Experimental Hematology

Experimental Hematology 2019;75:31–52

PSC-RED and MNC-RED: Albumin-free and low-transferrin robust erythroid differentiation protocols to produce human enucleated red blood cells

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(Received 23 April 2019; revised 23 May 2019; accepted 24 May 2019)

Many methods have been developed to produce cultured red blood cells (cRBCs) in vitro but translational applications have been hampered by high costs of production and by low rates of enucleation. We have developed R6 and IMIT, two chemically defined culture media and combined them into robust erythroid differentiation (RED) protocols to differentiate induced-pluripotent stem cells (iPSCs) and peripheral blood mononuclear cells (MNCs) into enucleated erythroid cells. The RED protocols do not require any albumin or animal components and require ten- to twentyfold less transferrin (Tf) than previously, because iron is provided to the differentiating erythroblasts by small amounts of recombinant Tf supplemented with FeIII-EDTA, an iron chelator that allows Tf recycling to take place in cell culture. Importantly, cRBCs produced by iPSC differentiation using the long PSC-RED protocol enucleate at much higher rates than with previous protocols, eliminating one of the impediments to the use of these cells to produce clinically useful cRBCs. The absence of albumin, the reduced amounts of Tf, the improved reproducibility associated with the elimination of all animal components, and the high yield on the RED protocols decrease the cost of production of cultured red blood cells. RED protocols should therefore help to make translational applications of cultured RBCs more economically realistic. © 2019 Published by Elsevier Inc. on behalf of ISEH – Society for Hematology and Stem Cells.

Cultured red blood cells (cRBCs) are useful in studying erythroid disease mechanisms and hold great promise as reagent cells to diagnose allo-immunization and as a potential source of invaluable cells carrying rare blood groups that are necessary to transfuse allo-immunized patients [1,2]. Genetically modified cRBCs that express therapeutic proteins [1,3,4] are another highly promising avenue of research because relatively small numbers of such cells could be clinically useful, greatly decreasing the technical barriers to translation associated with transfusion applications [5].

Fibach et al. [6] were the first to produce cRBCs using a two-step procedure in which cytokines were provided in serum and conditioned medium. Since then, many authors have reported that erythroid cells can be produced by expansion of primary hematopoietic stem and progenitor cells from multiple sources using various combinations of recombinant cytokines and small molecules, including stem cell factor (SCF), erythropoietin (EPO), interleukin-3 (IL3), FMS-like tyrosine kinase 3 ligand (FLT-3L), insulin-like growth factor 1 (IGF1), hydroxycortisone, and dexamethasone (Dex) [6–11]. The SED cocktail composed of SCF, EPO, and Dex has often been used to increase the yield of cRBCs because it induces multiple self-renewal divisions of stress erythroid progenitors, which retain their capacity for terminal differentiation and enucleation [12,13]. Erythroblasts of all developmental ages exhibit some self-renewal potential but human adult peripheral blood erythroblasts stimulated by the SED cocktail can

Author contributions SZ, ZY, SS, KR, and KW performed some of the experiments and contributed to experimental design and data interpretation. ENO performed some of the experiments, contributed to experimental design, data interpretation, and writing of the article. EEB supervised the project and contributed to experimental design, data analysis, writing of the article, and final approval of the article.

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Supplementary data related to this article can be found online at https://doi.org/10.1016/j.exphem.2019.05.006.

0301-472X/
https://doi.org/10.1016/j.exphem.2019.05.006

proliferate for only about 2 weeks, while embryonic stem cell-derived embryonic erythroblasts [14] and mouse primary embryonic erythroblasts [15] can proliferate for several months. Using a variation of the SED cocktail, the Migliaccio lab has developed a procedure termed human erythroid massive amplification (HEMA) that yields high numbers of human cRBCs that are antigenically very similar to RBCs produced in vivo [11].

The Douay group was the first to achieve a high level of enucleation in culture using a feeder layer of mouse MS-5 mesenchymal cells in the final stages of differentiation [7]. Subsequently, Miharada et al. reported that high rates of enucleation of cord blood-derived cRBCs could be obtained without the use of feeder layers by optimization of the culture conditions [8]. Building on this work, Timmins et al. recently obtained 90% enucleation and very high yields corresponding to 500 units of cRBCs per initial unit of cord blood in a stirred bioreactor setting using a simple dilution feeding protocol and a humanized medium containing SCF, EPO, and IL3 [16]. Together, these studies provided a proof of principle that producing RBCs in vitro could be economically viable.

Human cRBCs can also be produced from immortal cells such as embryonic stem cells [17–19], induced pluripotent stem cells (iPSCs) [3,14,20–23], and self-renewing [15] or immortalized [24–26] progenitors. We have focused on producing cRBCs from iPSCs as they are more amenable to industrialization than primary cells because, in addition to being immortal and easy to produce from any donor under chemically defined conditions [27], they are karyotypically stable [28] and genetically modifiable. Despite these advantages, the cost and complexity of iPSC differentiation procedures and the low rates of enucleation (<5%) [23] have so far precluded the translational use of iPSC-derived cRBCs.

To decrease costs and simplify the differentiation procedure, we have developed new albumin-free, low-transferrin (Tf), chemically defined culture media. Albumin, which is generally included in serum-free cultures as a carrier and as a source of small molecules, was eliminated because of its cost and because it introduced unnecessary variability, as the composition of each batch of albumin is unique. Tf concentration, which is very high (200 and 500 μg/mL) in all erythroid cell culture protocols because of the large amounts of iron required to synthesize heme, the oxygen-binding component of hemoglobin [29], was decreased about tenfold to 20 μg/mL. This was made possible by the introduction of an iron chelator, which allows Tf recycling to occur in cell culture.

These novel culture media were combined into robust erythroid differentiation (RED) protocols to differentiate human iPSCs and human primary hematopoietic stem and progenitor cells into large numbers of enucleated RBCs. Importantly, the resulting PSC-RED protocol yielded very high rates of enucleation, solving one of the major issues that precluded the use of iPSCs for translational applications.

Methods

Samples

Peripheral blood (PB) cells were obtained from healthy donors under a protocol approved by the Albert Einstein College of Medicine institutional review board.

Mononuclear cells (MNCs) from whole blood were purified with Histopaque (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions, and residual RBCs were lysed by addition of 5 vol of cold (4°C) RBC lysing buffer ($NaHCO_3$ 790 mg/L and NH_4Cl 7.7 g/L) for 10 min. Aliquots containing about 3 million cells were then frozen and stored in liquid nitrogen.

Reagents

The suppliers for all reagents are provided in Supplementary Table E1 (online only, available at www.exphem.org).

Culture conditions

All cultures were performed at 37°C in 5% or 10% CO_2 depending on the presence of stable glutamine in the medium. All experiments described above were performed in 5% oxygen, in accordance with previously published results [30].

Induced-pluripotent stem cells

Induced PSCs were reprogrammed from peripheral blood mononuclear cells using the Sendai virus approach (CytoTune-iPS 2.0 Sendai Reprogramming Kit, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Five lines of iPSCs (NY22, OM1, OM2, OM3. and OM4) and three sublines of OM1, all generated from healthy controls, were used during these experiments. The vast majority of experiments were performed with lines NY22 and OM1. All lines enucleated at a high rate although we noted differences that might have been associated with different growth rates between lines.

Pluripotent stem cell culture

Human pluripotent stem cells (hPSCs) were maintained undifferentiated in E8 medium [27] on vitronectin (Life Technologies, Carlsbad, CA) and passaged using ethylenediaminetetraacetic acid (EDTA) every 3–4 days depending on their confluence stage.

Differentiation of iPSCs into erythroid cells: Short PSC-RED protocol

Day −1. Three-day-old hPSC colonies were dissociated with 5 mmol/L EDTA in phosphate-buffered saline (PBS) for 6 min. The EDTA was then removed and replaced with 5 mL of E8 medium, and the well was thoroughly flushed with a 5-mL serological pipet by pipetting up and down 10 times. Small clumps were generated to produce small colonies of about 50 cells on day 0. The cells were then plated at $1–2 \times 10^5$ cells/well in 2 mL/well E8 medium on vitronectin in tissue culture-treated six-well plates (Falcon),

which are used throughout the protocol. After plating, the cells were allowed to attach overnight.

Day 0. Differentiation was induced by replacing the E8 medium with IMIT medium, containing supplement 1 (bone morphogenic protein 4 [BMP4, 10 ng/mL], vascular endothelium growth factor 165 [VEGF, 10 ng/mL], basic fibroblast growth factor [bFGF, 10 ng/mL], Wnt3A [5 ng/mL], Wnt5A [5 ng/mL], activin A [5 ng/mL], and GSK3β inhibitor VIII [2 μmol/L] [22])

Before induction of differentiation, the culture was inspected to ascertain that most of the colonies contained about 50 or fewer cells. One well of the culture was sacrificed for cell counting to calculate the yield of cells at the end of the experiments.

Day 2. Six-times-concentrated supplement 2 in IMIT was added to each well to bring the final concentration of fresh cytokines to 20 ng/mL BMP-4, 30 ng/mL VEGF, 5 ng/mL Wnt3A, 5 ng/mL Wnt5A, 5 ng/mL activin A, 2 μmol/L GSK3β inhibitor VIII, 10 ng/mL bFGF, 20 ng/mL SCF, and 0.4 ng/mL β-estradiol.

Day 3. The cells were dissociated with TrypleSelect 1 × for 5–10 min at 37°C. After the addition of 10 mL of PBS, cells were centrifuged for 3 min at 250*g*, the supernatant was discarded, and the cells were resuspended in fresh IMIT medium containing supplement 3 (BMP4 [20 ng/mL], VEGF [30 ng/mL], bFGF [20 ng/mL], SCF [30 ng/mL], insulin-like growth factor 2 [IGF2, 10 ng/mL], thrombopoietin [TPO, 10 ng/mL], SB431542 [3 μmol/L], heparin [5 μg/mL], 3-isobutyl-1-methylxanthine [IBMX, 50 μmol/L], and β-estradiol [0.4 ng/mL]} and plated at 1×10^5 cells/mL in a tissue culture-treated six-well plate (3 mL/well).

Day 6. The cells were centrifuged for 3 min at 250*g* and resuspended at 5×10^5/mL in fresh IMIT medium containing supplement 3 without SB431542 but with 30 nmol/L UM171.

Between days 6 and 10, the cells were diluted to 0.5×10^6/mL any time they reached more than 1.5×10^6 cells/mL by addition of the same medium and supplement. An additional dose of supplement 3 (provided from a 6 × concentrated stock) was added at day 8 to fully renew the cytokines and small molecules.

Day 10. The cells were plated at 0.66×10^5 cells/mL in IMIT containing the SED supplement (SCF [100 ng/mL], EPO [4 U/mL], IBMX [50 μmol/L], Dex [1 μmol/L]). From day 10 to day 17, the cells were diluted to 0.5×10^6/mL any time they reached more than 1.5×10^6 cells/mL by addition of the same medium and supplement. In addition, 6 × concentrated SED supplement in IMIT was added every 2 days to fully renew the cytokines and small molecules.

Day 17. The cells were centrifuged for 3 min at 250*g* and plated at a density of about 2×10^5/mL IMIT containing the SER supplement (SCF [50 ng/mL], EPO [4 U/mL], and RU486 [1 μmol/L]). From days 17 to 24, the cells were diluted to 0.5×10^6/mL any time they reached more than 1.5×10^6 cells/mL by addition of the same medium and supplement. In addition, 6 × concentrated SER supplement in IMIT was added every 2 days to fully renew the cytokines and small molecules.

Day 24. The cells were plated at 2×10^5/mL in R6 medium with the SER2 supplement (SCF [10 ng/mL], EPO [4 U/mL] and RU486 [1 μmol/L]). From days 24 to 31, the cells were diluted to 0.5×10^6/mL any time they reached more than 1.5×10^6 cells/mL by addition of the same medium and supplement. In addition, 6 × concentrated SER2 supplement in R6 was added every 2 days to fully renew the cytokines and small molecules.

Day 31. The cells were centrifuged for 3 min at 250*g* and maintained in R6 medium alone for up to 8 days.

Long differentiation protocol

This long protocol is identical to the short protocol but an additional HPC expansion step is added after day 10. This step consists of plating the day 10 cells in IMIT at 2×10^5/mL in the presence of supplement 4 (bFGF [5 ng/mL], SCF [15 ng/mL], VEGF [5 ng/mL], TPO [10 ng/mL], IGF2 [10 ng/mL], platelet-derived growth factor [PDGF, 5 ng/mL], angiopoietin-like 5 [ANGPTL5, 5 ng/mL], chemokine ligand 28 [CCL28, 5 ng/mL], IBMX [30 μmol/L], heparin [5 μg/mL], and UM171 [30 nmol/L]) for 1 or 2 weeks. As described above, the concentration of cells was kept below 1.5×10^6 cells/mL at all times, and cytokines are refreshed every 2 days by adding 6 × concentrated supplement. Cells kept for 2 weeks under these conditions were centrifuged and transferred to fresh plates after 7 days to eliminate any attached cells.

After this additional step, the differentiation resumes according to the short protocol day 10. A one-time addition of granulocyte–macrophage colony-stimulating factor (G-MCSF, 20 ng/mL) and granulocyte-stimulating factor (G-CSF, 20 ng/mL) is, however, necessary to induce maximal proliferation of the HPCs in the SED supplement.

Differentiation of peripheral blood mononuclear cells into day 14 erythroblasts and into cRBCs

Standard albumin-containing protocol. Frozen aliquots of PB mononuclear cells were seeded in the following four-step protocol: In the first step (days 0–7), cultures were initiated at a density of 500,000 cells/mL with serum-free StemSpan SFEM (StemCell Technologies, Vancouver, BC, Canada) supplemented with hydrocortisone (1 μmol/L), IL3 (7 ng/mL), FLT3L (17 ng/mL), SCF (50 ng/mL), and EPO (1.3 U/mL) for 7 days.

In the second step (days 7–14), the cells were transferred to StemSpan medium supplemented with hydrocortisone (1μM), IL3 (7 ng/mL), SCF (20 ng/mL), EPO (3.3 U/mL) and IGF-1 (20 ng/mL) for 7 days. Cell density was kept below two million cells per mL at all times by adding fresh medium every 2 or 3 days as needed.

In the third step (days 14–17), the day 14 erythroblasts thus generated were plated in RIT (RPMI; β-mercaptoethanol [0.1 mmol/L]; ethanolamine [1.6 mmol/L]; lipids [1/200]; insulin [10 μg/mL]; holo-Tf [200 μg/mL] in the presence of 10% hAB serum, the SEII cocktail [3 U/mL], EPO, and low concentrations of SCF [4.4 ng/mL]; IGF-1 [4.4 ng/mL], and

IL3 [1.5 ng/mL]) for 3 days and subsequently incubated for 10–15 days in RIT alone.

For all experiments described in Figures 2 and 3, MNCs that had been frozen at day 11 of culture were expanded until day 14 to test variations of the last 2 weeks of the protocol.

In Figure 2, condition 1, the cells were grown exactly as described for the standard protocol. Under condition 2, the SED cocktail was added at day 14 instead of the SEII cocktail, and the cells were cultured for 7 days prior to induction of differentiation, as in condition 1.

Under condition 3, the cells were grown in R8 medium supplemented with the SED cocktail for 7 days, followed by 7 days in R8 medium alone.

Under condition 4 and in the experiments described in Supplementary Figure E6 (online only, available at www.exphem.org), the cells were grown as described under condition 3 except that the various components of the SED cocktail were systematically omitted.

Under condition 5, the cells were grown as in condition 4 except that 1 μmol/L RU486 was added from days 14 to 28.

In the experiment illustrated in Supplementary Figure E7 (online only, available at www.exphem.org), the day 14 erythroblasts were grown as described under condition 5 of Figure 2 (1 week in SCF, EPO, and RU486 [SER], followed by 1 week in RU486) but the base medium was either R5, R8, or a modification of these media as indicated.

In the experiment illustrated in Figure 3, the cells were grown in R5 medium plus SER for 7 days, followed by 7 days in R5 alone (because we had shown at that point in time that RU486 was not necessary after day 21). The source of iron was varied as indicated. Optiferrin was dissolved in water and used as provided by the manufacturer (without preloading it with iron).

In Figure 7 and Supplementary Figure E12 (online only, available at www.exphem.org), MNCs from three healthy individuals were differentiated into cRBCs using either the standard protocol described above or the MNC protocol described next.

MNC-RED protocol. Frozen aliquots of PB mononuclear cells were seeded in the following four-step protocol:

1. Cultures were initiated at a density of 5×10^5 cells/mL and incubated for 7 days in IMIT supplemented with IL3 (7 ng/mL), FLT3L (17 ng/mL), SCF (50 ng/mL), EPO (1.3 U/mL), and hydrocortisone (1 μmol/L).
2. The cells were replated at a density of $3–5 \times 10^5$ cells/mL and incubated for 7 days in IMIT supplemented with IGF-1 (20 ng/mL), IL3 (7 ng/mL), SCF (20 ng/mL), EPO (3.3 U/mL), and hydrocortisone (1 μmol/L). Cell density was kept below 2×10^6 cells/mL at all times by diluting the cells to $3–5 \times 10^5$ cells/mL in fresh medium plus cytokines every 2 or 3 days as needed.
3. The cells were replated at 5×10^5 cells/mL and incubated for 1 week in R6 supplemented with SCF (50 ng/mL), EPO (3 U/mL) and 1 μmol/L RU486 (1 μmol/L). Cytokines and medium were renewed as described above.
4. Cells were replated at 5×10^5 cells/mL and allowed to enucleate by incubation for 7–12 days in R6 alone (in the absence of RU486).

Analysis and characterization

Cell enumeration. Cells were counted with a Luna-FL dual-channel automated cell counter (Logos Biosystems, Gyeonggi-do, South Korea) using acridine orange to visualize the live cells and propidium iodide to exclude the dead cells. Alternatively, the cells were counted manually using a hemocytometer.

Flow cytometry. Induced PSCs undergoing differentiation were evaluated by fluorescence-activated cell sorting (FACS) using antibodies against CD34, CD36, CD43, CD45, CD71, and CD235a, also known as glycophorin A (BD Biosciences, La Jolla, CA, and eBioscience, San Diego, CA).

Enucleation. The enucleation rate was measured using the DRAQ5 DNA nuclear stain (ThermoFisher) after exclusion of dead cells with propidium iodide. The cells were analyzed with a BD FACS Calibur flow cytometer (BD Biosciences) or a DPX10 (Cytek, Cerritos, CA) flow cytometer, and the flow cytometry data were analyzed with Flowjo software. In some experiments, the rate of enucleation was also measured by manual enumeration (see below).

Cytological staining. Erythroid differentiation and enucleation were assessed microscopically by rapid Romanowsky staining [31] of cytospin preparations using the HEMA-3 kit from Fisher Scientific as recommended by the manufacturer. Cell sizes were estimated on a Nikon TE-2000S microscope using software provided by the manufacturer. Alternatively, cells were stained with Wright–Giemsa stain from ThermoFisher.

RBC filtration. To eliminate the nuclei and most of the nucleated cells at the end of the experiments, RBCs were filtered using PALL Acrodisc 25-mm WBC filters as recommended by the manufacturer. Filtered cRBCs were stored for up to 1 month with little signs of hemolysis in Alsever's solution (Sigma-Aldrich).

HPLC analysis. Cells were washed twice with PBS and lysed in water by three rapid freeze–thaw cycles in dry ice and a 37°C water bath. Debris was eliminated by centrifugation at 16,000*g*, and the lysates were stored at −80°C. High-performance liquid chromatography (HPLC) was performed as described [32]. Briefly, a few microliters of lysate containing about 50 μg of protein in about 100 μL of 40% acetonitrile and 0.18% TFA was filtered and loaded on a VYDAC C4 column. The globin chains were then eluted with increasing concentrations of acetonitrile during an period of about 80 min. The starting elution buffer was programmed to be 80% buffer A and 20% buffer B and to rise to 50% buffer B in 50 minutes. Buffer A = 36% acetonitrile and 0.18% TFA, and buffer B = 56% acetonitrile and 0.18% TFA. Globin chain elution was monitored by measuring the optical density at 220 nm.

Transferrin analysis. Holo-Tf purchased from Sigma-Aldrich is almost 100% saturated with iron and was used as provided by the manufacturer. Optiferrin (InVitria) purchased from Fisher Scientific is 10%–30% iron saturated. Except when indicated, it was used as provided by the manufacturer in most experiments. Preloading with iron proved unnecessary because Optiferrin is rapidly loaded with iron at 37°C in the presence of an appropriate iron chelator.

Transferrin and Optiferrin iron loading was determined in 6 mol/L polyacrylamide gel electrophoresis (PAGE) as described previously [33]. About 2–5 μg of protein was loaded in each well. When necessary the Tf and Optiferrin were concentrated from the culture medium by centrifugation through 0.5-mL 10KD MWCO Amicon ultracentrifugal filters according to the manufacturer's instructions (Merck Millipore, Burlington, MA).

Lipids. All of the albumin-free media used were supplemented with lipids using a chemically defined mixture of lipids commercially available from Gibco (Fisher Scientific; Catalog No. 11905). We also tested lipid mixtures from Sigma-Aldrich (Lipid Mixture 1, Chemically Defined L0288) and from Peprotech (Rocky Hill, NJ; Lipid Mixture Solution cat # LM-200) and did not observe any major differences in cell yield. However, membrane stability was not characterized in detail. Additional experiments are necessary to clarify what is the optimal source of lipids for erythroid cell cultures performed under chemically defined conditions.

Statistical analysis. Significance was assessed using GraphPad (San Diego, CA) Prism 7 for Windows by performing Student *t* tests analyzing each row individually. *Q* values were calculated using the False Discovery approach (two-stage step-up method of Benjamini, Krieger, and Yeketieli as recommended by GraphPad) using a threshold of 5% FDR.

Results

Methods used to produce cRBCs from iPSCs require multiple steps generally including mesoderm induction and hematopoietic progenitor specification and expansion, followed by erythroid specification and expansion, and culminate in erythroid maturation and enucleation steps. Methods for the production of cRBCs from MNCs are similar except that the first steps are omitted because the starting cells are already specified as hematopoietic. In the studies reported below we have re-examined most of these steps. Because we previously reported that erythroid cells produced from iPSCs or from MNCs can generally grow in the same media [9], initial experiments to optimize each step of the protocol were performed either on adult MNCs or on iPSC-derived cells and then applied to the other cell source.

Production of enucleated cRBCs from iPSCs

Olivier et al. [22] previously published an efficient method for producing cRBCs from iPSCs that relies on commercial medium StemLine II to differentiate iPSCs into successive hematopoietic progenitor cells (HPCs) and erythroblasts, and IBIT, a serum-free IMDM-based medium containing bovine serum albumin (BSA), insulin, and Tf, to terminally differentiate the erythroblasts into cRBCs. However, the use of animal-derived components increases costs and decreases the reliability of the method. We therefore decided to develop a chemically defined protocol that would not require any albumin or other animal components and revisited several steps of the differentiation protocol.

Elimination of embryoid body formation. To eliminate all animal components from the protocol, we started with iPSCs grown under chemically defined conditions, instead of iPSCs grown in Stempro hESC, an albumin containing medium, as described previously [22]. Because embryoid bodies (EBs) do not form readily when iPSCs are grown under chemically defined conditions [34], we developed a two-dimensional mesoderm induction procedure instead. This first step relies on 0.5 mmol/L EDTA in PBS to dissociate iPSCs grown on E8/vitronectin into small clumps, and on plating these small clumps directly into mesodermal differentiation conditions that involve two successive cytokine supplements termed S1 and S2 (Table 1). The size of the iPSC clumps at this stage is important in maximizing contact of the differentiation factors with the cells while avoiding the protective colony effect on the cells in their centers. Supplementary Figure E1 (online only, available at www.exphem.org) illustrates the morphology of the cells during the first days of differentiation.

Elimination of albumin. To eliminate albumin from our medium, we devised IMIT, a medium similar to previously published IBIT but in which albumin is replaced by Trolox, an antioxidant, and methyl-β-cyclodextrin to help solubilize lipids and lipid soluble molecules [35]**.** Ethanolamine, an important membrane component, was added because it has been reported to improve cellular growth in serum-free media developed for hybridoma culture [36–38]. The medium was also supplemented with a commercially available mixture of chemically defined lipids to replace the lipids that were previously provided by some batches of albumin.

Comparison of IMIT and IBIT at different stages of the differentiation process, revealed that a significantly larger number of cells were obtained when IMIT was used at all stages during the culture instead of IBIT (t test p values < 0.0001 at days 25, 32, and 38, $n = 3$), suggesting that IMIT could perform better than IBIT (Figure 1A) in differentiating iPSCs into HPCs. However, in these experiments, the cells did not go further than the orthochromatic erythroblast stage with both media.

Table 1. Media and supplements[a]

IBIT
IMDM with 1 mmol/L glutamine
BSA 1%
Insulin 10 μg/mL
Transferrin 200 μg/mL
β-Mercaptoethanol 0.1 mmol/L
Chemically defined lipids (1 ×)
Ethanolamine

IMIT
IMDM with 1 mmol/L glutamine
Methyl-β-cyclodextrin 0.1 mg/mL
Trolox 50 μmol/L
Insulin 10 μg/mL
Optiferrin 50 μg/mL
FeIII-EDTA 4 μmol/L
Chemically defined lipids (1.5 ×)
Ethanolamine

RIT
RPMI-1640 with 1 mmol/L glutamine
Insulin 10 μg/mL
Transferrin 200 μg/mL
β-Mercaptoethanol 0.1 mmol/L
Chemically defined lipids (0.5 ×)
Ethanolamine

R8
RPMI-1640
Ethanolamine 1.6 mmol/L
L-ascorbic acid 220 μmol/L
Chemically defined lipids 0.5 ×
Sodium chloride 1 g/L
Sodium zelenite 74 nmol/L
Insulin 10 μg/mL
Transferrin 200 μg/mL

R5
RPMI-1640
L-ascorbic acid 220 μmol/L
Insulin 10 μg/mL
Transferrin 200 μg/mL
Chemically defined lipids 0.5 ×

R6
RPMI-1640
L-Ascorbic acid 220 μmol/L
Insulin 10 μg/mL
Optiferrin 20 μg/mL
FeIII-EDTA 4 μmol/L
Chemically defined lipids 0.5 ×

S1
BMP4 10 ng/mL
VEGF 165 10 ng/mL
Wnt3A/5A 5 ng/mL each
Activin A 5 ng/mL
Inhibitor VIII 2 μmol/L
bFGF 10 ng/mL

S2
BMP4 20 ng/mL
VEGF 165 30 ng/mL
Wnt3A/5A 5 ng/mL each
Activin A 5 ng/mL
Inhibitor VIII 2 μmol/L
bFGF 10 ng/mL
SCF 20 ng/mL
β-Estradiol 0.4 ng/mL

S3
BMP4 20 ng/mL
VEGF 165 30 ng/mL
bFGF 20 ng/mL
SCF 30 ng/mL
TPO 10 ng/mL
IGF2 10 ng/mL
β-Estradiol 0.4 ng/mL
SB431542 3 μmol/L on day 3 only
IBMX 50 μmol/L
UM171 30 nmol/L after day 6
Heparin 5 μg/mL

S4
VEGF165 5 ng/mL
bFGF 5 ng/mL
SCF 15 ng/mL
TPO 10 ng/mL
IGF2 10 ng/mL
IBMX 30 μmol/L
PDGF AB 5 ng/mL
ANGPTL5 5 ng/mL
CCL28 5 ng/mL
UM171 30 nmol/L
Heparin 5 μg/mL

SED
SCF 100 ng/mL
EPO 4 U/mL
IBMX 50 μmol/L
Dexamethasone 1 μmol/L

SER
SCF 50 ng/mL
EPO 4U/mL
RU486 1 μmol/L

SER2
SCF 10 ng/mL
EPO 4 U/mL
RU486 1 μmol/L

R
RU486 1 μmol/L

Week 1 (W1)
StemSpan SFEM
Hydrocortisone 1 μmol/L
SCF 50 ng/mL
FLT3L 16.7 ng/mL
IL3 6.67 ng/mL
EPO 1.33 U/mL

Week 2 (W2)
StemSpan SFEM
Hydrocortisone 1 μmol/L
SCF 20 ng/mL
IGF1 20 ng/mL
IL3 6.67 ng/mL
EPO 2 U/mL

SEII cytokines
SCF 4.4 ng/mL
EPO 3 U/mL
IGF1 4.4 ng/mL
IL3 1.5 ng/mL

[a]S3* and S4* in some of the figures refers to supplements S3 and S4 without UM171 that were used during development of the PSC-RED protocols.

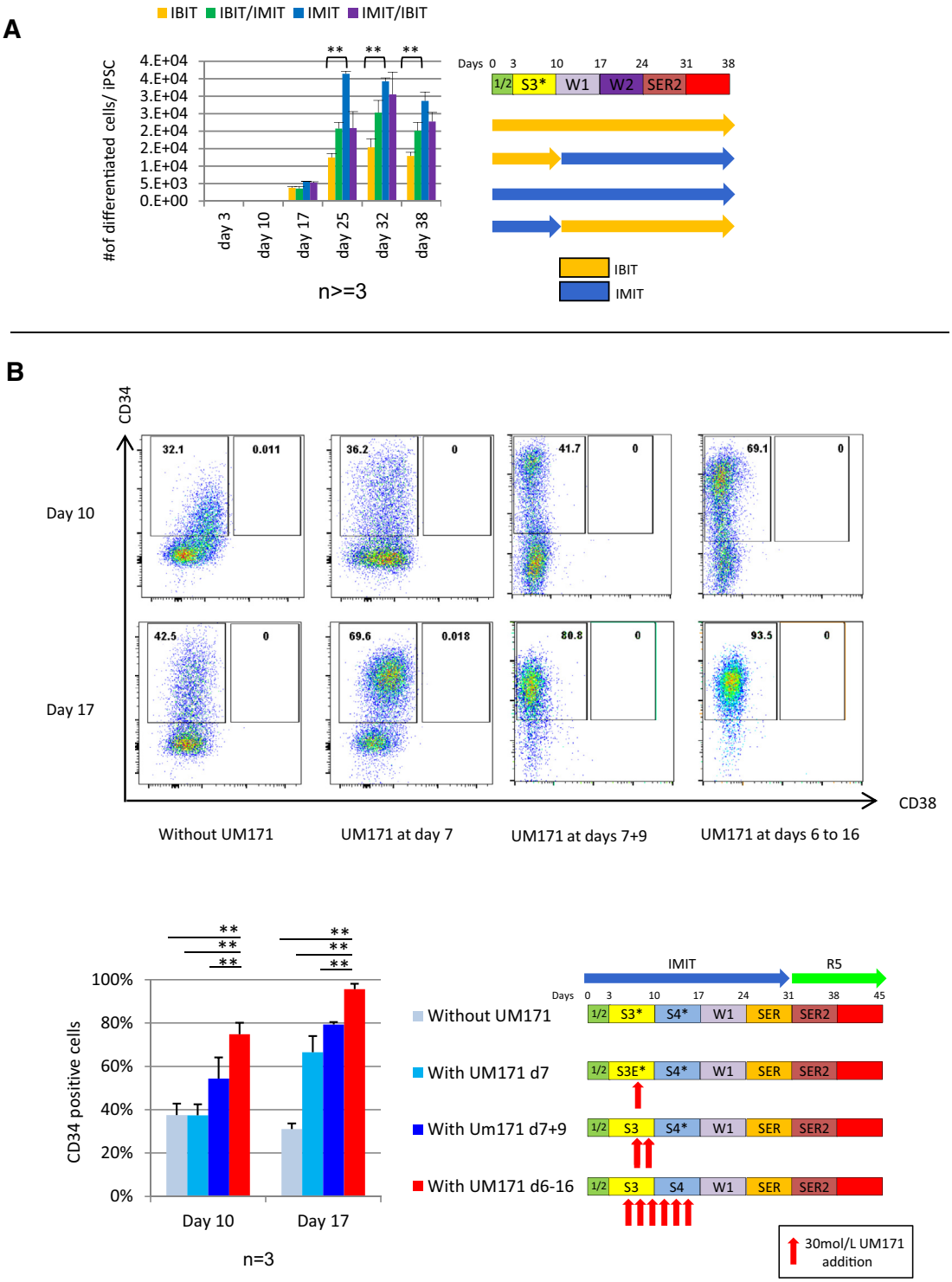

Figure 1. Development of IMIT and optimization of the HSC expansion steps. **(A)** Right: Graph illustrating the number of cells/iPSCs observed in albumin-free IMIT or BSA-containing IBIT medium during the differentiation process ($n = 3$). Left: Culture conditions. 1/2 and S3: supplements S1/S2 and S3*. The asterisk indicates that the S3 did not include UM171 (Table 1). Culture periods symbolized by boxes without text did not include any cytokines. The highest yield of cells was obtained when the IMIT medium was used at all stages of differentiation. **(B)** Supplement S4. Top: Dotplots illustrating flow cytometry analysis of the expression of CD34 and CD38, before and after incubation in supplement S4 with or without stimulation with UM171. Bottom right: Timing of the addition of UM171 and culture conditions are summarized in the culture schematic. Bottom left: Graph illustrating the proportion of CD34+ cells in the various conditions tested ($n = 3$). In the presence of UM171, cells retain a much more undifferentiated phenotype, as determined by the proportion of cells that are CD34+CD38− at day 17. The proportion of CD34+CD38− cells at day 17 significantly increases with increasing number of additions of UM171. When UM171 was added starting at day 6 in S3 and incorporated into supplement S4 (at every feeding), more than 95% of the cells were CD34+CD38− at day 17. $^{**}p$ value < 0.01.

We then focused on the first steps of the protocol and tested whether Trolox and methyl-β-cyclodextrin were necessary in IMIT and whether RPMI could be used instead of IMDM (RMIT medium). This revealed that Trolox was essential and that RPMI could not be substituted for IMDM at this stage, because all cells were dead by day 10 in the absence of Trolox or in the presence of RPMI (Supplementary Figure E2A, online only, available at www.exphem.org). Methyl-β-cyclodextrin had a small, but not significant, positive effect on the yield of cells (Figure S2A). Despite the lack of significance, we kept methyl-β-cyclodextrin in the formulation to increase the solubility of hydrophobic compounds in IMIT because these features may increase the versatility of the culture medium in future applications.

Addition of SB431512. The first steps of the differentiation process are very sensitive to minor variations in the culture conditions and crucial for the rest of the procedure as poor differentiation at this step results in a very low overall yield. It was previously reported that blocking activin A signaling after mesoderm induction favors definitive over primitive hematopoiesis [39,40]. To determine if SB431512, an activin signaling inhibitor [41], could improve the early steps of our protocol, we tested if a one-time addition of this compound to supplement S3 immediately after the dissociation step would increase the yield of the method. This revealed that the number of cells obtained between days 0 and 10 was about threefold higher in the treated than in the nontreated controls independently of whether IMIT or IBIT was used (Supplementary Figure E2B).

In vitro expansion of iPSC-derived HPC. To improve on this protocol, we hypothesized that the HPCs produced in IMIT would become more developmentally mature if they could be expanded without losing markers CD34, CD90, and CD49f, which are three markers often used to isolate cord blood HSCs [42]. To test this hypothesis, we attempted to modify S3 by addition of either SR1 [43] or UM171 [44], two small molecules that allow self-renewal of cord blood HSCs, and designed S4, a supplement intended to favor stem and progenitor cell expansion without differentiation. The initial formulation of S4 contained seven cytokines, isobutyl methyl xanthine (IBMX), and optionally either SR1 or UM171 (Table 1). Expansion of day 10 HPCs produced with or without adding SR1 to S3 at day 7 or 9 or after day 10 in the S4 supplement was not successful because in IMIT, SR1 was relatively toxic and the HPCs could barely be maintained for 5 additional days under hematopoietic conditions (not shown). By contrast, adding a single pulse of UM171 at day 7 in S3 was not toxic and significantly increased the yield of erythroid cells (t-test p value = 0.008, $n = 2$; Supplementary Figure E3A, online only, available at www.exphem.org).

Importantly, at day 17, after 7 days of culture in supplement S4, the percentage of CD34+ cells in the UM171 culture was 66.5 ± 7% (SD); significantly higher than the 23.4 ± 4% obtained without exposure to UM171 (p value at day 17 = 0.0004, $n = 3$; Figure 1B), and the majority of the CD34+ cells had retained CD90 + and CD49f+ expression (Supplementary Figure E3B), suggesting that UM171 can help expand iPSC-derived HPCs in an undifferentiated state.

Additional testing demonstrated that adding UM171 at days 7 and 9 in S3 or between days 6 and 16 in S3 and S4 had even more significant effects on the percentage of cells that retained expression of CD34+, because in the latter case, CD34 expression at day 17 averaged 95 ± 2.4% as compared with 31.5 ± 2.3 in the absence of UM171 (p value < 0.0001 at days 10 and 17, $n = 3$; Figure 1B). Differentiation of the cells obtained after growth in supplement S4 suggested that these cells had retained their erythroid differentiation potential (see below). The protocol integrating the S4 expansion step will thereafter be referred to as the long version of the protocol to differentiate it from the shorter version without the S4 supplement.

Development of the R6 cell culture medium

Having improved the hematopoietic specification step of the iPSC differentiation protocol, we then focused on the erythroid differentiation part of the protocol. To save time and efforts, we performed initial experiments using day 14 erythroblasts derived from adult peripheral blood mono-nuclear cells (PB MNCs) and subsequently applied the results to the erythroid differentiation of iPSC-derived HPCs.

Day 14 erythroblasts express CD71 and CD36 at more than 95% and CD235a at about 50%, and are morphologically mostly at the pro- and basophilic erythroblast stages of differentiation (Supplementary Figure E4, online only, available at www.exphem.org). They are obtained from PB MNCs using a previously published standard protocol (see Methods) and can be terminally differentiated into enucleated RBCs using a serum-containing protocol. We re-examined this terminal differentiation protocol because it had several major issues. First, the number of enucleated cRBCs produced was ≤50% of the number of input 14-day erythroblasts because of massive cell loss during differentiation. Second, high rates of enucleation could be obtained only in the presence of serum or albumin, which is problematic because these components are expensive and undefined. Third, it was expensive because of the massive amounts of Tf necessary to obtain enucleation.

To improve on this protocol we designed R8, a serum- and albumin-free medium that contains insulin, Tf, ethanolamine, L-ascorbic acid, lipids, sodium chloride, and sodium selenite (Table 1). R8 was modeled on the E8 medium originally developed to grow pluripotent stem cells [27] but the base medium was switched from DMEM-F12 to RPMI, which is better suited for erythroid cells production (not shown).

To determine if our day 14 erythroblasts had reached their maximal glucocorticoid-induced proliferation, we attempted to amplify them further using the SED cocktail in either a serum-containing medium, or in R8, a serum-free medium. Culturing day 14 erythroblasts for 7 days in serum-containing medium plus the SED cocktail, followed by 7 days of culture in the absence of cytokines, did not produce any improvement in cell yield as compared with our standard protocol (Figure 2A, conditions 1 and 2).

By contrast, day 14 erythroblasts grown for 7 days in R8 + SED, followed by 7 days in R8 alone (condition 3), proliferated significantly more than in our standard conditions (Figure 2A; Supplementary Figure E5A, online only, available at www.exphem.org). Examination of differentiation after Romanowsky staining revealed that incubation in the SED cocktail in the absence of serum retarded differentiation, as there were significantly more pro- and basophilic erythroblasts and fewer polychromatic and orthochromatic erythroblasts between days 21 and 28 (Figure 2C; Supplementary Figure E5B). However, under these conditions, enucleation was completely blocked with orthochromatic erythroblasts accumulating at day 28 and eventually dying by days 32–35.

To determine if one of the components of SED might be responsible for the lack of enucleation, we systematically omitted components of the SED cocktails. This revealed that day 14 erythroblasts grown in R8 containing either EPO, SCF, or Dex alone, EPO and Dex, or SCF and Dex did not grow well and/or died rapidly (Supplementary Figure E6, online only, available at www.exphem.org). However, cells grown in R8 without Dex, but with SCF and EPO (condition 4), proliferated significantly more than the control cells (Figure 2A; Supplementary Figure E5A), and enucleation was restored at a rate of about 20% (Figure 2C; Supplementary E5B). Because these experiments suggested that enucleation was improved by removing Dex, we added the glucocorticoid antagonist RU486 to SCF and EPO (condition 5). This confirmed that Dex was somehow affecting enucleation because addition of RU486 had no major effect on proliferation but restored enucleation levels to that under our standard conditions (Figure 2C; Supplementary Figure E5A, B). Assessment of enucleation by flow cytometry, a method that is somewhat more accurate than manual enumeration, because many more cells can be evaluated, confirmed that there were no significant differences in the rate of enucleation between conditions 1 and 5 (Figure 2D).

To refine this improved terminal differentiation protocol, we determined which of the components of medium R8 were necessary. Experiments in which single components were systematically removed from R8 suggested that ethanolamine at 1.6 mmol/L was associated with a small but significant decrease in yield, that lipids and L-ascorbic acid were necessary, and that sodium selenite had no effect (Supplementary Figures E5C and E7, online only, available at www.exphem.org). To confirm these results, we formulated medium R5, which contains only RPMI, lipids, Tf, insulin, and L-ascorbic acid and verified the above results by either removing either the L-ascorbic acid, the lipids or by adding sodium selenite. This confirmed that the presence of L-ascorbic acid and lipids was associated with a significantly higher yield and that sodium selenite had no effect (Supplementary Figures E5C and E7). Finally, we directly compared the R5 and R8 media. This revealed that R5 yielded significantly more cells than R8 and that both media yielded similar rates of enucleation (Figure 2E; Supplementary Figure E5C).

Because there is no addition of glucocorticoids after day 14, we wondered whether RU486 was still necessary after day 21. To test this hypothesis, we compared cell yields and rates of enucleation with or without RU486 added after day 21. This revealed that this compound was not necessary after day 21 (not shown).

Iron chelators. In vivo, holo-Tf is internalized after binding to its receptor, re-exported from the cells as apo-Tf, reloaded with iron, and eventually re-utilized [45]. However, in vitro, apo-Tf recycling does not occur in erythroid cell culture. To decrease cost, we tested several iron sources to allow the culture of erythroid cells in small amounts of Tf.

To find an appropriate source of iron, we compared the yield of cells and the rate of enucleation when day 14 erythroblasts were grown in R5 containing 50 μg/mL holo-Tf, which is not sufficient for erythroid differentiation, in the presence of iron chelators iron citrate, aurintricarboxylic acid [46] (ATA), eriochrome cyanine R [47], or FeIII-EDTA [48]. A mixture of 220 nmol/L $Fe(NO_3)_3$ and 3.2 μmol/L $Fe_2(SO_4)$, an iron source that is classically used in erythroid culture, was also tested [7].

This revealed that the ferric nitrate/ferrous sulfate mixture and the iron citrate were not effective as a source of iron (data not shown). By contrast, the three other iron chelators were effective. Supplementation with 50 μg/ml Tf with either 3 μmol/L eriochrome cyanine or FeIII-EDTA completely restored cell growth, as we observed no significant difference from the control culture containing 200 μg/mL holo-Tf (Figure 3A; Supplementary Figure E6D). In the case of 3 μmol/L ATA, the rescue was only partial, because the yield was higher than without supplementation, but significantly

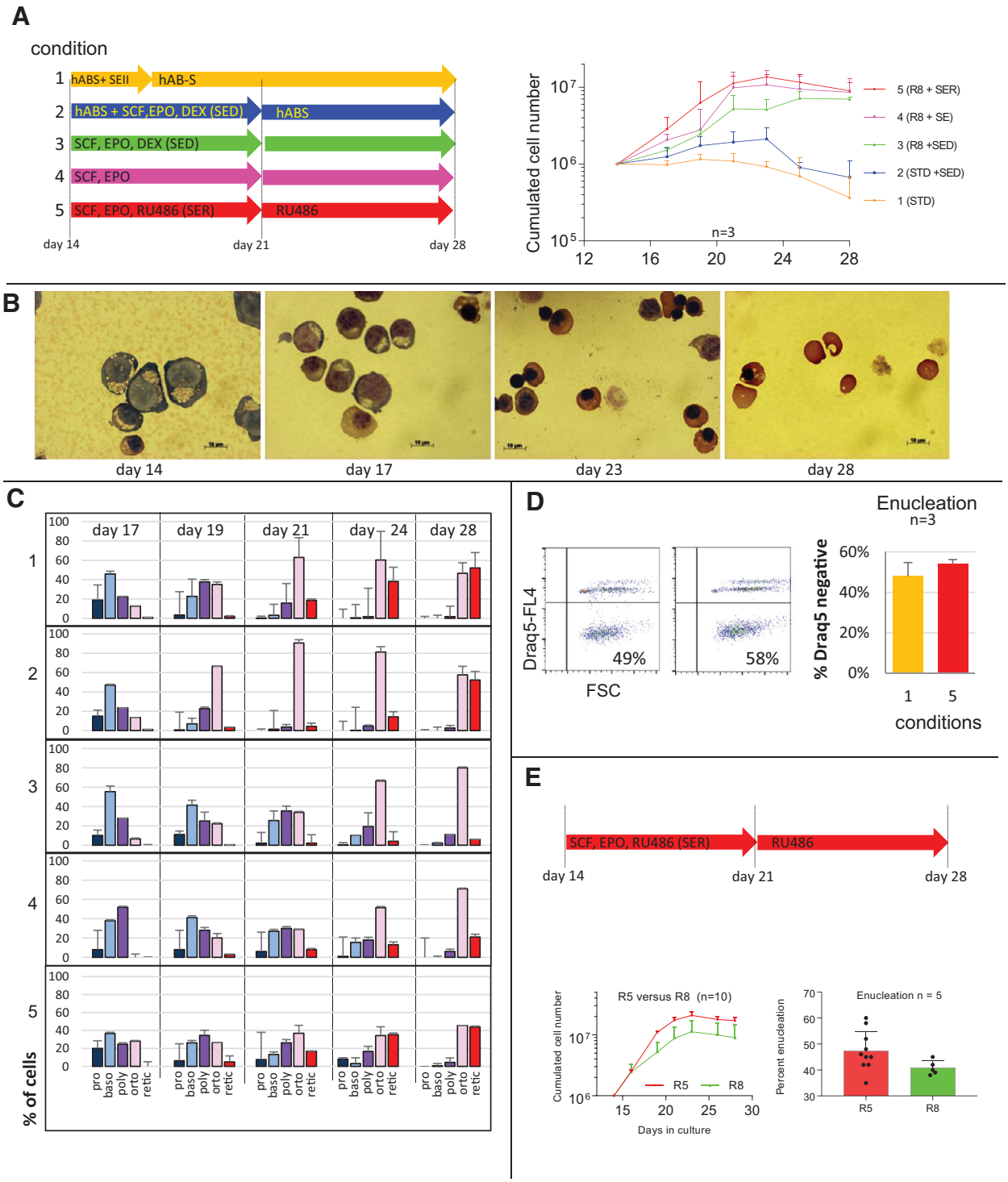

Figure 2. Development of R6 medium. **(A)** Two-point-one million day 14 erythroblasts, which had been obtained by growing PB MNCs for 7 days in week 1 medium, followed by 7 days in week 2 medium, were plated for 14 days either in our standard human AB serum-containing protocol (condition 1), or in four other conditions (conditions 2 to 5) as depicted in diagram on the top left. Details of the culture conditions are provided in the method section. Composition of the cytokine and small molecule cocktails (SEII cytokine, SED, SE, SER, and R) are provided in Table 1. The line graph on the right summarizes the averages and standard deviations ($n = 3$) of the number of live cells observed during the culture as determined by using a hemocytometer after trypan blue staining. Day 14 erythroblasts grown in condition 5 proliferated ten- to thirtyfold more than under our standard conditions (condition 1). **(B)** Micrograph illustrating the typical morphology of the erythroid cells observed at days 14, 17, 23, and 28 of culture after rapid Romanowsky staining. The majority of cells were pro- or basophilic erythroblasts at day 14, basophilic or polychromatophilic erythroblasts at day 17, orthochromatic erythroblasts at day 23, and orthochromatic erythroblasts and reticulocytes (young enucleated RBCs) at day 28. **(C)** Graph illustrating the quantification of erythroid differentiation under the conditions depicted above. About 100,000 cells were cytospinned on microscope slides and stained with the rapid Romanowsky method. The cells were

different from that of the 200 μg/mL holo-Tf control (Figure 3A; Supplementary Figure E6D). Additional experiments suggested that combinations of the three chelators had no advantage over the use of a single chelator (data not shown). Although there was no significant difference between eriochrome cyanine R and FeIII-EDTA, the latter compound had yielded the highest average number of cells. We consequently decided to focus on that molecule for the rest of the study.

Optiferrin. Holo-Tf is purified from human plasma. It is therefore expensive and a potential source of variation. To determine if it could be replaced by Optiferrin, a commercially available recombinant Tf produced in rice, we cultured day 14 erythroblasts in the presence of 3 μmol/L FeIII-EDTA and either 50 μg/mL holo-Tf or decreasing amounts of Optiferrin. This revealed that there was no significant difference between the yields of cells obtained with 50 μg/mL holo-Tf and as little as 12.5 μg/mL Optiferrin (Figure 3B). The day 14 erythroblasts could also grow in 6.25 μg/mL Optiferrin but the yield was significantly lower at day 28 (Figure 3B; Supplementary Figure E6E). Enucleation rates in the presence of 50 μg/mL holo-Tf or at least 12.5 μg/mL Optiferrin were not significantly different. To maintain an excess of Optiferrin at all times in these cultures, we decided to use a concentration of 20 μg/mL in subsequent experiments.

To determine the optimal concentration of FeIII-EDTA, we titrated it between 0 and 8 μmol/L. This revealed that the yield of cRBCs and the enucleation rates did not significantly differ when the day 14 erythroblasts were cultured in the presence of 2, 4, or 8 μmol/L FeIII-EDTA (Figure 3C). By contrast, the day 14 erythroblasts grew poorly when the concentration of FeIII-EDTA was <2 μmol/L. An additional experiment revealed that there was no obvious toxicity when the concentration of FeIII-EDTA was increased to 20 μmol/L (not shown). We decided to use 4 μmol/L FeIII-EDTA in future experiments and reformulated medium R5 by replacing the 200 μg/mL holo-Tf with 20 μg/mL Optiferrin and 4 μmol/L FeIII-EDTA, creating medium R6, which is significantly cheaper to produce than R5 or R8.

Tf recycling. To determine if, as hypothesized, the positive effect FeIII-EDTA was associated with Tf recycling, we directly measured Optiferrin iron loading by PAGE in 6 mol/L urea as described previously [33]. We first found that FeIII-EDTA could deliver iron to Optiferrin by preparing R6 medium containing 20 μg/mL Optiferrin, as provided by the manufacturer and either no additional iron or 0.22 μmol/L $Fe(NO_3)_3$ and 3.2 μmol/L $Fe_2(SO_4)$, or various amounts of FeIII-EDTA. After 2 hours of incubation at 37°C, the media were concentrated, and 2 μg of Optiferrin was examined by PAGE (Figure 3D). Staining with Coomassie blue revealed that the Optiferrin provided by the manufacturer was mostly in the apo conformation, but that it was fully loaded with iron in the presence of 4 or 20 μmol/L FeIII-EDTA and partially loaded with a 2 μmol/L concentration of the iron chelator (Figure 3D). The combination of $Fe(NO_3)_3$ and $Fe_2(SO_4)$ was completely ineffective to load Optiferrin with iron in the context of the R6 medium, explaining why these compounds are not effective as a source of iron in erythroid culture.

To determine if Optiferrin could be recycled in the presence of FeIII-EDTA, about 2×10^6 day 14 erythroblasts were incubated for 4 days without any feeding in R6 medium containing various amounts of iron (and 20 μg/mL Optiferrin). As expected, cells proliferated in all conditions containing at least 2 μmol/L FeIII-EDTA. The spent medium was then recovered, concentrated, and analyzed by 6 mol/L urea 5% PAGE as described above. This revealed that Optiferrin in media containing concentrations of FeIII-EDTA ≤4 μmol/L was now almost completely in its apo form, suggesting that the iron had been completely consumed by the cells (Figure 3D). By contrast, Optiferrin in the medium containing 20 μmol/L FeIII-EDTA was still in its holo form, indicating that FeIII-EDTA can be used as a source of iron to allow Optiferrin recycling to take place in erythroid cell cultures.

Erythroid differentiation of iPSC-derived HPCs

Having developed the R5 and R6 media and improved our terminal differentiation protocol, we attempted to differentiate iPSC-derived HPCs into cRBCs.

classified according to their morphology. proE = pro-erythroblasts; basoE = basophilic erythroblast; polyE = polychromatophilic erythroblast; orthoE = orthochromatophilic erythroblast; retic = reticulocytes (enucleated cRBCs). Graphs illustrate the frequency (average ± SD) of each erythroid precursor during the course of the experiments ($n = 3$). Differentiation in condition 3 was significantly delayed relative to differentiation in condition 2, as illustrated by the smaller amounts of orthochromatic erythroblasts and reticulocytes and the larger amounts of basophilic and polychromatophilic erythroblasts at day 21 in condition 3. The p and q values are provided in Supplementary Figure E2B. Enucleation in condition 3 was very poor (<5% reticulocytes), but was partly restored (21% reticulocytes) in condition 4 and completely restored (44% reticulocytes) in condition 5. These differences were statistically significant (Supplementary Figure E2B). **(D)** Enucleation as measured by flow cytometry: To confirm the rate of enucleations, the percentage enucleation at day 28 was also determined by flow cytometry using Draq5 staining on cells that had been grown under conditions 1 and 5. Rates of enucleation determined by this method were not significantly different from the rates measured after Romanowsky staining. **(E)** Comparison of R5 and R8 media. Top: Diagram illustrating the cytokine cocktails used in the experiments. Bottom: Three million day 14 erythroblasts were plated for 7 days in either R5 or R8 with the SER cytokine cocktail for 7 days, followed by 7 days in R5 or R8 + RU486. The graph on the left illustrates the number of live cells. The yield of cells was significantly greater in R5 at every time point monitored ($n = 5$). Student t-test p values and q values for an FDR of 5% are provided below the graph. The graph on the right illustrates the number of enucleated cells determined after Romanowsky staining. There was no significant difference between the two media.

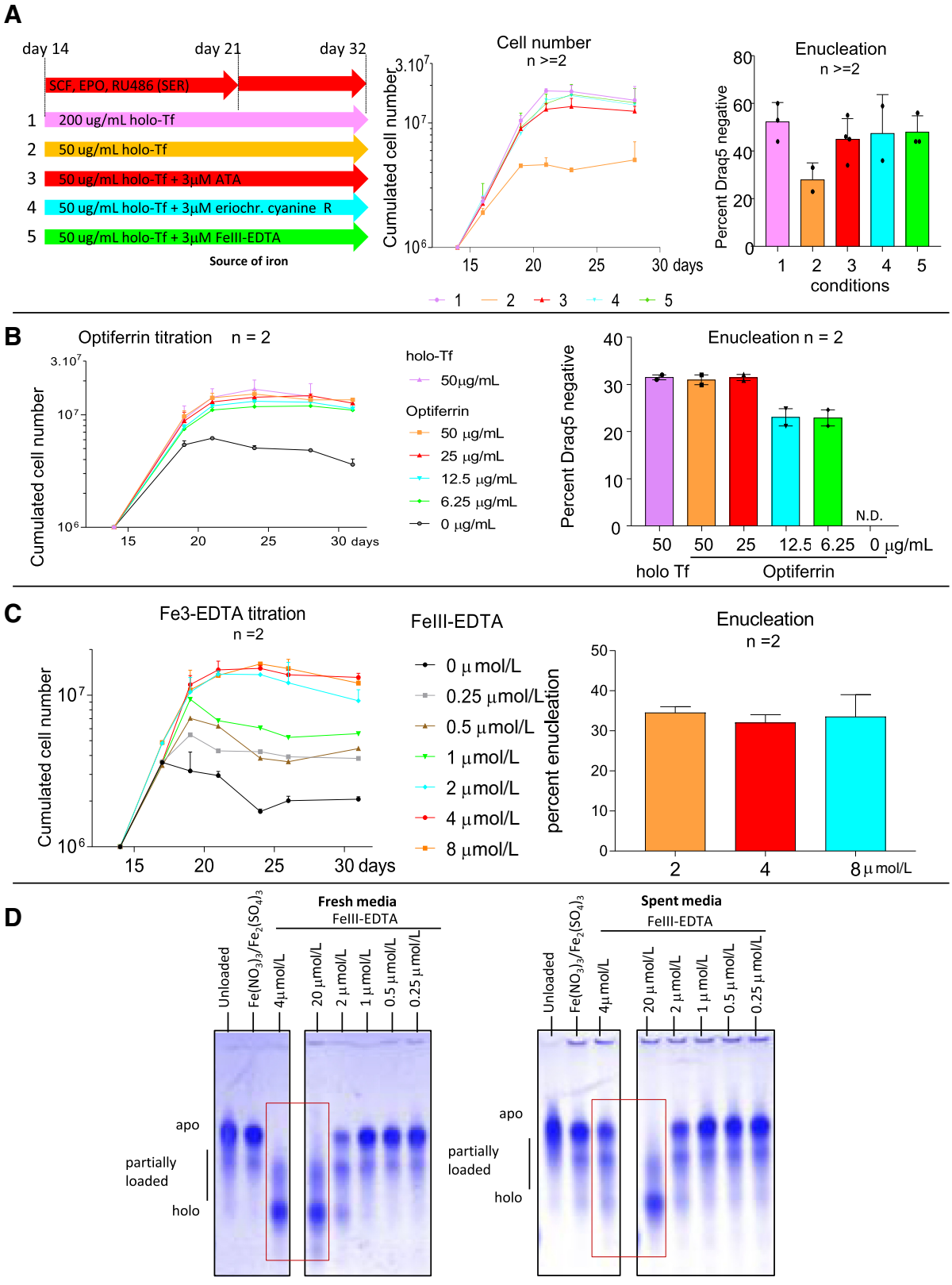

Figure 3. Iron source. **(A)** Iron chelators. Left: Diagram illustrating the cytokine cocktail used in the experiments (top row) and the source of iron in conditions 1 to 5. Middle: Three million day 14 erythroblasts were plated for 7 days in either R5 medium supplemented with the SER cytokine cocktail for 7 days, followed by 7 days in R5 medium alone. The graph illustrates the number of live cells at different days in the culture in the presence of different amounts of human holo-Tf and different iron chelators. Cell numbers were determined using a hemocytometer

We first compared our published conditions, culture of the HPCs in HSA containing Stemline II in the presence of the week 1/week 2 cytokine cocktails (Table 1), with culture of the HPCs in the SED cocktail, and its derivative, the SER cocktail (SCF, EPO, RU486), in the IMIT and R5 media.

Initial experiments revealed that the yield of cells was significantly higher (p value < 0.05 at days 25 through 38, $n = 2$) when day 10 iPSC-derived HPCs were cultured in IMIT + SED (condition 2) between days 10 and 17 as compared with IMIT plus the week 1 cocktails (condition 1) (Figure 4A) and, therefore, that the additional cytokines present in the week 1 cocktail were not essential. Further experiments revealed that growing day 10 iPSC-derived HPCs in condition 4 (IMIT/SED [days 10–17], IMIT/SER [days 17–24], R5/SER2 [day 24/31], and R5/R [days 31–38]) yielded significantly more cells than growing the cells in either condition 2 (p value = 0.0006 at day 32, $n = 2$) or condition 3 (p value = 0.009 at day 32, $n = 2$) (Figure 4A). Thus, similarly to adult 14-day erythroblasts, iPSC-derived erythroid progenitors expanded in the presence of SCF, EPO, and a glucocorticoid proliferated optimally when the glucocorticoid was removed before EPO and SCF (as shown when comparing conditions 4 and 3).

Additional experiments confirmed that using the IMIT medium until day 24 yielded significantly more cells than switching to the R5 medium at day 17 (p value at day 31 = 0.0058, $n = 6$; Figure 4B), and that HPCs expanded for an additional week in supplement S4 in the long protocol also yielded significantly more cells when they were expanded in condition 4 rather than using our previous protocol (p values = 0.043 at day 33 and <0.0001 at days 39 and 45, $n = 6$) (Figure 4C).

Use of small amounts of recombinant Tf

Additional experiments revealed that 200 μg/mL Optiferrin supplemented with FeIII-EDTA could replace 200 μg/mL holo-Tf (Figure 5A, B) because there were no significant differences between the yields of cells in both conditions. Reducing the Optiferrin concentration tenfold to 20 μg/mL decreased the yield by about a third (p value = 0.06, $n = 2$), which is an acceptable trade-off because it reduces the cost of the medium by a factor close to 10 (Figure 5A). Further experiments indicated that using 50 μg/mL Optiferrin with 4 μmol/L Fe3-EDTA in IMIT did not significantly differ from using 200 μg/mL (not shown) while decreasing the cost of the medium by 70% according to the catalog prices of both Holo-Tf and Optiferrin.

To determine if the source of iron and the type of Tf used affected differentiation, we analyzed the cells by FACS at day 31. This revealed that cells produced with holo-Tf or Optiferrin were almost all erythroid and had a phenotypic profile very similar to that previously published (Olivier et al, 2016) (Figure 5B), suggesting that the source of iron and Tf had no major effect on differentiation. We concluded that Optiferrin can be used throughout the differentiation protocol and that medium R6 (R5 in which holo-Tf is replaced with Optiferrin and FeIII-EDTA; see Table 1) is optimal for the final differentiation steps of iPSC-derived erythroblasts.

Robust erythroid differentiation protocols

Combining these experiments, we formalized albumin-free, chemically-defined protocols that we termed short and long pluripotent stem cell robust erythroid differentiation (PSC-RED) protocols (Figure 6A) and characterized the cRBCs obtained. Similarly to our previous protocols, the cells obtained using the short version of the PSC-RED protocol were almost 100% orthochromatic erythroblasts but enucleated very poorly (Supplementary Figure E8, online only, available at www.exphem.org). Importantly, the week of expansion in S4 in the long version of the protocol resulted in the production of cells that exhibited

after trypan blue staining. Cells grew poorly with only 50 μg/mL holo-Tf in the medium (compare conditions 1 and 2). Cell growth in medium containing 50 μg/mL holo-Tf was completely restored in the presence of all FeIII-EDTA and eriochrome cyanine R and partially restored in the presence of ATA ($n = 3$, p and q values in Figure S2D). Right: Graph illustrating enucleation (determined by flow cytometry) in the five conditions tested. There were no significant differences in the rates of enucleation ($n = 2$). **(B)** Optiferrin titration. Three million day 14 erythroblasts were cultured with the same cytokines as in (A) in R5 supplemented with 3 μmol/L FeIII-EDTA and the indicated concentrations of Optiferrin. The graph on the left illustrates the number of live cells at different days in the culture in the presence of different amounts of Optiferrin. Concentrations of Optiferrin as low as 6.25 μg/mL supported the growth of day 14 erythroblasts ($n = 2$, p and q values are in Supplementary Figure E2E). The graph on the right illustrates the rate of enucleation under the different conditions tested. Rates of enucleation were not significantly different ($n = 2$). We decided to use 20 μg/mL Optiferrin in subsequent experiments. **(C)** FeIII-EDTA titration. Three million day 14 erythroblasts were cultured with the same cytokines as in (A) in R5 supplemented with 20 μg/mL Optiferrin and the indicated concentration of FeIII-EDTA. The graph on the left illustrates the number of live cells at different days in the culture in the presence of different concentrations of FeIII-EDTA. The graph on the right illustrates the rates of enucleation. There were no significant differences between the yields of cells or the rates of enucleation in the presence of 2, 4, or 8 μmol/L FeII-EDTA ($n = 2$). Cells grew poorly in the presence of ≤1 μmol/L FeIII-EDTA. We decided to use 4 μmol/L FeIII-EDTA in subsequent experiments. **(D)** Optiferrin recycling: Fresh or spent R6 medium containing 20 μg/mL Optiferrin and various concentrations of FeIII-EDTA was analyzed on a 6 mol/L urea 5% polyacrylamide gel. In fresh medium (left), Optiferrin was in the apo form in the absence of iron, in the presence of $Fe(NO_3)_3/Fe_2(SO_4)$ (0.22 μmol/L/3.2 μmol/L), or with less than <2μM FeIII-EDTA, but was in the holo form in the presence of 4 or 20 μmol/L FeIII-EDTA, indicating that FeIII-EDTA can deliver iron to Optiferrin. In the spent media (right), Optiferrin was in the apo form in the presence of 4 μmol/L FeIII-EDTA, suggesting that the iron was depleted, but was still in its holo form in the 20 μmol/L condition, suggesting that iron recycling can occur in the R6 medium. Gels are representative of the results of two independent experiments.

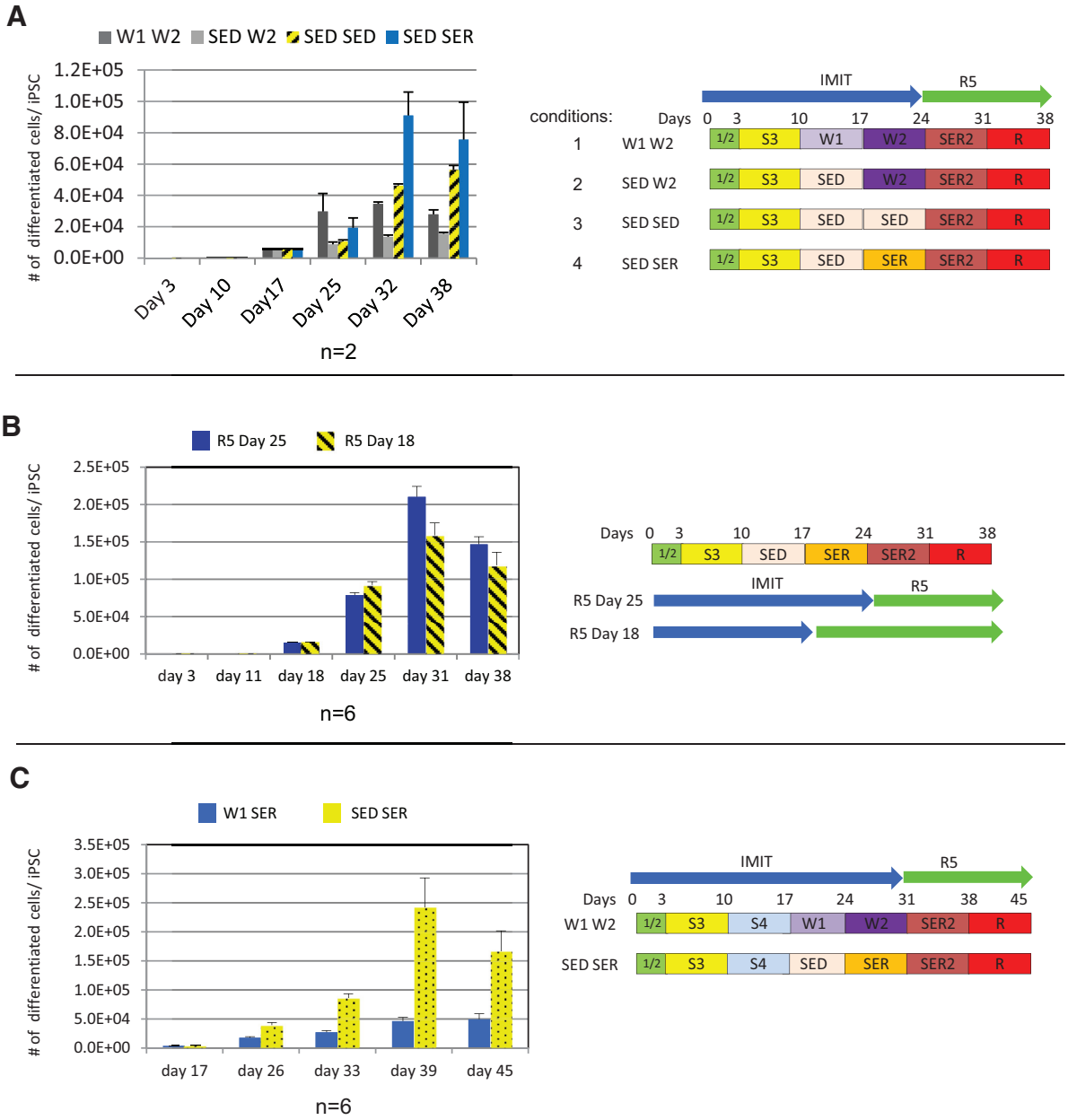

Figure 4. Optimization of the erythroid expansion and maturation steps (days 10–38). (A) Left: Diagram illustrating the number of cRBCs/iPSCs produced with different cytokine combinations to determine the most efficient formulation and the appropriate timing to switch from glucocorticoid stimulation to glucocorticoid inhibition ($n \geq 2$). Right: Schematic of culture conditions schematic (short protocol). **(B)** Left: Diagram illustrating the number of cRBCs/iPSCs produced as a function of the expansion medium timing switch, indicating that switching from IMIT to R5 at day 25 when using the SED/SER cytokine combination yields the most cells ($n \geq 4$). Right: Schematic of culture conditions (short protocol). **(C)** Left: Diagram illustrating the number of cells/iPSCa produced using the SED/SER cytokine combination in the long version of the protocol as compared with incubation in week 1/week 2 from days 17 to 31 ($n \geq 2$). Right: Schematic of culture conditions (long protocol). As in (A) and (B), erythroid differentiation for successive weeks with cytokine cocktails SED, SER, SER2, and R was most efficient.

rates of enucleation reaching more than 50% in some experiments (Figure 6B, C) and that averaged 41.8 ± 1.1% ($n = 10$ independent experiments), as compared with <5% enucleation for the short protocol. Rates of enucleation >75% ($n = 2$) (Figure 6C) could be obtained by adding a second week of culture in S4 but at the expense of a reduction in the yield of cRBCs (24000 ± 3000 cRBCs/iPSC), which significantly decreased the total number of cRBCs produced (see below). Similarly high rates of enucleation of RBCs derived from pluripotent stem cells have been published before [49] but never with a methodology and a production yield compatible with biomedical applications.

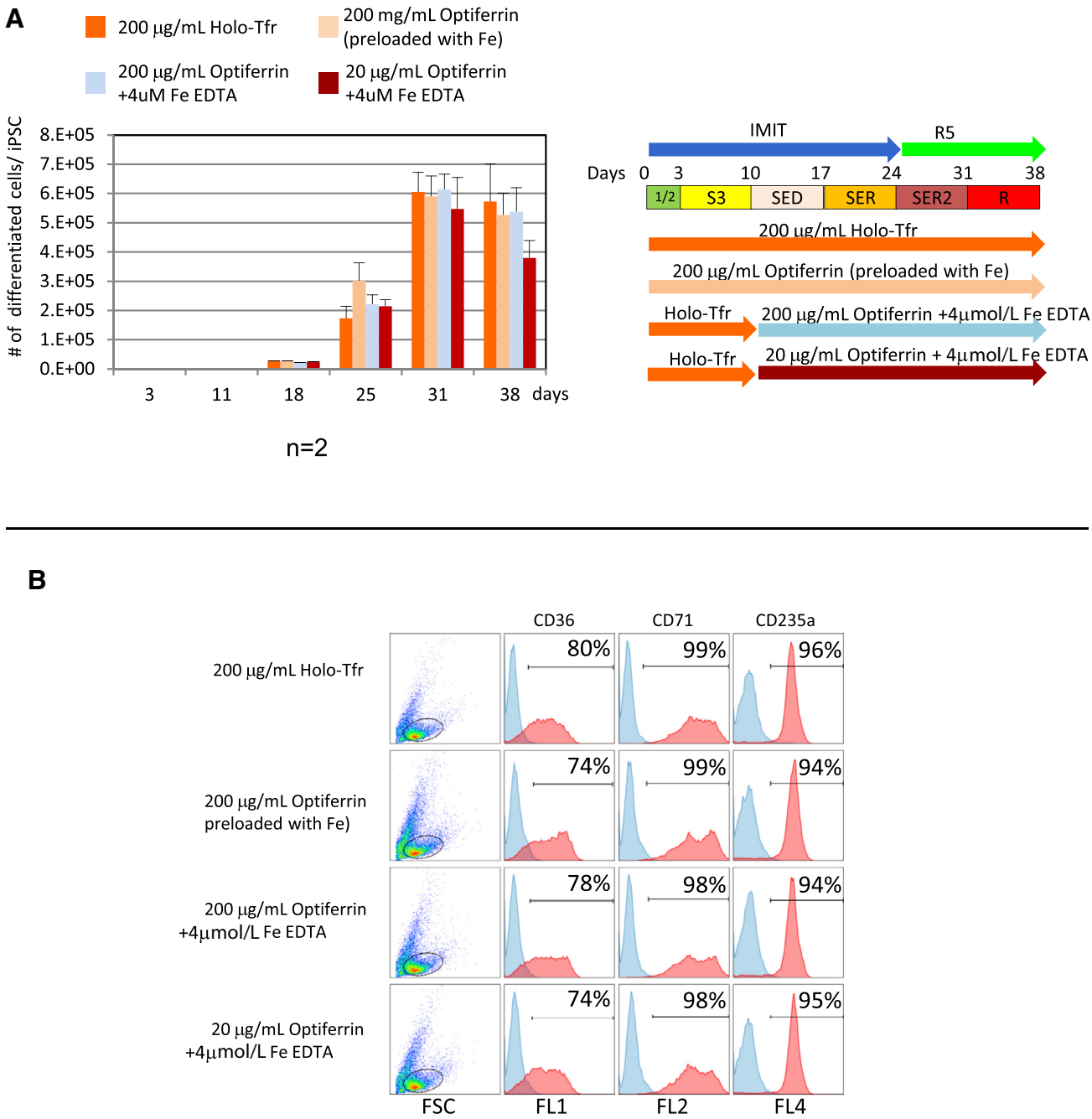

Figure 5. Substitution of animal-derived Tf by recombinant Tf (Optiferrin). **(A)** Left: Diagram illustrating the number of cells/iPSCs observed after substituting human serum-derived holo-Tf with Optiferrin. Optiferrin 200 μg/mL (pre-loaded or supplemented with iron) can replace holo-Tf 200 μg/mL. Optiferrin 20 μg/mL + FeIII-EDTA4 μmol/L can also replace holo-Tf but with a small decrease in yield ($n = 2$). Right: Culture conditions schematic. **(B)** FACS analysis. Erythroblasts obtained with Tf or Optiferrin were analyzed by FACS at day 31. The use of Optiferrin has no effect on the immunophenotype of the cRBCs, as expression of CD36, CD71, and CD235a is similar in all cases.

These important observations resolve one of the major impediments to the production of cRBCs by differentiation of iPSCs, as rates of enucleation greater than 40% are sufficient to produce pure populations of enucleated cRBCs on an industrial scale. Indeed, separation by filtration of the enucleated from the nucleated cells produced using the long PSC-RED protocol (1 week in S4) yielded a 99% pure population of enucleated cells (Figure 6B).

Estimation of the size of enucleated iPSC-derived cRBCs revealed that they were about 15% larger than adult RBCs produced in vivo (Figure 6B) either because they were produced in culture or because they had a more fetal phenotype.

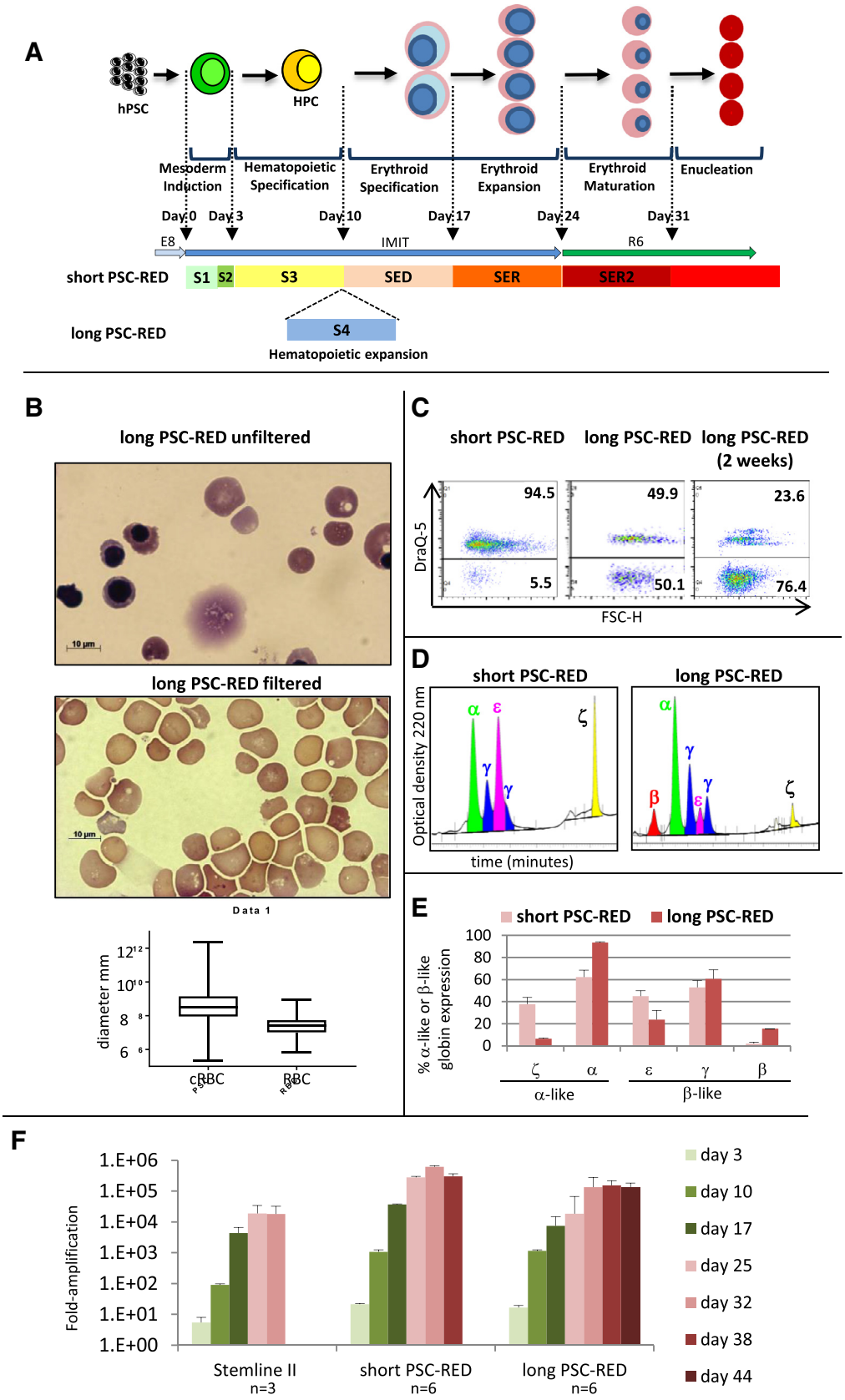

Figure 6. Summary of PSC-RED protocols. The long PSC-RED protocol generates enucleated cRBCs from iPSCs. **(A)** Graph illustrating short and long PSC-RED protocols used to produce enucleated cRBCs from iPSCs. Media and supplement compositions can be found in Table 1.

High-performance liquid chromatography analysis of the cRBCs produced with the short and long PSC-RED protocols revealed major differences: the short protocol yielded cells that expressed a mixture of embryonic and fetal hemoglobin (Figure 6D, E), whereas the long protocol yielded cells that expressed mostly γ-globin and 15.5 ± 0.16% (average ± SD) β-globin. Cells produced with the long PSC-RED protocol were therefore more developmentally mature than the cells produced with the short version of the protocol. HPLC analysis of additional experiments in which day 10 HPCs were grown in S4 for 0, 3, or 7 days and then expanded in erythroid conditions supported these results, as expression of embryonic globin decreased and that of fetal and adult globin progressively increased with increasing time under HSC expansion conditions (Supplementary Figure E9, online only, available at www.exphem.org).

Fluorescence-activated cell sorting analysis of the cells obtained throughout the differentiation with antibodies against CD45, CD43, CD34, CD36, CD71, and CD235a confirmed the fundamentally different nature of the cells obtained with the short and long protocols (Supplementary Figure E10, online only, available at www.exphem.org). Expression of CD235a during the short protocol is detected at day 10 in most cells and continues uninterrupted until the end of the differentiation, reflecting expression of this marker in CD34+CD43+ primitive HPCs [50]. This developmentally primitive phenotype is also illustrated by the lack of expression of CD45 in accordance with previous reports that the first wave of hematopoietic cells produced during pluripotent stem cell differentiation is CD45 negative [19]. By contrast, in the long protocol, CD235a expression almost completely disappears at day 17, reflecting the generation in supplement S4 of more definitive CD34+CD235a− HPCs, which differentiate into CD45+ cells by day 24 and into erythroid precursors that re-express CD235a starting at day 31. Very high levels of expression of CD36, CD71, and CD235a at the end of the differentiation confirmed that both protocols produce virtually pure populations of erythroid cells.

In terms of cell numbers, the short PSC-RED protocol generated significantly more nucleated cRBCs than our previously published procedure (p value <0.00001, $n \geq 3$) as it yielded up to 1×10^6 cRBCs/iPSC in the best experiment and an average (±SD) of 299,900 ± 72,444 cRBCs/iPSC as compared with 18,091 ± 2475 for the StemLine II protocol (Figure 6F). Because there are about 5×10^6 iPSCs per well of a six-well plate, this protocol could theoretically generate more than 10^{12} cells from a single well of iPSCs.

The long PSC-RED protocol was slightly less efficient but nevertheless yielded 135,131 ± 47,147 nucleated cRBCs/iPSC at day 45, which is significantly more than produced by the StemLine II protocol (p value = 0.00007, $n \geq 3$) (Figure 6F). On average, the long PSC-RED protocol yielded about 60,000 enucleated cRBCs/iPSC at day 45. Testing of the long PSC-RED protocol on four additional iPSC lines that had been derived from the peripheral blood cells of four different individuals yielded similar numbers of cells and similar rates of enucleation (Supplementary Figure E11, online only, available at www.exphem.org).

MNC-RED protocol

To determine if we could differentiate adult PB MNCs under chemically defined conditions, we designed MNC-RED (Figure 7A). In this protocol, MNCs are differentiated into day 14 erythroblasts using the same cytokine cocktails as in our standard protocol, but StemSpan is replaced with IMIT and the day 14 erythroblasts are then differentiated into enucleated RBCs in R6 medium using the optimal conditions described above.

Expanding and differentiating MNCs from three healthy individuals in albumin-free, chemically defined conditions proved highly efficient (Figure 7B) as the MNC-RED protocol yielded 1293 ± 739 cRBCs/MNC (average ± SD) as compared with 465 ± 156.5 cRBCs/MNC when cells from the same individuals were grown using our standard protocol. The difference in yield was statistically significant at days 21 and 25 (Supplementary Figure E12, online only, available at www.exphem.org). At day 32, the rate of enucleation with the R6 medium reached 94% in one individual and averaged 79.3 ± 18.46% (average ± SD), as compared with 57.1 ± 20.75% when albumin-containing

(B) Micrographs of rapid Romanowsky stains illustrating the morphology of iPSC-derived enucleated cRBCs before and after filtration through a PALL Acrodisc WBC filter to eliminate the nucleated cells and the expelled nuclei. Box-and-whisker plots on the bottom illustrate the diameter of the iPSC-derived cRBCs as compared with in vivo produced adult RBCs. The *boxes* represent the 25% and 75% percentiles; the *line inside the box,* the mean; and the *whiskers*, the extremes of the distribution. **(C)** FACS plots illustrating enucleation rates as determined by DNA content measurement observed with the short and long PSC-RED protocols and an extra-long protocol during which the cells were incubated for 2 weeks in supplement S4. Rates of enucleation increase dramatically when iPSC-derived hematopoietic progenitors are allowed to proliferate in an undifferentiated state in supplement S4. **(D, E)** Histograms and HPLC chromatograms illustrating globin expression of cRBCs obtained using the short or long PSC-RED protocols. Cells obtained after the long PSC-RED protocol are more developmentally mature (see also Supplementary Figure E9). Globin expression phenotype is represented as percentage α-like or β-like globin. Percentage α-like = 100 * (α or ζ)/($\alpha + \zeta$). Percentage β-like =100 * (ε, (Gα + Aγ), or β)/(ε + Gγ + Aγ + β). Data are expressed as the average ± SEM of two independent experiments. **(F)** Histograms illustrating the number of cRBCs/iPSCs observed during differentiation in albumin-containing media (Stemline II and IBIT) or using the short or long PSC-RED protocols. Data are expressed as the average ± SEM of three to six independent experiments. Both PSC-RED protocols yield significantly more cRBCs than the albumin-containing media.

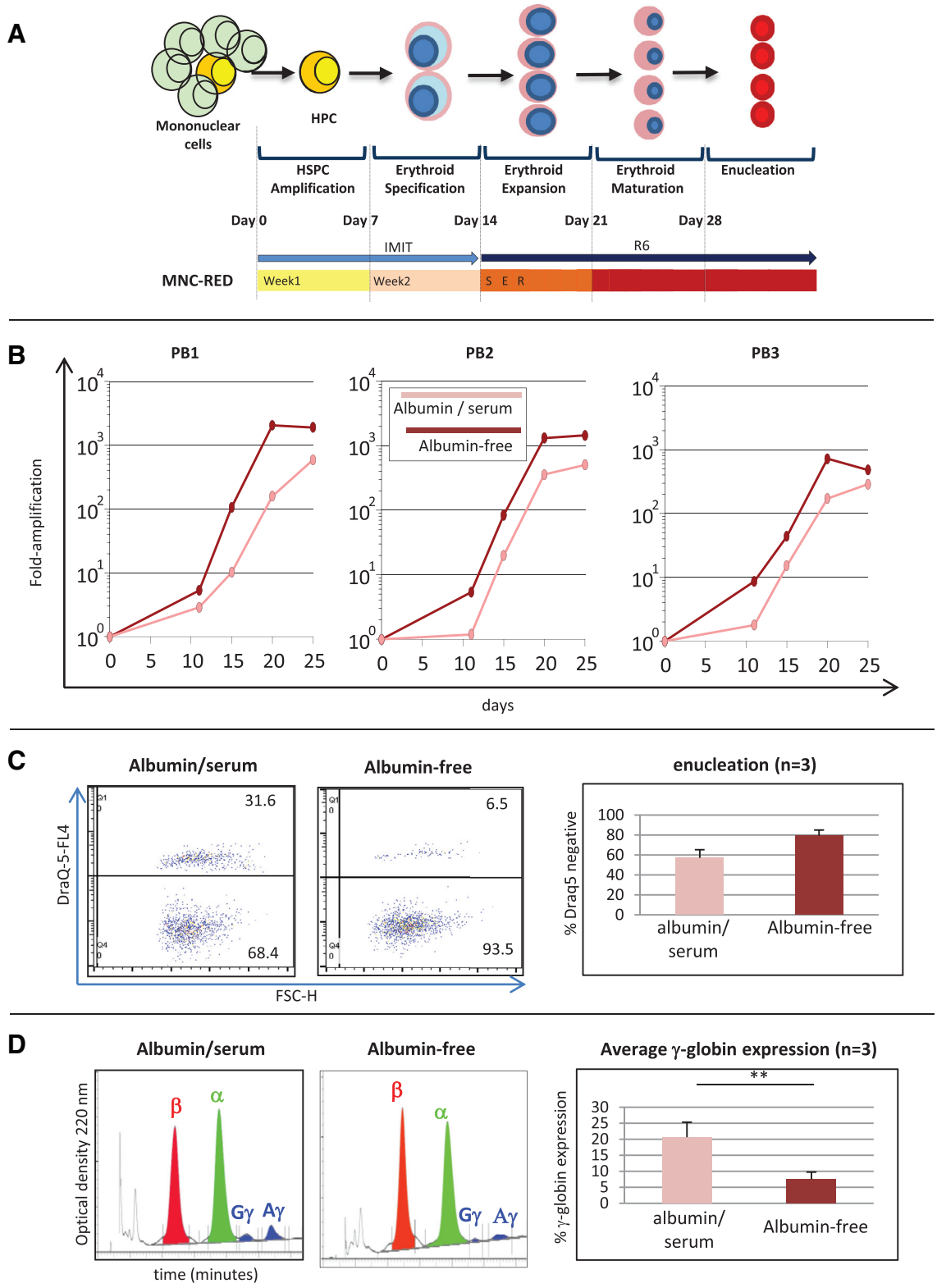

Figure 7. MNC-RED protocol used to generate enucleated cRBCs from PB MNCs. (A) Graph summarizing the MNC–RED protocol. Compositions of media and supplements can be found in Table 1. **(B)** Scatterplots illustrating the growth of PB MNC cells collected from three healthy individuals and incubated for 28 days using the MNC-RED protocol or in albumin-containing media using the standard protocol. The yield of enucleated erythroid cells in the MNC-RED protocol was higher for all three individuals. When the data from the three individuals were averaged, the difference between the two protocols was significant (Supplementary Figure E5). **(C)** cRBCs produced using either the albumin-containing or albumin-free protocol were analyzed by flow cytometry on day 32 of the protocol to assess the rate of enucleation using nuclear stain

medium was used (Figure 7C). This difference, however, did not reach statistical significance (*t*-test *p* value = 0.23).

To determine if globin expression was affected when MNCs were differentiated using either the MNC-RED or our standard serum-containing protocol, globin expression was analyzed by HPLC. This revealed that the cRBCs produced using MNC-RED expressed mostly HbA and an average of 7.3 ± 3.8% HbF (average ± SD), whereas the cells produced using our standard protocol expressed 20.6 ± 8.0% HbF. This difference was significant (*t*-test *p* value = 0.008; Figure 7D). We concluded that the cells produced using MNC-RED express smaller amounts of HbF than cells produced with the standard protocol. The former cells are therefore more similar to cells produced in vivo, but are still overexpressing HbF, because in vivo produced cells generally express <1% HbF.

Discussion

We have developed R6 and IMIT, two chemically defined cell culture media suitable for iPSC differentiation and erythroid culture. R6 and IMIT are inexpensive because they contain no albumin and low concentrations of Optiferrin. They are nevertheless suitable to produce cRBCs because the presence of FeIII-EDTA allows the recycling of Optiferrin. We have combined these media into RED protocols for the production of large numbers of enucleated cRBCs from iPSCs and MNCs. The RED protocols should have increased reproducibility compared with previously published methods because the variability associated with the unpredictable quality of animal- or human-derived components is eliminated. Despite the very small number of components used in these protocols, they yield more cells than did our previous protocols at most steps of the differentiation.

Terminal differentiation

We have observed that terminal differentiation can be improved by withdrawing the glucocorticoid stimulation before removing SCF and EPO. Bauer et al. reported that the glucocorticoid receptor is required for stress erythropoiesis but not steady-state erythropoiesis [51]. Stellacci et al. found that the glucocorticoid receptor directly interacts with the EPO receptor and that combined Dex and EPO stimulation inhibits some but not all of the effects of EPO, which leads to a delay in pro-erythroblast maturation and erythroid expansion [52]. Eliminating Dex while maintaining EPO and SCF stimulation, in cells that have already been subject to a long exposure to glucocorticoids, might allow stress erythroblasts to slowly re-enter the normal erythroid differentiation pathway and resume their terminal differentiation in a more efficient manner than when all three components of the SED cocktails are removed at the same time.

In serum-free culture, one of the functions of albumin is to provide lipids which are contaminants of partially purified albumin preparation. In both R6 and IMIT, lipids are provided by a commercially available chemically defined lipid emulsion. Recent publications have reported that the lipid composition of the culture media has a profound effect on hematopoietic cells in culture [53]. In particular, it was recently suggested that the presence of dexamethasone predisposes human erythroblasts to impaired lipid metabolism [54]. A possible influence of glucocorticoid stimulation on terminal differentiation was also supported by a 2006 study that found that RU486 was useful in obtaining cRBCs that enucleated at a high rate [8]. The improved cell yield that we observed by withdrawing the glucocorticoid stimulation before the Epo and SCF stimulation might therefore also be caused by a greater cell membrane stability of the resulting cRBCs.

Hemoglobin expression

We previously reported that longer iPSC differentiation protocols are associated with the production of fetal hemoglobin rather than embryonic hemoglobin, recapitulating some of the globin switches that occur early in development [23]. HPLC analysis of globin expression of cRBCs obtained with the short and long PSC-RED protocols confirm these early observations, and clearly indicate that a week-long expansion of HPCs in S4 is sufficient to obtain HbF-expressing cRBCs.

The MNC-RED protocol produces cells that express relatively small amounts of HbF as compared with serum-containing protocols, maybe because, as previously reported, elimination of undefined components from erythroid cultures can yield cRBCs with more physiological levels of HbF [55].

Improved enucleation

The mechanisms by which PSC-RED improves the rate of enucleation are not clear. The observation that cells produced with the short protocol do not enucleate suggests that the increase in the rate of enucleation reflects

Draq5 (and propidium iodide to exclude the dead cells). FACS plots illustrating the enucleation rate for individual PB1 (measured at day 32). Right: Histogram illustrates the rates of enucleation (±SEM) in albumin-free and albumin-containing media averaged for the three individuals ($n = 3$). For all three individuals, cells grown using the MNC-RED protocol exhibited a higher rate of enucleation but the difference did not reach significance. **(D)** Chromatograms illustrating globin chain expression in the cRBCs derived from individual PB1 in albumin-containing and albumin-free media. Right: Histogram illustrates the significant difference in the percentage expression of gamma globin in albumin-free and albumin-containing media for the three individuals tested ($n = 3$; paired *t*-test *p* value = 0.0345). Percentage γ-globin expression was calculated as $100 * (G\gamma + A\gamma)/(G\gamma + A\gamma + \beta)$.

the emergence, or the preferential amplification, of more definitive progenitors during the week of culture in S4. The increased overall length of the protocol might also select for more developmentally mature cells, as primitive cells likely differentiate and die before the end of the culture. Finally, elimination of inhibitory compound(s) present in undefined medium components might also contribute to the increased rate of enucleation.

Cost of producing cRBCs

The cost of cRBC production for clinical applications has been considered to be a major limitation of the technology. Most of this cost occurs at the end of the protocol because of the exponential growth of culture volumes. Transferrin and albumin were particularly expensive components of previous protocols and represented 30% to 40% of the total cost of cRBC production because they are required in large amounts up to the end of the culture [3]. The last few weeks of the RED protocols require only a commonly used base medium, a few small molecules, small amounts of recombinant Tf, and two cytokines, EPO and SCF. These two cytokines are in the public domain and relatively simple to produce in large amounts. When purchased in bulk, they represent about 36% of our current cRBC production reagent costs. All other necessary cytokines and small molecules, which are required in much smaller amounts, represent 7% of reagent costs.

The RED protocols are scalable and compatible with high-cell-density fed-batch bioreactor systems because they do not require any feeder layers, because changes in medium composition can be achieved by simple medium addition, and because the cells grow in suspension for the entire culture period in the case of MNC-RED and starting at day 10 in the case of PSC-RED.

In summary, the low cost and scalability of the RED protocols should make testing applications of cRBCs in small clinical trials more economically feasible.

Potential application of the RED protocols

The MNC-RED and PSC-RED protocols will likely prove useful in the study of RBC disease mechanisms [56] and in helping to decipher transduction and enucleation mechanisms during erythroid differentiation because the complete elimination of all undefined components provides an ideal platform for mechanistic studies.

A very promising application of the long PSC RED protocol might be the generation of drug-carrying cRBCs because it yields enucleated cRBCs starting from iPSCs, an immortal cell type that can be easily and precisely genetically modified to differentiate into cRBCs that carry a useful cargo. As discussed in the introduction, genetically engineered cRBCs are highly attractive applications of the technology because delivering drugs through therapeutic cRBCs requires much fewer cells than transfusion applications.

Acknowledgments

SZ, ENO, ZY, SZ, KR, KW, and EEB were supported in part by grants NYSTEM C030135, C029154; NIH HL130764; and Doris Duke Foundation 2017087. We thank Connie Westhoff from the New York Blood Center for providing samples.

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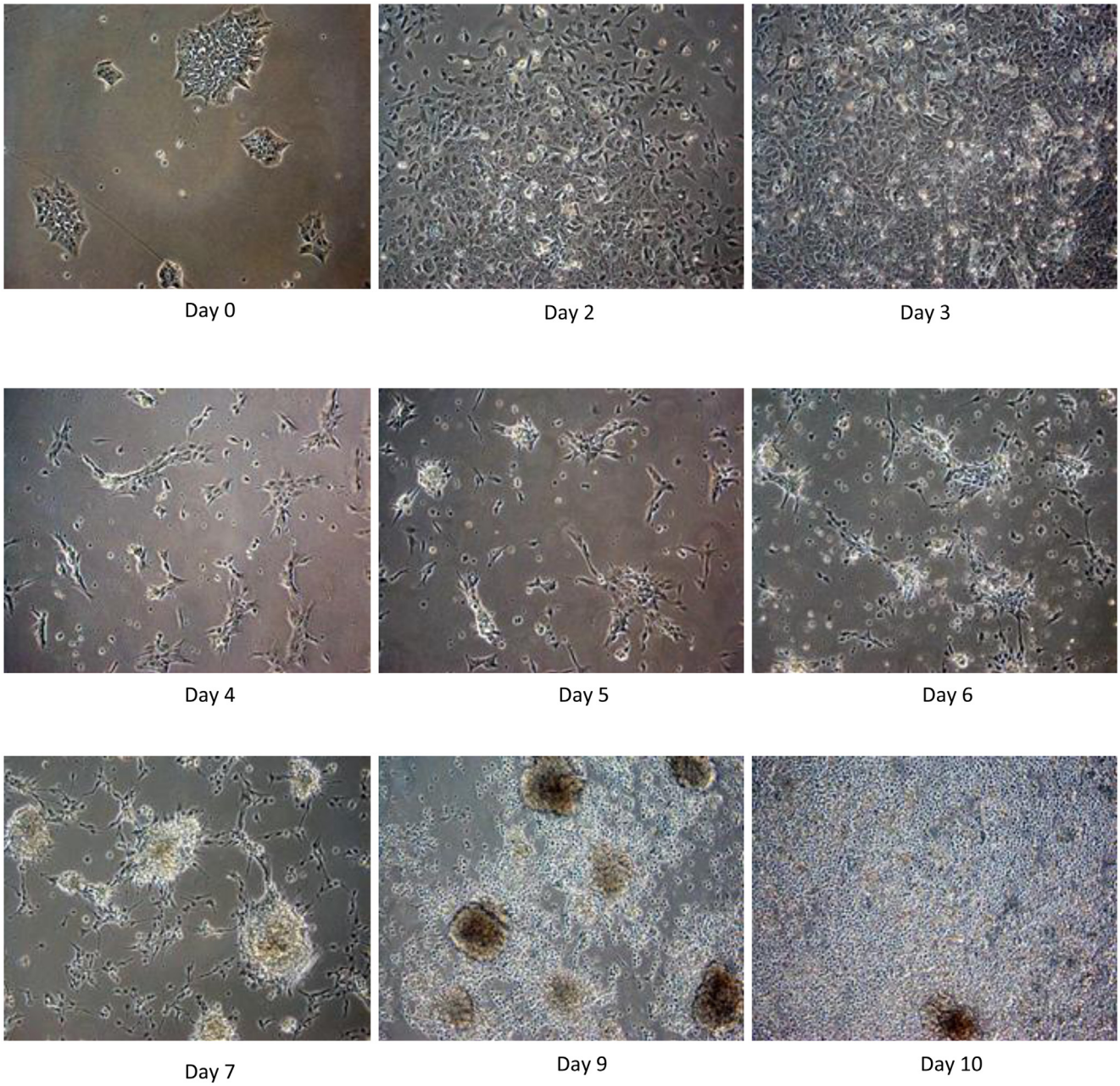
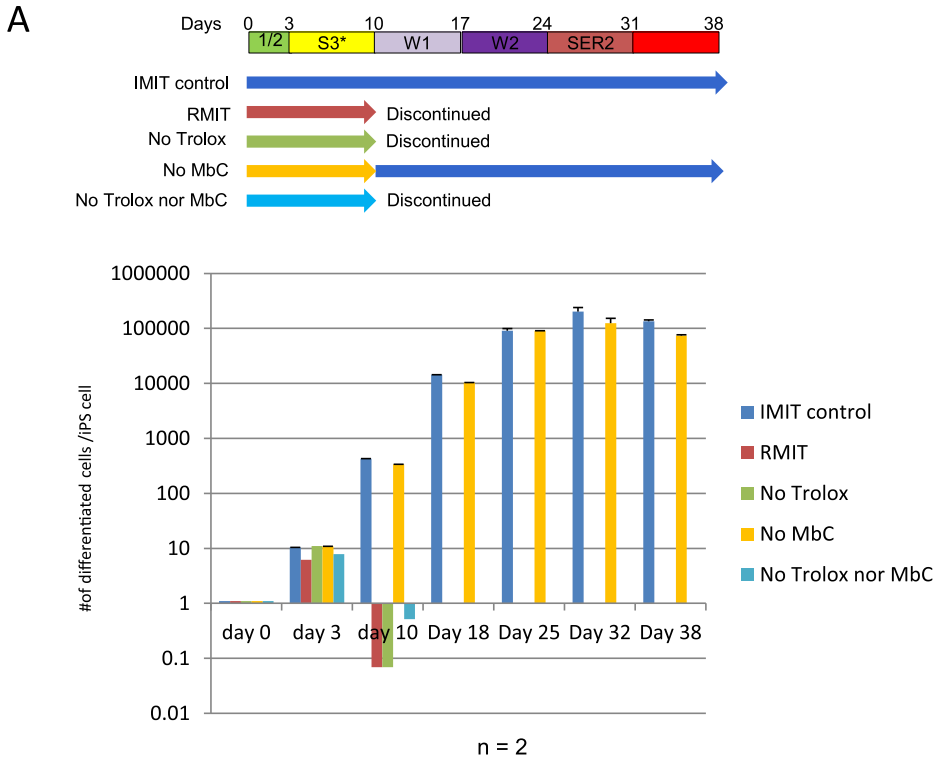


Figure E1. Phase contrast micrographs (x40 magnification) of cells in culture during the first 10 days of differentiation. At day 0 the cells are small colonies of iPSC undifferentiated, by day 2 they already exhibit a changed morphology with larger and more elongated cells. The differentiation continues on day 3 with the emergence of endothelial looking cells. On day 4 after dissociation and replating the cells arrange in small clusters of elongated cells, from these cluster on days 5, 6 and 7, emerge round and bright cells. The round and bright cells divide very actively. Tens of millions of cells can be obtained by day 10 when starting with 100,000 iPSCs in 1 well at day 0.



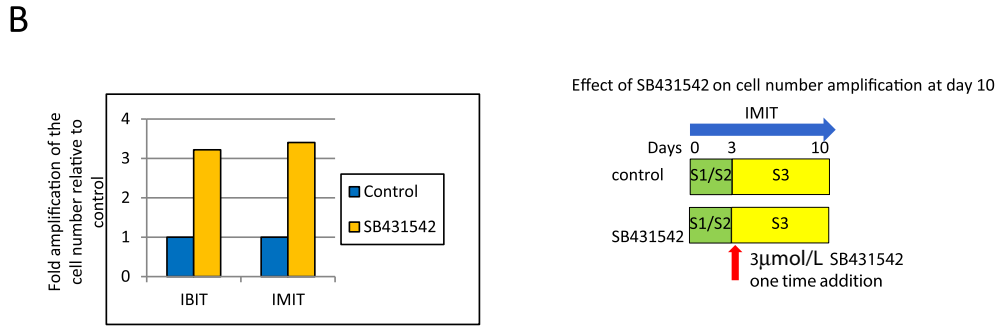


Figure E2. Development of IMIT. **A:** Components of the IMIT medium were tested for their importance in supporting iPSC differentiation toward erythroid cells. Top: Schematic of the differentiation conditions. Bottom: bar graph illustrates the number of differentiated cells/iPSC obtained after 38 days of differentiation (n=2). Trolox and the use of IMDM (rather than RPMI) are absolute requirements since all cells were dead after day 10 (compare IMIT control, with RMIT or no Trolox). Methyl-β-cyclodextrin (MbC) had a noticeable but non-significant effect on the yield of cells. **B: Left,** bar graph illustrating the increased number of cells at day 10 after a single pulse of SB431542 at day 3 whether IBIT or IMIT is used as expansion medium. **Right:** Schematic of the differentiation conditions.

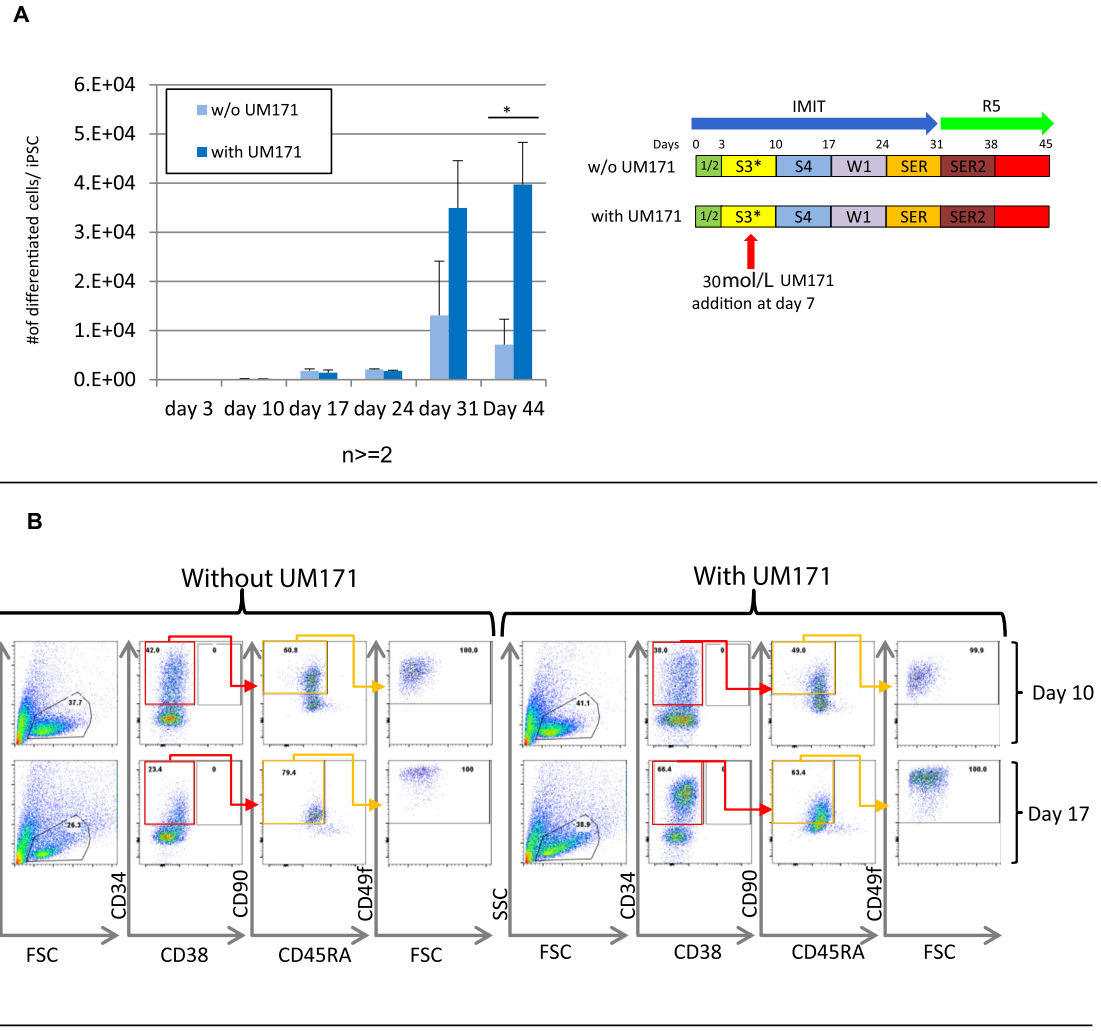


Figure E3. Optimization of the HSC expansion steps **A: Left** Diagram summarising the effect of a single addition of UM171 on the number of cells/iPSC produced in an early version of the long differentiation protocol, n≥2. **Right:** Culture condition schematic. **B**: Dot plots illustrating flow cytometry analysis of the expression of CD34, CD38, CD45RA, CD90 and CD49f after a single dose of UM171 at day 7 as described in the graph above. In the presence of UM171, cells retain a much more undifferentiated phenotype, as determined by the proportion of cells expressing CD34, CD49f and CD90 at day 17, data representative of 2 or more experiments.

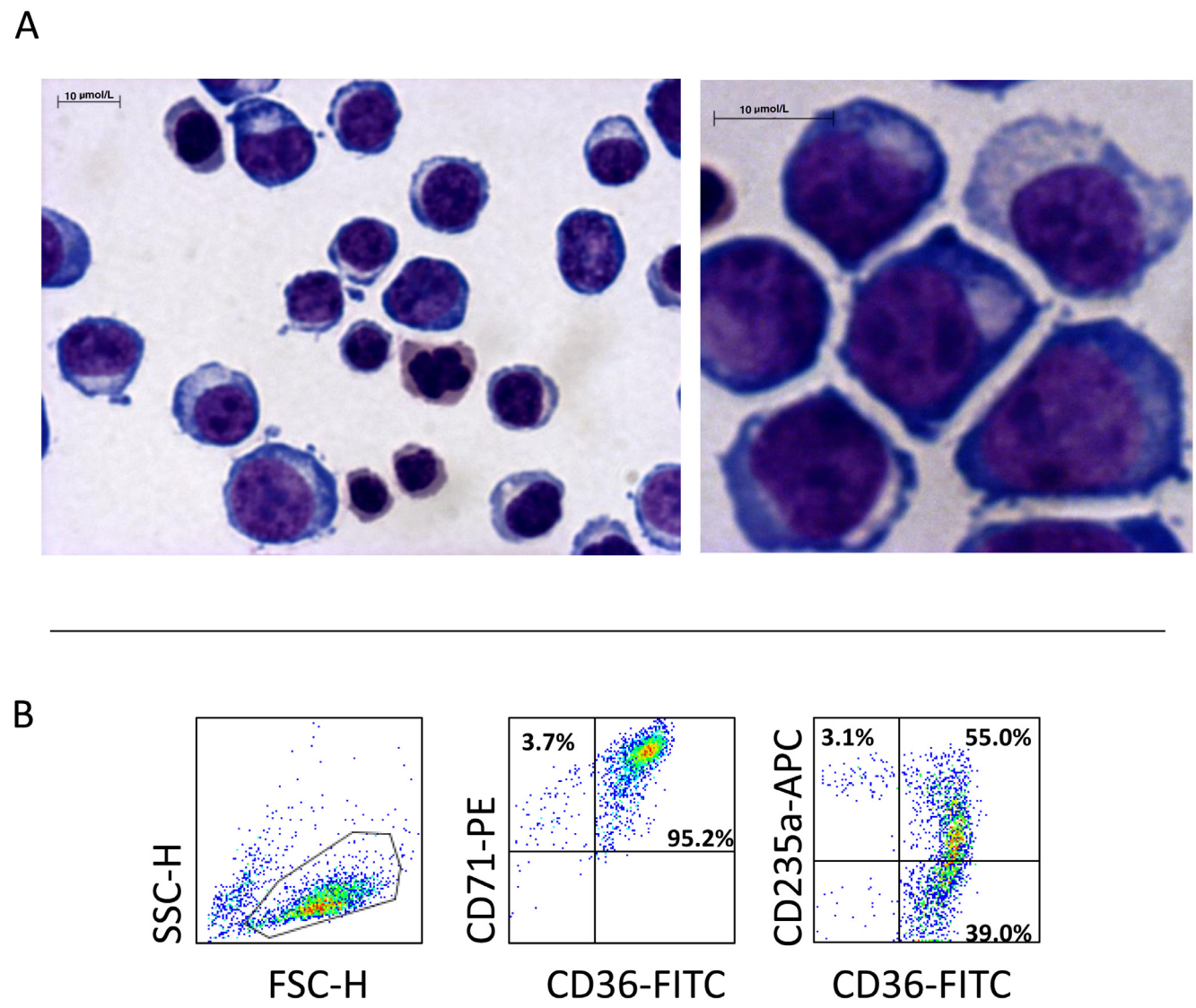


Figure E4. day-14 erythroblasts. A: Micrograph illustrating the typical morphology of day-14 erythroblasts after rapid Romanowsky staining. Most cells are pro-and basophilic erythroblasts but up to 15% of the cells are more mature polychromatophilic and orthochromatophilic erythroblasts. B: Flow cytometry. Dotplot illustrating a representative example of expression of CD71, CD36 and CD235a in day-14 erythroblasts. About 98.9% of the cells express CD71, 95.2% CD36 and 58.1% CD235a (n = 2). This data supplement the Romanowsky staining and confirms that most of the cells are committed to the erythroid pathway at this point of time in the culture.

A

	days	p-value	q-value
1 vs 3 STD vs SED	17	0.0107	0.0168
	19	0.0039	0.0119
	21	0.0447	0.0470
	23	0.0153	0.0193
	25	0.0057	0.0119
	28	0.0003	0.0016
1 vs 4 STD vs SE	17	0.0093	0.0584
	19	0.3093	0.3248
	21	0.0639	0.0806
	23	0.0387	0.0609
	25	0.0294	0.0609
	28	0.0319	0.0609
1 vs 5 STD vs SED	17	0.0552	0.0232
	19	0.1707	0.0597
	21	0.0024	0.0019
	23	0.0000	0.0001
	25	0.0034	0.0019
	28	0.0037	0.0019
2 vs 3 (STD +SED) vs SED	19	0.1219	0.1280
	21	0.0899	0.1180
	23	0.0587	0.1027
	25	0.0299	0.0784
	28	0.0005	0.0026
2 vs 5 (STD +SED)vs SER	17	0.0906	0.0381
	19	0.2131	0.0746
	21	0.0036	0.0030
	23	0.0001	0.0003
	25	0.0173	0.0091
	28	0.0042	0.0030
3 vs 5 SED vs SER	17	0.1289	0.2103
	19	0.2737	0.2874
	21	0.0424	0.1113
	23	0.0021	0.0108
	25	0.1602	0.2103
	28	0.3480	0.3045

Figure E5. p-and q-value for Figure 1A, 1C, 2, 3A, 3B and S4. t-test p-values were calculated for each pair of conditions. The q-values were calculated using the Two-stage step-up method of Benjamini, Krieger and Yekutieli using a desired FDR of 5%. P- and q-values were calculated systematically for all combination of conditions. For each experiment, conditions in which none of the row were significant are not shown. A: In figure 1A, the growth rate in condition 1 was significantly different from that of conditions 3 and 5 at most of the days tested. Condition 2 was significantly different from condition 5 at days 21 to 25; (n = 3). B: In figure 1C, the proportion of the various erythroid precursor during the time course of the experiment was significantly different at multiple days between conditions 1 and 3, 1and 4, 1 and 5, 2 and 3, 2 and 4 and 2 and 5. The number of enucleated cells (retic) was also significantly different between conditions 3 and 5. C: In figures 2 and S4, Ethanolamine proved to be toxic (n = 2) while acid ascorbic (n = 5) and lipids (n = 2) improved cell yields. R5 yielded more cells that R8 (n >= 6). D: In figure 3A, the growth rate of the cells was significantly different at multiple days between conditions (1 and 2), (1 and 3), (2 and 3), (2 and 4) and (2 and 5); (n = 3). We concluded that eriochrome cyanine and FeIII-EDTA could both be used to provide iron in erythroid culture and allow the growth and differentiation of the cells with lower amounts of transferrin (n = 2). E: In figure 3B, cells could not grow in the absence of Optiferrin and the yield of cells was lower when the concentration of Optiferrin was 6.25μg/mL (n = 2).

B

	day 17		day 19		day 21		day 24		day 28	
	p-value	q-value	p-value	q-value	p-value	q-value	p-value	q-value	p-value	q-value
1 vs 3										
proE	0.5028	0.6534	0.1284	0.4026	0.0095	0.0150	0.1778	0.1400		
basoE	0.6223	0.6534	0.2576	0.4026	0.0068	0.0150	0.0002	0.0007	0.1778	0.1467
polyE	0.3257	0.6534	0.3067	0.4026	0.2640	0.1733	0.0132	0.0208	0.0012	0.0006
orthoE	0.4414	0.6534	0.2877	0.4026	0.2751	0.1733	0.6035	0.3802	0.0080	0.0028
Retic	0.5415	0.6534	0.6302	0.6617	0.1133	0.1190	0.0454	0.0477	0.0011	0.0006
1 vs 4										
proE	ND	ND	ND	ND	ND	ND				
basoE	ND	ND	ND	ND	ND	ND	0.0000	0.0001		
polyE	ND	ND	ND	ND	ND	ND	0.0027	0.0029	0.6636	0.2323
orthoE	ND	ND	ND	ND	ND	ND	0.2243	0.1178	0.0181	0.0095
Retic	ND	ND	ND	ND	ND	ND	0.0389	0.0272	0.0138	0.0095
1 vs 5										
proE	0.4868	0.8520	0.2057	0.4704	0.0002	0.0006	0.1037	0.1452		
basoE	0.8464	0.8887	0.2431	0.4704	0.0038	0.0060	0.0394	0.0828	0.3559	0.2803
polyE	0.4266	0.8520	0.3205	0.4704	0.2901	0.1828	0.0002	0.0009	0.0240	0.0378
orthoE	0.7996	0.8887	0.3584	0.4704	0.1342	0.1409	0.5027	0.4223	0.0519	0.0545
Retic	0.4366	0.8520	0.7888	0.8282	0.2516	0.1828	0.1498	0.1573	0.0087	0.0274
2 vs 3										
proE	0.5065	0.6808	0.0447	0.1174			0.2722	0.2481		
basoE	0.6483	0.6808	0.0098	0.0513	0.0182	0.0064	0.0002	0.0008	0.2722	0.1429
polyE	0.5203	0.6808	0.9033	0.9485	0.0028	0.0026	0.0817	0.1715	0.0097	0.0155
orthoE	0.4851	0.6808	0.1338	0.2341	0.0049	0.0026	0.1408	0.1971	0.2631	0.1429
Retic			0.4544	0.5964	0.4085	0.1072	0.2953	0.2481	0.0147	0.0155
2 vs 4										
proE	ND	ND	ND	ND	ND	ND				
basoE	ND	ND	ND	ND	ND	ND	0.0002	0.0002		
PolyE	ND	ND	ND	ND	ND	ND	0.0190	0.0066	0.6876	0.7220
orthoE	ND	ND	ND	ND	ND	ND	0.0031	0.0016	0.3999	0.6299
Retic	ND	ND	ND	ND	ND	ND	0.7182	0.1885	0.0506	0.1593
2 vs 5										
proE	0.6023	0.9459	0.0115	0.0181	0.0007	0.0003	0.1830	0.2402		
basoE	0.9009	0.9459	0.0077	0.0181	0.0068	0.0018	0.0820	0.1435	0.4366	0.6112
polyE	0.5664	0.9459	0.8187	0.5362	0.0003	0.0002	0.0215	0.0565	0.1658	0.3481
orthoE	0.7742	0.9459	0.1463	0.1536	0.0000	0.0000	0.0103	0.0541	0.7418	0.7789
Retic			0.8512	0.5362	0.1109	0.1233	0.7606	0.7986	0.0230	0.0965
3vs 5										
proE	0.8479	0.9013	0.4343	0.9583	0.0133	0.0700	0.3753	0.5254		
basoE	0.5874	0.9013	0.9126	0.9583	0.2320	0.3046	0.9999	0.8400	0.3552	0.1865
polyE	0.8584	0.9013	0.8093	0.9583	0.0878	0.1536	0.8922	0.8400	0.0571	0.0400
orthoE	0.7843	0.9013	0.7706	0.9583	0.7087	0.7441	0.1679	0.3527	0.0136	0.0143
retic			0.5149	0.9583	0.0796	0.1536	0.0059	0.0246	0.0038	0.0080

Figure E5. Continued.

C

	days	p-value	q-value
Ethanolamine in R8 1.6 mmol/Lvs 0 mmol/L	17	0.521698	0.273891
	21	0.000043	0.000045
	23	0.000546	0.000382
	25	0.000004	0.000008
R5 vs R5 - acid ascorbic	16	0.000000	0.000000
	19	0.006416	0.004491
	21	0.014597	0.007663
	23	0.029402	0.012349
	26	0.050052	0.017518
	28	0.004295	0.004491
R5 vs R5 - lipids	17	0.516746	0.180861
	19	0.006330	0.002658
	21	0.000077	0.000040
	23	0.000001	0.000001
	25	0.000004	0.000004
	28	0.000035	0.000025
R5 versus R8	16	0.81292	0.24388
	19	0.00003	0.00006
	21	0.00052	0.00055
	23	0.00147	0.00103
	26	0.00375	0.00158
	28	0.00342	0.00158

D

	days	p-value	q-value
1 vs 2 200 vs 50 µg/mL holo-Tf	16	0.8053	0.1691
	19	0.0048	0.0012
	21	0.0000	0.0000
	23	0.0000	0.0000
	28	0.0000	0.0000
1 vs 3 200 µg/mL holo-Tf vs 50 µg/mL holo-Tf +3 µmol/L ATA	16	0.9437	0.5945
	19	0.3285	0.2587
	21	0.0010	0.0033
	23	0.0051	0.0080
	28	0.0563	0.0591
2 vs 3 50 µg/mL holo-Tf vs 50 µg/mL holo-Tf +3 µmol/L ATA	16	0.7752	0.1628
	19	0.0012	0.0003
	21	0.0000	0.0000
	23	0.0000	0.0000
	28	0.0000	0.0000
2 vs 4 50 µg/mL holo-Tf vs 50 µg/mL holo-Tf +3 µmol/L ATA Eryochrome cyanide	16	0.4411	0.0926
	19	0.0007	0.0002
	21	0.0000	0.0000
	23	0.0000	0.0000
	28	0.0000	0.0000
2 vs 5 50 µg/mL holo-Tf vs 50 µg/mL holo-Tf +3 µmol/L FeIII-EDTA	16	0.8456	0.3551
	19	0.0651	0.0342
	21	0.0009	0.0007
	23	0.0001	0.0001
	28	0.0011	0.0007

Figure E5. Continued.

E

	days	p-value	q-value
Optiferrin 50 mg/mL vs 6.25 mg/mL	19	0.1441	0.1513
	21	0.0970	0.1357
	24	0.0862	0.1357
	28	0.2715	0.2280
	31	0.0034	0.0142
Optiferrin 50mg/mL vs 0 mg/mL	19	0.0454	0.0477
	21	0.0215	0.0282
	24	0.0057	0.0123
	28	0.0070	0.0123
	31	0.0010	0.0053
Optiferrin 25mg/mL VS 0 mg/mL	19	0.0823	0.0173
	21	0.0261	0.0069
	24	0.0066	0.0023
	28	0.0045	0.0023
	31	0.0037	0.0023
Optiferrin 12.5mg/mL vs 0 mg/mL	19	0.1043	0.0219
	21	0.0379	0.0100
	24	0.0091	0.0048
	28	0.0073	0.0048
	31	0.0146	0.0051
Optiferrin 6.5mg/mL vs 0 mg/mL	19	0.1259	0.0529
	21	0.0532	0.0280
	24	0.0159	0.0111
	28	0.0126	0.0111
	31	0.0019	0.0041
0mg/mL vs 50mg/mL (Holo-Tf)	19	0.0681	0.0179
	21	0.0124	0.0061
	24	0.0048	0.0051
	28	0.0174	0.0061

Figure E5. Continued.

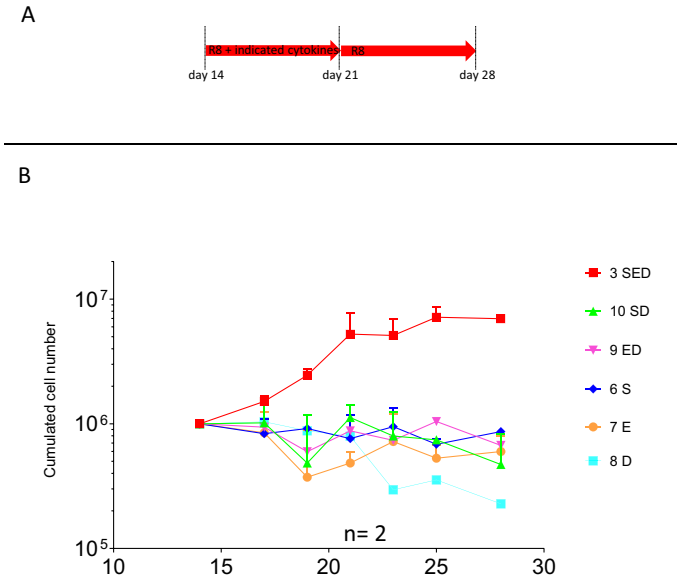


Figure E6. Effects of the SED components. A: Culture conditions: 14-days erythroblasts were grown in R8 for 7 days in the presence of the indicated cytokines followed by 7 days in R8 alone. B: Growth rates observed in the presence of the SED cocktail, of SCF alone (S), EPO alone (E), Dex alone (D), SCF and Dex (SD) or EPO and Dex (ED). 14-days erythroblasts expanded in the presence of the SED cocktail (see Figure 1A), but very little or not at all, in all other conditions tested in this figure. We concluded that in the absence of serum, 14-day erythroblasts can significantly expand and terminally differentiated without significant loss of cells in the presence of SCF and EPO, but not if either of these cytokines is absent (see main text).

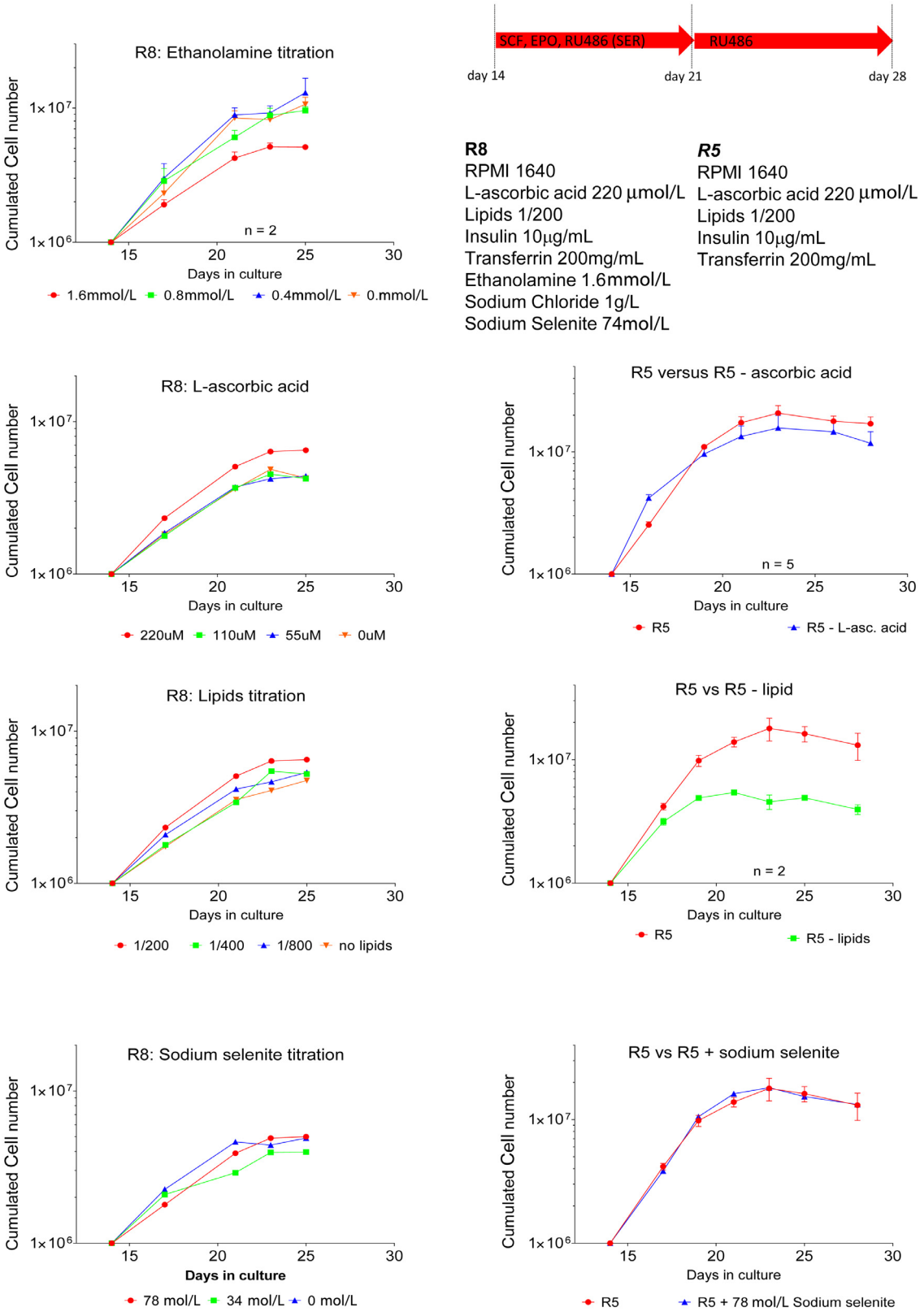


Figure E7. Analysis of the component of the R8 medium. Diagram on the top right illustrating the cytokine cocktails used in the experiments. 3.10^6 day-14 erythroblasts, were plated for 7 days in various base media (derived either from R5 or R8) in the SER cytokine cocktail for 7 days, followed by 7 days in the same conditions but without SCF or EPO. All other graphs illustrate the cumulated number of live cells as a function of days in cultures. Top left: Cells grown in R8 with 1.6mM ethanolamine grew less rapidly that with no ethanolamine (n=2, q-value

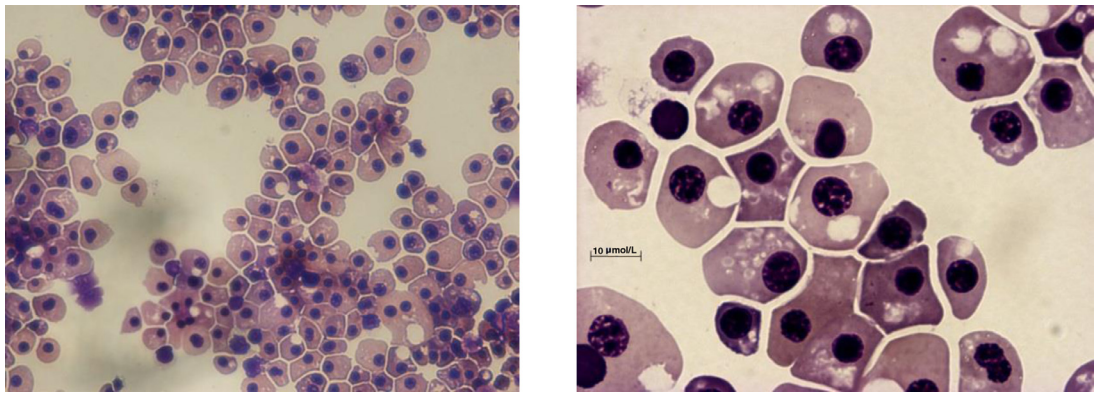


Figure E8. Micrographs of rapid Romanowsky stain illustrating the morphology of iPSC-derived cRBCs obtained at the end of the short PSC-RED protocol. Most of the cells are orthochromatic erythroblasts and have a primitive morphology.

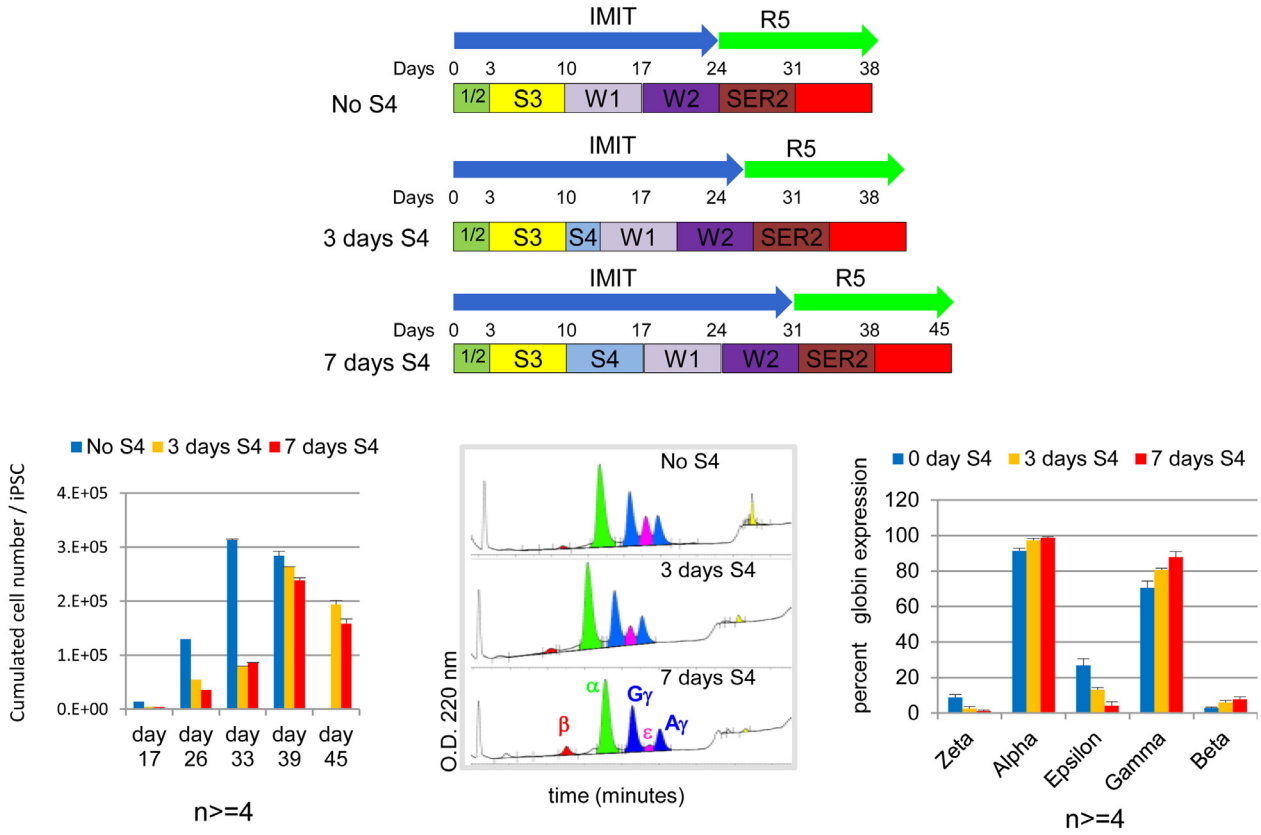


Figure E9. Expansion and globin expression as a function of time in S4. **Top:** Culture conditions schematic. **Bottom left:** Diagram illustrating the yield of cells/iPSC for up to 45 days either without, or with 3 or 7 days incubation in supplement S4 (containing UM171). **Middle**, HPLC chromatograms, and **right** bar graph illustrating the proportion of embryonic (epsilon and zeta), fetal (gamma and alpha) and adult globin (beta and alpha) produced as a function of time in S4 supplement. Progenitors cultured the longest in S4, exhibit a more mature phenotype characterized by a sharp decrease in epsilon- and zeta-globin chain expression, and an increase in alpha-, gamma- and beta-globin chain expression, n≥4.

<0.001 9FDR < 5%) for days 21 to 28). Second row: Cells were grown in R8 containing decreasing amount of L-ascorbic acids (left) or in R5 with or without L-ascorbic acid. Cells grown in R5 without L-ascorbic acid grew significantly less than in the presence of L-ascorbic acid (n=5, q-value <0.001 at days 21 to 28 (FDR <5%). Third row: Cells were grown in R8 containing decreasing amount of lipids (left) or in R5 with or without lipids (right) (n=2, q-value <0.001 at days 21 to through 28). In both cases grew better in the presence of lipids. Last row: Cells were grown in R8 containing decreasing amount of Sodium selenite (left) or in R5 with or without sodium selenite

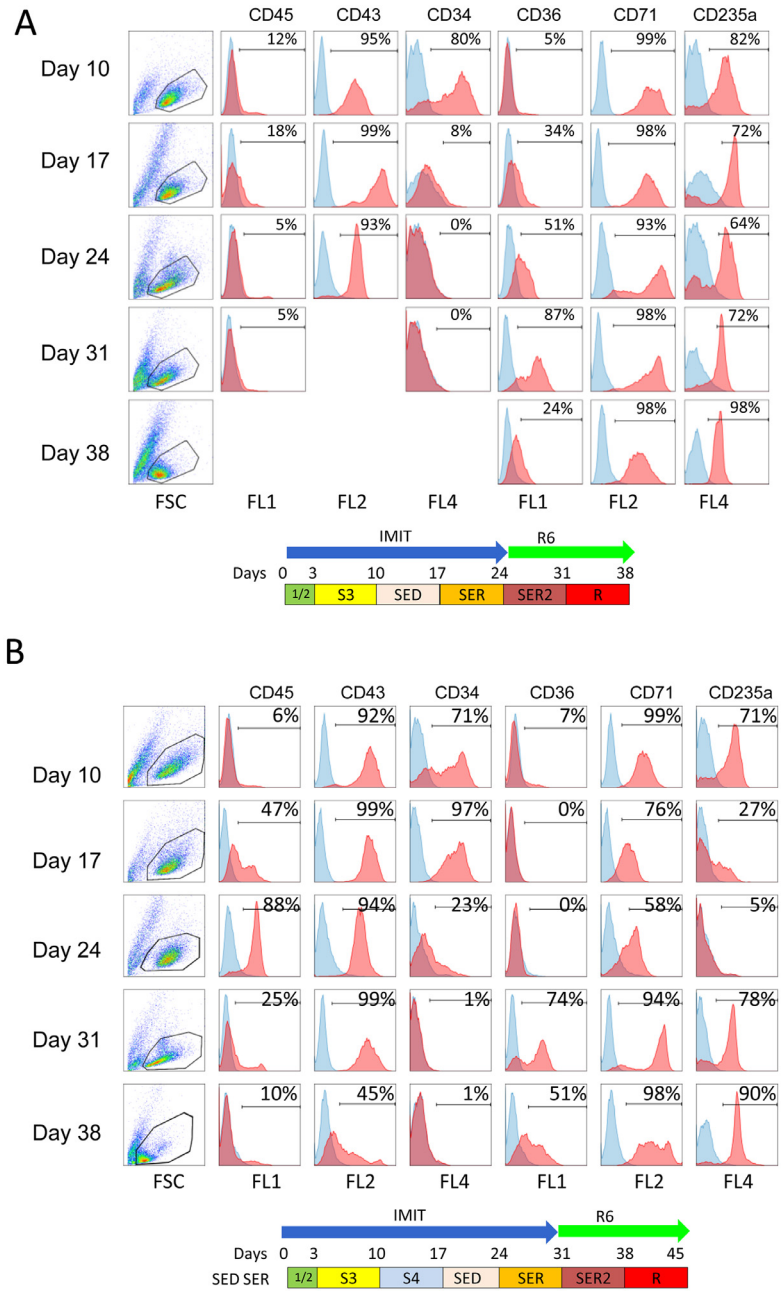
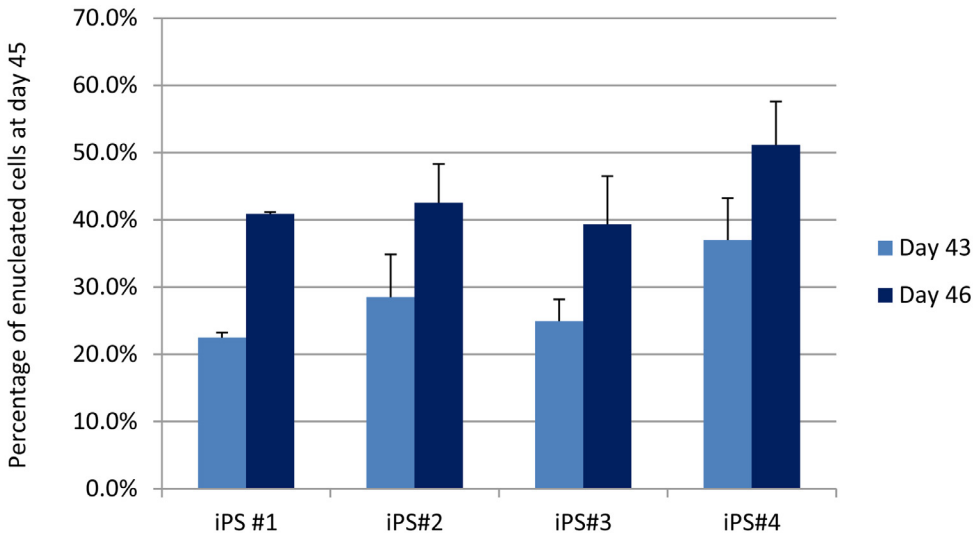
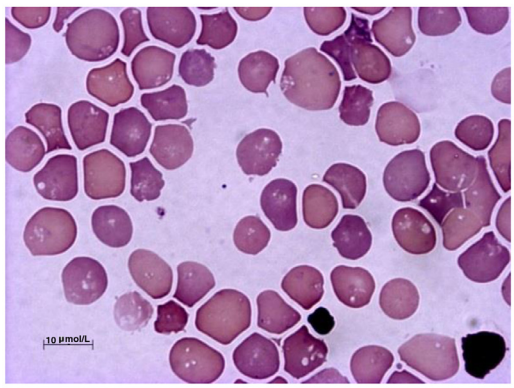


Figure E10. Phenotypic analysis of the cell markers during the PSC-RED protocols. **A: Flow cytometry analysis during the Short PSC-RED protocol:** Representative flow cytometry plots depicting the expression kinetic of hematopoietic and erythroid markers during the short PSC-RED protocol. Cells are gated by size (FSC) and granularity (SSC) to exclude debris, dead and dying cells. For each marker the corresponding isotype control is shown in blue. At day 10, the cells are almost all hematopoietic as indicated by CD34 and CD43. During the differentiation, CD235a is expressed on most cells at all time and expression of CD45 never takes off. CD36 expression culminates at day 31 before receding during the cell maturation process. At day 38 the cells are almost all erythroid as indicated by CD36, CD71 and CD235a expression. The culture conditions schematic is shown under the plot and the data are representative of 3 or more experiments. **B: Flow cytometry analysis during the long PSC-RED protocol:** Representative flow cytometry plots depicting the expression kinetic of hematopoietic and erythroid markers during the long PSC-RED protocol. Cells are gated by size (FSC) and granularity (SSC) to exclude debris, dead and dying cells. For each marker the corresponding isotype control is shown in blue. Cells are almost 100% CD45 positive at day 24 but loose expression of this marker as erythroid differentiation progresses. Starting at day 10, most cells in the culture are hematopoietic as determined by expression of CD43. Expression of CD34 peaks at day 17, when it reaches almost 100%. Expression of this marker decreases rapidly when the cells are placed in the SED conditions. CD36 expression is first detected at day 31 and decreases at day 38 as the cells enter their final maturation. CD71 expression is complex because this marker is expressed in all cells at low levels, at very high levels in erythroid cells except in orthochromatic erythroblasts and reticulocytes where expression is low again. CD235a expression is high in day 10 HPCs, low or absent at day 17 and 24 and high again at day 31 and 38. Combined expression of CD36, 71 and 235a demonstrates that almost all cells are erythroid at day 38. The culture conditions schematic is shown under the plot and the data are representative of 3 or more experiments.





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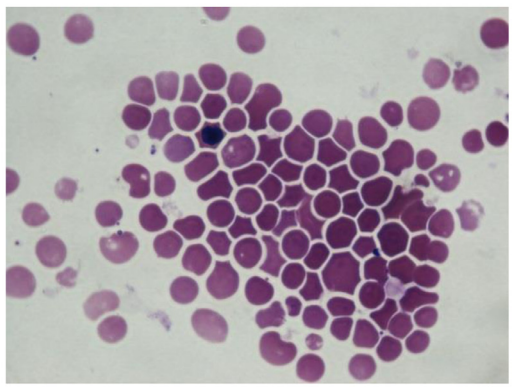

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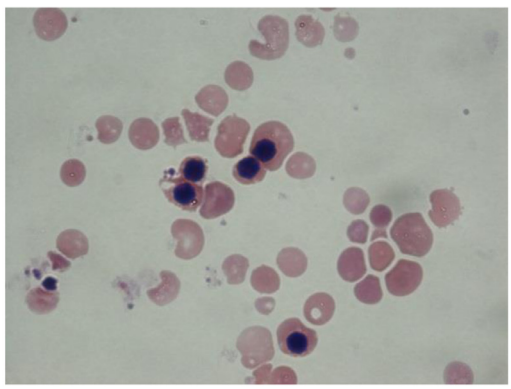

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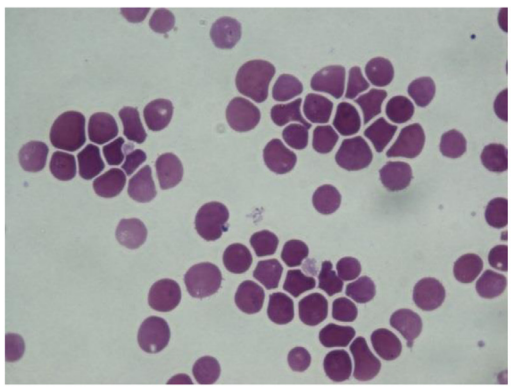
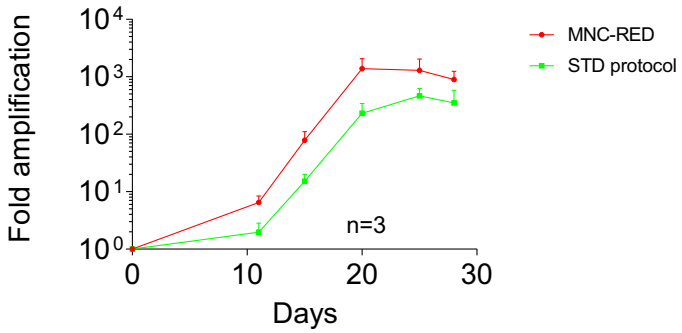


iPS#OM4

Figure E11. Production of enucleated cRBCs from 4 different iPSC lines. **A:** Diagram illustrating the percentage of enucleated cells at days 43 and 45 (n=2) as measured by Draq5 staining. Cells were obtained by differentiation of 4 different iPSC lines generated from four different individuals using the long PSC-RED protocol. B: Micrographs of rapid Romanowsky stain illustrating the morphology of the cRBCs obtained from the four iPSC lines at the end of the long PSC-RED protocol after filtration through a Pal Acrodisc 25mm WBC filter.



Days	p-value	q-value
15	0.8096	0.7000
20	0.0002	0.0008
25	0.0041	0.0087
28	0.0474	0.0664

Figure E12. MNC RED protocol: data from the three individuals tested in Figure 5B were averaged. The difference between the two protocols is significant at days 21 and 25 and almost significant at days 28.The graph illustrates the average fold amplification (± S.D.).

Table E1. Reagents

Reagent	Provider	Catalog Number
IMDM with 1mM Glutamine	Biochrom	FG0465
RPMI 1640 with 1mM Glutamine	Gibco	61870
StemSpan SFEM	Stemcell Technologies	09650
Methyl- β -Cyclodextrin	Sigma	C4555
Trolox	Sigma	238813
Insulin	Sigma	I9218
Chemically defined Lipids 200X	Gibco	11905
Ethanolamine	Sigma	E0135
BSA	Sigma	A1653, A3311, A3782 or A7906
BSA	Gibco	From Kit A1000701
β-mercapto-ethanol 1000X	Gibco	21985
L-ascorbic acid	Sigma	A8960
Holo-Transferrin	R&D Systems/Biotechne	2914-HT
Optiferrin	FisherScience	NC9954311
FeIII-EDTA	Sigma	E6760
BMP4	R&D Systems/Biotechne	314-BP
VEGF165	Peprotech	100-20
Wnt3A	R&D Systems/Biotechne	5036-WN
Wnt5A	R&D Systems/Biotechne	645-WN
Activin A	Peprotech	120-14
GSK3β Inhibitor VIII	Calbiochem/EMD Millipore	361549
aFGF	Peprotech	100-17A
bFGF	Peprotech	100-18B
SCF	Peprotech	300-07
β-Estradiol	Sigma	E2758
TPO	Peprotech	300-18
IGF1	Alfa Aesar	BT-106
IGF2	Alfa Aesar	BT-107
SB431542	Tocris/Biotechne	1614
UM171	Stemcell Technologies	72912
IBMX	Sigma	I5879
PDGF AB	Peprotech	100-00AB
ANGPTL5	R&D Systems/Biotechne	6675-AN
CCL28	Peprotech	300-57
Heparin	Sigma	H3149
EPO	Amgen	NDC 55513-126-10
Dexamethasone	Sigma	D4902
RU486	Sigma	M8046
Hydrocortisone	Sigma	H0888
FLT3L	Peprotech	300-19
IL3	Peprotech	200-03
GM-CSF	Peprotech	300-03
G-CSF	Peprotech	300-23