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Strategy for erythroid differentiation in *ex vivo* cultures: Lentiviral genetic modification of human hematopoietic stem cells with erythropoietin gene

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If cultured in appropriate conditions, such as supplementing culture media with costly cytokines and growth factors, hematopoietic stem/progenitor cells (HSPCs) from different origins have shown to be an adequate source of erythroid cells. This requirement turns erythroid cells production into a complicated process to be scaled-up for future applications. The aim of our work was to genetically modify HSPCs with human erythropoietin (hEPO) sequence by lentiviral transgenesis in order for cells to secrete the hormone into the culture medium. Initially, we evaluated erythroid differentiation in colony forming units (CFU) assays and further analyzed cell expansion and erythroid differentiation throughout time in suspension cultures by flow cytometry and May-Grünwald-Giemsa staining. Additionally, we studied hEPO production and its isoforms profile. The different assessment approaches demonstrated erythroid differentiation, which was attributed to the hEPO secreted by the HSPCs. Our data demonstrate that it is possible to develop culture systems in which recombinant HSPCs are self-suppliers of hEPO. This feature makes our strategy attractive to be applied in biotechnological production processes of erythroid cells that are currently under development.

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[**Key words:** Hematopoietic stem/progenitor cells; Erythroid cells; Erythropoietin; Lentiviral vectors; *Ex vivo* culture]

Red blood cells (RBCs) transfusion is commonly used for supportive care surgery, trauma, and therapy for solid and haematological malignancies, and is one of the major treatments for hereditary anemias (1,2).

However, even in developed countries blood transfusion presents limitations such as the cumbersome crossmatch tests, the limited preservation time, the immunomodulation of the recipient, and the risk of pathogen transmission (3). Some patients groups go through chronic shortages of blood due to the substantial polymorphism of blood group antigens (4,5). Considering these drawbacks, alternative transfusion products have been explored with disappointing performance of stabilized or recombinant hemoglobins, limited indications of oxygen transporters (perfluorocarbons), and slow development of “universal” RBCs. Therefore, there is a need for complementary sources of RBCs for transfusion, and *in vitro* clinical-grade RBCs generated from hematopoietic stem and progenitor cells (HSPCs) are a potential adequate surrogate (6,7).

Numerous research works have demonstrated the feasibility of *ex vivo* generation of erythroid cells, particularly RBCs from HSPCs. This approach aims to provide a safe and appropriate supply of RBCs in those cases of rare phenotypes, hemoglobinopathies and poly-transfused patients with poly-immunization (8), and it has been proven to be clinically applicable (9).

Substantial progress has been made in the biotechnological processes intended for production of RBCs (10–12), but cost effectiveness is still a major challenge to make an impact on medical care. It is necessary to consider that for RBCs production, the complete medium is too expensive, with growth factors representing more than 90% of the price, and hEPO alone represents 33% of the total medium price (8,13). Up to this point, the low yields of fully mature cells obtained make the process costly and inefficient (5,8,14).

In the meantime, other three more realistic applications could be achieved with the currently available culture systems: reagent RBCs for antibody identification, drug discovery and drug delivery (5). The use of reagent RBCs for antibody identification in diagnostic kits/devices requires low numbers of these cells derived from donors with common and rare blood group phenotypes/genotypes in order to identify adequate transfusion matches and alloimmunized individuals (5,15). Extraction of blood from rare donors is frequently restricted but RBCs could be generated *in vitro* from mononuclear cells usually discarded during the leukoreduction process (5). Utilization of primary erythroid cells for drug discovery and evaluation is appropriate for drugs targeting diseases with variable clinical responses, which depend on age, sex and genetic polymorphisms (e.g., assays that measure proliferation/maturation rates of erythroblasts have been proposed for screening of inducers of hemoglobin F production in Thalassemia and Sickle Cell Anemia, cellular-based antimalarial therapies and erythropoiesis stimulators for myelodysplastic syndrome, among other applications) (5).

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In addition, proof-of-principle for the use of erythroid cells for systemic drug delivery has been established in a mouse model of hemophilia, in which factor IX was produced specifically by modified erythroid cells (5,16).

The various potential applications of erythroid cells production from HSPCs have encouraged us to develop a strategy aiming to reduce the usage of costly growth factors in the culture process. Thus, in this work we have genetically modified hematopoietic stem/progenitor cells by third generation lentiviral transgenesis with the sequence of hEPO and we have assessed the effects of this approach over erythroid differentiation *in vitro*.

MATERIALS AND METHODS

Cells and culture media The use of blood samples (peripheral and umbilical cord blood samples) was approved by the Ethics and Research Security Advisory Committee of the School of Biochemistry and Biological Sciences, the Research Ethics Committee of the Hospital José María Cullen and the Bioethics Committee of the Province of Santa Fe.

Six umbilical cord blood units were obtained from normal full-term deliveries attended in the Hospital José María Cullen (Santa Fe, Argentina). Mothers have previously signed the informed consent. All samples were normal and did not show variability in the hematologic parameters (no evidence of hematologic diseases was observed). Within 24 h after collection, mononuclear cells (MNCs) from these samples were obtained and cryopreserved, and three of them were used as source of HSPCs. Two peripheral blood units were obtained with the donors' informed consent and used immediately after collection.

Light-density MNCs from umbilical cord blood and peripheral blood units were separated by Ficoll–Paque PLUS (GE Healthcare, Little Chalfont, Buckinghamshire, UK) centrifuged and cryopreserved until use. Then, $CD34^+$ cells were enriched by supermagnetic microbead selection using direct CD34 progenitor isolation beads and Mini-MACS columns (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany).

HSPCs were cultured in semisolid medium with methylcellulose (MACS HSC-CFU basic, Miltenyi Biotec) and erythroid commitment medium (ECM), adapted from a published report (9), constituted by Iscove's modified Dulbecco's medium (IMDM) (Gibco, Waltham, MA, USA), glutamine (Gibco) 2 mM, human holo-transferrin 330 μg/ml (Sigma–Aldrich, Saint Louis, MO, USA), human recombinant insulin 10 μg/ml (Sigma–Aldrich), heparin 2 IU/ml (Riveparin, Rivero, Buenos Aires, Argentina) and fresh frozen plasma 5% (V/V). Fresh frozen plasma was prepared in our laboratory from peripheral blood of a single donor: immediately after collection, the whole blood treated with heparin was centrifuged at 1700 ×g during 5 min at 20°C, then the platelet-rich plasma was centrifuged at 4800 ×g during 5 min at 4°C, in order to eliminate platelets from plasma. The plasma was complement-inactivated 30 min at 56°C and stored at −20°C until use.

Culture media were supplemented with hydrocortisone (Sigma–Aldrich), commercial hEPO (specific biological activity 132,772 IU/ml, Zelltek, SA, Santa Fe, Argentina), hSCF (Miltenyi Biotec) and hIL-3 (Miltenyi Biotec), according to each particular experiment.

Cell culture Initially, cultures in semisolid media were performed. Peripheral blood HSPCs were pre-stimulated in ECM (without human holo-transferrin), supplemented with hydrocortisone 10^{-6} M, hSCF 100 ng/ml, hIL-3 5 ng/ml and commercial hEPO 3 IU/ml (22.6 ng/ml) during seven days. Then, cells were transduced with lentiviral vectors containing the hEPO sequence under the control of EF-1 alpha promoter. A control of non-transduced cells was performed. Cultures of non-modified and hEPO-modified cells were carried out during eight days in semisolid medium with methylcellulose supplemented with hSCF 50 ng/ml and hIL-3 10 ng/ml, both in the presence and in the absence of hEPO 3 IU/ml (22.6 ng/ml). The cultures were maintained at 37°C in 5% CO_2 atmosphere. After culture, the percentage of erythroid colonies (CFU-E and BFU-E), the total number of colonies and hEPO concentration in culture supernatants were assessed.

Then, suspension cultures were carried out. Mononuclear and $CD34^+$ cells from umbilical cord blood were separated as described in the Cells and culture media section. After preparing a pool of progenitor cells from three different blood samples, purity was assessed by flow cytometry (95% of $CD34^+$ cells).

The pool was pre-stimulated in IMDM supplemented with fresh frozen plasma 5% (V/V), glutamine 2 mM, heparin 2 IU/ml, hSCF 50 ng/ml and hIL-3 5 ng/ml during five days for further lentiviral transduction.

Cultures of non-modified and hEPO-modified cells were analyzed. After lentiviral transgenesis, the culture procedure was based on a two-step protocol. This protocol was identical for non-modified and hEPO-modified cells, although commercial hEPO was not added to the hEPO-modified cells medium (ECM-E^-, ECM without hEPO) in none of the steps.

In the first step (day 0 to day 7), 7.5 × 10^4 non-modified cells/ml were cultured in ECM with hydrocortisone 10^{-6} M, hSCF 50 ng/ml, hIL-3 5 ng/ml and hEPO 3 IU/ml (22.6 ng/ml). In the second step (day 7 to day 18), the cells were resuspended at 4.5 × 10^5 cells/ml and 7.5 × 10^5 cells/ml on days 7 and 11, respectively, in ECM supplemented only with EPO (ECM-E^+ medium, ECM containing hEPO).

Non-modified cells were also cultured in ECM-E^- as additional control culture. The cultures were maintained at 37°C in 5% CO_2 atmosphere.

Cultures samples were taken on days 0, 7, 11, 15 and 18 for morphological analyses by May-Grünwald-Giemsa staining, cell counting and immunophenotypic analyses by flow cytometry.

Lentiviral vectors assembly The transfer plasmids pLV-EF-1 alpha-hEPO and pLV-EF-1 alpha-GFP were generated by introducing the hEPO and GFP sequences, respectively, into pLV-PLK vector already available in our laboratory (17) and then, CMV promoter was replaced by EF-1 alpha promoter. GFP, which can be qualitatively and quantitatively measured by flow cytometry, was used as a surrogate that allowed controlling the feasibility of genetic material delivery in HSPCs. Briefly, cells were seeded at 4 × 10^6/100 mm culture dish and transfected 24 h later. Research grade self-inactivating lentiviral vectors were generated via Lipofectamine 2000 (Invitrogen, Carlsbald, CA, USA) cotransfection of HEK293T/17 cells with four plasmids: the Rev-expressing plasmid pRSV-Rev (18), the packaging plasmid pMDLg/pRRE (18), the envelope plasmid expressing the glycoprotein G of the Vesicular Stomatitis Virus (VSV-G) (pMD2.G) (19) and the transfer vectors pLV-EF-1 alpha-hEPO or pLV-EF-1 alpha-GFP. They were used at a ratio of mass of 5:13:7:20. Viral supernatants were harvested after 48 h, clarified and filtered through 0.45 μm pore size. hEPO-containing supernatants were also concentrated via centrifugation at 65,000 ×g during 3 h. Viral titers were determined by p24 sandwich ELISA, using the kit QuickTiter Lentivirus Titer Kit (Lentivirus-Associated HIV p24) (Cell Biolabs, Inc., San Diego, CA, USA), according to the supplier's instructions. Lentiviral vectors were successfully assembled: hEPO-lentivirus presented a final titer of 10^7 Transduction Units (TU)/ml and GFP-lentivirus, a titer of 5 × 10^5 TU/ml.

Lentiviral transduction of hematopoietic progenitors After five days of pre-stimulation, cells were transduced with hEPO-lentiviral vectors. Controls of non-transduced cells (no lentiviral vector added) and GFP-transduced cells were performed. The transductions were carried out in StemMACS HSC-CFU basic 25% (V/V) in IMDM with hIL-3 17 ng/ml and hSCF 33 ng/ml at MOI 4 TU/cell (three independent transduction experiments were executed in each case). After a three-day exposure, the non-modified and the hEPO-modified cells were washed twice with IMDM and resuspended in the corresponding culture medium. GFP-transduced cells were washed twice with IMDM, resuspended in phosphate-buffered saline (PBS) and analyzed by flow cytometry in order to assess transduction feasibility in the utilized conditions.

Flow cytometry For phenotyping, cells were labeled with Anti-CD34-APC, Anti-CD45-PECy5, Anti-CD71-APC and Anti-CD235a-PE (BD Biosciences, San Jose, CA, USA) diluted 1/20 or 1/10 in BSA 1% (W/V) in PBS, during 20 min at 4°C. Unbound antibodies were removed by washing with PBS. For transduction feasibility evaluation, intracellular GFP expression was studied. Analyses were performed on a BD FACSAria III flow cytometer (BD Biosciences) with BD FACSDiva 6.1.2 (BD Biosciences) and FlowJoX 10 (Tree Star Inc., Ashland, OR, USA) softwares.

May-Grünwald-Giemsa staining Approximately 2–3 × 10^5 cells were harvested from each culture replicate at different times (0, 7, 11, 15 and 18 days), pelleted at 300 ×g for 5 min, resuspended in a reduced volume and deposited in glass slides. When visibly dry, cells were heat fixed and slides were covered gently with May-Grünwald solution (Biopack, Buenos Aires, Argentina) during 3 min. Then the same amount of water was added and mixed gently over the glass during 3–5 min. The glass was washed with water, covered with Giemsa solution (Biopack) for 12 min, and washed again. Morphological analysis was performed through classification of cells in random non-overlapping microscope fields in Nikon Eclipse Ti-S inverted microscope (Nikon, Tokyo, Japan).

Sandwich ELISA hEPO quantification in culture supernatants was performed by sandwich ELISA. Briefly, 96-well plates were coated with 50 ng per well of rabbit anti-hEPO polyclonal antibody (capture molecule) in 50 mM carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. After blocking for 1 h at 37°C with BSA 1% (W/V) in PBS, plates were incubated with 1:2 serial dilutions of hEPO (Zelltek SA) from 10 ng/ml to 0.078 ng/ml or 1:2 serial dilutions of test samples for 1 h at 37°C. Then, plates were incubated with an appropriately diluted biotinylated-rabbit anti-hEPO polyclonal antibody for 1 h at 37°C. Both polyclonal antibodies were developed in our laboratory. The reagent AMDEX Streptavidin Horseradish Peroxidase conjugate (GE Healthcare) was added and incubated during 1 h at 37°C. Plates were incubated with substrate solution (*o*-phenylenediamine 0.5 mg/ml, H_2O_2 0.015% (V/V) in phosphate-citrate buffer 50 mM). Reaction was stopped by the addition of H_2SO_4 2 N. Absorbance was measured (λ = 492 nm) in a LabSystems Multiskan MCC/340microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) after color developing. Tween 20 0.05% (V/V) in PBS was used as washing buffer between every step.

Isoelectric focusing and western blotting In order to detect the main isoforms of erythropoietin produced by the hematopoietic progenitor/precursors, supernatants taken at the end of the hEPO-modified cell cultures were concentrated 158 times and desalted by diafiltration in Amicon Ultra-0.5 ml centrifugal filters

(Merck Millipore, Billerica, MA, USA). Isoelectric focusing (IEF) was performed in 1-mm thick 8% (W/V) polyacrylamide gel containing urea 7 M, 3–5 ampholytes (Fluka, St. Louis, MO, USA) and 5–7 ampholytes (Sigma–Aldrich) (3:1). The gel was prefocused at 30 W, 250 V and 50 mA for 30 min, using glicine 2 g/l as catholyte and glutamic acid 0.018 g/l and aspartic acid 0.017 g/l as anolyte. The samples were focused at 30 W, 2000 V and 50 mA during 90 min. Then, IEF-separated components were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). Blocking was carried out with milk 5% (W/V) in TBS overnight at 4°C and washing was done with Tween 0.05% (V/V) in Tris-buffered saline (TBS) three times for 3 min. For the immunochemical reaction, the membrane was incubated with rabbit anti-hEPO specific polyclonal antibody during 1 h at room temperature in agitation, washed and further incubated with P 0448 polyclonal goat anti-rabbit immunoglobulins antibody conjugated to horseradish peroxidase (Dako, Santa Clara, CA, USA) in the same conditions. Immunoreactive bands were visualized using an ECL Chemiluminescent Western Blotting Analysis System (GE Healthcare).

RESULTS

Preliminary assay in semisolid medium We used a methylcellulose based-semisolid medium to evaluate the clonogenic capacity of the hematopoietic progenitors after lentiviral transduction, and to assess whether hEPO produced by the modified cells could have a considerable effect on erythroid differentiation, compared to that of commercial hEPO on the non-modified cells.

The preliminary assay showed that erythroid colonies development was higher for hEPO-modified cells, cultured both in the presence and in the absence of commercial hEPO (62% and 70% over total colonies, respectively), whereas non-modified cells only showed colonies development in cultures supplemented with hEPO (16.3% erythroid colonies over total). In cultures without hEPO only small clusters of 2–8 cells were observed (Fig. 1).

Increased erythroid differentiation in hEPO-modified cell cultures correlated with higher hEPO concentrations in culture supernatants (7 ng/ml for cultures with commercial hEPO and 5 ng/ml for cultures without commercial hEPO). hEPO quantification was not possible in cultures of non-modified cells, because its concentration was lower than the detection limit (Table 1).

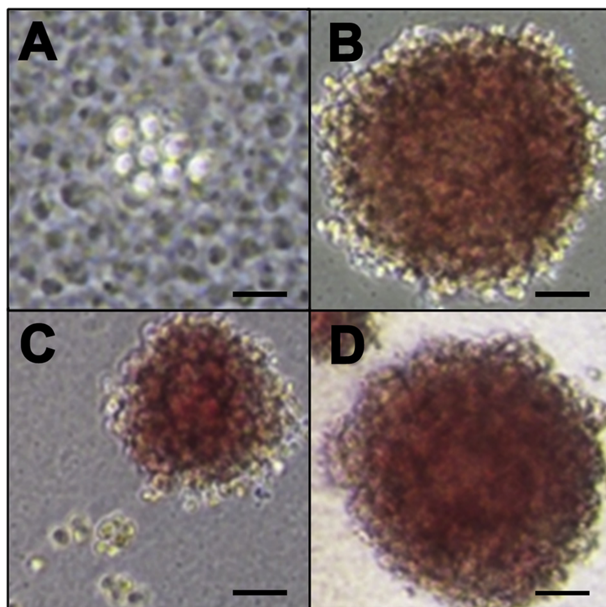

FIG. 1. Representative images of colonies grown in different culture conditions. (A) Non-modified cells in medium without commercial hEPO. The image shows no typical colony, but a small cluster of cells. (B–D) CFU-E obtained in cultures of (B) hEPO-modified cells in medium without commercial hEPO, (C) non-modified cells in medium with commercial hEPO, and (D) hEPO-modified cells in medium with commercial hEPO. The bars indicate 20 μm (magnification ×100).

TABLE 1. Colony forming units assay for non-modified and hEPO-modified cells.

	Non-modified cells		hEPO-modified cells	
	EPO$^-$	EPO$^+$	EPO$^-$	EPO$^+$
Total colony number	n.d.	257	501	406
Erythroid colonies (%)	n.d.	16	70	62
hEPO concentration in culture supernatants (ng/ml)	n.d.	n.d.	5	7

Evaluation of clonogenic capacity, erythroid differentiation and hEPO concentration in cultures after lentiviral transduction. Results are presented for cultures of non-modified and hEPO-modified cells in absence or presence of exogenous hEPO (3 IU/ml), indicated as EPO$^-$ and EPO$^+$, respectively (n.d., non-detectable).

Our hypothesis was that lentiviral transgenesis of HSPCs with hEPO gene would allow cells to produce and secrete the hormone into the culture medium. It could reduce the usage of this growth factor as medium supplement in the processes aiming to produce erythroid cells. Thus, these preliminary results set the basis to support this hypothesis and encouraged us to further investigate it in suspension cultures.

Suspension cultures: expansion, differentiation and characterization of grown cells Cultures were carried out as mentioned above, with 3 biological replicates derived from 3 independent transduction events obtained from a pool of hematopoietic cells from three different umbilical cord blood samples. Before initiating the suspension cultures, transduction feasibility was checked by flow cytometry using the cells derived from three transduction events with lentiviral vectors containing the GFP sequence. The aim of this approach was to assess whether lentiviral particles as well as the protocol used for transduction were effective in modifying HSPCs, considering that authors state that these primary cells present intrinsic barriers that limit lentiviral transduction efficiency (20,21). Even though expression levels reached by GFP- and hEPO-transduced cells are not necessarily the same, using GFP as a surrogate for the gene of interest allows to evidence whether the procedure yields a positive cell population. This information is easily obtained by flow cytometry or fluorescence microscopy:

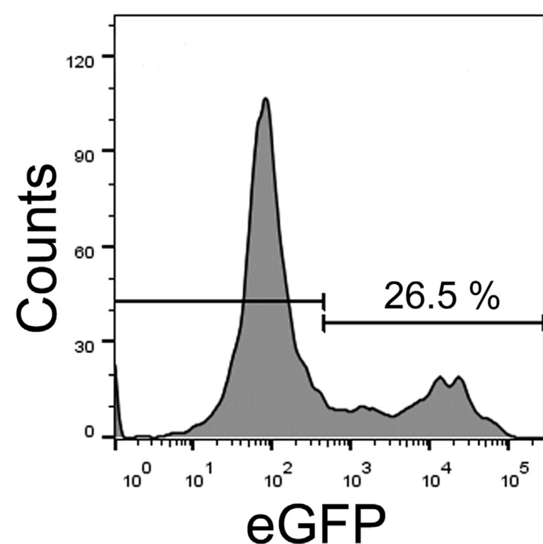

FIG. 2. Flow cytometry analysis of control HSPCs transduced with lentiviral vectors containing the sequence of GFP. This protein was used as a surrogate to estimate the feasibility of genetic material transfer. The histogram shows the percentage of GFP$^+$ cells for one representative transduction experiment.

cytometric analyses revealed that 26.6 ± 0.5% of the cells expressed GFP (Fig. 2).

hEPO-modified cells were cultured in ECM-E$^-$, meanwhile non-modified cells cultures were supplemented with hEPO (ECM-E$^+$). For modified cells, cell expansion reached a maximum of a 953-fold mean amplification (range, 616-1510-fold) by day 15 (post-transduction). Additionally, non-modified cells showed a maximum fold-expansion of 369-fold mean amplification (range, 201-570-fold) by day 11, which decreased towards day 18. No significant differences were evidenced between the maximum expansion reached by the cultures (two samples *t*-test, *p*-value = 0.124). Regarding the control cultures of non-modified cells in ECM-E$^-$, the expansion only occurred during the first week (Fig. 3); after that, the total number of cells decreased and no erythroid differentiation was observed in the morphological analyses (Fig. 4A). During the first week, the cells presented morphology of progenitor/precursor cells, and erythroblasts were not observed. After that time, most of the cells progressively reduced their size and did not show changes in dyes affinity from basophilia to acidophilia, which is characteristic of erythroid differentiation.

On day 0 of culture (post-transduction), both non-modified and hEPO-modified cells were granule-free homogenous MNCs (90% and 98%, respectively), with a slight proportion of non-progenitor/precursor cells (10% and 2%, respectively). As early as day 7, differential cell count evidenced erythroid differentiation in both culture lines (Fig. 4A and B). At this point the prevalent stages were proerythroblasts/basophilic erythroblast with large nuclei and basophilic cytoplasm (64 ± 2.6% for non-modified cells cultured in ECM-E$^+$ and 78 ± 8.9% for hEPO-modified cells), whereas from day 11 onwards cultures were almost exclusively erythroid. Frequency of HSPCs detected by morphological analyses decreased from day 0 to day 7, when 31.7 ± 6.1% of non-modified cells and 7.7 ± 5.9% of the hEPO-modified cells were HSPCs; this maturation state was almost absent by day 11 (5.7 ± 6.7% and 0%, respectively). By day 15 and 18 the culture was mainly composed of orthochromatic erythroblasts, smaller and with small eccentric nuclei. This population was 81.7 ± 3.1% in the case of non-modified cells and 80.0 ± 9.5% for hEPO-modified cells, by day 18.

The morphological analysis was confirmed by immunological characterization by flow cytometry (Fig. 5). In both cell lines, the

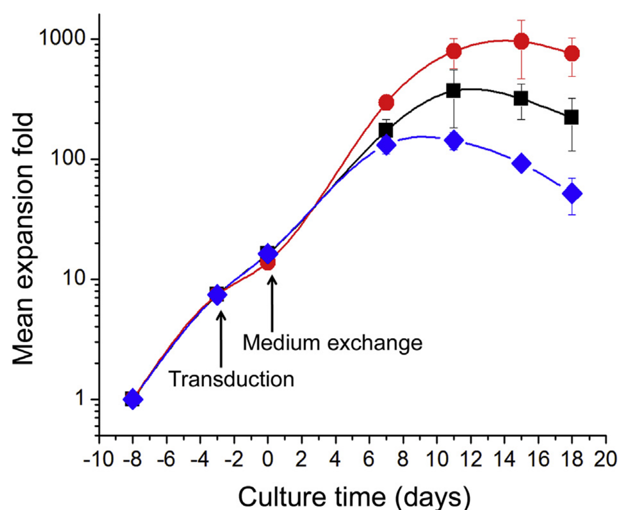

FIG. 3. Mean cell amplification. Cell expansion of non-modified cells cultured in ECM-E$^+$ (black squares) and ECM-E$^-$ (blue rhombus) and amplification of hEPO-modified cells grown in ECM-E$^-$ (red circles), expressed as fold increase of cell number. Values are mean ± S.D. in log scale (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CD34 hematopoietic progenitor marker rapidly decreased its expression; by day 7 the levels reached the minimum (Fig. 5A and 5C), although cells with HSPCs morphology were still visible by May-Grünwald-Giemsa staining. In addition, the percentage of CD45$^+$ cells (leukocyte marker, weakly expressed in early erythroblasts) (Fig. 5B and C) for hEPO-modified cells declined from an initial value of 78 ± 1% to 23 ± 3% by day 7, and to 4.4 ± 0.4% by day 11 (n = 3). For non-modified cells, the decline of this marker was slower: the initial percentage of 88 ± 1% diminished to 54 ± 8% by day 7 and dropped to 8.0 ± 1.5% by day 15. The progressive loss of CD45 antigen agrees with maturation from progenitor cells to predominant early erythroblasts during the first week; the loss is nearly complete by day 11, when polychromatic and orthochromatic erythroblasts were the prevalent maturation states.

Regarding CD71 (transferrin receptor) (11,22) and CD235a (glycophorin A, a specific marker of mature erythroid cells) (11), the cytometric analysis showed that the amount of CD71$^+$CD235a$^-$ cells (colony forming unit erythroid precursors) (23) (Fig. 5D) rapidly diminished in the case of hEPO-modified cells, meanwhile the reduction in the percentage was less abrupt in the case of non-modified cells. It was observed that the CD71$^+$ population progressively acquired the CD235a marker (23) (Fig. 5E and G), concomitantly with progressive maturation of precursors. Thus, the amount of double positive cells (intermediate erythroblasts) increased during the lapse of culture in both cell lines, but the increment was quicker for hEPO-modified cells until day 7. After that, the frequency of these erythroblasts appeared to be comparable in both lines. The CD71$^-$CD235a$^+$ populations (Fig. 5F), phenotype characteristic of mature red blood cells (23), reached their maximum proportion by day 11 (mean of 21 ± 1% for hEPO-modified cells and 7.8 ± 0.4% in the case of non-modified cells). In the last culture period these percentages slightly decreased.

Analysis of rhEPO produced by modified progenitors/precursors: quantification and isoforms profile We quantified rhEPO produced by modified cells in suspension cultures throughout the whole culture time by sandwich ELISA. The concentrations of the hormone in culture supernatants were detected at ng/ml levels, with a maximum of 35.5 ± 6.9 ng/ml (range, 30–43 ng/ml) on day 7. This mean level was higher than the initial input of hEPO in the non-modified cells cultures (22.6 ng/ml). Afterwards, levels decreased (day 11, 6.3 ± 1.5 ng/ml; day 15, 0.7 ± 0.2 ng/ml; day 18, 3.1 ± 1.3 ng/ml) in cultures of hEPO-modified cells (Fig. 6A).

Predominant isoforms of erythropoietin expressed by hEPO-modified cells could be determined by isoelectric focusing followed by western blotting. Specificity of the antibody on western blotting was demonstrated in preliminary experiments, which revealed that no supernatant components are detected other than hEPO (data not shown).

As a control we used the hEPO employed to supplement cultures of non-modified cells (Zelltek SA). This commercial protein is produced in CHO cells and enriched in the most acidic isoforms by several chromatographic separation steps. Therefore, it contains mainly acidic isoforms with isoelectric points between approximately 3 and 4.5, placed at the more acidic pH range zone of the membrane (24). As it can be observed in Fig. 6B, both commercial hEPO and secreted hEPO were located in the more acidic part of the membrane (considering that the pH range obtained with the ampholytes used is approximately 3.5–5.5), reflecting their predominant acidic isoforms.

Low concentration of hEPO produced by modified cells could have prevented us from detecting less abundant isoforms. However, in Fig. 6B, the four isoforms observed in lane 2 are slightly less acidic than those observed in lane 1 (control), which may be associated with small differences in sialic acid content. This could

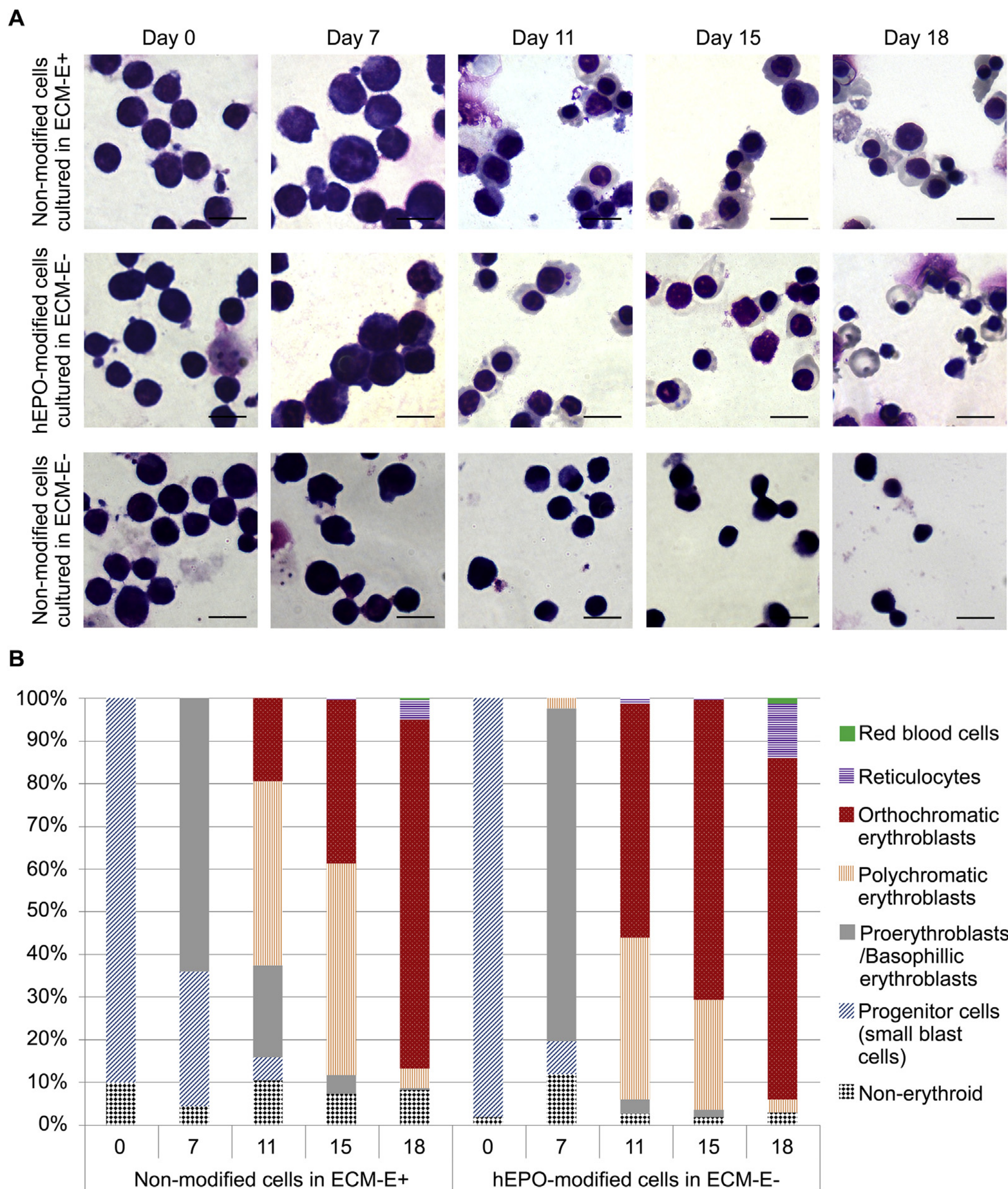

FIG. 4. (A) Morphological analysis of the cells. May-Grünwald-Giemsa stainings from three representative cultures (non-modified cells cultured in ECM-E$^+$, hEPO-modified cells cultured in ECM-E$^-$ and non-modified cells cultured in ECM-E$^-$) on days 0, 7, 11, 15 and 18. The bars indicate 20 μm (magnification ×1000). (B) Frequencies of different maturation stages of the cells found in cultures of non-modified cells cultured in ECM-E$^+$ and hEPO-modified cells cultured in ECM-E$^-$ on days 0, 7, 11, 15 and 18 (mean percentage values, n = 3).

be attributed to the different hosts in which the two recombinant proteins were produced (hamster vs. human). It has been described that glycosylation patterns and particularly the amount of sialic acid are host and tissue dependent. Distinct glycosylation enzymatic machinery could be a potential explanation for the differences found (25).

Both hEPO analyses (ELISA and isoelectric focusing followed by western blotting) confirm that transduced cells were able to produce erythropoietin with predominantly acidic isoforms.

DISCUSSION

In vitro erythroid cells production presents applications in cell therapy for transfusion practices, drug testing and delivery, and in the identification of transfusion matches. However, in order to make this technology applicable it is mandatory to find means of producing adequate quantities of cells at a cost that allows practical usage.

With this reference point, in this work we describe the generation of erythroid cells using *ex vivo* culture of human hematopoietic stem/progenitor cells (HSPCs) genetically modified with hEPO sequence by lentiviral transgenesis. In this case hEPO was not added as a culture supplement because we expected we could make HSPCs secrete their own erythropoietin. Additionally, we compared these cultures with non-modified HSPCs cultures in which hEPO was added exogenously.

We report erythroid lineage commitment (assessed by flow cytometry and May-Grünwald-Giemsa staining) due to the effect of erythropoietin produced by the HSPCs. The expression of CD34 stemness marker as well as CD45 leukocyte marker decreased considerably during the first week of culture, in correlation with

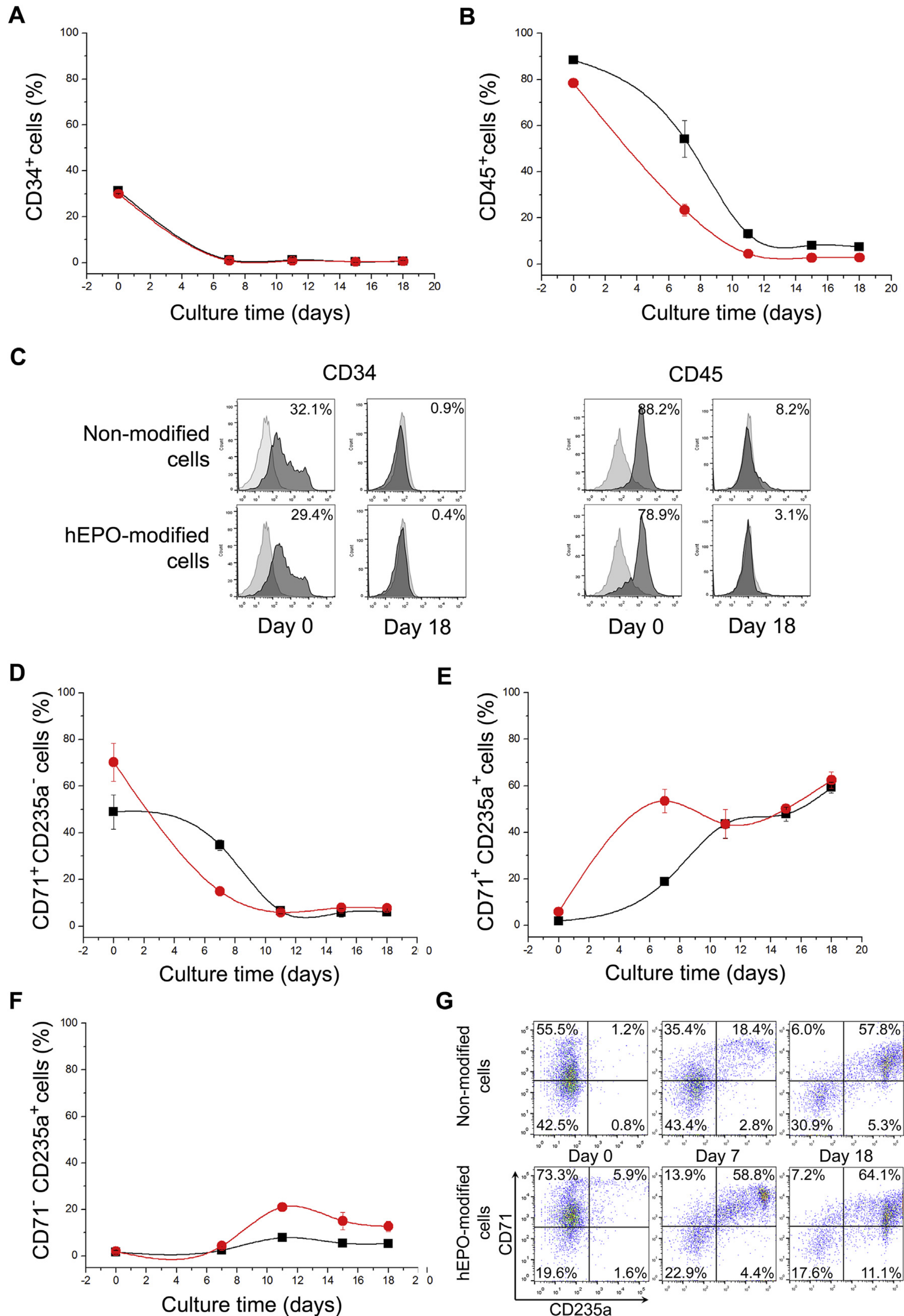

FIG. 5. Flow cytometry immunophenotypic characterization. Panels show percentages of cell populations obtained from non-modified cells (black squares) and hEPO-modified cells (red circles) throughout cultures. Results are expressed as mean percentage ± S.D. ($n = 3$). (A) $CD34^+$ cells; (B) $CD45^+$ cells; (D) $CD71^+CD235a^-$ cells; (E) $CD71^+CD235a^+$ cells and (F) $CD71^-CD235a^+$ cells. (C) Expression of CD34 and CD45 in the beginning and in the end of cultures of both cell lines (one representative experiment is shown). Gates statistics are given in each histogram. Dark histograms represent relevant mAbs and light histograms represent controls. (G) Progression (days 0, 7 and 18) on CD71 and CD235a expression in one representative experiment for both cell lines. Quadrant statistics are given in each dot plot. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

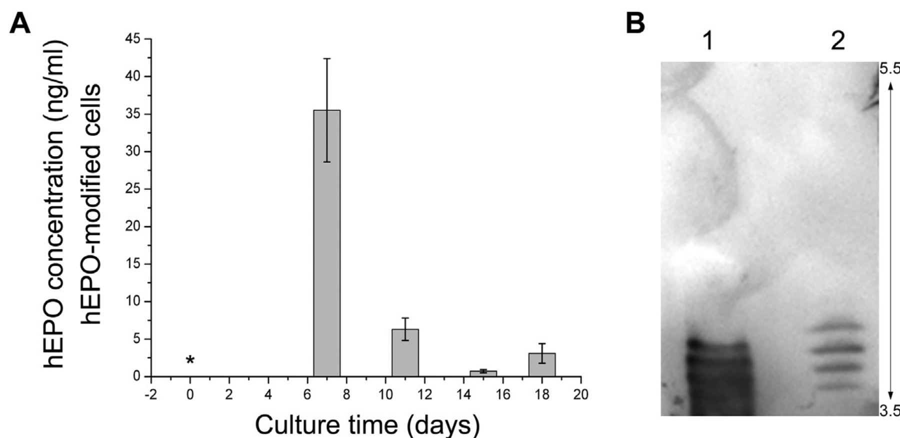

FIG. 6. (A) ELISA quantification of hEPO in culture supernatants of hEPO-modified cells on days 0, 7, 11, 15 and 18. Bars indicate mean concentration of the hormone (ng/ml) ± S.D. (n = 3). Asterisk means non-detectable. (B) hEPO isoforms analysis by isoelectric focusing followed by western blotting. Lane 1, commercial hEPO; lane 2, culture supernatant (concentrated and desalted) from hEPO-modified cell cultures.

the appearance of early erythroblasts. CD45 antigen continued decreasing until day 11, concomitantly with the appearance of polychromatic and orthochromatic erythroblasts. Both in hEPO-modified cells as well as in non-modified cells, immunophenotyping revealed that erythroid differentiation was characterized by the increase in CD235a and CD71 expression (marker expressed at the early erythroid and early reticulocyte stages) (23,26). Together with immunophenotypic changes, additional events which characterize erythroid development were observed: morphology study showed clear nuclear condensation due to chromatin condensation. It was also possible to evidence changes in dyes affinity, progressing from basophilia caused by the high RNA content in more immature cells to acidophilia, a phenomenon directly related to hemoglobin storage (27). The modified cells, as well as the non-modified cells (cultured in the presence of commercial hEPO) reached a similar maturation state. In the preliminary assay in semisolid medium the colonies were normal in size and morphology. Additionally, it is known that hEPO is required for committed CFU-E and erythroblasts survival and maturation, but it is not required in the orthochromatic erythroblast stage for final steps of erythroid differentiation (28,29).

In these experiments, secretion of erythropoietin was demonstrated by sandwich ELISA and isoelectric focusing followed by western blotting. Secreted hEPO reached its maximum level by day 7, when proerythroblasts/basophilic erythroblasts were found to be the predominant maturation stages. It can be inferred that during the first week progenitors produced the hormone and thus stimulated proliferation and differentiation towards erythroblasts. hEPO level decline beyond the first week would not be detrimental for expansion and differentiation of erythroblasts, given that maturation stages reached at that culture time would have had less hEPO responsiveness than during the stages present earlier in the culture and could autonomously mature with reduced hEPO stimulation.

The drop in secreted hEPO levels can be explained by dilutions made on days 7 and 11 of culture. However, cells present at this stage were not capable of counterbalancing the reduction in concentration by synthesis of more hormone, probably due to reduction of ribosome numbers (30), and heterochromatinization associated with maturation (27,30–32). It is known that nuclear activity decreases until the orthochromatic stage, when the nucleus is completely inactive and unable to synthesize either DNA or RNA (32).

Isoelectric focusing followed by western blotting showed similarity between the isoforms present in the commercial hEPO and in the HSPCs-secreted hEPO, although the second presented isoforms slightly less acidic than the first. Comparison with non-modified cell cultures allowed us to observe high resemblance in the final differentiation state of populations of both lines of culture. The obtained results imply that the hEPO secreted by the modified cells would be enough to induce expansion and differentiation of hematopoietic progenitors comparably to commercial hEPO action over non-modified cells.

In order to translate the erythroid cells generation technologies to practice, processes will need to be scaled-up. This projection brings about the cost effectiveness evaluation. Previous publications have reviewed this issue over the existing culture protocols. The aftermath of RBCs production process pointed that the recombinant growth factors IL-3, SCF and EPO together represent 57.5% of the price of the complete medium; EPO alone, the main cytokine required for erythroid differentiation, contributes to one third of the total cost (8,13). But our data demonstrates that it is possible to develop culture systems in which recombinant HSPCs are self-suppliers of hEPO.

The practical use of these genetically hEPO-modified cells in large-scale RBCs production processes appears not to be impossible due to the considerable progress made both in HSPCs culture procedures and large-scale lentiviral production. The main hurdles towards the use of recombinant lentivirus as a gene therapy vector or in cell bio-engineering are the low titer at which it is produced as well as the difficulty to purify it at an acceptable level without degrading it (33). Lentiviral vectors based in HIV-1 are more frequently required in translational, preclinical and clinical research, so large-scale closed systems such as hollow fiber bioreactors are gaining impulse as production devices to obtain high quantities of high titer vectors (34). Moreover, lentiviral vector assembly processes involve good manufacture practices (35–37) which make this gene delivery technology suitable for the purpose we are claiming.

In conclusion, HSPCs genetically modified with hEPO sequence were self-induced to differentiate in *in vitro* conditions, in absence of commercial hEPO, into populations highly enriched in erythroid cells. This result was demonstrated to be achieved because of the hEPO that was secreted by the HSPCs themselves. The final maturation state reached by these cells showed high similarity with the one of non-modified HSPCs cultured in presence of commercial hEPO.

This strategy will allow reducing the amount of hEPO added into cultures, and the consequent associated cost. Additionally, it could be explored as a way for expression of other relevant proteins in different contexts.

Altogether, these arguments make our strategy interesting to be applied in biotechnological processes intended for the *in vitro* production of erythroid cells, which are currently in development and optimization stages. The generation of recombinant HSPCs lines modified to produce their own proliferation and differentiation factors represents a standardized platform to produce precursor cells. We consider that our results could substantially contribute to future practical improvements.

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L.A.C. designed and performed the experiments, analyzed the data and wrote the manuscript. C.C.P. conceived the study, coordinated sample collection, contributed to experiments design and supervised the manuscript and research. M.E. and R.K. supervised the research and manuscript. All authors have approved the final manuscript.

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