁴ Myeloid cell presence was low in all cultures. All cultures finally consisted of greater than 90% erythroid cells. The colony forming capacity was not diminished at least until up to 10 culture days, indicating that *in vivo* erythroid proliferation and differentiation could be possible after 10 days of expansion. The increased HbF expression during culture was expected in view of the erythropoietic stress conditions present during culture and was also shown in the adult cultures.²⁵ The cell smears at the end of the culture showed a few proerythroblasts and all stages of erythroblasts through normoblast. We did not observe reticulocytes or RBCs. We also observed signs of dyserythropoiesis or stress erythropoiesis, which may be explained due to the continuous drive to proliferate in the cultures.

Recently the literature on *ex vivo* expansion of CD34^{pos} and MNCs has been summarized, showing that MNCs from both adult and CB could generate on average 10 to 100 times more erythroblasts than the corresponding CD34^{pos} cells.¹⁸ Compared to other studies, we generated a lower number of erythroblasts aiming for a single-step culture method suitable for standard blood bank conditions in closed systems. Only a minority of the studies, reviewed by Migliaccio and colleagues¹⁸ used xenofree medium. The fold increase varied between factors of 50 and 1.10^6 depending on the cell source or growth factor combination used; enucleation rate was either not given or ranged between 30 and 100%.^{10,11,18,26} Baek and coworkers⁹ also used non xenogeneic proteins but these cells were cultured upon human mesenchymal stem cells. They reached approximately 10^4 -fold increase with an enucleation rate of greater than 60%. The combination IL-3, SCF, and EPO was used in all of these studies and seems to be fundamental for *ex vivo* expansion.^{9-11,26} Furthermore, an extensive set of steroids and cytokines, addition of lipids or other components like d-mannitol, trace elements, glucocorticoid antagonists, or multiple

culture phases resulted in complex procedures.^{10,11,18,26} Of these, the highest fold expansion was reached by Miharada and coworkers¹⁰ who used vascular endothelial growth factor and insulin growth factor-2. With our approach, we did not reach a 100% CD235a positive cell population, in view of the remaining lineage negative cells. For autologous use, however, this is not an obstacle. Expanded erythroblasts have been suggested as an alternative for transfusion.^{12,27} Most *in vivo* mice models, however, are not all suitable for studying expanded human erythroblast maturation and enucleation, as human erythroid cells are removed by the mouse spleen.^{21,22,28} Although the splenectomized NOD/SCID/ IL2R γ null mice model seems more suitable for *in vivo* human erythroblast tracking, in this model human RBC counts in peripheral mouse blood also remained low. Alongside improving our culture method, our production process of autologous CB products should be adapted. During processing approximately 60% of the total WBCs remained in the RBC product¹⁶ and, vice versa a relatively large amount of autologous RBCs remained in the buffy coats. A better separation method, preferentially by filtration and recovery of WBCs from the filter²⁹ would lead to a larger product for autologous transfusion and an increased proportion of nucleated cells suitable for *ex vivo* expansion.

Hence, next to salvage of CB RBCs to prepare an autologous transfusion product, we provide preliminary results indicating the potential use of the remaining progenitor cells to obtain an additional RBCs in the early neonatal period during which most transfusions are required. Despite apparently suboptimal harvest and culture conditions, our results suggest that it may be worthwhile to put effort in further optimization and generation of clinical-grade culture conditions and *in vivo* differentiation and functionality testing. It should be emphasized, however, that the expansion experiments we performed merely indicate the possible feasibility of this approach. Obviously, major hurdles must be overcome before *ex vivo* expanded CB cells can actually supplement autologous CB RBCs in the treatment of anemia of prematurity. The use of premature CB for autologous RBC transfusion purposes has been critically summarized.³⁰ Nevertheless both our previous and current feasibility study suggest that complete avoidance of allogeneic RBC transfusions for anemia of prematurity may be in reach in the future when combining a more efficient RBC separation with a larger availability of residual CD34^{pos} and progenitor cells that can be used for both transfusion and *ex vivo* expansion. Where there is a will, there will be a way. In our opinion, the full potential of premature CB for autologous use has not been fully explored yet and offers ample room for progress.

Reference list

1. Silverman TA, Weiskopf RB. Hemoglobin-based oxygen carriers: current status and future directions. *Transfusion* 2009;49:2495-515.
2. Buehler PW, D'Agnillo F, Schaer DJ. Hemoglobin-based oxygen carriers: from mechanisms of toxicity and clearance to rational drug design. *Trends Mol Med* 2010;16:447- 57.
3. Ohlsson A, Aher SM. Early erythropoietin for preventing red blood cell transfusion in preterm and/or low birth weight infants. *Cochrane Database Syst Rev* 2006;(3): CD004863.
4. García-Erce JA, Cuenca J, Haman-Alcober S, Martínez AA, Herrera A, Muñoz M. Efficacy of preoperative recombinant human erythropoietin administration for reducing transfusion requirements in patients undergoing surgery for hip fracture repair. An observational cohort study. *Vox Sang* 2009;97:260-7.
5. Carless PA, Henry DA, Moxey AJ, O'Connell D, Brown T, Fergusson DA. Cell salvage for minimising perioperative allogeneic blood transfusion. *Cochrane Database Syst Rev* 2010;(4):CD001888.
6. Neildez-Nguyen TM, Wajzman H, Marden MC, Bensidhoum M, Moncollin V, Giarratana MC, Kobari L, Thierry D, Douay L. Human erythroid cells produced ex vivo at large scale differentiate into red blood cells in vivo. *Nat Biotechnol* 2002;20:467-72.
7. Giarratana MC, Kobari L, Lapillonne H, Chalmers D, Kiger L, Cynober T, Marden MC, Wajzman H, Douay L. Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. *Nat Biotechnol* 2005;23:69- 74.
8. Fujimi A, Matsunaga T, Kobune M, Kawano Y, Nagaya T, Tanaka I, Iyama S, Hayashi T, Sato T, Miyanishi K, Sagawa T, Sato Y, Takimoto R, Takayama T, Kato J, Gasa S, Sakai H, Tsuchida E, Ikebuchi K, Hamada H, Niitsu Y. Ex vivo largescale generation of human red blood cells from cord blood CD34+ cells by co-culturing with macrophages. *Int J Hematol* 2008;87:339-50.
9. Baek EJ, Kim HS, Kim S, Jin H, Choi TY, Kim HO. In vitro clinical-grade generation of red blood cells from human umbilical cord blood CD34+ cells. *Transfusion* 2008;48: 2235-45.
10. 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;24:1255-6.
11. Migliaccio G, Sanchez M, Masiello F, Tirelli V, Varricchio L, Whitsett C, Migliaccio AR. Humanized culture medium for clinical expansion of human erythroblasts. *Cell Transplant* 2010;19:453-69.
12. Migliaccio AR, Whitsett C, Migliaccio G. Erythroid cells in vitro: from developmental biology to blood transfusion products. *Curr Opin Hematol* 2009;16:259-68.
13. Valieva OA, Strandjord TP, Mayock DE, Juul SE. Effects of transfusions in extremely low birth weight infants: a retrospective study. *J Pediatr* 2009;155:331-7.
14. Fabres J, Wehrli G, Marques MB, Phillips V, Dimmitt RA, Westfall AO, Schelonka RL. Estimating blood needs for very-low-birth-weight infants. *Transfusion* 2006;46:1915-20.
15. Khodabux CM, von Lindern JS, van Hilten JA, Scherjon S, Walther FJ, Brand A. A clinical study on the feasibility of autologous cord blood transfusion for anemia of prematurity. *Transfusion* 2008;48:1634-43.
16. Khodabux CM, Van Beckhoven JM, Scharenberg JG, El Barjiji F, Slot MC, Brand A. Processing cord blood from premature infants into autologous red-blood-cell products for transfusion. *Vox Sang* 2011;100:367-73.
17. 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;95:1594-8.
18. Migliaccio AR, Masselli E, Varricchio L, Whitsett C. Ex-vivo expansion of red blood cells: how real for transfusion in humans? *Blood Rev* 2012;26:81-95.
19. 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;94:34-43.
20. Tsuji T, Sakata T, Hamaguchi Y, Wang F, Houwen B. New rapid flow cytometric method for the enumeration of nucleated red blood cells. *Cytometry* 1999;37: 291-301.

21. Ghinassi B, Ferro L, Masiello F, Tirelli V, Sanchez M, Migliaccio G, Whitsett C, Kachala S, Riviere I, Sadelain M, Migliaccio AR. Recovery and biodistribution of ex vivo expanded human erythroblasts injected into NOD/SCID/ IL2Rg mice. *Stem Cells* 2011;2011:673752.
22. Hu Z, Van Rooijen N, Yang YG. Macrophages prevent human red blood cell reconstitution in immunodeficient mice. *Blood* 2011;118:5938-46.
23. Kie JH, Jung YJ, Woo SY, Ryu KH, Park HY, Chung WS, Seoh JY. Ultrastructural and phenotypic analysis of in vitro erythropoiesis from human cord blood CD34+ cells. *Ann Hematol* 2003;82:278-83.
24. Schipper LF, Brand A, Fibbe WE, Van Hensbergen Y. Functional characterization of TPO-expanded CD34+ cord blood cells identifies CD34- CD61- cells as platelet producing cells early after transplantation in NOD/SCID mice and rCD34+ cells as CAFC colony-forming cells. *Stem Cells* 2012;30:988-96.
25. Stamatoyannopoulos G, Veith R, Galanello R, Papayannopoulou T. Hb F production in stressed erythropoiesis: observations and kinetic models. *Ann N Y Acad Sci* 1985; 445:188-97.
26. Carlile GW, Smith DH, Wiedmann M. Caspase-3 has a nonapoptotic function in erythroid maturation. *Blood* 2004;103:4310-6.
27. Kobari L, Yates F, Oudrhiri N, Francina A, Kiger L, Mazurier C, Rouzbeh S, El-Nemer W, Hebert N, Giarratana MC, François S, Chapel A, Lapillonne H, Luton D, Bennaceur- Griscelli A, Douay L. Human induced pluripotent stem cells can reach complete terminal maturation: in vivo and in vitro evidence in the erythropoietic differentiation model. *Haematologica* 2012;97:1795-803.
28. Zhang X, Luo YM, Ma YN, Zhang JW. In vitro maturation of erythroid progenitors from human umbilical cord blood and patterns of globin gene expression: serum from different developmental stage plays important roles in liquid culture. *Biochem Biophys Res Commun* 2005;336: 42-8.
29. Takahashi TA, Rebull P, Armitage S, van Beckhoven J, Eichler H, Kekomäki R, Letowska M, Wahab F, Moroff G. Multi-laboratory evaluation of procedures for reducing the volume of cord blood: influence on cell recoveries. *Cytotherapy* 2006;8:254-64.
30. Strauss RG, Widness JA. Is there a role for autologous/ placental red blood cell transfusions in the anemia of prematurity? *Transfus Med Rev* 2010;24: 125-9.

