SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 405429, 10 pages
doi:10.4061/2011/405429

Research Article

Human Fetal Liver: An *In Vitro* Model of Erythropoiesis

Guillaume Pourcher,[1,2,3] Christelle Mazurier,[1,4] Yé Yong King,[1] Marie-Catherine Giarratana,[1,5] Ladan Kobari,[1,5] Daniela Boehm,[1,5] Luc Douay,[1,4,5] and Hélène Lapillonne[1,5]

[1] *Prolifération et Différenciation des Cellules Souches: Application à la Thérapie Cellulaire Hématopoïétique, INSERM, UMR_S938, CDR Saint-Antoine, 75012 Paris, France*
[2] *Service de chirurgie générale, AP-HP Hôpital Antoine Béclère, 92141 Clamart, France*
[3] *Université Paris sud XI, Orsay, France*
[4] *Etablissement Français du Sang Ile de France, 94200 Ivry-sur-Seine, France*
[5] *Service d'Hématologie et immunologie biologique, AP-HP Hôpital Saint Antoine/Armand Trousseau, 75012 Paris, France*

Correspondence should be addressed to Hélène Lapillonne, helene.lapillonne@trs.aphp.fr

Received 5 May 2011; Accepted 24 May 2011

Academic Editor: Anna Rita Migliaccio

We previously described the large-scale production of RBCs from hematopoietic stem cells (HSCs) of diverse sources. Our present efforts are focused to produce RBCs thanks to an unlimited source of stem cells. Human embryonic stem (ES) cells or induced pluripotent stem cell (iPS) are the natural candidates. Even if the proof of RBCs production from these sources has been done, their amplification ability is to date not sufficient for a transfusion application. In this work, our protocol of RBC production was applied to HSC isolated from fetal liver (FL) as an intermediate source between embryonic and adult stem cells. We studied the erythroid potential of FL-derived $CD34^+$ cells. In this *in vitro* model, maturation that is enucleation reaches a lower level compared to adult sources as observed for embryonic or iP, but, interestingly, they (i) displayed a dramatic *in vitro* expansion (100-fold more when compared to CB $CD34^+$) and (ii) 100% cloning efficiency in hematopoietic progenitor assays after 3 days of erythroid induction, as compared to 10–15% cloning efficiency for adult $CD34^+$ cells. This work supports the idea that FL remains a model of study and is not a candidate for *ex vivo* RBCS production for blood transfusion as a direct source of stem cells but could be helpful to understand and enhance proliferation abilities for primitive cells such as ES cells or iPS.

1. Introduction

The cell therapy approach which consists of generating cultured RBC (cRBC) *in vitro* after amplification of stem cells (SC) makes sense in a context of chronic difficulties of obtaining blood supplies. More than blood supplies, hematopoietic differentiation of human stem cells into RBC has important therapeutic implications, including the possibility to produce virus-free units or tailor-designed phenotypes for transfusion purposes. Our team has established an experimental procedure to reproduce *in vitro* terminal erythropoiesis from adult hematopoietic stem cells (HSC) from diverse sources (peripheral blood (PB), bone marrow (BM), and cord blood (CB)) [1, 2]. This protocol, using appropriate cytokines and a specific microenvironment (including stromal murine MS5 or Mesenchymal Stem Cells (MSC)) in a serum-free medium, allows HSC proliferation and terminal differentiation in mature and functional enucleated RBC containing adult haemoglobin [2]. Major advances have allowed towards the *in vitro* production of RBCs from diverse sources in a few years. Nevertheless, their transfusional future will become a reality only if we are able to produce functional transfusable RBCs at a large scale. This will of course require not only the conception of appropriate industrial tools, but also finding the best source of stem cells. To date, the most accessible and proliferative source of HSC in a quantitative aspect is cord blood (CB) [3].

However, this cell source is dependent on donations and *ex vivo* production of RBC is restricted to a system of production in batches, using the finite quantity of HSC available in a CB unit. Such are its limits.

Consequently, our present efforts are focused on establishing conditions to produce RBC using a permanent and inexhaustible source of stem cells. Human embryonic stem cells or recently discovered induced pluripotent stem cells (iPS) [4, 5] are the natural candidates. As other teams, we have shown the possibility to reconstitute *in vitro* erythropoiesis starting from very primitive stem cells [6–13], and we were the first to report the erythroid differentiation and maturation of iPS lines (from fetal and adult fibroblasts) into mature enucleated RBC (4% to 10% compared to 52% to 66% from human ES cell line (H1)), which synthesized functional fetal hemoglobin [9].

The demonstration that primitive cells can be differentiated *in vitro* to cells similar to their natural counterpart was the first step for a potential therapeutic application. But there is still a major problem for a hypothetical application in transfusion: their amplification. Indeed, in our conditions, 1 ES or 1 iPS cell can generate 5000 or 1000 RBC, respectively, [9] whereas 1 $CD34^+$ from CB can give up to 2.5×10^5 RBCs [2]. This crucial difficulty of amplification of ES or iPS has not yet been solved by any team.

Rollini et al. have demonstrated that fetal liver (FL) could be an alternative source of HSC despite a limited total cell number per tissue, due to their high proliferative capacity [14]. Indeed, the site of erythropoiesis migrates during development. It takes place first in the yolk sac and the para-aortic region. Erythropoiesis then migrates to the fetal liver between the 8th and the 22nd week of gestation to finally take place in the BM [15]. In the present work we investigated the capacity of $CD34^+$ cells isolated from human FL, an ontogenic source intermediate between very primitive cells (ES and iPS) and adult stem cells, to expand and differentiate into erythroid lineage cells.

We cocultured FL-derived $CD34^+$ cells over an *in vitro* reconstituted bone marrow (BM) microenvironment. As observed for hES or iPS, only a fraction of erythroid cells reached terminal differentiation into mature RBC (15%). These few RBCs contained nearly exclusively fetal hemoglobin at the protein level. However, as compared to adult HSC, we could observe a dramatic expansion of FL-derived erythroid cells, 100-fold higher when compared to CB $CD34^+$.

FL is an alternative model that might be relevant to understanding mechanisms both implied in amplification and differentiation of HSC in erythropoiesis and could be used as a model for other differentiation pathways.

2. Materials and Methods

2.1. Fetal Liver. Fetal livers were surgically obtained from 6 aborted fetuses (after 12–14 weeks of amenorrhoea) after informed consent of patients was given. All experiments and procedures were in agreement with the guidelines from the French Health ministry and the Agence de la Biomédecine. FL was dissociated within less than 4 hours after collection and flushed through a 70 μm filter and preserved in a PBS/BSA 10% (v/v) (Sigma Aldrich, Lyon, France) solution until CD34 cell immunomagnetic separation, which was performed within a maximal 1 hour delay.

2.2. CD34 Cell Isolation. FL $CD34^+$ cells were isolated by immunomagnetic separation according to the manufacturer's guidelines (Miltenyi Biotech, Bergisch Gladbach, Germany). $CD34^+$ purity was evaluated by flow cytometry.

2.3. FL-Derived Mesenchymal Stem Cells (MSCs). The CD34 negative fractions were used for MSC generation according to the technique described by Doucet et al. [16]. Briefly, cells were plated at a density of 2×10^5 cells/cm^2 and cultured in alpha-MEM medium (Biological Industries, ATGC Biotechnologies, Noisy le Grand, France) supplemented with 5% (v/v) platelet-enriched plasma lysate (PL) and 2 IU/mL heparin (Roche Diagnostics, Basel, Switzerland). Cultures were fed every 3-4 days with fresh medium. At confluence cells were replated at 6×10^3/cm^2. The grown cells had a fibroblast-like morphology. After 2 passages, cells were assessed for immunophenotyping, and their phenotype corresponded to MSCs, that is, $CD73^+$, $CD105^+$, $CD90^+$, $CD45^-$.

2.4. Generation of Red Blood Cells Ex Vivo. $CD34^+$ cells were cultured in a three-step procedure in a serum-free medium as previously described [2] with some modifications such as the source of serum or the use of FL mesenchymal stem cells (MSCs) as stroma. 2×10^4 $CD34^+$ cells/mL were first cultured in IMDM medium (Biological Industries, ATGC Biotechnologies) supplemented either with 1% BSA or 1% human AB plasma, 10 μg/mL insulin (Sigma), and 120 μg/mL iron-saturated human transferrin (Sigma). In the first step (days 0–8), 2×10^4/mL $CD34^+$ cells were cultured in the presence of 10^{-6} M hydrocortisone (Sigma), 100 ng/mL stem cell factor (SCF, Peprotech, Neuilly sur Seine, France), 5 ng/mL IL-3 (R&D Systems), and 3 IU/mL erythropoietin (Eprex, kindly provided by Janssen-Cilag). On day 4, one volume of cell culture was diluted in four volumes of fresh medium containing hydrocortisone, SCF, IL-3, and erythropoietin. In the second step (days 8–10), the cells were replated at 5×10^4/mL and cocultured on an adherent stromal layer in fresh medium supplemented with erythropoietin. In the third step (days 10–18), cells were cultured on an adherent stromal layer in fresh medium without cytokines. The cultures were maintained at 37°C in 5% CO_2. The adherent cell layer consisted of either the MS-5 stromal cell line (provided by K. Mori) or fetal mesenchymal stromal cells (MSCs) established from the CD34 negative fraction of FL liver samples.

2.5. Colony Assay. Colony-Forming Unit Granulocyte Macrophage (CFU-GM), Colony-Forming Unit Erythroid cells (CFU-E), Burst-Forming Unit Erythroid cells (BFU-E) and Colony-Forming Unit Mix (CFU-Mix) were assayed in a semisolid methylcellulose medium as previously described [17].

2.6. Flow Cytometry Analyses. All immunophenotyping analyses were performed using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest Pro software. Immunophenotyping of fetal

MSCs was carried out with the following antibodies: FITC-conjugated anti-CD45 (Beckman Coulter, Imunotech, Roissy en France, France), anti-CD105 (Serotec, Cergy Saint Christophe, France), PE-conjugated anti-CD90, and anti-CD73 (Pharmingen-Becton Dickinson Franklin Lakes, USA). Immunophenotyping of fetal RBCs was carried out with the following antibodies: FITC-conjugated anti-CD36 and PE-conjugated anti-CD71 (Beckman Coulter, Imunotech). Immunophenotyping of fetal mononuclear cells was carried out with the following antibodies: FITC-conjugated anti-CD38 (DAKO, Glostrup, Denmark), anti-Glycophorin A, anti-CD36, anti-CD71, anti-CD45, PE-conjugated anti-CD117, anti-CD33, anti-CD34, anti-CD2, anti-CD3, anti-CD8, (Beckman Coulter-Imunotech), anti-CD19, anti-CD4, anti-CD14, and anti-CD56 (Becton Dickinson). Appropriate negative controls (mouse anti-human irrelevant control) were performed for each FACS analysis.

2.7. Cells and Hemoglobin Characterisation

Hematological Staining. Cell differentiation was monitored throughout culture by morphological analysis of the cells after cytocentrifugation and May-Grünwald-Giemsa (MGG) staining.

Hemoglobin Analysis. The pattern of hemoglobin (Hb) synthesis was analyzed by high-performance liquid chromatography (HPLC) (Biorad variant II, BioRad Laboratories, Munich, Germany) with a high-resolution betathalassemia program.

2.8. Semiquantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Using the protocol of erythroid differentiation described above, we generated RBCs from $CD34^+$ cells isolated from different ontogenic sources (i.e., from CB, BM, and FL) in order to compare the kinetics of the transcriptomic profile of genes known to be involved in erythroid differentiation (GATA-1, FOG 1, SOX6, GFI 1b, STAT5a, FOXO3a, and $\alpha, \beta, \gamma, \varepsilon, \zeta$ globin chains).

Total RNA was prepared from cells collected at different times of RBCs culture (days 8, 11, 15, 18). RNA was extracted using the Trizol method according to the manufacturer's instructions (Invitrogen, Paisley, Scotland). 1 μg of DNase-treated RNA was transcribed into cDNA using 200 units of SuperScript II reverse transcriptase (Invitrogen) and 150 ng of random primers (Invitrogen). The resulting cDNA was aliquoted to avoid repeat freeze/thaw cycles. Real-time PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif, USA). The 18S gene was used as an internal reference. All primers and probes were from Applied Biosystems.

Other genes were amplified using SYBRgreen chemistry, and the GAPDH gene was used as an internal reference. All primers were from Eurogentech (Liege, Belgium).

For both chemistries, PCR were performed in duplicate using either the TaqMan Master Mix or SYBR green Master Mix with 10 ng of cDNA and 300 nM of primers in a final volume of 25 μL. After 2 minutes incubation at 50°C, the AmpliTaq Gold was activated by 10-minute incubation at 95°C. A total of 40 amplification cycles were run with an annealing temperature of 60°C. Calibration curves were established to check that PCR efficiency was similar for all target genes and references. The relative expression of a given target gene was given by the $2^{-\Delta\Delta Ct}$ method [18], where $\Delta\Delta Ct$ represents the difference of an unknown sample (days 8, 11, 15, 18 of culture) versus a control (day 0 of culture) for a target gene normalized to a control gene (18S or GAPDH). This calculation method is suitable for investigating physiological changes in gene expression levels.

Table 1: Flow cytometric analysis of fetal liver cells before $CD34^+$ immunomagnetic separation. Fetal liver was dissociated, and the mononuclear cells were isolated by density-gradient centrifugation and analyzed by flow cytometry for different cell surface markers. Results are presented as the percentage of positive cells. CD: cluster of differentiation.

Phenotypical analysis of mononuclear cells	
CD45	29%
CD117	20%
CD38	15%
CD14	2.2%
CD13	14.5%
CD33	11%
CD2, CD3, CD19, CD56	Negative
CD36	72%
GlycoA	68%
CD71	78%
CD34	**22**%

3. Results

Starting from FL-derived $CD34^+$ cells, we used a three-step stimulation protocol. Firstly, cell proliferation and erythroid differentiation were induced with stem cell factor (SCF), interleukin-3 (IL3), and erythropoietin (Epo). Secondly, the cells were cocultured with additional Epo alone on an *in vitro* reconstituted BM microenvironment (human fetal MSC or murine MS5 stromal cell line). In the third step, all exogenous factors were withdrawn, and the cells were incubated on the stroma alone.

3.1. Massive Expansion and Commitment of Human Erythroid Cells from FL-Derived $CD34^+$ Cells. In the starting FL population, smear examination showed that a majority of cells belonged to the erythroid lineage, more than 50% being proerythroblasts. As analyzed by flow cytometry, most cells expressed markers of the erythroid lineage (69% double positive for CD235a/CD36 and 74% for CD235a/CD71), and $22 \pm 2.8\%$ were $CD34^+/CD38^-$ HSC. Other cells belonged to the myeloid lineage while no lymphoid B or T cells were detected (Table 1). After $CD34^+$ immunomagnetic separation, we obtained a purity of over 90%. The three-step protocol resulted in a dramatic amplification of $CD34^+$

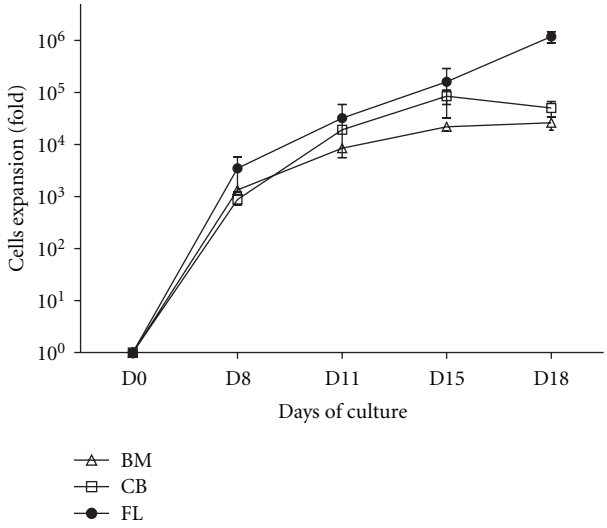


Figure 1: Amplification of CD34$^+$ FL cells during erythroid differentiation. CD34$^+$ hematopoietic stem cells from fetal liver (FL) were grown in liquid culture according to the three-step protocol described in Section 2, and total cells were counted at the indicated times. Mean values with the standard deviation for three experiments are shown, and the results are compared to those for amplification of cells from cord blood (CB) and bone marrow (BM).

	Day of culture		
Type of colony	D3	D8	D10
BFU-E	228	100	20
CFU-E	32	18	31
CFU-GM	167	22	3
Mix (CFU-GM + BFU-E)	91	14	2
Total	518	154	56

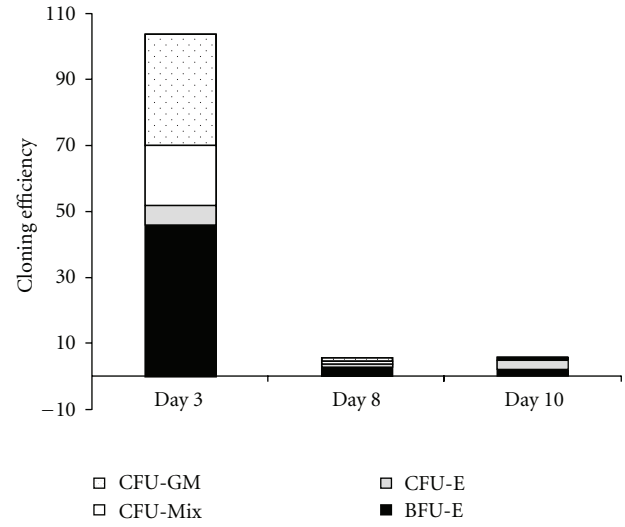


Figure 2: Colony formation of FL-derived erythroid cells. The colony forming ability of FL-derived erythroid cells was measured on days 3, 8, and 10 of liquid culture. Aliquots of nonadherent cells were grown in methylcellulose medium in the presence of SCF, GM-CSF, IL3, and erythropoietin. CFU-E colonies were counted on day 7 and BFU-E, CFU-GM, and CFU-mix colonies on day 14 of semisolid culture.

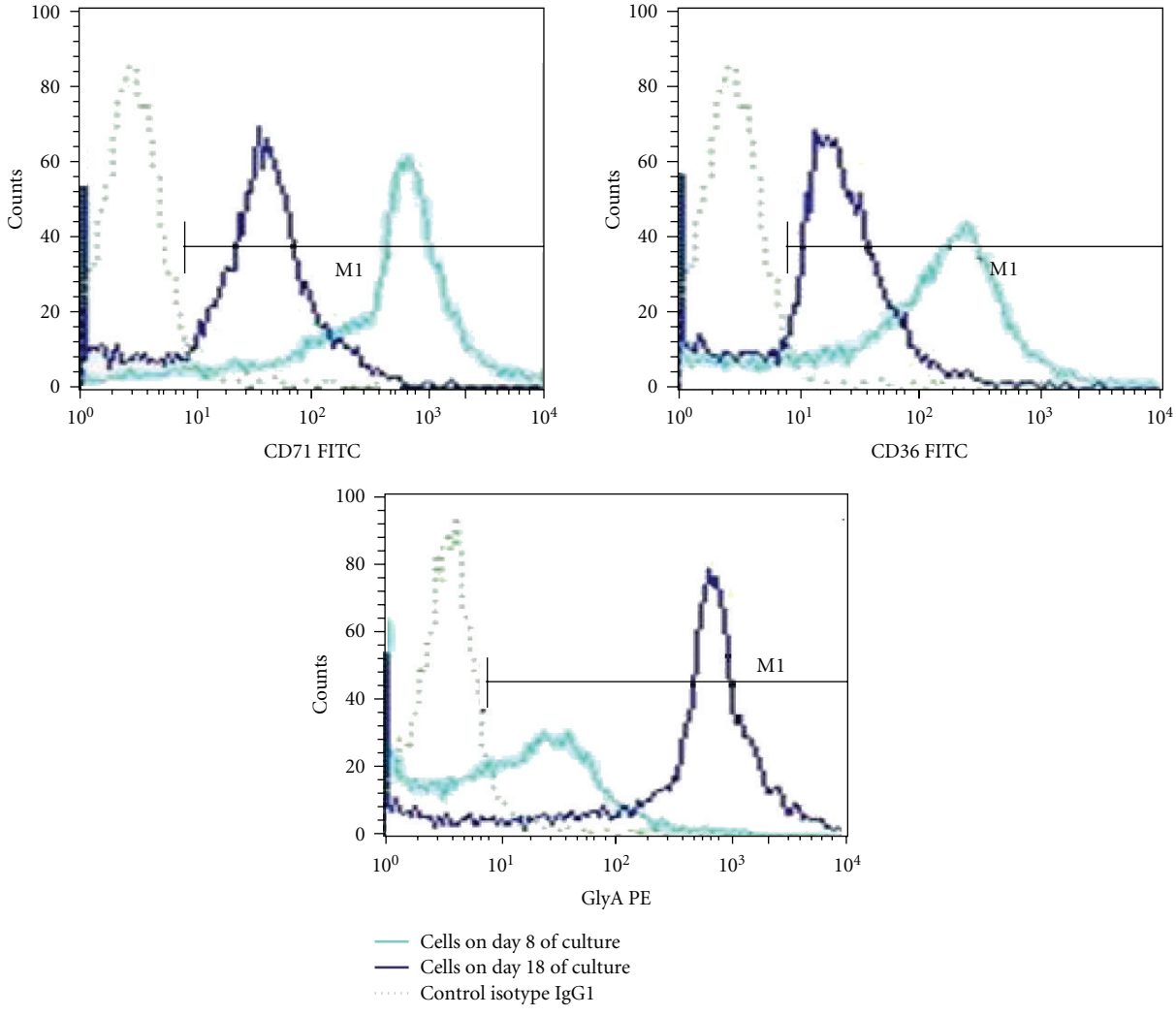
stem cells by $1.2 \pm 0.3 \times 10^6$-fold (mean of six different liver samples) by day 18. This amplification represents 1 or 2 logs higher expansion as compared to CB ($8 \times 10^4 \pm 2 \times 10^4$-fold, $n = 3$) and BM ($2.6 \times 10^4 \pm 0.8 \times 10^4$-fold, $n = 3$) cells, respectively, (Figure 1).

The development of a large and almost pure erythroid cell population can probably be attributed to the preferential proliferation of the generated erythroid progenitors and their subsequent differentiation and maturation. On day 3, 100% of the cells were clonogenic, most belonging to the erythroid lineage (44% BFU-E, 7% CFU-E, 33% CFU-GM and 16% CFU-mix). On day 8, 5% of the cells were clonogenic, 90% of these being BFU-Es. Late erythroid CFU-E progenitors proliferated extensively and constituted 57% of the clonogenic cell population by day 10 (Figure 2). Cells in culture lost their immaturity as early as day 8 as assessed by CD34, CD117, and CD33 negativity, while their erythroid commitment was confirmed by CD235a, CD71, and CD36 positivity. Day 18 cultured RBCs were CD235a$^+$. These cells still expressed CD36 and CD71 markers, as was also observed in our model using CB and BM sources (Figure 3(a)).

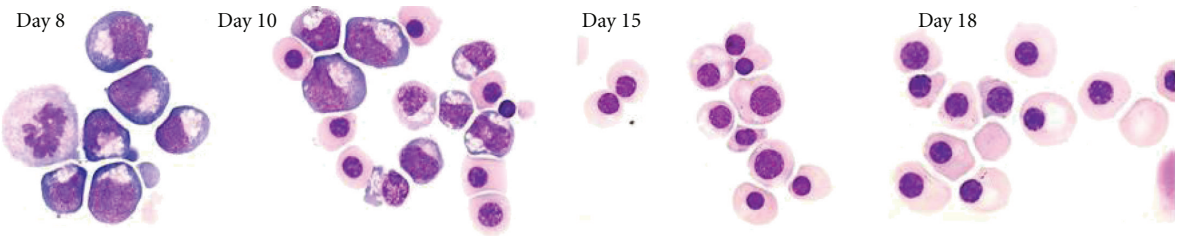
On an MS5 stromal layer, FL CD34$^+$ cells displayed the same erythroid maturation kinetics as CD34$^+$ cells from other sources (CB, BM, or peripheral blood, data not shown) up to day 15. However, their erythroid maturation was blocked by day 18 at the acidophilic stage, and very little nucleus expulsion was observed (3 to 15%) (Figure 3(b)). On the hypothesis that a fetal microenvironment would provide a more suitable niche to achieve complete maturation, we established cocultures over autologous or allogeneic MSCs grown from the FL CD34 negative fractions, but with no improvement in the enucleation efficiency even by extending culture up to day 24.

3.2. Hemoglobin Analysis of FL-Derived Erythroid Cells. Quantitative real-time PCR analysis of total RNA at different times of culture (days 8, 11, 15, and 18) showed that, except for the β globin gene whose expression is constant regardless of the stage of the culture, the expression level of mRNA coding for the other globin genes is upregulated during the erythroid differentiation process.

More particularly, the globin gene expression of the FL-derived erythroid cells showed to be mainly embryonic (ζ and ε globin chains) and fetal (γ globin chains). No adult β globin gene expression could be detected. BM-derived erythroid cells showed an adult globin profile as they mainly expressed β globin chains and to lesser extent fetal (α, γ) globin chains. No embryonic genes (ζ- and ε-globin chains) could be detected. CB-derived erythroid cells showed a similar gene profile but had a stronger expression of the fetal γ-globin chains; embryonic genes (ζ- and ε-globin chains) could be detected but with a variable and low expression (Figure 4(a)).



(a)



(b)

Figure 3: Maturation of FL-derived erythroid cells. (a) FACS analyses of transferrin receptor (CD71), CD36 and glycophorin A (CD235a) expression on days 8 and 18 of liquid culture. Data are from one representative experiment and the green, light blue and dark blue, tracings represent the negative control and cells from days 8 and 18 of culture, respectively. (b) Photographs of the cells on days 8, 10, 15, and 18 of liquid culture after May-Grünwald-Giemsa staining. Every stage of erythroid maturation is represented: proerythroblast, basophilic erythroblast, polychromatophilic erythroblast, orthochromatic erythroblast, and RBCs (magnification ×50).

HPLC analysis ($n = 4$) performed on erythroid cells derived from FL revealed mainly fetal hemoglobin (Hb) regardless of the time of culture. On day 8, the cells contained 68% (59–77%) fetal hemoglobin (HbF) including an acetylated fraction. A very early migrating subfraction was detected and could be identified as Bart Hb, which corresponds to the association of four γ globin chains. At the end of culture (days 19–24), 86% (80–89%) of the cells contained HbF and less than 2% (1.2–1.8%) adult HbA (Figure 4(b)).

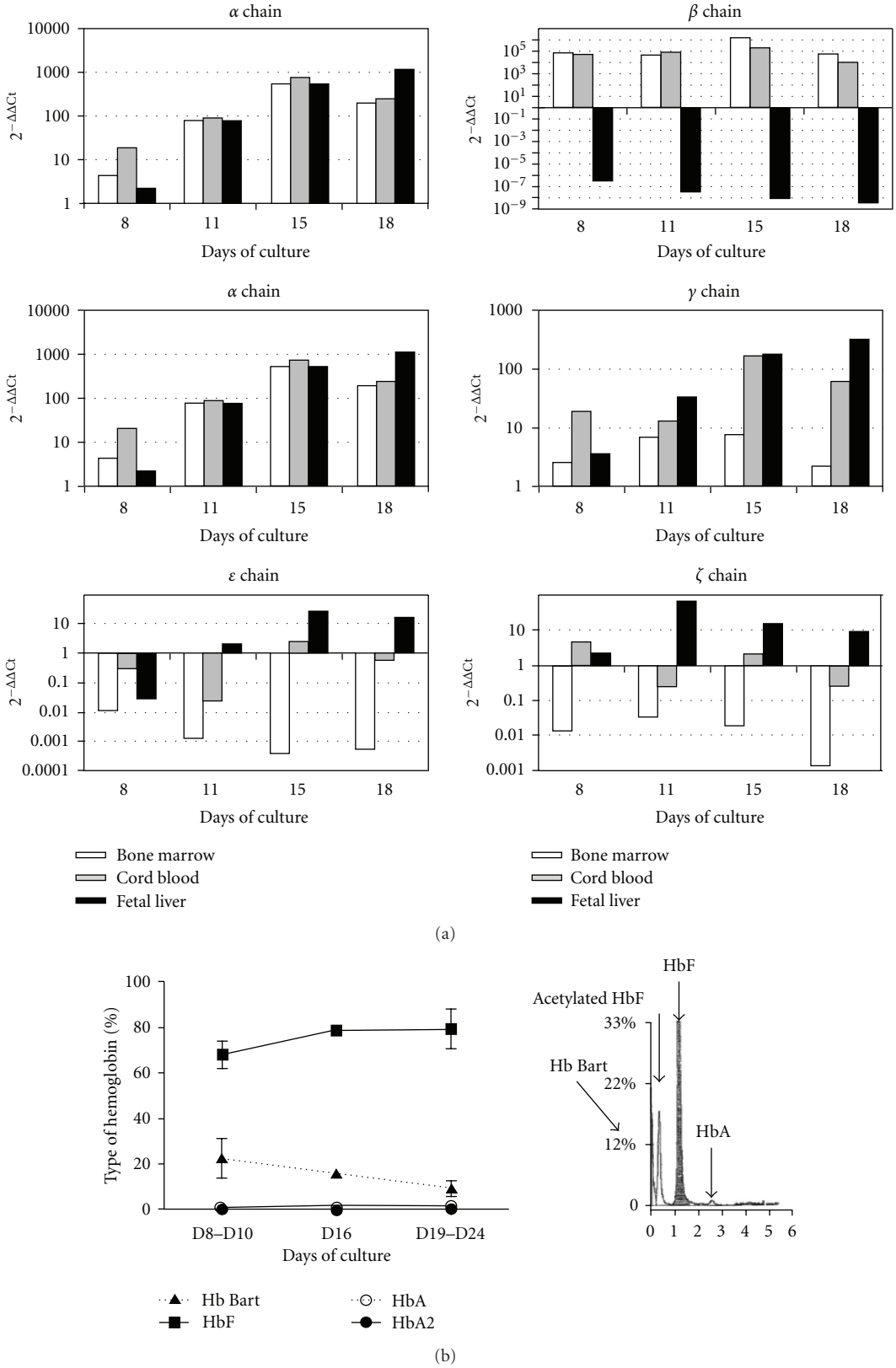


Figure 4: Analysis of the hemoglobin produced by FL-derived erythroid cells. (a) Quantitative real-time PCR analysis of the globin chains at different times of culture (days 8, 11, 15, and 18). Relative gene expression is given by the $2^{-\Delta\Delta Ct}$ method where $\Delta\Delta Ct$ represents the difference of an unknown sample (days 8, 11, 15, and 18 of culture) versus a control (day 0 of culture) for a target gene (globin genes) normalized to a control gene (18S). (b) HPLC analysis of the hemoglobin (Hb) produced on days 8 ($n = 6$), 16 ($n = 2$), and 19–24 ($n = 5$) of erythroid differentiation. Graph and profile show mainly fetal hemoglobin (HbF) including an acetylated fraction.

3.3. Gene Expression of Factors Involved in Definitive Erythropoiesis. In an attempt to explain the blockage at the acidophilic stage of the FL-derived erythroid cells, we analyzed the expression pattern of factors known to be involved in definitive erythropoiesis. We compared PCR results with those obtained for BM and CB samples taken at the same time of erythroid culture (days 8, 11, 15, 18). The $2^{-\Delta\Delta Ct}$ method was applied to establish relative expression of genes (Figure 5).

As compared to BM or CB samples, RT-PCR revealed a strong downregulation of all genes for FL-derived cells regardless of the stage of the culture. BM and CB had the same expression pattern of factors except for STAT5a factor (for which CB showed the same profile as FL). We also observed that the levels of gene expression were higher for BM than for CB.

4. Discussion

A large variety of ontogenic sources of HSC have been explored for cellular therapies. Because of constantly increasing demand in blood supplies, one particular field of interest is transfusion. Thus, we and others have described experimental procedures which allow the massive expansion of $CD34^+$ cells from various sources and the generation of mature RBCs [1, 2, 19–22]. To date, the most proliferative and accessible source of stem cells for generating cultured RBCs (cRBCs) is cord blood. It presents, nevertheless, the drawbacks of being restricted to voluntary donations and is not unlimited; 1 CB unit gives one batch of cRBC production.

Because they are an unlimited source of stem cells, many studies are being carried out with ES cells and more recently with iPS. Critical advances have allowed progress towards the *in vitro* production of functional RBC from these sources in a few years. However their limited ability to expand *in vitro* is a limitation to large-scale production.

In human development, FL is a rich source of hematopoietic progenitors between the 8th and the 22nd week of gestation as it is the exclusive site of erythropoiesis. Although "conventional" sources of HSC for generating cRBC include both adult (mobilized peripheral blood or CB) and primitive stem cells (ES or iPS cells), evidence suggests that FL may represent a rich alternative source of "early" HSCs, displaying multipotency, possessing high proliferative and repopulating potential [23–26]. Indeed, it has been proven to be efficient in transplantation [27–29].

In the present work, we addressed the question of the possibility of generating *in vitro* mature cells from HSC of such an intermediate ontogenic source (FL cells) between ES cells and adult cells. In our experimental conditions, FL cell maturation is mainly blocked at the acidophilic stage. Indeed, only few cells (3–15%) achieved terminal differentiation. We made the hypothesis that the blockade of maturation observed *in vitro* was the consequence of an inappropriate microenvironment. On the assumption that a microenvironment from the same ontogenic stage would better support erythroid differentiation, we cocultured FL-derived $CD34^+$ cells on FL-derived MSC instead of the murine MS5 stromal cell line. However, no difference was observed in terms of terminal maturation, and the produced cells were mostly nucleated, whereas in the same culture conditions adult stem cells could generate fully enucleated cRBC. When we analyzed by RT-PCR globin transcripts of FL-derived erythroid cells, the transcriptomic profiles were mainly fetal (γ-globin chains) and embryonic (ζ- and ε-globin chains). In the same way, BM-derived erythroid cells showed an adult globin profile as they mainly expressed β-globin chain and to lesser extent fetal chains (α-, γ-globin chains). No embryonic genes (ζ- and ε-globin chains) could be detected. In comparison, CB-derived erythroid cells showed a similar gene profile but had a stronger expression of the fetal γ-globin chains and embryonic genes (ζ- and ε-globin chains) could be detected but with a variable and low expression. By analyzing the globin genes expression, we could observe that our system of culture mimics what happens during development and leads to globin gene expression patterns in accordance with the cells' ontogeny.

The precise molecular mechanisms that regulate the erythroid differentiation process remain to be fully elucidated despite many genes and pathways have been described to be essential in erythropoiesis. In order to understand the reason for the enucleation deficiency observed with FL cells, we analyzed by RT-PCR the expression of some genes known to be involved in definitive erythropoiesis (i.e., GATA-1, FOG 1, SOX6, GFI 1b, STAT5a, FOXO3a) upon the following rationale. GATA-1 is found exclusively in hematopoietic cells and is particularly important in erythroid and megakaryocytic lineages. *In vitro*, in the absence of GATA-1, a maturation deficit can be observed, indicating its crucial role in terminal erythroid differentiation [30]. FOG-1-null embryos present a defect both in primitive and definitive erythropoiesis with a blockage of maturation at the stage of proerythroblast [31].

The signal transducer and activator of transcription STAT5 is responsible for the control of proliferation, differentiation, and apoptosis, via its effect on gene expression. STAT5 proteins [32] are suggested to play an important role in hematopoiesis, but there are numerous contradictory reports on the role of STAT5 in normal hematopoietic cell development. A recent study [33] shows that overexpression of an activated mutant of (STAT5) induces erythropoiesis and increases long-term proliferation of human hematopoietic stem/progenitor cells. siRNA silencing of FOXO3a as well as the inactivation of GFI 1b led to erythroid differentiation blockage [34, 35]. Finally, it has been reported that, in $CD34^+$ from CB, SOX6 accelerated the kinetics of erythroid maturation and increased the number of cells that reached the final enucleation step [36].

Our results clearly show that, in our culture conditions, all the genes we studied are strongly downregulated in FL cells, whereas they are upregulated both in BM and CB cells, notably in BM cells. The pattern of gene expression that we observed was in concordance with the enucleation defect observed in FL but could not fully explain it. Indeed this molecular analysis is qualitative and not functional. Nevertheless, it suggests that genes involved in terminal differentiation are differently regulated in the function of the cell ontogeny and might at least in part explain the

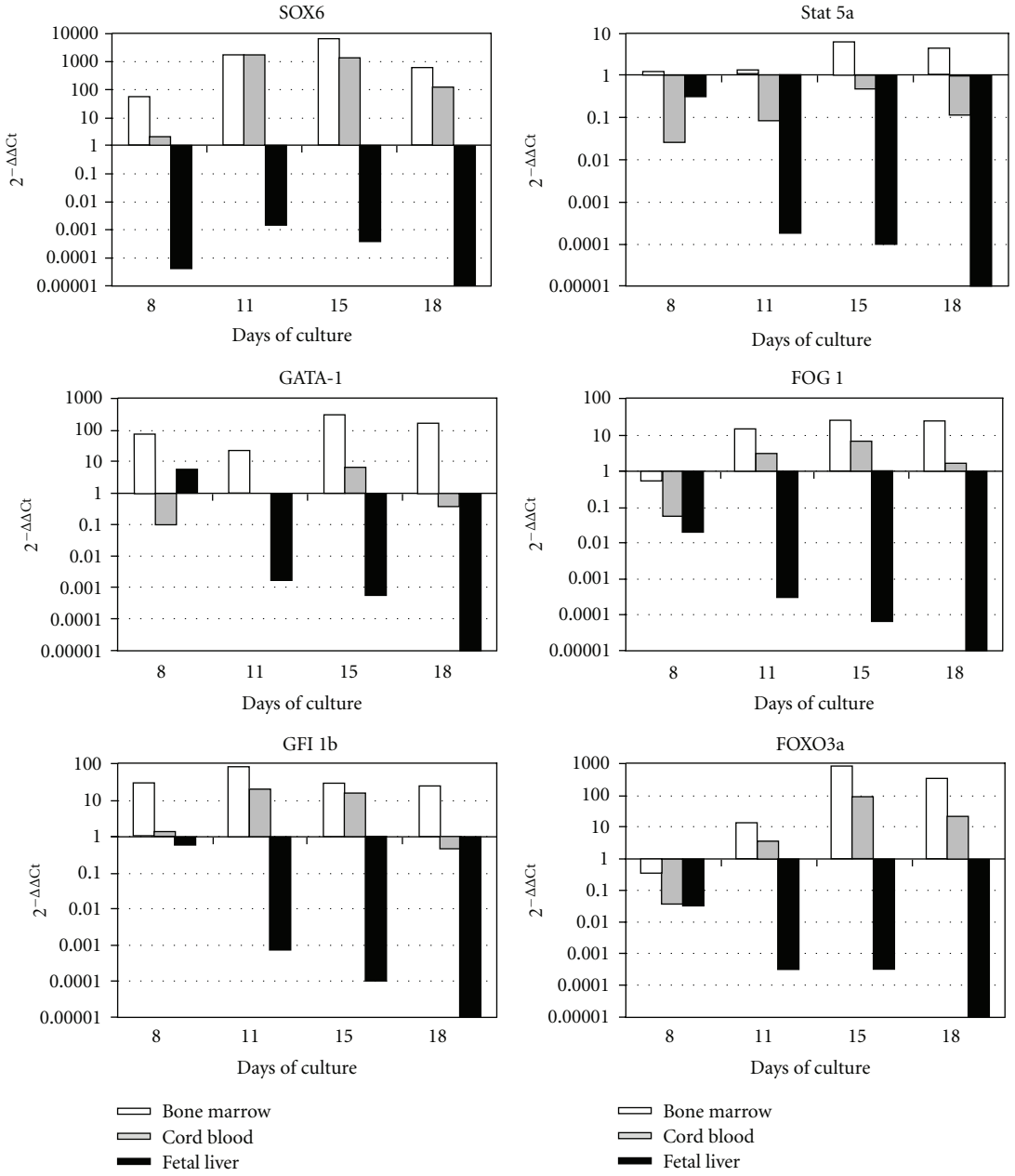


Figure 5: Gene expression of factors involved in definitive erythropoiesis. Quantitative real-time PCR analysis of genes known to be implied in definitive erythropoiesis at different times of culture (days 8, 11, 15, and 18). Relative gene expression is given by the $2^{-\Delta\Delta Ct}$ method where $\Delta\Delta Ct$ represents the difference of an unknown sample (days 8, 11, 15, and 18 of culture) versus a control (day 0 of culture) for a target gene normalized to a control gene (GAPDH).

differences observed in the ability to generate RBC *in vitro*. For many reasons, ES or iPS cells would be the natural candidates for the regenerative medicine of tomorrow. Through this model of erythropoiesis, one can understand how different cells behave according to their ontogeny. As it is titled in the publication of Williams et al. "Children are not little adults: just ask their hematopoietic stem cells" [37]. Extensive analysis on models explaining these differences would be of great interest to fully control the differentiation pathway of stem cells and would be helpful for the next generation of stem cell therapy.

Even if the generation of RBCs from ES cells or iPS has been quite routinely achieved by several teams [6–13], none succeeded in a 100% enucleated population whatever the culture conditions. Primitive cells seem to underperform in terms of production yield of fully mature cRBCs *in vitro*. The question whether this is due to their ontogeny or related to inappropriate culture conditions remains open.

If FL has no advantage in terms of final maturation over pluripotent stem cells, we observed an interest in terms of amplification. As far as the hematopoietic pathway is concerned, FL is known to contain a compartment of

colony-forming cells with a high proliferative potential [14, 23–25]. These observations were confirmed by our findings of (i) a strong expansion of $CD34^+$ FL cells and (ii) 100% cloning efficiency in hematopoietic progenitor assays after 3 days of erythroid induction, as compared to 10–15% cloning efficiency for adult $CD34^+$ cells [17]. The proliferation capacity of stem cells from various sources is partly related to their ontogenic origin. ontogeny-related changes in proliferative potential of human hematopoietic cells *in vitro* have already been suggested in the literature, and analyses have shown, by comparing bone marrow, peripheral blood, umbilical cord blood, and fetal liver, that FL appears to represent a good target for *ex vivo* stem cell expansion [38, 39].

We reproduced this observation in our culture conditions. Indeed, we observed a 100,000- and 25,000-fold expansion for cells from CB, BM, respectively. Strikingly, for FL-derived $CD34^+$ cells, in the same culture conditions, the expansion reached more than 1.2×10^6-fold.

FL remains a model of study and is not a candidate as a direct source of stem cells for *ex vivo* RBCs production for blood transfusion. On the contrary, because iPS cells can proliferate indefinitely, they are obviously the best candidate to set up complementary sources of RBCs for transfusion. But their clinical application will need a drastic increase of their ability to proliferate *in vitro*. The present observations relating to erythropoiesis from FL could pave the way for the design of new strategies to generate induced pluripotent stem cells. This idea is supported by the work of Kim et al. [40]. In a murine model, these authors observed that iPS harbor residual methylation signatures of their somatic tissue of origin, which favors their differentiation into lineages related to the donor cells while restricting alternative cell fates. According to this logic, we could imagine that a cell with a high proliferative capacity thereby would be optimal for the production of RBC.

Acknowledgments

This work was supported by grants from the Association Combattre La Leucémie (CLL) and the Etablissement Français du Sang (EFS). G. Pourcher and C. Mazurier equally contribute this work.

References

[1] T. M. A. Neildez-Nguyen, H. Wajcman, M. C. Marden et al., "Human erythroid cells produced ex vivo at large scale differentiate into red blood cells in vivo," *Nature Biotechnology*, vol. 20, no. 5, pp. 467–472, 2002.

[2] M. C. Giarratana, L. Kobari, H. Lapillonne et al., "Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells," *Nature Biotechnology*, vol. 23, no. 1, pp. 69–74, 2005.

[3] L. Douay and M. C. Giarratana, "Ex vivo generation of human red blood cells: a new advance in stem cell engineering," *Methods in Molecular Biology*, vol. 482, pp. 127–140, 2009.

[4] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[5] J. Yu, M. A. Vodyanik, K. Smuga-Otto et al., "Induced pluripotent stem cell lines derived from human somatic cells," *Science*, vol. 318, no. 5858, pp. 1917–1920, 2007.

[6] C. Qiu, E. Hanson, E. Olivier et al., "Differentiation of human embryonic stem cells into hematopoietic cells by coculture with human fetal liver cells recapitulates the globin switch that occurs early in development," *Experimental Hematology*, vol. 33, no. 12, pp. 1450–1458, 2005.

[7] F. Ma, Y. Ebihara, K. Umeda et al., "Generation of functional erythrocytes from human embryonic stem cell-derived definitive hematopoiesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 35, pp. 13087–13092, 2008.

[8] S. J. Lu, Q. Feng, J. S. Park et al., "Biologic properties and enucleation of red blood cells from human embryonic stem cells," *Blood*, vol. 112, no. 12, pp. 4475–4484, 2008.

[9] H. Lapillonne, L. Kobari, C. Mazurier et al., "Red blood cell generation from human induced pluripotent stem cells: perspectives for transfusion medicine," *Haematologica*, vol. 95, no. 10, pp. 1651–1659, 2010.

[10] K. D. Choi, J. Yu, K. Smuga-Otto et al., "Hematopoietic and endothelial differentiation of human induced pluripotent stem cells," *Stem Cells*, vol. 27, no. 3, pp. 559–567, 2009.

[11] C. Lengerke, M. Grauer, N. I. Niebuhr et al., "Hematopoietic development from human induced pluripotent stem cells," *Annals of the New York Academy of Sciences*, vol. 1176, pp. 219–227, 2009.

[12] Q. Feng, S. J. Lu, I. Klimanskaya et al., "Hemangioblastic derivatives from human induced pluripotent stem cells exhibit limited expansion and early senescence," *Stem Cells*, vol. 28, no. 4, pp. 704–712, 2010.

[13] J Dias, M Gumenyuk, H Kang et al., "Generation of red blood cells from human induced pluripotent stem cells," *Stem Cells and Development*, vol. 20, no. 9, pp. 1639–1647, 2011.

[14] P. Rollini, E. F. V. Hull, S. Kaiser, U. Kapp, and S. Leyvraz, "Phenotypic and functional analysis of human fetal liver hematopoietic stem cells in culture," *Stem Cells and Development*, vol. 16, no. 2, pp. 281–295, 2007.

[15] W. G. Wood, J. B. Clegg, and D. J. Weatherall, "Developmental biology of human hemoglobins," *Progress in Hematology*, vol. 10, pp. 43–90, 1977.

[16] C. Doucet, I. Ernou, Y. Zhang et al., "Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications," *Journal of Cellular Physiology*, vol. 205, no. 2, pp. 228–236, 2005.

[17] L. Kobari, F. Pflumio, M. C. Giarratana et al., "In vitro and in vivo evidence for the long-term multilineage (myeloid, B, NK, and T) reconstitution capacity of ex vivo expanded human CD34+ cord blood cells," *Experimental Hematology*, vol. 28, no. 12, pp. 1470–1480, 2000.

[18] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[19] G. Migliaccio, A. R. Migliaccio, and J. W. Adamson, "In vitro differentiation of human granulocyte/macrophage and erythroid progenitors: comparative analysis of the influence of recombinant human erythropoietin, G-CSF, GM-CSF, and IL-3 in serum-supplemented and serum-deprived cultures," *Blood*, vol. 72, no. 1, pp. 248–256, 1988.

[20] E. Fibach, D. Manor, A. Oppenheim, and E. A. Rachmilewitz, "Proliferation and maturation of human erythroid progenitors in liquid culture," *Blood*, vol. 73, no. 1, pp. 100–103, 1989.

[21] G. Migliaccio, R. Di Pietro, V. Di Giacomo et al., "In Vitro mass production of human erythroid cells from the blood

of normal donors and of thalassemic patients," *Blood Cells, Molecules, and Diseases*, vol. 28, no. 2, pp. 169–180, 2002.

[22] K. Miharada, T. Hiroyama, K. Sudo, T. Nagasawa, and Y. Nakamura, "Efficient enucleation of erythroblasts differentiated in vitro from hematopoietic stem and progenitor cells," *Nature Biotechnology*, vol. 24, no. 10, pp. 1255–1256, 2006.

[23] M. O. Muench, J. Cupp, J. Polakoff, and M. G. Roncarolo, "Expression of CD33, CD38, and HLA-DR on CD34+ human fetal liver progenitors with a high proliferative potential," *Blood*, vol. 83, no. 11, pp. 3170–3181, 1994.

[24] S. F. A. Weekx, D. R. Van Bockstaele, J. Plum et al., "CD34++CD38− and CD34+CD38+ human hematopoietic progenitors from fetal liver, cord blood, and adult bone marrow respond differently to hematopoietic cytokines depending on the ontogenic source," *Experimental Hematology*, vol. 26, no. 11, pp. 1034–1042, 1998.

[25] P. Rollini, S. Kaiser, E. Faes-Van'T Hull, U. Kapp, and S. Leyvraz, "Long-term expansion of transplantable human fetal liver hematopoietic stem cells," *Blood*, vol. 103, no. 3, pp. 1166–1170, 2004.

[26] S. Nava, M. Westgren, M. Jaksch et al., "Characterization of cells in the developing human liver," *Differentiation*, vol. 73, no. 5, pp. 249–260, 2005.

[27] G. Lucarelli, T. Izzi, and A. Porcellini, "Fetal liver transplantation in 2 patients with acute leukaemia after total body irradiation," *Scandinavian Journal of Haematology*, vol. 28, no. 1, pp. 65–71, 1982.

[28] R. P. Gale, "Fetal liver transplantation in aplastic anemia and leukemia," *Thymus*, vol. 10, no. 1-2, pp. 89–94, 1987.

[29] C. Royo, J. L. Touraine, P. Veyron, and A. Aitouche, "Survey of experimental data on fetal liver transplantation," *Thymus*, vol. 10, no. 1-2, pp. 5–12, 1987.

[30] M. J. Weiss, C. Yu, and S. H. Orkin, "Erythroid-cell-specific properties of transcription factor GATA-1 revealed by phenotypic rescue of a gene-targeted cell line," *Molecular and Cellular Biology*, vol. 17, no. 3, pp. 1642–1651, 1997.

[31] A. P. Tsang, J. E. Visvader, C. A. Turner et al., "FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation," *Cell*, vol. 90, no. 1, pp. 109–119, 1997.

[32] T. Nosaka, T. Kawashima, K. Misawa, K. Ikuta, A. L. F. Mui, and T. Kitamura, "STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells," *EMBO Journal*, vol. 18, no. 17, pp. 4754–4765, 1999.

[33] A. T. J. Wierenga, E. Vellenga, and J. J. Schuringa, "Down-regulation of GATA1 uncouples STAT5-induced erythroid differentiation from stem/progenitor cell proliferation," *Blood*, vol. 115, no. 22, pp. 4367–4376, 2010.

[34] W. J. Bakker, M. Blázquez-Domingo, A. Kolbus et al., "FoxO3a regulates erythroid differentiation and induces BTG1, an activator of protein arginine methyl transferase 1," *Journal of Cell Biology*, vol. 164, no. 2, pp. 175–184, 2004.

[35] L. Garçon, C. Lacout, F. Svinartchouk et al., "Gfi-1B plays a critical role in terminal differentiation of normal and transformed erythroid progenitor cells," *Blood*, vol. 105, no. 4, pp. 1448–1455, 2005.

[36] Z. Yi, O. Cohen-Barak, N. Hagiwara et al., "Sox6 directly silences epsilon globin expression in definitive erythropoiesis.," *PLoS Genetics*, vol. 2, no. 2, article e14, 2006.

[37] D. A. Williams, H. Xu, and J. A. Cancelas, "Children are not little adults: just ask their hematopoietic stem cells," *Journal of Clinical Investigation*, vol. 116, no. 10, pp. 2593–2596, 2006.

[38] P. M. Lansdorp, W. Dragowska, and H. Mayani, "Ontogeny-related changes in proliferative potential of human hematopoietic cells," *Journal of Experimental Medicine*, vol. 178, no. 3, pp. 787–791, 1993.

[39] S. Huang, P. Law, D. Young, and A. D. Ho, "Candidate hematopoietic stem cells from fetal tissues, umbilical cord blood vs. adult bone marrow and mobilized peripheral blood," *Experimental Hematology*, vol. 26, no. 12, pp. 1162–1171, 1998.

[40] K. Kim, A. Doi, B. Wen et al., "Epigenetic memory in induced pluripotent stem cells," *Nature*, vol. 467, no. 7313, pp. 285–290, 2010.