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Large-scale production of embryonic red blood cells from human embryonic stem cells

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Objective. **To develop a method to produce in culture large number of erythroid cells from human embryonic stem cells.**

Materials and Methods. **Human H1 embryonic stem cells were differentiated into hematopoietic cells by coculture with a human fetal liver cell line, and the resulting CD34-positive cells were expanded in vitro in liquid culture using a three-step method. The erythroid cells produced were then analyzed by light microscopy and flow cytometry. Globin expression was characterized by quantitative reverse-transcriptase polymerase chain reaction and by high-performance liquid chromatography.**

Results. **CD34-positive cells produced from human embryonic stem cells could be efficiently differentiated into erythroid cells in liquid culture leading to a more than 5000-fold increase in cell number. The erythroid cells produced are similar to primitive erythroid cells present in the yolk sac of early human embryos and did not enucleate. They are fully hemoglobinized and express a mixture of embryonic and fetal globins but no β-globin.**

Conclusions. **We have developed an experimental protocol to produce large numbers of primitive erythroid cells starting from undifferentiated human embryonic stem cells. As the earliest human erythroid cells, the nucleated primitive erythroblasts, are not very well characterized because experimental material at this stage of development is very difficult to obtain, this system should prove useful to answer a number of experimental questions regarding the biology of these cells. In addition, production of mature red blood cells from human embryonic stem cells is of great potential practical importance because it could eventually become an alternate source of cell for transfusion.**

Mammalian erythroid cells are a model system to study biochemistry, structural membrane biology, human genetics, transcriptional regulation, and more recently computational biology because of multiple technical advantages associated with this lineage. Erythroid cells are abundant, grow in suspension, and both the mature and the progenitor cells are relatively easy to harvest. In addition, red blood cells express few genes and a large number of genetic disorders have been identified. Study of the globin genes has always been of particular interest in part because reversing or preventing the γ- to β-globin gene switch could cure or ameliorate sickle cell disease and the thalassemias. The earliest human erythroid cells, the nucleated primitive erythroblasts, are not very well characterized because experimental material at this stage of development is very difficult to obtain.

The availability of human embryonic stem cells (hESCs) offers an opportunity to produce large number of erythroid cells in vitro and fill some of the knowledge gap in the biology of early human erythroid cells. In addition, production of mature red blood cells from hESCs is of great potential practical importance because it could eventually become an alternate source of cell for transfusion. We therefore developed a method to produce large number of erythroid cells from hESCs.

Humans sequentially produce three main types of hemoglobin associated with two successive waves of

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hematopoiesis. The first erythroblasts are produced in the yolk sac from the third to the eighth week of gestation and are megaloblastic and nucleated. These primitive erythroblasts first produce embryonic ($\zeta_2\epsilon_2$) hemoglobin and then a mixture of embryonic and fetal hemoglobins ($\alpha_2\gamma_2$) [1]. Definitive hematopoietic cells can first be detected in the dorsal aorta, gonads, and mesonephros region of the embryo proper [2–6] a few days after the onset of primitive erythropoiesis; they migrate first to the liver, and around birth to the bone marrow. Early definitive erythroid cells produced in the liver express large amounts of fetal hemoglobin and a small amount of adult hemoglobin ($\alpha_2\beta_2$). A gradual transition to cells expressing primarily adult hemoglobin culminate around birth. The contribution of yolk sac–derived cells to definitive hematopoiesis is not clear.

Hematopoietic differentiation of hESCs has been achieved using embryoid body formation [7–11] and coculture with stromal cells [12–16]. Both of these methods produce sufficient numbers of hematopoietic cells to perform colony assays but not functional or biochemical experiments. We recently developed a procedure to produce hematopoietic cells by coculturing hESCs with fetal human liver clone B (FH-B-hTERT) cells[17], a telomerase immortalized human fetal liver cell line [18]. We demonstrated that we could reproducibly obtain about 150,000 CD34 cells per five million hESCs and that some of these CD34-positive cells were hematopoietic as they yielded erythroid and myeloid colonies in methylcellulose assays. We also demonstrated that increasing the time of coculture of the hESCs from 1 to 3 weeks was associated with a partial globin switch, reminiscent of the switch observed in early human development. We report here that the hematopoietic cells produced by coculture on FHB-hTERT can be greatly expanded in vitro into fully mature primitive erythroid cells.

Methods

Cell culture

hESC line H1 cells between passages 30 and 70 were maintained undifferentiated by coculture with irradiated mouse embryonic fibroblast (MEF) cells (80 cGy) as described by Thomson et al. [19]. MEF, FH-B-hTERT, and MS-5 cells were grown in Dulbecco's modified Eagle's medium high-glucose (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 u/mL of penicillin sodium and streptomycin sulphate (P/S). FH-B-hTERT cells and MEF were irradiated (80 Gy using a cesium-137 shepherd Mark I irradiator, JL Sheperd, San Fernando, CA, USA) before attachment onto gelatin-coated six-well plates.

Hematopoietic differentiation and liquid culture for large amplification and differentiation of hematopoietic progenitor cells

We followed five steps for hematopoietic differentiation and liquid culture.

Step 1. Undifferentiated H1 cells were passaged onto irradiated FH-B-hTERT feeder layers and cultured with differentiation medium composed of LGlu DMEM supplemented with 20% FBS (Invitrogen), 100 μM MEM-nonessential amino acids, and 100 u/mL peni/100 mg/ml staepto. Medium was changed every 2 to 3 days. On day 15, differentiated H1 cells on FH-B-hTERT were dissociated into single-cell suspensions by treatment with collagenase IV followed by trypsin/ethylenediamine-tetraacetic acid (Invitrogen) supplemented with 5% chick serum (catalog number 16110-082, Invitrogen). CD34 cell separations were performed using magnetic beads according to the manufacturer's instructions (Milteny Biotec, Auburn, CA, USA).

Step 2. Sorted $CD34^+$ cells were seeded at a density of 1000,000 cells/mL in serum-free medium Stemspan (Iscove Modified Dulbecco Medium [IMDM], bovine serum albumin, insulin, transferrin, 2-β-mercapto-ethanol) (StemCell Technologies, Vancouver, BC, Canada) supplemented with hydrocortisone (10^{-6} M), interleukin-3 (IL3; 13 ng/mL), BMP-4 (13 ng/mL), Flt-3L (33 ng/mL), stem cell factor (SCF) (100 ng/mL), and erythoprotein (Epo) (2.7 U/mL) for 7 days.

Step 3. Cells were collected washed with phosphate-buffered saline (PBS), and reseeded on StemSpan supplemented with hydrocortisone (10^{-6} M), IL3 (13 ng/mL), BMP4 (13 ng/mL), SCF (40 ng/mL), Epo (3.3 U/mL), and insulin like growth factor-1 (IGF-1) (40 ng/mL) for 7days. Every 2 to 3 days, 1 to 2 mL of the same fresh medium was added to keep the cell density below 500,000 cells/mL.

Step 4. Cells were collected, washed with PBS, and seeded at 200,000 cells/mL in flasks containing a confluent MS-5 feeder layer in StemSpan medium supplemented with Epo (3 U/mL) and 25 μM hemin for 3 days. Hemin was prepared as described by Fibach et al. [20].

Step 5. Cells were collected washed with PBS and seeded on a confluent MS-5 feeder for up to an additional 7 days in StemSpan medium supplemented by 25 μM hemin.

Cord blood hematopoietic cells from normal deliveries were obtained after gaining proper approval by the Internal Review Board of the Albert Einstein College of Medicine. Light density mononuclear cells were separated by centrifugation on Histopaque-1077 using the protocol recommended by the manufacturer (Sigma-Aldrich, Saint Louis, MO, USA) and the CD34-positive cells were isolated using the Easy-Sep system also as recommended by the manufacturer (StemCell Technologies). The purified CD34-positive cells were then grown in the same liquid culture system used for the hESC-derived CD34-positive cells, starting at step 2 (see above).

Flow cytometry and cell sorting

Single-cell suspensions from different steps were washed with calcium - and magnesium-free PBS supplemented with 2% FBS and 0.1% sodium azide and cells were labeled with CD34-phycoerythrin (PE; clone 581, Beckman Coulter Immunotech, Massielle, France), CD45-fluorescein isothiocyanate (FITC; clone J33, Beckman Coulter Immunotech), CD71-FITC (clone T56/14, ebioscience, San Diego, CA, USA), CD235a-PE (clone CLB-ery-1

[AME-1], Caltag Invitrogen, Carsbad, CA, USA), and their corresponding immunoglobulin 1 controls. Dead cells were gated out based on morphology or 7-AAD exclusion.

Total mRNA extraction
Total mRNA was extracted by trizol method (Invitrogen) following the manufacturer's instructions.

High-performance liquid chromatography
One to 5 million cells from step 5 were collected, washed twice with PBS, resuspended in water and freeze-thawed three times in a dry ice/ethanol bath. Cellular debris was then eliminated by centrifugation. The supernatants were used for hemoglobin measurement and globin separation by high-performance liquid chromatography (HPLC) as described [21].

Cytospin and Giemsa-staining
Colonies picked from methylcellulose were spun onto slides using a cytospin apparatus (Cytospin 2; Thermo Shandon, Pittsburg, PA, USA). After drying for a minute, slides were stained with Wright-Giemsa reagents (Hema 3 stain, Fisher Scientific, Pittsburgh, PA, USA) following the manufacturer's instructions. The various stages of erythroid differentiation were defined as follows.

Proerythroblast. Cell size is large, cytoplasm is blue, and nuclear chromatin is granular or branny, and reddish purple color.

Basophilic erythroblast. Cell size is smaller than the proerythroblast, and cytoplasm is dark blue color, a perinuclear halo may be prominent, and condensed chromatin is more darkly purple and more coarsely clump than proerythroblasts.

Polychromatophilic erythroblast. Cell size becomes smaller, cytoplasm is muddy blue color, nucleus is smaller, and has more condensed chromatin with chromatin arranged in evenly spaced chunky aggregation with dark purple color.

Orthochromatic erythroblast. Cell becomes smaller than the other erythroid cells, cytoplasm is eosinophilic, and nucleus shows pyknotic purple black color.

Erythroid cell size measurement
Cell diameters were estimated on cytospun, Giemsa-stained cells using a Nikkon Eclipse TE 2000-S microscope (Nikon Corporation, Tokyo, Japan) and the ACT-2U imaging software. Peripheral blood red blood cells (RBCs (Nikon Corporation, Tokyo, Japan)) were processed exactly as the cord blood and hESC-derived RBCs and used to control for the size distortion introduced by the cytospin and fixation procedures assuming an average adult RBC size of 7.8 μm [22].

Globin expression analysis by quantitative real-time reverse-transcriptase polymerase chain reaction
Quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR) reactions were performed using a single-tube hot start RT-PCR Kit (Quantitech probe RT-PCR kit, Qiagen, Valencia, CA, USA) on a LightCycler real-time PCR instrument (Roche, Indianapolis, IN, USA). Standard curves were established using cDNA clones for each globin. The concentration of each purified template was carefully quantified using the Picogreen dsDNA Quantitation Reagent kit (Molecular Probes, Eugene, OR, USA) and by spectrophotometry using the NanoDrop ND-1000 micro-spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The detection limit for all primer-probe combinations was under 100 copies of mRNA.

The primers and probes for each specific globin were:

α-forward: CGGTCAACTTCAAGCTCCTAAG; α-reverse: CCGCCCACTCAGACTTTATT

β-forward: TACATTTGCTTCTGACACAAC; β-reverse: ACA GATCCCCAAAGGAC

γ-forward: CTTCAAGCTCCTGGGAAATGT; γ-reverse: GCA GAATAAAGCCTACCTTGAAAG

ϵ-forward: GCCTGTGGAGCAAGATGAAT; ϵ-reverse: GCG GGCTTGAGGTTGT

ζ-forward: CGGTGAAGAGCATCGACG; ζ-reverse: GGAT ACGACCGATAGGAACTTGT

Hemoglobin concentration determination
Optical density at 415 nm of washed cell freeze-thaw lysates was measured using a NanoDrop ND1000 instrument (NanoDrop Technologies). The concentration of hemoglobin was calculated assuming an extinction coefficient for oxygenated hemoglobin of 500 mM at 415 nM.

Results

In an effort to obtain larger number of erythroid cells starting from hESCs, we have modified serum-free liquid culture protocols, originally created to expand and differentiate cord and peripheral blood hematopoietic stem cells into enucleated RBCs [23,24]. The procedure that we have developed requires five steps. In the first step, hESCs are cocultured for 2 weeks on FH-B-hTERT to induce their differentiation into hematopoietic stem cells. CD34-positive cells are then sorted using immunomagnetic beads to a purity greater than 90 or 95%. In the second step, these hESC-derived CD34-positive cells are induced to proliferate by incubation with Flt-3L, SCF, Epo, BMP-4, and IL3 for 7 days. BMP-4 was added to the cocktail because this growth factor [25,26] promotes hematopoietic differentiation. In the third step, the erythroid progenitors are stimulated for 7 days with the same medium with the exception of IGF-1 in place of Flt-3-L because IGF-1 has been shown to be necessary for normal erythroid differentiation in serum-free culture [27,28] and because *FLt-3l* is not necessary at this step [23]. In the fourth step, terminal erythroid maturation is induced by coculture with MS-5 cells, a mouse bone marrow stromal cell line [29], in serum-free medium containing Epo and hemin. Finally, in the fifth step, the cells are plated in serum free medium on the MS-5 feeder layer in the presence of hemin. High concentrations of hemin were added to steps 4 and 5 because it greatly decreases the vacuolization that is often observed in RBCs produced in vitro. The mechanism of action of hemin is not clear but could be related to improved iron transport or metabolism or to the effect of this compound on transcription and translation [30]. MS-5 was used as a feeder layer because these stromal cells have been shown

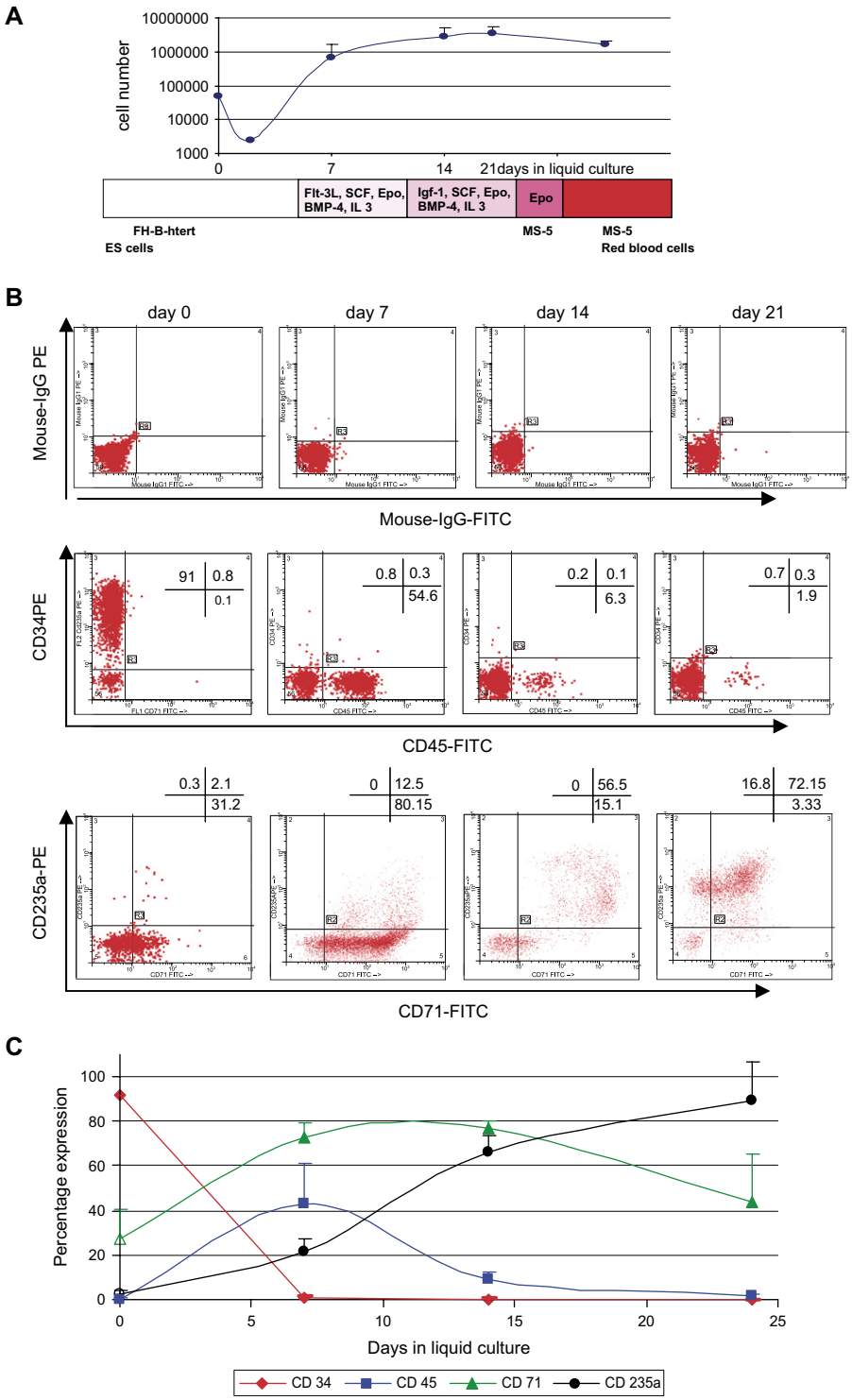
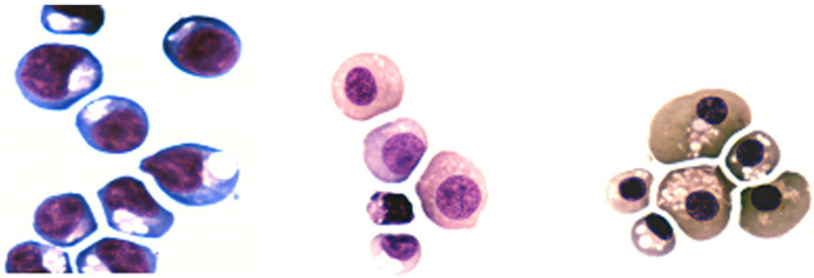


Figure 1. Amplification of erythroid cells in liquid culture. **(A):** Fifty thousand hESC-derived CD34-positive cells were seeded in liquid culture and live cells were periodically counted using a hematocytometer. After the death of the nonhematopoietic cells preset in the population, a 5000- to 10,000-fold amplification of the cells can be observed. The results of three experiments (average and standard deviation) are plotted. **(B):** Dot plots illustrating the evolution of antigen expression of hESCs-derived CD34-positive cells after purification using immunomagnetic beads (day 0) or after 7, 14, or 21 days in liquid culture. Expression of CD 34, CD45, CD71, and CD235a was analyzed by flow cytometry using directly labeled antibodies. Dead cells were gated out using 7-AAD exclusion and morphologic parameters (forward scatter and side scatter. **(C):** Plot summarizing the expression profiles of the CD 34, CD45, CD71, and CD235a antigens observed at different time point in three independent experiments. Results are the average and standard deviation of the percentage of cells expressing each antigen.

A



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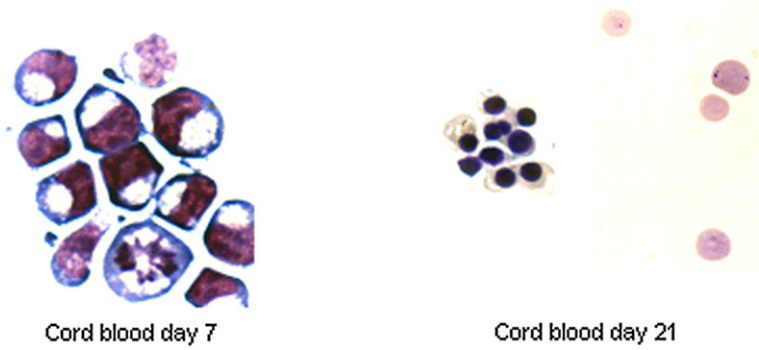

C

	cord blood-derived RBCs		hESC-derived RBCs	
	relative diameter* (%)	estimated size (μm)	relative diameter* (%)	estimated size (μm)
enucleated	119 ± 19.6	9.4 ± 1.5	NA	NA
orthochromatic	135± 25.6	10.6 ±1.9	222 ± 43.4	17.4 ± 3.4

*: Relative to mature peripheral blood RBCs

D

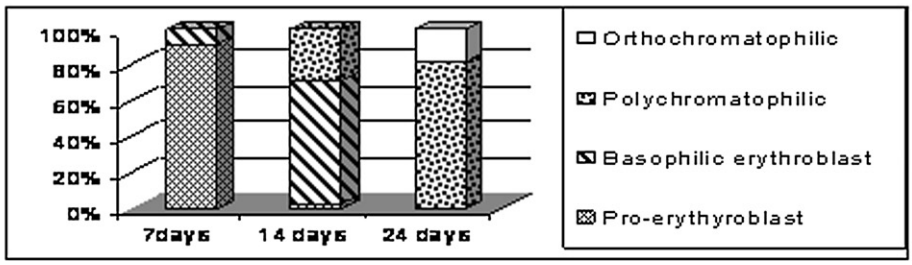

Figure 2. Morphologic analysis. Wright-Giemsa staining of cells obtained at different time after liquid culture of CD34-positive cells derived either from hESCs **(A)** or cord blood (magnification 10× 1000 **(B). (C):** Table summarizing the diameter of cord blood and hESC-derived RBCs (see methods). N/A = not applicable (because there is no enucleated cells produced in the cultures of hESC-derived CD34-positive cells). **(D):** Histograms illustrating the proportion of pro-, basophilic, polychromatophilic, and ortho-chromatophilic erythroblasts at different times in the culture of hESC-derived CD34-positive cells. The hESC-derived proerythroblasts are morphologically similar to cord blood–derived proerythroblasts at day 7 but the hESC-derived nucleated megaloblastic orthochromatic erythroblasts observed at days 21 are very different from the small orthochromatic erythroblasts and the reticulocytes that are obtained with cord blood.

to promote terminal differentiation and enucleation of erythroid cells produced in culture [24].

The evolution of the number of cells during the 24-day liquid culture period is shown in Figure 1A. In a typical experiment, 50,000 sorted CD34-positive cells yield about 5 million erythroid cells. This corresponds to a 5000- to 10,000-fold amplification of the hematopoietic cells produced at the end of step 1 as only a few percent of the cells

recovered with the CD34 magnetic beads are hematopoietic (data not shown). Most of the nonhematopoietic CD34-positive cells seeded in the liquid culture die rapidly as illustrated by the severe drop in cell number in the first 2 days of the culture.

Figure 1B illustrates the flow cytometry profile of the cells throughout the culture for the CD34, CD45, CD71 (tranferrin), and CD235a (glycophorin A) antigens. As expected, the CD34 antigen is rapidly downregulated. The CD45 antigen, which was almost absent at the beginning of the liquid culture, is maximally expressed at day 7 and is then downregulated as the cells mature to terminally differentiated erythroblasts that do not express CD45 [31]. CD71 and CD235a antigen expression also mimic the patterns known to occur during erythroid maturation with a gradual increase in CD235a expression along with a peak of expression of CD71 before the final differentiation divisions [31]. At the end of the culture, up to 95% of the cells are glycophorin A–positive (average 87.6% ± 5.3%) with less than 1 to 2% CD45-positive cells (average 1.5% ± 0.85%) detectable, demonstrating that the vast majority of the cells produced are erythroid. Extension of steps 2 and 3 to 10 days each (instead of 7) led to population of cells that were more than 99.5% erythroid as judged by glycophorin A staining (data not shown). These fluorescein-activated cell sorting profiles also show that the cultures are relatively synchronized probably because of the sequential addition and withdrawal of critical cytokines.

Figure 2A illustrates Wright Giemsa staining of the cells obtained at different time points during the liquid culture. For comparison purposes, CD34-positive cells isolated from cord blood were placed in the same culture conditions and processed in parallel with the hESC-derived CD34-positive cells (Fig. 2B). This revealed first, that hESC-derived RBCs do not enucleate although cord blood-derived erythroid cells cultured in the same conditions enucleated at more than 50%, and second, that the hESC-derived RBCs were much larger than cord blood-derived RBCs (Fig. 2C). This strongly suggests that in these culture conditions, the RBCs produced from hESCs are similar to cells that are produced in the human yolk sac rather than to cells produced later in development, which are smaller and enucleated [32]. Enumeration of the cells at each stage of erythroid maturation during the culture on the slides stained with Wright-Giemsa (Fig. 2D) confirmed the partial synchrony of the culture that we detected by flow cytometry. At the end of step 5, a few cells with the morphology of erythroid progenitors or macrophages could be detected after Wright-Giemsa staining. These cells probably

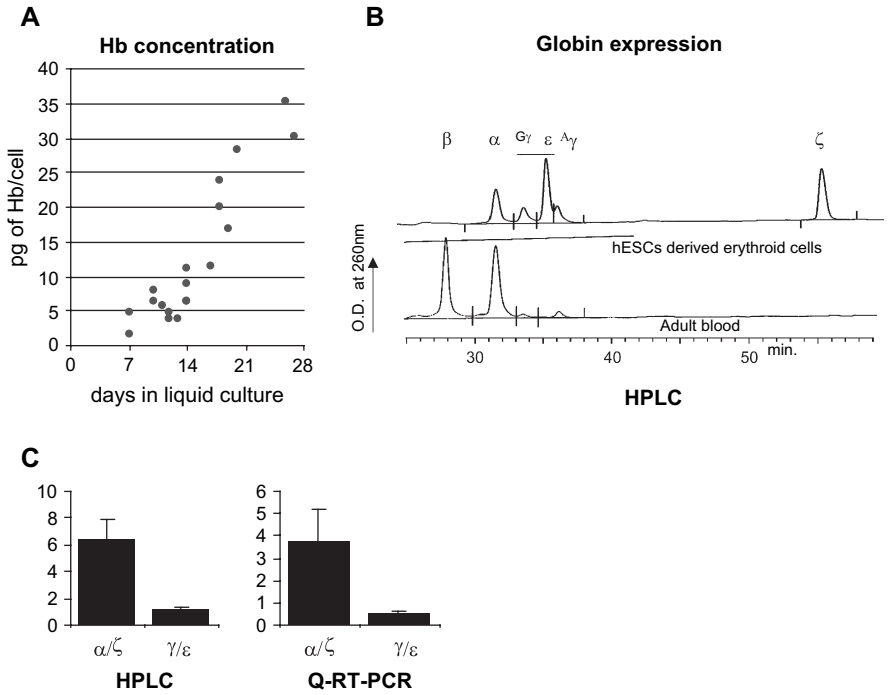

Figure 3. Hemoglobin characterization. **(A):** X-Y scatter plot illustrating hemoglobin concentration determined by spectrophotometry at different time points during the liquid culture phase. The concentration of hemoglobin reached at day 26 to 28 is similar to the concentrations of hemoglobin observed in peripheral blood RBCs (about 30 pg of Hb/cell). The results of five independent experiments are plotted **(B).** Chromatograms illustrating analysis of globin expression at the protein level by HPLC. The top chromatogram was obtained with a hESC-derived erythroid lysate. The bottom control histogram was obtained with a peripheral-blood derived lysate. **(C):** Histograms illustrating the α/ζ and γ/ϵ globin ratio at the end of the culture period (day 24) determined by HPLC or by quantitative real-time RT-PCR. The results are the average and standard deviation of three independent experiments. Both methods revealed that the RBCs derived from hESCs produce a mixture of embryonic and fetal globin and that they do not express any β-globin gene.

correspond to the less than 2% CD45-positive cells detected by flow cytometry at the end of the culture.

Figure 3A illustrates the hemoglobin concentration measured by spectrophotometry at different time points in the culture. The concentration of hemoglobin per cell reached (30 pg of hemoglobin/cell) is close to the hemoglobin concentration in peripheral blood RBCs. This suggests that many of the cells generated in culture are fully mature nucleated erythroid cells. To confirm that these cells were indeed of the primitive rather than the definitive erythroid lineage, quantitative real time RT-PCR on total RNA and HPLC analysis on cell lysates were performed on RBCs obtained at the end of the procedure. This revealed that the hESC-derived erythroid cells produce exclusively embryonic and fetal globin chains (Fig. 3B) and globin mRNAs (Fig. 3C) and that no adult globin chains or mRNA could be detected even when a sensitive quantitative real-time PCR assay was used. These experiments therefore confirmed the embryonic nature of these cells.

Discussion

We have developed a new method to produce relatively large numbers of human erythroid cells in liquid culture from undifferentiated hESCs. Because the cells grow in suspension, the procedure should be scalable to bioreactors. The cells obtained are megaloblastic, nucleated, well hemoglobinized and express a mixture of embryonic and fetal globins similar to the globins found in nucleated RBCs that are produced in the yolk sac and found in the circulation of early human embryos [33,34].

This new method should prove useful as an experimental system to study many questions related to human erythropoiesis such as the mechanism of globin switching, hemoglobinopathies, iron transport, and enucleation to name just a few possibilities. One considerable advantage of producing RBCs by directed differentiation of hESCs is that the cells produced are genetically homogenous and can be genetically manipulated either directly in the hESCs or using lentiviruses during the culture.

Another important aspect of this work is that it is a first step toward the production of RBCs for transfusion. The current system based on collection from donors is very safe but could be compromise by emerging pathogens. Production of RBCs could become one of the earliest practical applications of hESCs because the erythroid lineage has unique advantages. First, the lack of histocompatibility antigens expression on RBCs greatly simplifies the problems of immune rejection. Second, the complete absence of proliferation potential of fully mature RBCs practically eliminates the chances that they could become transformed and cause cancer. Third, there is a huge demand for RBCs, particularly with rare blood groups [35] and worldwide distribution networks. Genetic manipulations of transfusable erythrocytes produced in vitro would open the way for a myriad of applications such as the fabrication of carrier erythrocytes [36].

Acknowledgments

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