

Title: Phenotypic and molecular characterisation of a serum-free miniature erythroid differentiation system suitable for high throughput screening and single cell assays

Authors:

1. Sachith Mettananda ^{1,2}
2. Kevin Clark ¹
3. Chris A. Fisher ¹
4. Jackie A. Sloane-Stanley ¹
5. Richard J. Gibbons ¹
6. Douglas R. Higgs^{1,3}

Author affiliations:

¹ Medical Research Council (MRC) Molecular Hematology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DS, UK.

² Department of Paediatrics, Faculty of Medicine-University of Kelaniya, Thalagolla Road, Ragama 11010, Sri Lanka.

³ Oxford National Institute for Health Research Biomedical Research Centre, Blood Theme, Oxford University Hospital, John Radcliffe Hospital, Headington, Oxford OX3 9DS, UK.

Corresponding author's contact information:

Name: Professor Douglas R Higgs

Address: MRC Molecular Hematology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DS, UK.

Email: doug.higgs@imm.ox.ac.uk, liz.rose@imm.ox.ac.uk

Telephone: +44 1865 222393

Fax: +44 1865 222424

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Abstract

In vitro erythroid differentiation systems are used to study the mechanisms underlying normal and abnormal erythropoiesis and to test the effects of various extracellular factors on erythropoiesis. The use of serum or conditioned medium in liquid cultures, and seeding cultures with heterogeneous peripheral blood mononuclear cells confound the reproducibility of these systems. Newer erythroid differentiation culture systems have overcome some of these limitations by using a fully defined, serum-free, medium and initiating cultures using purified CD34+ cells. Although widely used in bulk cultures, these protocols have not been rigorously tested in high throughput or single cell assays. Here, we describe a serum-free erythroid differentiation system suitable for small-scale and single cell experiments. This system generates large numbers of terminally differentiated erythroid cells of very high purity. Here we have adapted this culture system to a 96-well format and also developed a protocol to grow erythroid colonies from single erythroid progenitors in minute culture volumes.

Key words: liquid culture, erythropoiesis, erythroid differentiation, haematopoietic stem cell, single cell assays

Introduction

In vitro erythroid differentiation systems are essential tools for understanding normal and abnormal human erythropoiesis and for testing extracellular factors affecting erythropoiesis. A number of techniques have been described for the production of erythroblasts in cultures of which many require stromal cell support (1). Since the initial description (2), several liquid culture systems that do not require supporting stroma were introduced (3). Most of these utilize animal serum or conditioned medium with variable composition affecting the consistency and reproducibility of such assays (4, 5). Furthermore, these systems use peripheral blood mononuclear cells (PBMC) to initiate the cultures and these contain a mixture of monocytes and lymphocytes and only a small fraction of haematopoietic stem and progenitor cells (HSPC)(5, 6). Consequently, these cultures are often contaminated with other lineages in addition to erythroid cells. To overcome these limitations and to improve reproducibility, erythroid differentiation cultures initiated from purified CD34+ HSPC and using fully defined serum-free growth medium were developed(1, 7, 8).

With the current need for high throughput platforms for screening small molecules affecting erythropoiesis, validated erythroid culture systems optimised for such small-scale assays are required (9). More recently the analysis of molecular biology in single cell experiments are revealing entirely new insights into the mechanisms underlying cell fate decisions, differentiation and maturation: hence there is also a need for robust, reproducible systems that can generate erythroid cells from a single HSPC in liquid cultures. To date, only a few culture systems have been tested at such small scales or in single cell experiments (10, 11). Here we describe a serum-

free erythroid differentiation system starting from human CD34+ HSPCs suitable both for high throughput and single cell assays. This culture system generates large numbers of terminally differentiated erythroid cells with very high purity. We have adapted such assays to 96-well plates to detect changes in globin gene expression by pharmacological methods and RNAi techniques. Finally, we used this system to generate erythroid cells from single HSPC in very small culture volumes. These newly developed assays will be of great value in developing our understanding of normal and abnormal erythropoiesis and in identifying new small molecules affecting this process.

Material and methods

Purification of CD34+ HSPCs: Human umbilical cord blood (UCB) and adult peripheral blood (APB) buffy coat residues (in the form of component donation leucocyte cones) were purchased from the National Blood Service (NHS) Blood and Transplant Service in Oxford, UK. Ethical approval for the study was obtained from North West Research Ethics Committee of NHS National Research Ethics Services (reference no. 03/08/097). Mononuclear cells were collected from the interface after fractionation on Histopaque®-1077 Hybri-Max (Sigma-Aldrich) according to manufacturer's protocol. CD34+ cells were purified using positive selection by magnetic activated cell sorting using CD34 MicroBead Kit (Miltenyl Biotech Cat. 130-046-702) according to manufacturer's instructions. To increase the purity, eluate containing CD34+ HSPCs was re-purified using a second magnetic column and purity confirmed by FACS. Purified CD34+ HSPCs were stored in liquid nitrogen in a freezing solution (90% fetal calf serum+10% Dimethyl sulfoxide) until required.

Erythroid differentiation culture from human CD34+ HSPCs: CD34+ HSPCs were cultured in a two-phase liquid culture system. In phase 1, HSPCs were thawed and seeded at a concentration of 5×10^4 cells/ml in serum free StemSpan™ SFEM II (Stem cell technologies) medium supplemented with 100U/ml penicillin/streptomycin (Gibco), 2mM glutamine (Gibco), 100ng/ml stem cell factor (Peprotech), 10ng/ml interleukin-3 (Peprotech), 10ug/ml cholesterol rich lipids (Sigma-Aldrich) and 0.5IU/ml erythropoietin. Cells were incubated at 37°C in 5% CO₂ for 7 days. The volume of cell cultures was doubled on day 3 and 5 by adding equal volumes of fresh medium. After 7 days, the cells were transferred into phase 2 medium at a

concentration of $1.5\text{-}2 \times 10^5$ cells/ml. Phase 2 medium was similar to phase 1 medium except for the addition of 0.5mg/ml iron saturated holotransferrin (Sigma-Aldrich) and higher erythropoietin concentration (3U/ml). During phase 2 (day 7 -21) the cell culture volume was serially diluted by adding fresh medium every 2-3 days to maintain cell concentration below 2×10^6 /ml. Cells were cultured in 96-well tissue culture plates in volumes of 200 μ L.

Erythroid differentiation culture from sorted single human CD34+ HSPCs:

Single CD34+ HSPCs were sorted by BD FACSAria Fusion cell sorter (BD Biosciences) into the wells in Terasaki multiwell plates into 20 μ l of culture medium which was the same phase 1 medium used in bulk cell cultures. Cells were incubated at 37°C in a 5% CO₂ environment in a tissue culture box kept in the incubator for 14 days without addition or change of medium.

Trypan blue test for viability: Equal volumes of cell suspension and 0.4% trypan blue (Sigma-Aldrich) were incubated for 3-5 minutes at room temperature. Blue stained (nonviable) and unstained (viable) cells were counted under the light microscope using a haemocytometer.

Benzidine stain for haemoglobin: A fresh solution was prepared by adding 1 part of 30% hydrogen peroxide (Sigma-Aldrich) to 100 parts of 0.2% 3,3',5,5'-tetramethyl benzidine (Sigma-Aldrich) in 5% acetic acid (Sigma-Aldrich). Equal volumes of this solution and cell suspension were incubated at room temperature for 2-3 minutes. The percentage of blue (haemoglobinised) cells was scored under light microscope using a haemocytometer.

Morphology analysis by cytopsins: Cytospins were prepared by centrifuging 200µl culture volume at 400rpm for 4 minutes onto a glass slide. The slides were air-dried and stained with modified Wright's stain using Hemateck slide strainer.

Flow cytometry: One hundred thousand cells were washed with phosphate buffered saline (PBS) (Gibco), re-suspended in 200µl of 2% bovine serum albumin (Sigma-Aldrich) and labelled for 20 minutes on ice with the following monoclonal anti-human antibodies; allophycocyanin (APC) conjugated anti-CD34 (Miltenyl Biotec Cat. 130-090-654; dilution 1:100), fluorescein isothiocyanate (FITC) conjugated anti-CD71 (BD Biosciences Cat. 555536; dilution 1:100) and phycoerythrin (PE) conjugated anti-CD235a (BD Biosciences Cat. 340947; dilution 1:500). Dead cells were identified by Hoechst 33258 pentahydrate nucleic acid stain (Invitrogen; dilution 1:10,000) and were excluded. Analysis was performed on Cyan ADP (Beckman Coulter) analyzer using Summit v4.3 and FlowJo V10 softwares.

RNA extraction and reverse transcription: Total RNA was purified using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA concentration was assessed using Nanodrop ND-1000 spectrophotometer (Thermo Scientific) and RNA quality was assessed using Agilent RNA 6000 Nano Kit and Agilent 2100 bioanalyser instrument (Agilent Technologies). Complementary DNA (cDNA) was prepared from 500ng of total RNA using high capacity RNA to cDNA kit (Applied Biosystems) in a reaction mixture of 10µL. In single cell clone experiments, from small number of cells, cDNA was prepared directly from cell lysate using TaqMan gene expression cells-to-CT kit (Life technologies).

Quantitative polymerase chain reaction (qPCR): Validated and inventoried TaqMan assays for human α -globin, β -globin, γ -globin and ribosomal protein L13A (*RPL13A*) were used in qPCR experiments (Applied Biosystems; TaqMan IDs: HBA2/HBA1-Hs00361191_g1, HBB-Hs00747223_g1, HBGHs00361131_g1 and RPL13A-Hs03043885_g1). All probes span exon junctions. qPCR was carried out in 7500 Fast Real Time PCR System (Applied Biosystems) using in 10uL reaction volume according to manufacturer's protocol. Data was analyzed by 7500 software v 2.0.6 using delta delta CT method. *RPL13A* was used as the housekeeping gene for normalization.

Nanostring gene expression assay: Nanostring is a novel technique that directly assesses the amount of messenger RNA quantity without having a reverse transcription or amplification. We designed a gene set panel to analyse the expression of number of erythroid-specific genes. Custom made capture probe set panel and the other consumables were purchased from Nanostring technologies. In each reaction, 100ng of total RNA was hybridised with capture probe set and reporter code set at 65°C in a thermocycler for 16 hours. The hybridised samples were processed using the nCounter Prep Station and nCounter Digital Analyzer (Nanostring Technologies) according to manufacturer's instructions. Raw data were normalised to internal positive spike-in control to normalise to all platform associated sources of variation.

Analysis of haemoglobin: Weak cation exchange high performance liquid chromatography (CE-HPLC) was performed using Bio-Rad VARIANTnbs system (Bio-Rad Laboratories) according to manufacturer's instructions.

Validation using compounds: Primary human erythroid cells at day 7 of the culture were incubated with compounds at the specified concentrations. Each compound was added to 10,000 cells in 200 μ L volume of phase 2 culture medium (cell concentration 50,000/ml) in 96-well flat bottom tissue culture plates. Cells were incubated for 72 hours without changing or adding medium and harvested on day 10.

Transfection of short-interfering RNA for validation: Commercially available, validated siRNAs were purchased from Qiagen. Transfections were performed using Amaxa human CD34⁺ cell nucleofector kit (Lonza) in Amaxa nucleofector I device (Lonza). Briefly, cells were spun at 1000rpm for 5 minutes. The cell pellet was resuspended in 100 μ l nucleofection solution and was nucleofected with 100pmol of siRNA in the provided cuvette using the U-08 program. Cell suspension was then transferred back in to the culture medium.

Statistical analysis: A two-tailed unpaired Student's t-test was used in statistical analysis between groups for normally distributed data. Differences corresponding to $P < 0.05$ were considered statistically significant.

Results

Cell expansion and viability

The erythroid differentiation protocol resulted in very good cell number expansion (Figure 1A&B). The expansion was slow during the first 3 days but increased exponentially thereafter until day 18. CD34⁺ cells of UCB origin demonstrated 4.7×10^5 -fold mean expansion at day 18 whereas cells from APB origin showed a mean expansion of 1.1×10^5 -fold. After day 18, the rate of cell expansion declined. Similar to previous reports (7, 10, 12), we observed a large variation in fold expansion in the later time points of cultures in different samples, most likely due to biological variability.

The cellular viability remained high throughout the first two weeks and mean proportions of viable cells on day 14 were 93.7% and 89.9% respectively for UCB and APB derived erythroid cells (Figure 1C&D). Viability declined thereafter and was 73.1% and 76.6% for UCB and APB derived cells on day 21. There was no significant difference in viability between cells from UCB and APB origin.

Morphological characterisation

Erythroid differentiation was morphologically evident from around day seven of the culture when the majority (80%) of cells were proerythroblasts (Figure 2A & B). After a few divisions these cells differentiated into smaller basophilic erythroblasts which constituted a majority (59%) of the cells by day 10. As the cells proceeded through differentiation, the cytoplasm gradually turned pink and the nucleus condensed to form polychromatic erythroblasts, which first appeared on day 14 and accounted for

40% of cells by day 18. The proportion of orthochromatic erythroblasts, the final nucleated stage of erythroblasts, increased gradually from day 14 (from 1% on day 14 to 55% on day 21). The proportions of non-erythroid cells were very low from day seven until the end of culture.

Immunophenotypical characterisation

Erythroid differentiation was further confirmed by immunophenotypical characterization of cell populations using antibodies to CD71, CD235a (also known as glycophorin A [GPA]) and CD34 cell surface markers. The expression of CD71 was first observed by flow cytometry on day three and was maximum by day 14 (Figure 3A). This was accompanied by later appearance of CD235a, from day seven onwards. After day 14, CD71 expression declined, while the expression of CD235a remained high, resembling normal erythropoiesis. The very high expression (98%) of CD235a at day 21 confirmed the presence of purely erythroid cells at the terminal stages of the culture. The expression of CD34 was high (>90%) during the initial three days, and demonstrated a gradual decline thereafter to very low levels (0.2%) by day 14 (Figure 3B&C). Furthermore, at later stages of the culture, almost all cells (over 99% on day 21) were negative for CD45, again confirming minimal non-erythroid cell contamination (data not shown).

Gene expression during erythroid differentiation

Levels of α -, β - and γ -globin mRNA steadily increased throughout the culture further validating erythroid differentiation since globin gene expression characteristically occurs in erythroid cells after the proerythroblast stage. In erythroid cells differentiated from UCB CD34⁺ cells, the expression levels of γ -globin were higher

than β -globin, throughout the culture (Figure 4A). In contrast, the cells differentiated from APB CD34⁺ cells predominantly expressed β -globin and the levels of γ -globin were comparatively low (Figure 4B). The expression of α -globin was similar in both cell types. This clearly demonstrates that globin gene expression in erythroid cells in culture closely resembles the developmental background of the initiating cells, further validating the system.

Next, gene expression was analysed using the more sensitive Nanostring technique, which directly quantifies the mRNA levels of each gene. In cells differentiated from UCB CD34⁺ cells, globin gene expression increased with erythroid differentiation. Expression of the α , β and γ -globin genes was 10 to 100-fold higher than expression of the embryonic globin genes (ζ and ϵ), μ -globin and θ -globin which were expressed at negligible levels throughout culture (Figure 4C). In addition to the globin genes, the expression levels of other erythroid specific genes (*AHSP*, *KLF1* and *FTH1*) demonstrated a gradual rise throughout the culture (Figure 4D). These findings confirm that this differentiation system recapitulates erythroid gene expression at the molecular level.

Characterisation of haemoglobin

Next, we characterised the cells at the protein level. Differentiating erythroid cells in culture produced haemoglobin from around day seven and the colour of the cell pellet changed gradually from white through pink to red at later stages. The haemoglobinisation was also assessed quantitatively using a benzidine wet stain which demonstrated a rise in the proportion of haemoglobinised cells from 7% on day 7 to over 90% on day 16. To characterize the relative proportions of

haemoglobin subtypes that are produced, cultured erythroid cells were lysed and the extracted haemoglobin was analysed by iso-electric focussing (IEF) and CE-HPLC (Figure 4E&F). Comparable results were observed with IEF and CE-HPLC showing the erythroid cells derived from UCB CD34+ cells predominantly produce foetal haemoglobin (HbF) and the cells differentiated from APB CD34+ cells mostly produce adult haemoglobin (HbA). The production of haemoglobin subtypes appropriate to the developmental stage is a further validation that the system recapitulates erythropoiesis at the molecular level in a developmental stage-specific manner. Also, no unaccounted bands in IEF or unusual elutes in CE-HPLC were seen confirming the absence of any unusual haemoglobin subtypes.

Validation using small molecules and RNA interference

Next we validated the miniaturised culture system to demonstrate its suitability to detect changes in gene expression using pharmacological and RNA interference techniques. Firstly, the effects of several small molecules which were previously shown to upregulate the expression of γ -globin were examined (11, 13). Incubation of cells for 72 hours at day seven of culture with sodium butyrate, vorinostat and valproic acid resulted in statistically significant upregulation of γ -globin compared to controls (Figure 5A and 5B). Furthermore, consistent with previous reports from *in vitro* culture models and in patients *in-vivo*, both hydroxyurea and sodium butyrate demonstrated dose-dependent increases in γ -globin expression and γ/β mRNA ratio in treated cells (Figure 5C&D) (11, 14). Next, cells were transfected with a pair of previously validated siRNAs targeting α -globin mRNA (Figure 5E). This, as expected, resulted in selective knockdown of α -globin expression by up to 80% compared to a negative control siRNA thus validating the use of siRNAs in our culture system.

Erythroid differentiation from sorted single CD34+ cells

Finally, we examined the validity of this culture system for single cell assays. Here, single CD34+ cells were sorted by flow cytometry into the wells of Terasaki multiwell tissue culture plates and cultured in 20 μ L medium for 14 days (Figure 6). Clones appeared in about half of the wells in each plate (mean efficiency-50.7%; SD \pm 7.0%; n=5). These clones demonstrated large cell expansions and grew in size to cover between half to full surface area of the well (Figure 6A). All cells were morphologically erythroid, and erythroid differentiation proceeded in the same sequence as in 96-well plates, albeit slightly advanced; by day 14 the majority of cells were orthochromatic erythroblasts (Figure 6B). Analysis of globin gene expression revealed mean increase of 52-fold and 92-fold in α - and β -globin expression at day 10 compared to day 3 of the culture (Figure 6C) and the clones were visibly red by day 14 indicating successful haemoglobinisation (Figure 6D).

Discussion

Here, we have presented the characterisation and validation of a serum-free miniature erythroid differentiation system which is suitable for both high-throughput and single-cell assays. This system has a number of advantages. Firstly, it utilises human CD34⁺ cells to initiate the culture, rather than PBMCs which contain an admixture of monocytes, and lymphocytes and a small fraction of HSPCs, as used in many previous liquid erythroid culture systems and which resulted in impure populations of erythroid cells with macrophage contamination (5, 6). Since CD34⁺ cells are enriched for HSPCs, the purity of erythroid cells generated from such cultures was high throughout. Secondly, erythroid differentiation occurred with reasonable synchrony, when characterised using CD71 and CD235a, the two most commonly used cell surface marker proteins in human erythroid differentiation to stage erythropoiesis. Thirdly, this system does not use animal or human serum or other conditioned medium, but instead uses a fully characterised serum-free culture medium to induce erythroid differentiation. Serum is a collection of unknown growth factors and cytokines which may vary in composition and the absence of serum greatly improves reproducibility and facilitates analysing the effects of external factors and small molecules on erythroid cells by minimising the unknown variables which might perturb the system.

An important feature of this culture system is its potential to generate a large number of cells. The mean fold expansion (4.7×10^5 -fold) was far in excess of what has been demonstrated by most of the previous liquid culture systems which reported expansion rates of less than 1000-fold (3, 15). Higher expansion rates of

approximately 1.4×10^5 -fold was reported by Giarratana et al.(1) by using a three-step stimulation protocol in which erythroid cells were co-cultured on an adherent stromal layer comprised of either MS-5 stromal cell line or mesenchymal stromal cells. In contrast Neildez-Nguyen et al. reported further higher expansion rates (2.0×10^5 -fold) by using a three-step protocol without stromal cell support (7). However, previous miniature erythroid cultures had not achieved higher expansion rates as seen in larger scales. In small-scale experiments, Cheung and colleagues only achieved 3000-fold expansion, 100-fold less than we report (10). Therefore, our system generates larger numbers of erythroid cells in small scale cultures which will be invaluable in providing sufficient cell numbers for a wide range of experiments and minimising the cost of high-throughput experiments. As reported previously (1), CD34+ cells of UCB origin underwent a higher degree of expansion compared to cells derived from APB (4.7×10^5 vs 1.1×10^5 -fold) possibly due to intrinsic factors unique to UCB cells.

One limitation of this system is the low frequency of enucleation and differentiation into reticulocytes seen in the terminal stages of erythropoiesis. Factors governing enucleation of erythroid cells are still poorly understood, however, interactions of erythroid cells with other cells like macrophages has been shown to induce enucleation (1). Consequently, the frequency of enucleation has always been low in most liquid culture systems that do not have supporting stromal cells which may explain the lower efficiency of enucleation in our culture system (7). Nonetheless, the lack of enucleation does not interfere with the assays for gene expression during erythropoiesis and in fact an intact nucleus is required to study changes in gene expression.

Novel molecular biology techniques increasingly use single cell assays to minimise inter-cell variations, therefore methods of differentiating erythroid cells from a single HSPC are immensely valuable. Here, we have miniaturised the erythroid differentiation system to the single cell level in 20 μ L volumes. Large clones were generated from single HSPCs using this model and notably, they complete the differentiation into pure orthochromatic erythroblasts. Erythroid differentiation was more rapid than was seen in the 96-well plates despite using a low erythropoietin medium throughout. This could be due to increased cell-cell interactions in smaller space, presence of cytokines secreted by adjacent cells promoting differentiation in conditioned medium (as fresh medium was not added) or to both. Nevertheless, this assay would undoubtedly add strength to research in single cell biology related to erythropoiesis.

Validation of the erythroid differentiation system, along with the detection protocol for globin expression, was performed using pharmacological compounds known to alter globin gene expression and RNAi techniques. Both hydroxyurea and sodium butyrate upregulated γ -globin expression in a dose dependent manner similar to previous reports (11, 16, 17) and a pair of siRNAs targeting α -globin demonstrated the expected down-regulation of α -globin expression suggesting the assay is a valid *in vitro* tool to assess changes in globin gene expression in human erythroid cells. Additionally, we utilised this system to screen for pharmacological compounds which alter globin gene expression in human erythroid cells (18) and to test a novel genome editing approach to down regulate human α -globin expression through editing of α -globin enhancer in single cell assays (19) further validating the system.

In conclusion, we have characterised and validated a small-scale serum-free erythroid differentiation system generating large numbers of pure and synchronous erythroid cells from pools of CD34+ HSPCs or single HSPCs. This system appears to recapitulate *in vivo* erythropoiesis in erythroid morphology, immunology and at the molecular level and was validated as a tool to assess changes in globin gene expression.

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Figure Legends

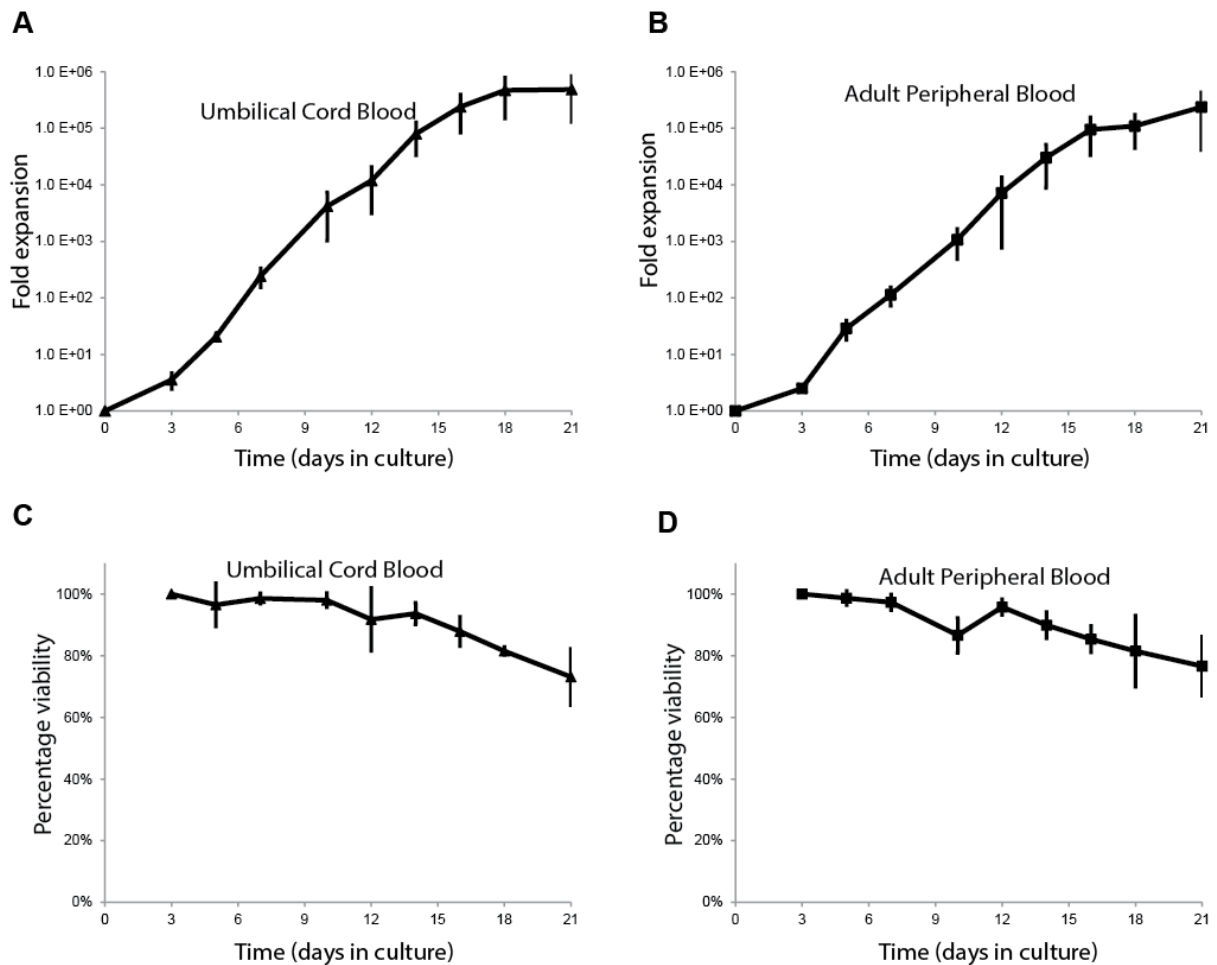


Figure 1 – Proliferation and viability of erythroid cells differentiated from human UCB and APB CD34+ cells. (A) Mean fold expansion during erythroid differentiation of UCB CD34+ cells; error bars represent SD (n=4). (B) Mean fold expansion during erythroid differentiation of APB CD34+ cells; error bars represent SD (n=4). (C) Mean percentage cell viability during erythroid differentiation of UCB CD34+ cells; error bars represent SD (n=4). (D) Mean percentage cell viability during erythroid differentiation of APB CD34+ cells; error bars represent SD (n=4).

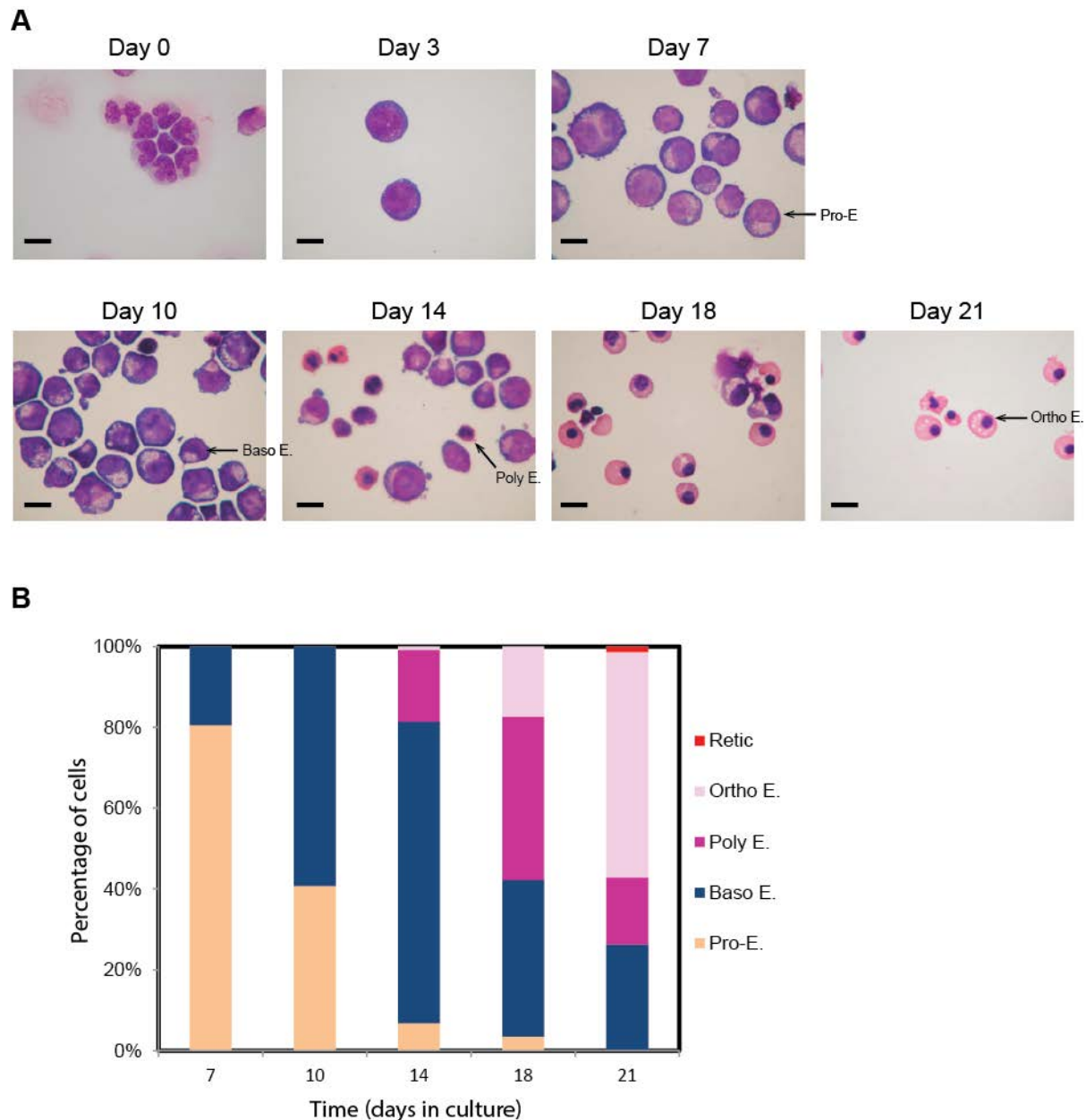


Figure 2 – Morphology of cells at different time points representing different stages of erythroid differentiation. (A) Representative cytopins stained using modified Wright's stain at different time points (day 0-21), in culture representing different stages of erythroid differentiation, demonstrating progression through stages of pro-, basophilic and polychromatic to orthochromatic erythroblasts; scale bar – 10µm. (B) Percentages of morphologically distinct erythroid precursors at different time points of culture. At each stage 300 cells (from three biological repeats) were counted to analyse the morphological stage. Abbreviations: Pro-E,

proerythroblasts; Baso E., basophilic erythroblast; Poly E., polychromatic erythroblast; Ortho E., orthochromatic erythroblast; Retic, reticulocyte.

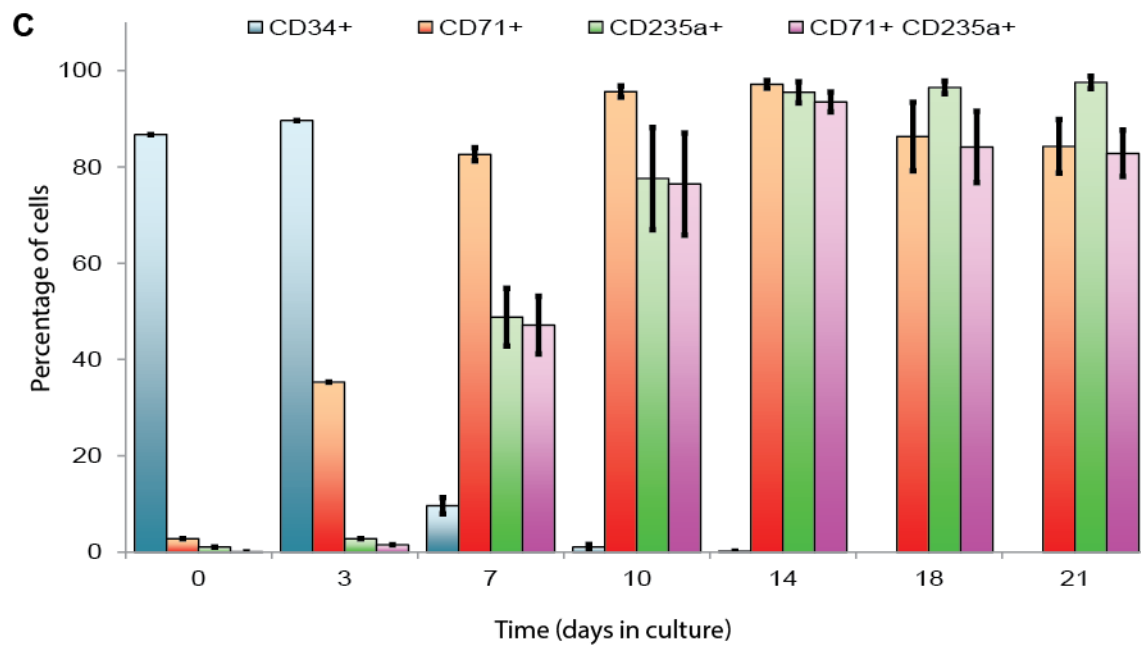
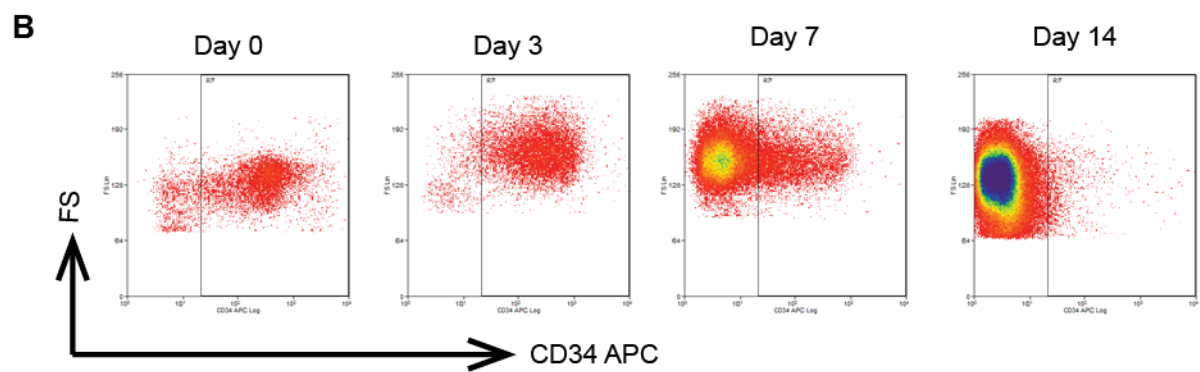
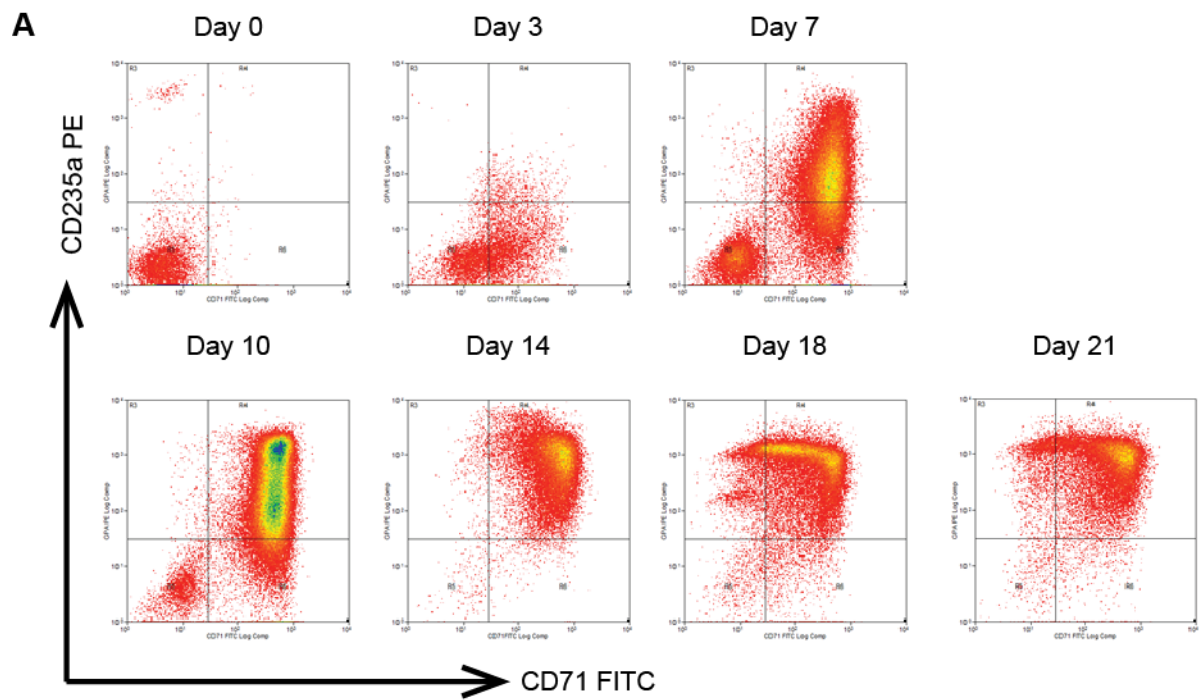


Figure 3 – Immunophenotypical characterisation of cells at different time points in culture. (A) Representative flow cytometry plots of cells stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD71 and phycoerythrin (PE)-conjugated anti-CD235a antibodies, demonstrating sequential expression of CD71 followed by CD235a and subsequent loss of CD71. (B) Representative flow cytometry plots of cells stained with APC-conjugated anti-CD34 antibody plotted against forward scatter demonstrating sequential loss of CD34. (C) Percentages of cells expressing CD34, CD71 and CD235a; means of 3 independent biological repeats are shown; error bars represent SD. Data on day 0 and 3 are from single experiments.

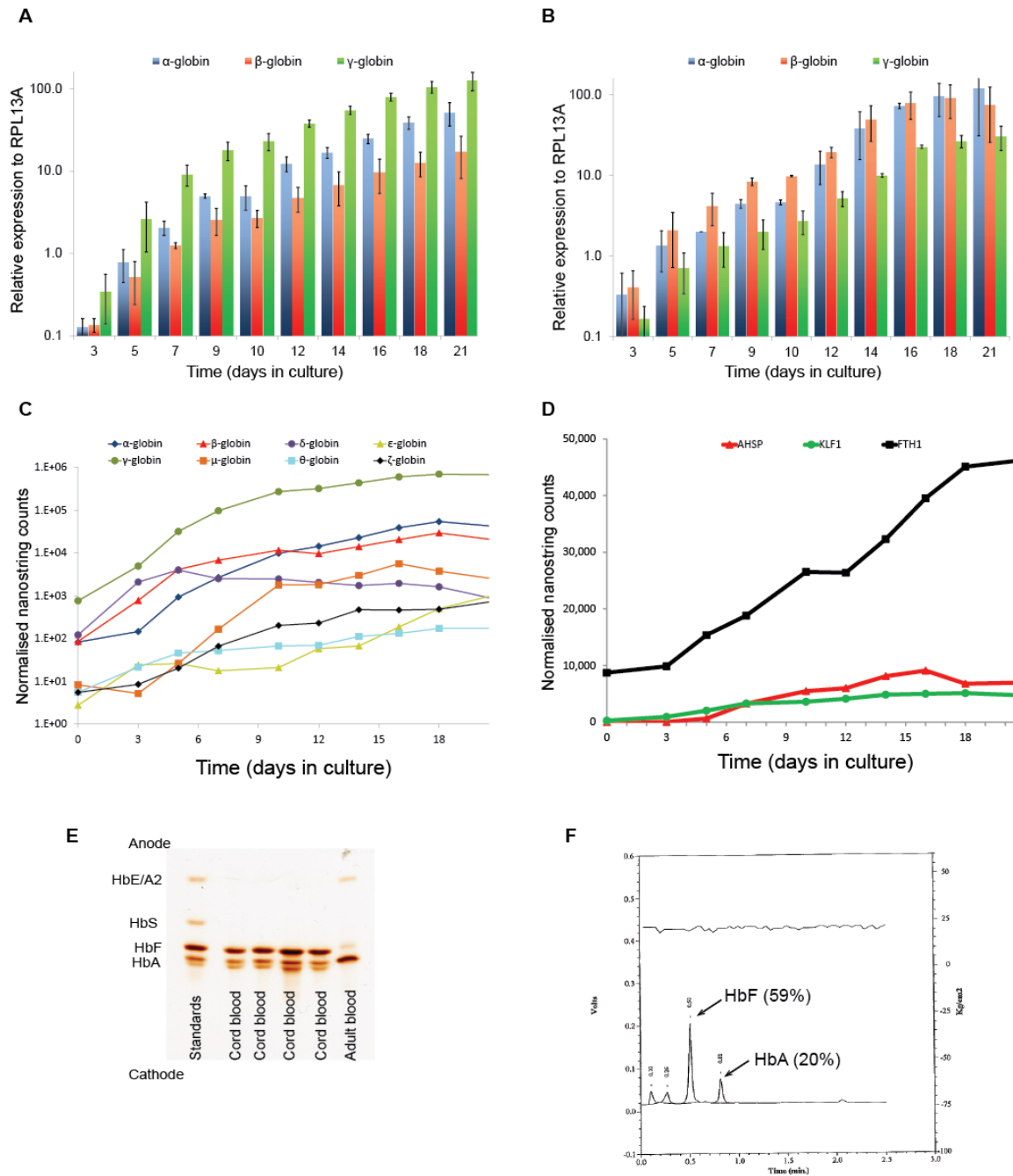


Figure 4 – Globin gene expression and haemoglobinisation. (A) Relative expression of α -, β - and γ -globin mRNA levels quantified by qPCR and normalized to the housekeeping gene *RPL13A* at different time points in culture (UCB CD34+ cells); error bars represent SD (n=3). (B) Relative expression of α -, β - and γ -globin mRNA levels quantified by qPCR and normalized to the housekeeping gene *RPL13A* at different time points in culture (APB CD34+ cells); error bars represent SD (n=3).

(C) Relative expression of globin mRNA levels quantified by nanostring and normalized to eight housekeeping genes (*RPL13A*, *RPL18*, *GAPDH*, *PABPC1*, *CA2*, *FTH1*, *PAIP2* and *LAPTM4A*) at different time points in culture (UCB CD34+ cells)

(D) Relative expression of other erythroid-specific gene mRNA levels quantified by nanostring and normalized to eight housekeeping genes (*RPL13A*, *RPL18*, *GAPDH*, *PABPC1*, *CA2*, *FTH1*, *PAIP2* and *LAPTM4A*) at different time points in culture (UCB CD34+ cells)

(E) Hemoglobin subtypes of the erythroid cells differentiated from UCB and APB CD34+ cells analyzed by isoelectric focusing. The samples were run against a commercial set of standards.

(F) Representative tracing of cation exchange –high performance liquid chromatography haemoglobin of cells differentiated from UCB CD34+ cells. Abbreviations: RPL13A, ribosomal protein L13a; AHSP, α -haemoglobin stabilising protein; KLF1, Kruppel-like factor 1; and FTH1, Ferritin, Heavy Polypeptide 1.

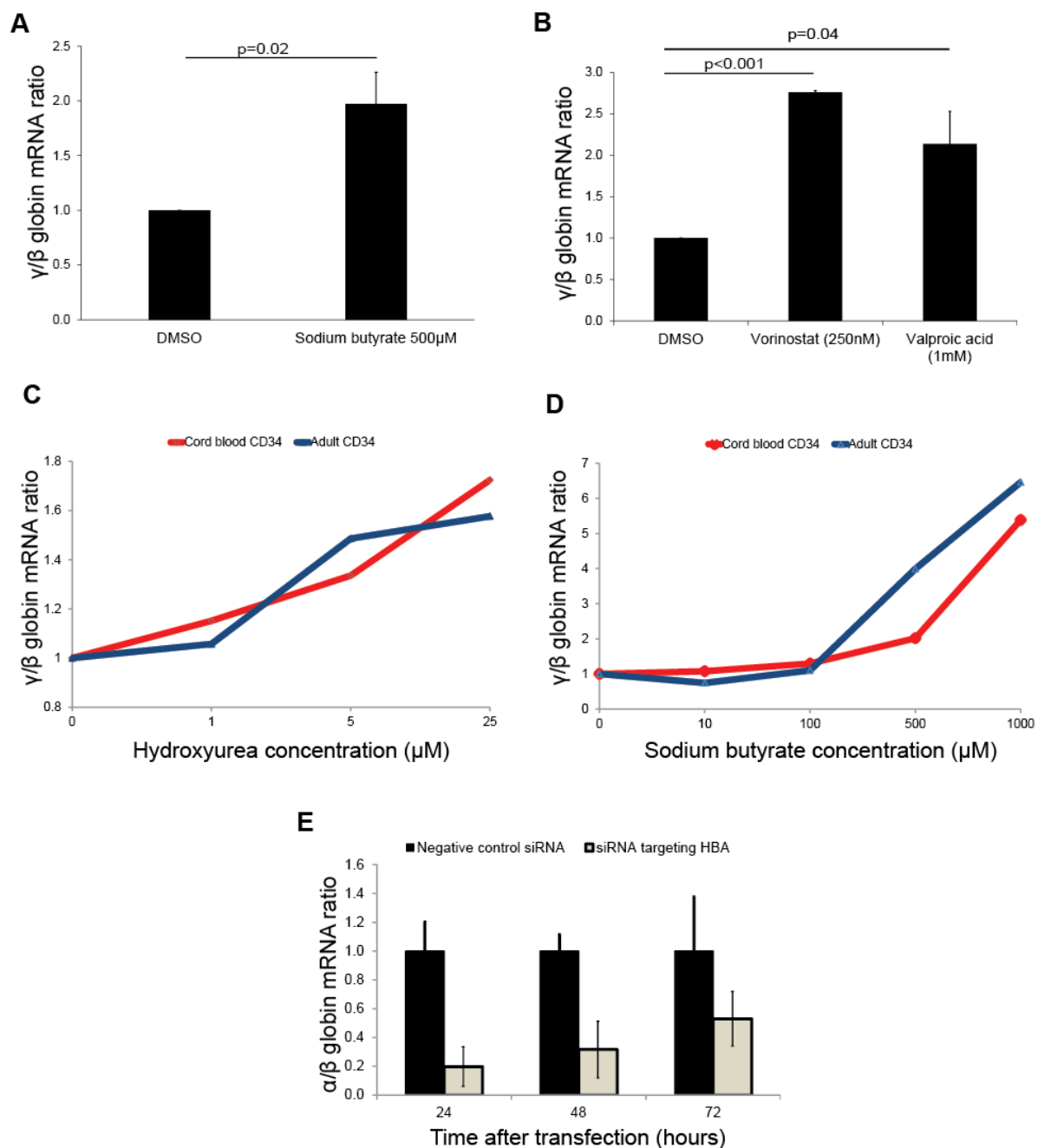


Figure 5 – Validation of the erythroid differentiation system. (A) γ/β mRNA ratio of sodium butyrate (500 μ M) treated erythroid cells (UCB derived) compared to DMSO control; error bars represent SEM (n=6). (B) γ/β mRNA ratio of vorinostat (250 nM) and valproic acid (1 mM) treated erythroid cells (UCB derived) compared to DMSO control; error bars represent SEM (n=3). (C) and (D) γ/β mRNA ratio after incubation of erythroid cells in a dose range of hydroxyurea (C) and sodium butyrate (D). Data on erythroid cells differentiated from UCB and APB CD34+ cells are

presented in red and blue respectively. Compounds were added to the liquid culture medium on day 7 of erythroid cell differentiation (corresponding to the proerythroblast stage), and the cells were then incubated in a 5% CO₂ atmosphere at 37°C for 72 hours. (E) α/β globin mRNA ratio of erythroid cells transfected with a pair of siRNAs targeting human α -globin and a negative control siRNA; error bars represent SD (n=2).

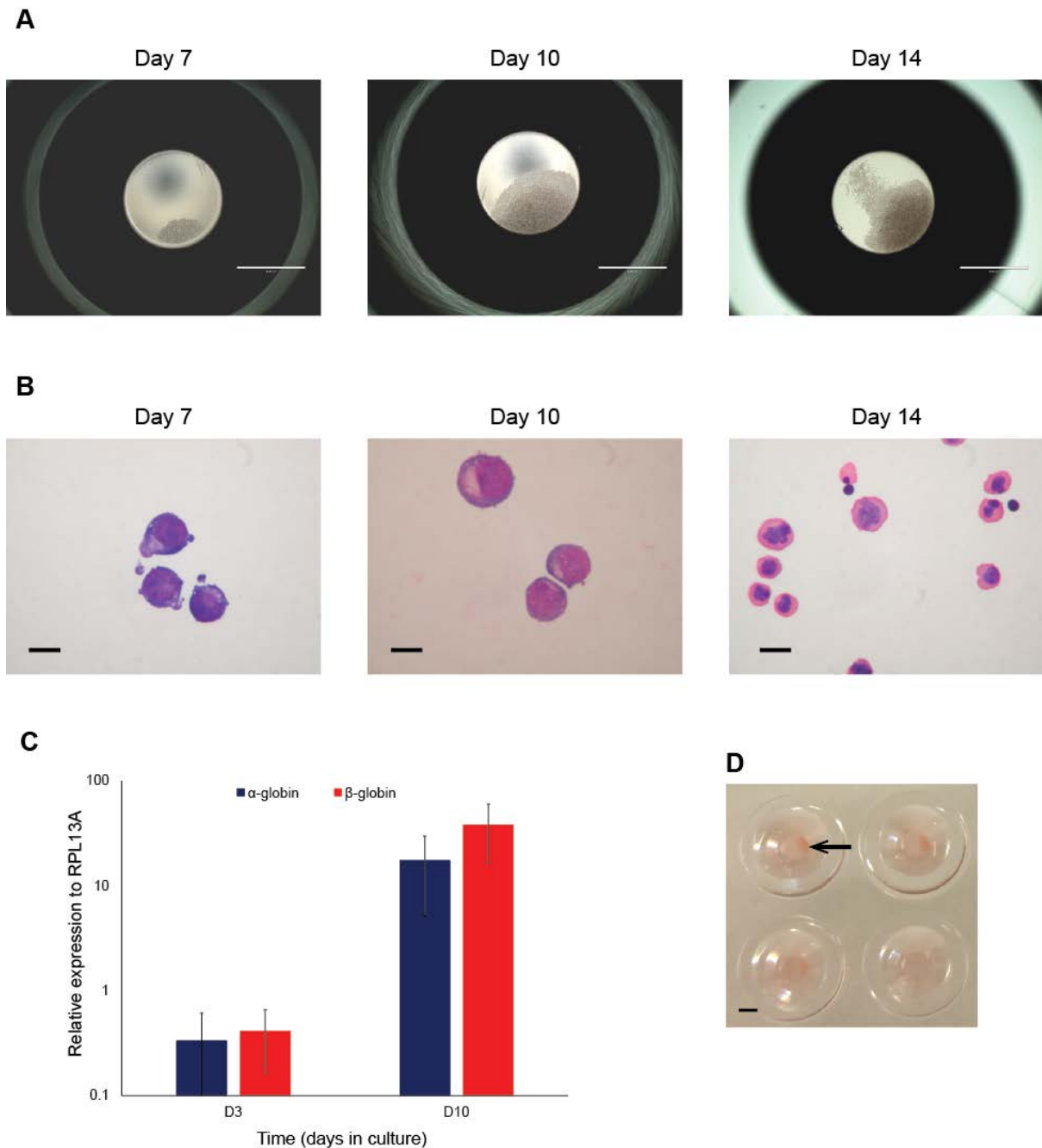


Figure 6 – Erythroid differentiation from single CD34⁺ cells. (A) Representative microscopic images of a single well in the Terasaki plate demonstrating the size of the clone at different time points; white scale bar – 1mm. (B) Representative cytopspins of the cells obtained from the clones and stained with modified Wright stain at different time points; black scale bar – 10 μ m. (C) Relative expression of α - and β -globin mRNA levels quantified by qPCR and normalized to the housekeeping gene *RPL13A* at different time points in culture (APB CD34⁺ cells); error bars represent

SD (n=3). (D) Clones grown and differentiated from single CD34+ cells were visibly red (arrow) after 14 days suggesting erythroid differentiation and successful haemoglobinisation; scale bar – 1mm.