Stem cell factor and erythropoietin-independent production of cultured reticulocytes

Emmanuel Olivier, Shouping Zhang, Zi Yan and Eric E. Bouhassira

Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York, NY, USA

Correspondence: E.E. Bouhassira
eric.bouhassira@einsteinmed.edu

Received: October 13, 2023.
Accepted: April 5, 2024.
Early view: April 11, 2024.

https://doi.org/10.3324/haematol.2023.284427

Abstract

Cultured reticulocytes can supplement transfusion needs and offer promise for drug delivery and immune tolerization. They can be produced from induced pluripotent stem cells (iPSC), but the 45-day culture time and cytokine costs make large-scale production prohibitive. To overcome these limitations, we have generated iPSC that express constitutive stem cell factor (SCF) receptor and jak2 adaptor alleles. We show that iPSC lines carrying these alleles can differentiate into self-renewing erythroblasts that can proliferate for up to 70 cell-doubling in a cost-effective, chemically-defined, albumin- and cytokine-free medium. These kitjak2 self-renewing erythroblasts retain the ability to enucleate at a high rate up to senescence. Kitjak2-derived cultured reticulocytes should be safe for transfusion because they can be irradiated to eliminate residual nucleated cells. The kitjak2 cells express blood group 0 and test negative for RhD and other clinically significant red blood cell antigens and have sufficient proliferation capacity to meet global red blood cell needs.

Introduction

Cultured reticulocytes (cRetic), which can mature into red blood cells (RBC) upon transfusion,[1] have the potential to supplement transfusion needs, to be used as drug carriers or as immune tolerization agents.[2] Several methods have been devised to amplify primary cord blood (CB) or peripheral blood (PB) hematopoietic progenitor cells (HPC) into cRetic.[3,4] These processes involve multi-stage culture protocols during which the HPC expand and differentiate into reticulocytes. Erythroid differentiation of HPC is typically achieved using various combinations of cytokines and small molecules, the most important being stem cell factor (SCF), erythropoietin (Epo), Interleukin-3 and dexamethasone (Dex). These methods can achieve up to 10^5-fold cell expansion of HPC into cRetic and offer a potential solution to relieve local blood shortages. However, they are not ideal for large scale production of cRetic because the primary cells required for this process must constantly be replenished.

In order to overcome this limitation, several immortalized cell lines have been developed. This was achieved by lentiviral transduction of CB, PB or bone marrow (BM) $CD34^+$ or mononuclear cells to overexpress the E6 and E7 proteins of human papilloma virus (HPV) 16,[5-9] sometimes combined with hTERT or Simian virus 40 T antigen.[10] Additionally, other erythroid cell lines have been generated by overexpression of cellular genes such as *c-myc* and *Bcl-XL*, *Spi1* or *Bmi-1*.[11,12] Many of these lines can be cultured for months and in some cases indefinitely and exhibit a phenotype that resembles certain stages of RBC development, generally colony-forming unit-erythroid (CFU-E) or pro-erythroblasts. Importantly, some of these cell lines retain the ability to differentiate into reticulocytes with rates of enucleation varying between <1% up to about 30%, even after long-term culture.[5,6,13] These cell lines are invaluable for research, particularly in studying the process of RBC formation. They also hold promise as a source of cells for blood transfusions and other applications involving cRetic. However, there are still challenges to be addressed. For instance, cell lines expressing the HPV E6/E7 proteins invariably show significant aneuploidy.[5] The cost of the culture media and cytokines needed to maintain these cells, and the low cell densities required for effective terminal differentiation are also hurdles that need to be overcome for these lines to be viable for large-scale RBC production for clinical use.

Induced pluripotent stem cells (iPSC) present an alternative source for cRetic production because they are inherently

immortal, maintain karyotypic stability and are easy to produce. Multiple laboratories have developed and refined protocols to differentiate iPSC into cRetic, first using a feeder layer and then using feeder-free protocols.[14,15] These experiments revealed that the cRetic derived from iPSC predominantly expressed embryonic and fetal globins rather than the adult variant and are larger in size than typical adult RBC.[16]

In an effort to decrease the cost of cRetic production from iPSC, we previously developed two chemically defined culture media, IMIT and R6, and combined them into PSC robust erythroid differentiation (PSC-RED) protocols which yield, in its long version, more than 100,000 cRetic/iPSC.[17] A notable feature of PSC-RED is its elimination of the need for albumin and a significant reduction in transferrin requirements - by 20-fold compared to previous methods. This efficiency is due to an iron chelator in the R6 media, which facilitates transferrin recycling in the cell culture.

However, despite these improvements, the PSC-RED protocol proved in our hands to be too expensive and too complex for large scale production. This is partly due to the lengthy differentiation process of iPSC into cRetic, which takes 40 to 45 days, and partly because of the high costs of SCF and Epo.

Independent of the cell source used, producing a single unit of RBC containing $2x10^{12}$ cells theoretically requires over 1,000 liters of medium in a static culture system where the maximum cell density is approximately $2x10^{6}$ cells/mL. Since these large volumes are unpractical and prohibitively expensive, investigators have sought to overcome these limitations by growing the cells in bioreactors which can theoretically support a much higher cell density. Griffith *et al*. were able to produce 3.10^{10} cRetic (about 5 mL of packed cells) in 24 liters at a density of $1\text{-}6.10^{6}$ cells/mL using a 2-liter stirred glass bioreactor.[18] A more recent study based on measurements of erythroblast O_2 uptake rate suggested that density greater than 10^{9} cells/mL might be achievable and that 500 liters of medium might be sufficient to produce one unit of cRetic.[19] Sivalingam *et.al*. were able to achieve complete differentiation of IPSC into cRetic in a perfusion bioreactor, reaching a cell density of $3.5x10^{7}$/mL.[20] Others studies have reported achieving very high cell densities using various small scale 3D bioreactor designs (reviewed in [21]). Collectively, these findings suggest that large scale production of cRetic is technically possible with existing bioreactor technology. However, each cell sources comes with inherent limitations and the high costs associated with these processes currently impede the widespread production of cRetic for transfusion.

In the case of the PSC-RED protocol, about 17 liters of culture medium are consumed during the 45-day PSC-RED protocol to produce 5.10^{9} cRetic, the equivalent of about 1 mL of blood. However, less than 50 mL of medium is consumed during the first 17 days because of the exponential proliferation of the cells (*Online Supplementary Figure S*1). Most of the cost of cRetic production is therefore incurred after day 17. Since SCF and Epo are the only cytokine required after day 17, we decided to test the hypothesis that the introduction of constitutive mutations in the SCF and Epo pathways would allow us to produce cRetic from iPSC without SCF or Epo.

In order to test this hypothesis, we selected the *kit*D816V mutation, often associated with mastocytosis, because it causes the *kit* gene (the SCF receptor) to become constitutively active.[22] Additionally, we chose the *jak2*V617F, frequently associated with polycythemia vera (PV), because it significantly reduces the need for Epo by enabling the jak2 kinase to signal in the absence of this cytokine.[23] The selection of the *kit*D816V mutation was supported by a report indicating that the Epo- and SCF-dependent HUDEP-2 transformed erythroid cell line,[13] could be rendered independent of SCF by introducing mutations around position 816 of the kit gene.[24] Importantly, the Epo dependence of some HUDEP-2 sublines described in this report was also reduced, possibly due to interactions between the SCF and Epo receptors.[25]

We report that iPSC lines carrying these alleles can differentiate into self-renewing erythroblast (SRE) that can proliferate for up to 70 cell doubling in a cost-effective, chemically-defined, albumin- and cytokine-free medium. These kitjak2 SRE are karyotypically stable and retain the ability to enucleate at a rate >50%. The development of these Kitjak2 SRE removes major obstacles to the production of large amounts of cRetic for translational applications.

Methods

Internal Review Board

All experiments involving human cells were performed under a protocol approved by the Albert Einstein College of Medicine Internal Review Board (Bronx, New York).

Induced pluripotent stem cells

The iPSC lines O1 and O2 have been described previously.[17]

Self-renewing erythroblasts

Differentiation of induced-pluripotent stem cells into kitjak2 self-renewing erythroblasts

Day 0: kitjak2 iPSC were differentiated into day-17 HPC according to the PSC-RED protocol (omitting SCF). Day 17: day-17 HPC were then cultured in IMIT combined with 1 mM Dex, 30 mM 3-isobutyl-1-methylxanthine (IBMX). After 10 to 14 days, greater than 98% of the cells in the culture had acquired the antigen profiles of SRE.

Differentiation of induced-pluripotent stem cells into kitD816V self-renewing erythroblasts

Day 0: *kit*D816V iPSC were differentiated into day-17 HPC

according to the PSC-RED protocol (omitting SCF). Day 17: day-17 HPC were then cultured in IMIT combined with 1 mM Dex, 30 mM IBMX and 1 unit[U]/mL Epo. After 10 to 14 days, greater than 98% of the cells in the culture had acquired the antigen profiles of SRE.

Long-term culture of self-renewing erythroblasts

kitjak2 SRE can be cultured for about 120 days in IMIT combined with 1 uM Dex and 30 uM IBMX. D816V SRE can be cultured for about 140 days in in IMIT combined with 1 uM Dex, 30 uM IBMX and 1 U/mL of Epo. They can also be cultured for about 45-55 days in the same medium without Epo.

All SRE were passaged every 3 to 5 days by dilution to 1.25 to 2.5x10^5 cells/mL once the culture concentration exceeded 1.5x10^6/mL. The passage frequency depended on the passage number because the rate of proliferation of the SRE diminishes gradually over time.

Terminal differentiation of self-renewing erythroblasts

Day 0: cells were centrifuged, rinsed once in phosphate-buffered saline (PBS) to eliminate all traces of Dex and IBMX and plated at about 1.5x10^5 cells/mL in R6 media containing 4 U/ml of EPO, 5% human AB plasma and 1 mM RU 486. Day 3: cells were diluted 1 to 2 to about 3.5x10^5 cells/mL in the same media without Epo. Days 5, 7 and 9: cells were diluted in pure RPMI to about 3 to 5x10^5 cells/mL. Flow cytometry: iPSC undergoing differentiation were evaluated by fluorescence-activated cell sorting (FACS) using a Cytek aurora spectral cytometer and a 15-color antibodies panel described in *Online Supplementary Table S3*. Data were analyzed with the FlowJo software using the FlowSOM[26] and UMAP[27] plugins, essentially as suggested by the manufacturer using the default parameters.

Low-pass sequencing

Genomic DNA was extracted and 1.5 Gb of sequence was obtained on an Illumina sequencer (2x150 bp configuration). Reads were aligned to the hg38 genome using the bwa aligner and copy-number variants were detected using the CNVKit software package.[28] A library of ten normal genomes sequenced to the same depth was used as a baseline control. iPSC genomic DNA were also compared with the DNA obtained from peripheral blood cells from the same donor.

Reagents

The suppliers for all reagents are provided in *Online Supplementary Table S5*.

Statistical analysis

Paired and unpaired *t* tests were used to assess significance using the GraphPad Prism software.

Detailed protocols for other procedures are provided in the *Online Supplementary Appendix*.

Results

Production of induced pluripotent stem cells carrying the D816V mutation

In order to generate cells with the *kit*D816V mutation, we transfected the iPSC clone O1[17] with *cas9* mRNA, a single-guide RNA (sgRNA) targeting exon 17 of the *kit* gene, and a 200 bp homology-directed recombination (HDR) donor oligonucleotide. Screening 24 iPSC clones showed a targeting frequency of about 50% (*Online Supplementary Figure S2*). We selected lines homozygous (B34) and hemizygous (A4) for the D816V mutation for further characterization.

Cultured reticolocytes can be produced without stem cell factor

During the PSC-RED protocol, iPSC differentiate into cRetic over a period of 45 days. Initially, from day 0 to day 17, the cells are sequentially cultured with four supplements (S1 to S4), resulting in a mixture of HPC (Figure 1A). These HPC are then directed towards early erythroid differentiation by being cultured in the presence of SCF, Epo, Dex, and IBMX between days 17 and 24. From days 24 to 38, the cells progress to the late erythroid stage when maintained in similar media, but without Dex or IBMX. In the final phase, these late-stage progenitors mature and enucleate when cultured from day 38 to 45 without any cytokines or small molecules.

In order to evaluate the phenotype of the *kit*D816V iPSC, we differentiated the A4, B34, and the unedited O1 iPSC lines using the PSC-RED protocol but omitting SCF, which is normally present from day 2 to 38, at all steps of the protocols (Figure 1A). As anticipated, the unedited O1 iPSC could not survive beyond day 17 in the absence of SCF (*data not shown*). In contrast, the A4 and B34 lines grew exponentially and exhibited a viability (assessed weekly using staining with propidium iodide) greater than 90% during the entire growth period, generating over 200,000 cells/ iPSC by day 38. Viability and yield were comparable to the cell production from control O1 iPSC differentiated in the presence of SCF (Figure 1B, black curve).

Analysis of the cells generated in these cultures through Romanowsky staining, a stain similar to Giemsa (*Online Supplementary Figure S3*) revealed that the *kit*D816V iPSC acquired a progressively mature erythroid phenotype during the differentiation process. Analysis through a 15-color flow cytometry assay demonstrated that the progression of cells during the PSC-RED protocol could be succinctly represented using the flowSOM and UMAP algorithms.[29,30] These algorithms categorized the cells into four distinct populations of HPC (HPC1 to HPC4) and four populations of erythroid cells (Ery1 to Ery4), based on the expression patterns of ten surface antigens (Figure 1C-E; *Online Supplementary Figure S4A, C*).

Examination of the data revealed that the erythroid populations became predominant earlier in the differentiation

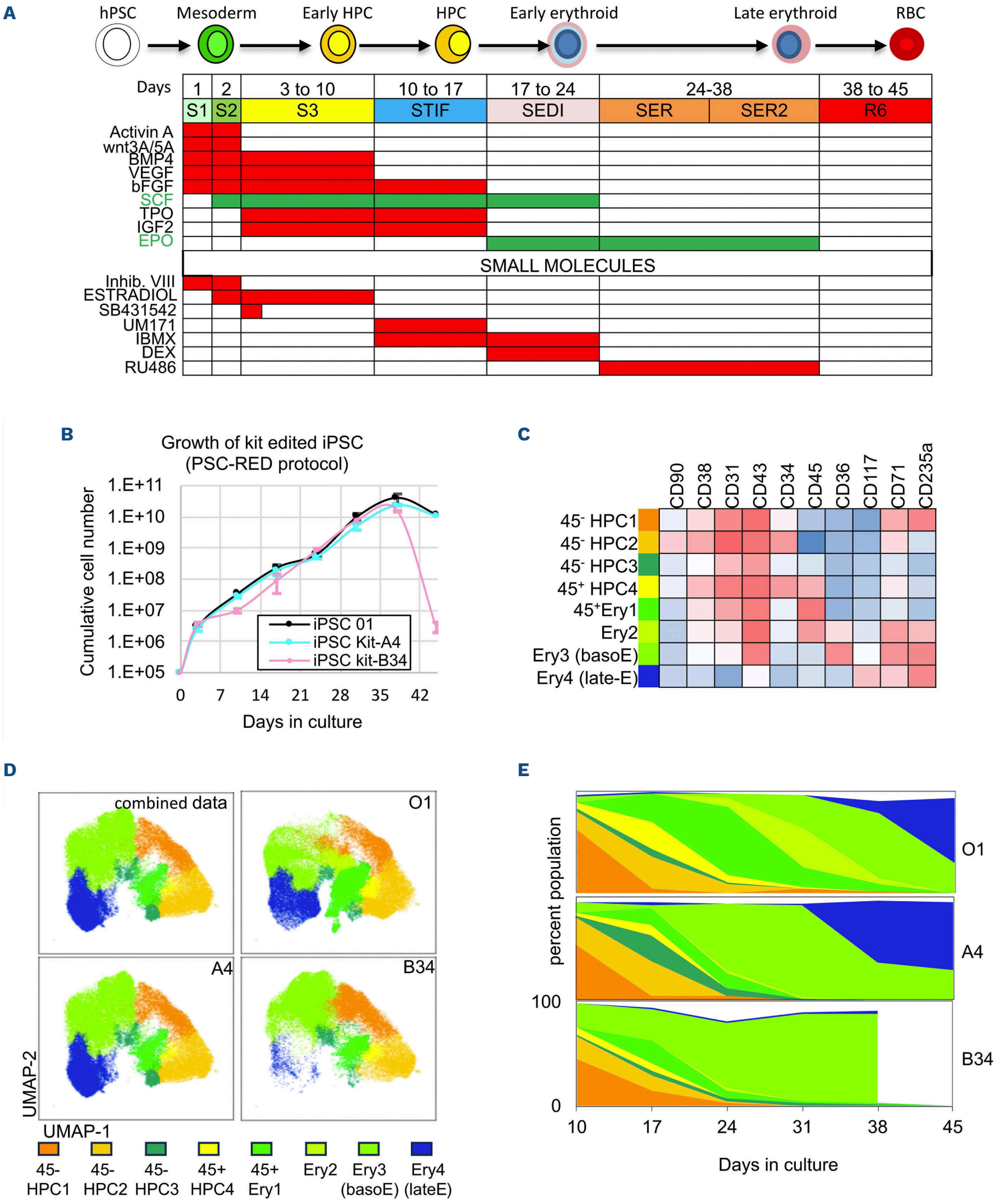


Figure 1. Differentiation of kitD816V induced pluripotent stem cells. (A) Diagram and table illus-trating the long chemically-defined pluripotent stem cell robust erythroid differentiation (PSC-RED) protocol to differentiate induced pluripotent stem cells (iPSC) into erythroid cells. STIF: S: stem cell factor (SCF); T: Tpo; I: IGF-2; F: bFGF. SEDI: S: SCF; E: erythropoietin (Epo); D: dexamethasone (Dex); I: 3-isobutyl-1-methylxanthine (IBMX). SER and SER2: S: SCF; E: Epo; R: RU486. Concentrations of all

Continued on following page.

components are provided in the online method section. The short version of the protocol is similar except that the expansion in STIF between day 10 and 17 is omitted. The short version yields red blood cells (RBC) that express more embryonic globin than the long protocol. (B) Growth curve of iPSC hemizygous (line A4) or homozygous (line B34) for the D816V mutation. The control O1 cells were differentiated according to the PSC-RED protocol in-cluding SCF. The A4 and B34 lines were differentiated according to the same protocol but SCF was omitted at all steps. (C) A 15-color flow cytometry assay was used to examine the antigen expression profiles of differentiating cells that we collected weekly between day 10 and 45. Dimensionality reduction analysis using flowSOM and UMAP revealed that dividing the cells into 8 major populations according to expression of 10 markers provided a useful summary of the evolution of the cells undergoing the PSC-RED protocol. Heatmap summarizes the relative expression of the 10 markers used to define the 8 populations. Populations expressing CD34 were labeled HPC1 to 4 while populations negative for this marker but positive for erythroid markers were labeled Ery1 to 4. (D) UMAP analysis. Data from the 15-color flow cytometry analysis of control A4 and B34 cells assessed weekly from day 10 to 45 was concatenated and analyzed using UMAP generating the pattern illustrated in (D). The concatenated data was then analyzed with flowSOM which segmented the data into 12 populations. Four populations representing each less than 1% of the cells and were eliminated. The UMAP graph was then colors with the 8 remaining major populations which represented more than 96% of all cells. (E) Chart summarizes the evolution of the 8 populations defined by FlowSOM dur-ing erythroid differentiation. At day 10, the phenotype of the A4 and B34 cells is similar to the control cells with populations of 43^{+}; 34^{+}; 45^{-} HPC1 and HPC2 (which differed by expression of CD235a) dominating the culture. At days 17 and 24, the control cells differentiated progressively into popula-tions of 45+ HPC4 and 45+ Ery1 cells. At day 31, Ery2 cells (34^{low}; 45^{+}; 36^{low}; 71^{+}; $235a^{+}$) resembling pro-erythroblasts and Ery3 cells (34^{-}; 45^{-}; 36^{+}; 71^{+}; $235a^{+}$) resembling basophilic erythroblasts became prominent and matured into Ery4 late erythrocytes (34^{-}; 45^{-}; 36^{-}; 71^{low}; $235a^{+}$) by day 38. In the kit mutated clones, the HPC2 and Ery1 cells did not amplify to the same degree as in the control cells, and the Ery2 cells were barely detectable. Instead, an HPC3 population with a phenotype intermediate between HPC1 and 2 briefly expanded and, most notably, the Ery3 population became prominent much earlier, particularly in the homozygous B4 cells. The Ery3 cells from the A4 cells eventually differentiated into Ery4 cells, but those from the B34 clone did not, resulting, at days 31 and 38, in cultures composed almost exclusively of Ery3 cells. Because of massive cell death of the B34 cells during the last week of differentiation, a fluorescence-activated cell sorting analysis was not performed at day 45 for these cells. hPSC: human pluripotent stem cells.

process in the presence of the *kit*D816V mutation than in the control cells. Multiple hypotheses might explain this observation. One possibility is that the constitutive activation of the SCF signaling pathways in the D816V cells allows the proliferation of early primitive HPC and erythroid cells that do not proliferate in the absence of the kit mutation because of the relatively low concentration of SCF (10 ng/mL) and the absence of Epo during the first 17 days of the PSC-RED protocol.

In order to gain further insight into the differentiation potential of the iPSC-derived HPC in the presence of the kit mutations we performed methyl-cellulose assays on cells obtained at days 10, 17 and 24 of the PSC-RED protocol. Control O1 cells were obtained in the presence of SCF, but this cytokine was omitted when generating the cells from the kit-mutated A4 and B34 iPSC (*Online Supplementary Figure S5*). For all three iPSC lines tested, the proportion of clonogenic cells was highest at day 10 and gradually decreased until day 24 (averaging 772±92/10,000 HPC at day 10, 162±196/10,000 HPC at day 17 and 70±23/10,000 HPC at day 24). Notably, both myeloid and erythroid colonies were obtained at all time points, the number of colony-forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) colonies decreased over time while the ratio of colony-forming unit-erythroid (CFU-E) to burst-forming unit-erythroid (BFU-E) increased. No significant difference in the distribution of the colonies between the control and *kit*D816V cells was observed.

Upon completion of the differentiation process, the hemozygous *kit*D816V A4 clone yielded a combination of basophilic erythroblasts (referred to as Ery3), orthochromatic erythroblasts, and enucleated cells (referred to as Ery4), a pattern similar to that of the control cells. In the case of the homozygous (B34) clone, the erythroid populations proliferated even earlier than those of the A4 clone. In addition, the B34 clone generated Ery3 cells but few Ery4 cells. Repeat of this differentiation experiment using new batches of antibodies confirmed that the erythroid cells dominated the culture earlier in the presence of the D816V mutations than in the control cells, and that the B34 iPSC produced fewer Ery4 cells than the A4 iPSC (*Online Supplementary Figure S4B*).

Erythropoietin is also dispensable for the production of induced pluripotent stem cell-derived *kit*D816V erythrocytes

In order to investigate the potential of iPSC-derived *kit*D816V cells to differentiate in the absence of both Epo and SCF, we induced the differentiation of the A4 and B34 iPSC lines omitting both cytokines (Figure 2A). This demonstrated that both lines could proliferate without Epo and SCF yielding a substantial number of cells (>200,000 cells/iPSC). Again fewer Ery4 cells were produced by the B34 clones than by the control and A4 clones (Figure 2B, C).

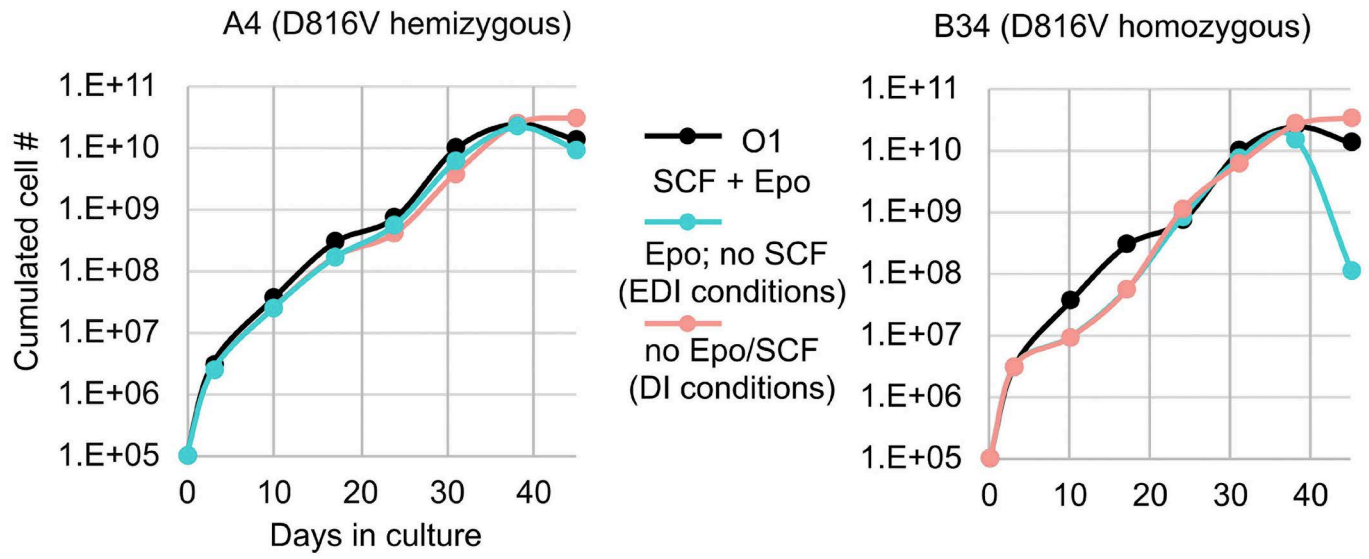
*Kit*D816V cultured reticulocytes express mostly fetal globins

In order to measure the enucleation rate, day-45 cells cultured without SCF and induced to terminally differentiate by discontinuing Epo on day 38 were analyzed by flow cytometry after staining with Draq5, a cell permeant DNA dye, and by light-microscopy following Romanowsky staining. In order to test the hypothesis that in this system constitutive activation of the SCF receptor might be detrimental

A

cells	cytokines	1	2	3 to 10	10 to 17	17 to 24	24-31	24-38	38 to 45
O1	Epo + SCF	S1	S2	S3	STIF	SEDI	SER	SER2	
A4 & B34	Epo	S1	S2*	S3*	TIF	EDI	ER	ER2	
	none					DI	D	R	

B



C

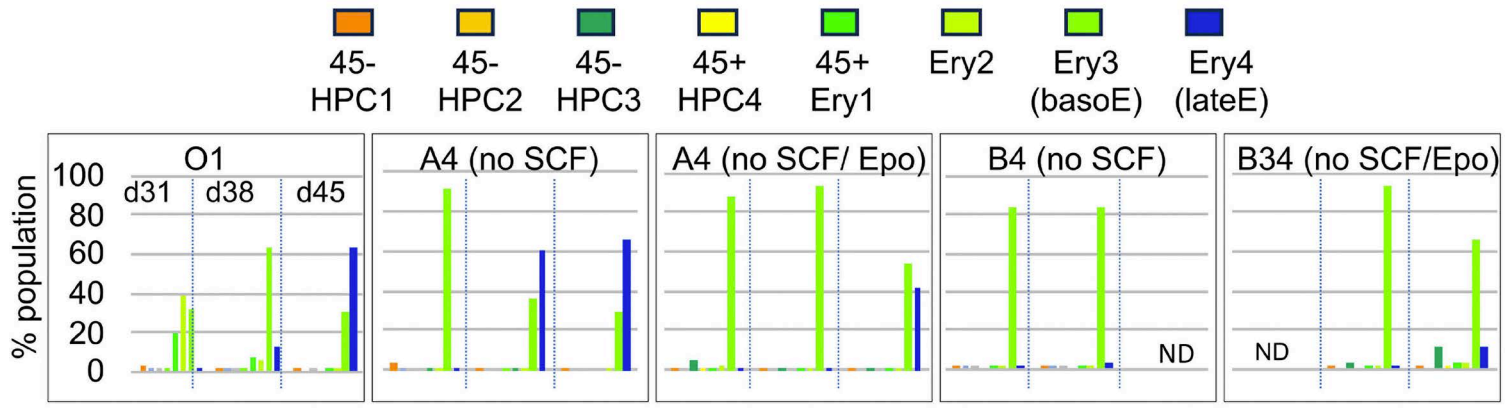


Figure 2. Differentiation in the absence of stem cell factor and erythropoietin. (A) Diagram illustrating the differentiation conditions. The * indicates the absence of stem cell factor (SCF) in supplement S2 and S3. See Figure 1A for details. (B) Growth curves illustrating the proliferation of the A4 and B34 in the absence of SCF and erythropoietin (Epo). Results of 1 experiment representative of 2 experiments are shown. Both hemizygous and homozygous D816V lines can differentiate in the absence of both SCF and Epo. In the absence of SCF and Epo, B34 Ery3 cells did not die as quickly upon withdrawal of dexamethasone (Dex) when Epo was absent from the culture media, and some of the cells were even able to complete their terminal differentiation and enucleate at a rate of about 5%. This suggests that the block of differentiation of the Ery3 cells in the B34 cells is likely due to overstimulation by the homozygous *kit*D816V, and that this overstimulation can be partly relieved by omitting Epo from the media. (C) Diagram illustrating the 15-color fluorescence-activated cell sorting analysis of differentiation in the absence of SCF and Epo.

to terminal erythroid differentiation, we also investigated the potential of dasatinib,[31] an inhibitor of the kinase activity of the *kit*D817V SCF receptor, to enhance terminal differentiation.

In the absence of dasatinib, the enucleation rate of A4 cells averaged 14.65±4.4%, similar to the control cells. This rate rose to 21.8±4.9 when 200 nM of dasatinib was added on day 31 (Figure 3A). Further analysis of Romanowsky-stained microscope slides revealed that dasatinib also accelerated the differentiation process (Figure 3B, C). Subsequently, the globin chain composition of cells obtained on day 45 was analyzed through reverse-phase HPLC. As previously reported,[17] reticulocytes and orthochromatic erythroblasts produced from the control O1 cells expressed mostly fetal β-like globin chains (Figure 3D). Cultured RBC obtained from the A4 line also expressed predominantly fetal globins, alongside detectable embryonic globin chains. Further experiments indicated that A4 cells grown without SCF and Epo enucleated at similar rates (*data not shown*).

The impact of the *kit*D816V mutation on erythroid differentiation can be replicated in induced pluripotent stem cells from a distinct donor

In order to replicate these findings and assess the phenotype of heterozygous *kit*D816V iPSC (which were not obtained in the experiments described above), CRSPR editing was repeated on cells from two donors (O1 and O2), mixing in an additional HDR donor oligonucleotide encoding the wild-type sequence in equal proportions with the *kit*D816V HDR oligonucleotide. This approach yielded multiple clones

heterozygous for the D816V mutation for both the O1 and O2 donors (*Online Supplementary Figure S6A*). Differentiation experiments using the PSC-RED protocol revealed that these clones proliferated at a high rate (slightly lower than the A4 hemizygous clones) and could produce enucleated cells (*Online Supplementary Figure S6B, C*). These results indicate that the *kit*D816V mutation represents a reliable and broadly applicable approach to generate iPSC lines capable of differentiating into RBC without the need for SCF.

Production of stem cell factor-independent self-renewing erythroblasts

It has long been known that the glucocorticoid receptor is a key regulator of the decision between self-renewal and

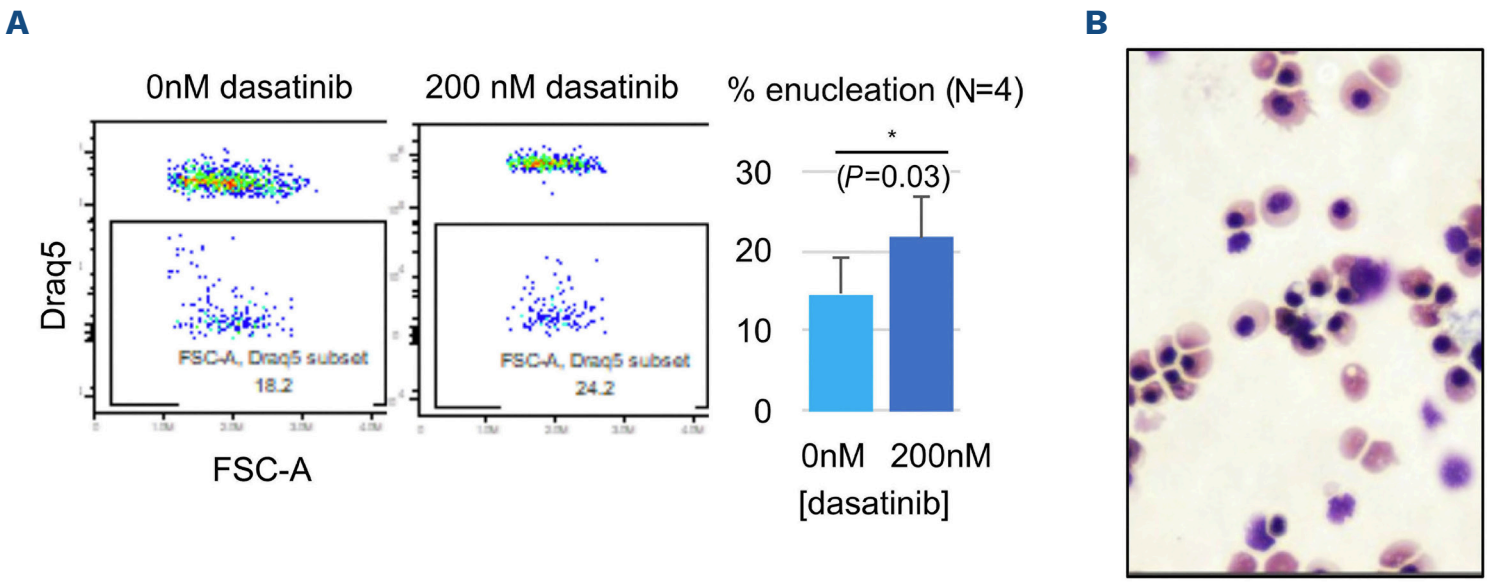


Figure 3. Terminal differentiation of *kit*D816V induced pluripotent stem cells. (A) Representative dotplots illustrating the enucleation of the A4 cells differentiated in the absence of stem cell factor (SCF), with or without 200 nM dasatinib (introduced on day 31 of differentiation). The experiment was performed in duplicate. (B) Micrograph illustrating the morphology of the cells obtained on day 45 of the pluripotent stem cell robust erythroid differentiation (PSC-RED) protocol (magnification =400x). (C) Graph illustrating the evolution of the cultures between day 31 to day 45 in the presence or absence of dasatinib. Cells were classified using morphological criteria after Romanowsky staining and light microscopy examination. Baso: basophilic erythroblasts; Poly: poly-chromatophilic erythroblasts; Ortho: ortho-chromatophilic erythroblasts; retic: reticulocytes. Dasatinib accelerates the differentiation and increases the rate of enucleation. One hundred cells were enumerated at each time point. (D) High pressure liquid chromatography (HPLC) profiles of erythroblasts obtained from clone A4 (in the absence of SCF). (E) Bargraph illustrating the averages (± standard deviation) globin chain expression measured in 3 experiments. Red blood cells (RBC) produced by clone A4 express mostly fetal hemoglobins.

differentiation in erythroid progenitors and that both human and mouse CFU-E/pro-erythroblasts can self-renew for a limited time when cultured in the presence of SCF, Epo and Dex.[4,32,33] In addition, we have previously shown that erythroblasts derived from human pluripotent stem cells could self-renew in culture for an extended period of time in the presence of the same factors.[14]

In order to investigate whether *kit*D816V erythroid progenitors also exhibit self-renewal capabilities, we used the PSC-RED protocol to generate day-17 HPC from the A4 and B34 iPSC lines and expanded them without cytokines but with Dex and IBMX (referred to as DI conditions). Both lines demonstrated the ability to expand under these conditions for approximately 45 to 55 days resulting in a greater than 1,000-fold expansion before reaching a plateau and ceasing to proliferate (Figure 4B). Flow cytometry analysis indicated that in the DI conditions the A4 and B34 day-17 HPC downregulated CD49f, CD45, CD38 and CD34 and upregulated expression of CD36, CD71, and CD235a (*Online Supplementary Figure S7A*). This transition from an HPC to an erythroblast phenotype, resembling iPSC-derived late CFU-E and pro-erythroblasts, was almost completed by day 24. After that time point the antigen profiles of both the A4 and B34 cells remained constant, suggesting that the cells were self-renewing. Romanowsky staining supported these findings since it showed that the *kit*D816V day-17 HPC cultured in DI conditions exhibited a very uniform CFU-E/pro-erythroblast phenotype at day 45 (*Online Supplementary Figure S7B*), while the same day-17 HPC cultured according to the PSC-RED protocol differentiated into orthochromatic erythroblasts and reticulocytes (*Online Supplementary Figure S3B, C*). We concluded from these experiments that *kit*D816V day-17 HPC were able to differentiate into erythroblasts with limited self-renewing capability when grown in the absence of SCF and Epo.

Since the A4 cells were easier than the B34 cells to differentiate, we focused subsequent experiments on the former cells. Crucially, further experiments, indicated that adding 1 unit/mL of Epo to Dex and IBMX (referred to as EDI conditions) enabled the A4 erythroblasts to proliferate without differentiation for over 25 passages (approximately 120 days), resulting in a 10^{20}-fold amplification (Figure 4C). Remarkably, the viability of these SRE, assessed by propidium iodide or by Annexin V staining, during this expansion phase was between 90% and 99% at all times (*Online Supplementary Figure S8*). These findings indicated that SRE capable of sustained growth without the need for SCF can be generated from *kit*D816V iPSC.

Production of stem cell factor- and erythropoietin-independent kitjak2 self-renewing erythroblasts

In order to investigate the possibility of obtaining cells capable of a similar long-term self-renewal without requiring Epo, we introduced the *jak2*V617F mutation through CRSPR-mediated mutagenesis in the A4 line using the method described earlier. Once again, over 50% of the screened clones had acquired the *jak2*V617F mutation (*Online Supplementary Figure S9A, B*). We termed the double-mutant, the kitjak2 lines. When subjected to differentiation using the PSC-RED protocol, iPSC lines hemizygous for the *kit*D816V mutation and either homozygous (lines G19 and H12) or hemizygous (lines H5 and H11) for the *jak2*V617F mutation demonstrated the ability to undergo erythroid expansion and differentiation in the absence of both SCF and Epo (Figure 4D; *Online Supplementary Figure S9C*).

Significantly, additional experiments demonstrated that day-17 HPC from the G19 and H12 kitjak2 SRE cultured in DI conditions proliferated exponentially in the absence of SCF and Epo for about 3 months before entering a state of senescence, suggesting that they had differentiated into SRE. Even more remarkable, the H5 and H12 cells proliferated in the same conditions for more than 4 months resulting in an amplification of greater than 10^{18}-fold (Figure 4E). Similarly to the A4 cells grown in EDI conditions, propidium iodide staining revealed that the viability of the kitjak2 SRE grown in DI conditions was very high (90-99%) until senescence (*Online Supplementary Figure S8*). Flow cytometry analysis confirmed that the A4 and kitjak2 SRE cultured in DI conditions, rapidly acquired a phenotype almost identical to the SRE phenotype of the A4 cells grown in EDI conditions (Figure 4F; *Online Supplementary Figure S10A, B*). Again, the transition between the day-17 HPC phenotype and the SRE phenotype was almost completed by day 24 and became stable after day 31 of culture. Remarkably, the antigen profile of all the SRE analyzed was strikingly similar and varied minimally over a span of 70 days. Romanowsky staining performed at early and late passages, revealed that all the SRE exhibit a uniform CFU-E/pro-erythroblasts erythroid morphology, with few signs of differentiation, confirming the FACS data (*Online Supplementary Figures S7, S10C*).

Kitjak2 cells can differentiate into cultured reticolocytes after extended culture without cytokines

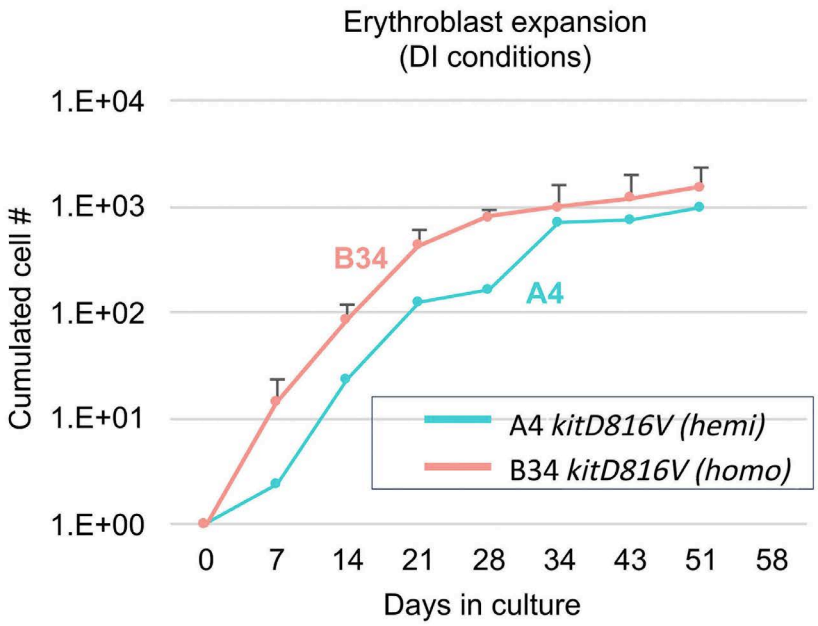
In order to investigate the potential of the A4 and kitjak2 SRE respectively expanded in EDI and DI conditions, we initiated their differentiation by withdrawing Epo, Dex and IBMX from the culture media. This resulted in a rapid 7-day differentiation which produced a few enucleated cells, but with a yield of cRetic/SRE of only about 0.1 to 0.01 due to high cell mortality (*data not shown*).

Epo serves as the primary cytokine shielding erythroblasts from apoptosis.[34] In order to enhance the differentiation of these cells, we administered a pulse of Epo (4 u/mL) to cushion the cells from the abrupt withdrawal of Dex and IBMX, and supplemented with 5% human plasma during the initial 5 days of differentiation (Figure 5A). These adjustments dramatically improved survival and lengthened the differentiation period to between 9 and 11 days. For both the A4 and the kitJak2 SRE, the cells expanded about 30- to 40-fold during the differentiation period (Figure

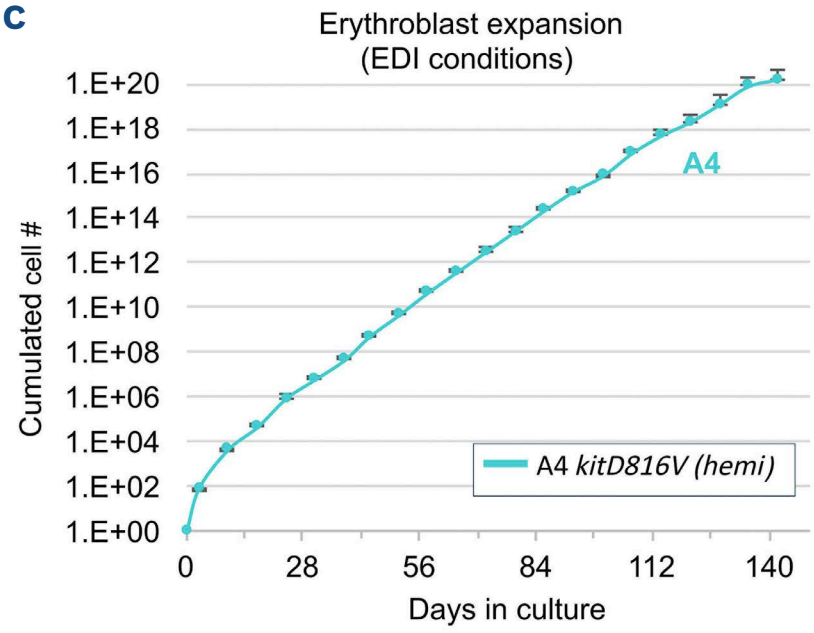
A

days	protocol	1	2	3-10	10-17	17-24	24-31	31-38	38 - indefinite
control	PSC-RED	S1	S2	S3	STIF	SEDI	SER	SER2	
No SCF		S1	S2*	S3*	TIF	EDI	ER	ER2	
No SCF/ EPO		S1	S2*	S3*	TIF	DI	R	R2	
No SCF	Self-renewal	S1	S2*	S3*	TIF	EDI			
No SCF/Epo		S1	S2*	S3*	TIF	DI			

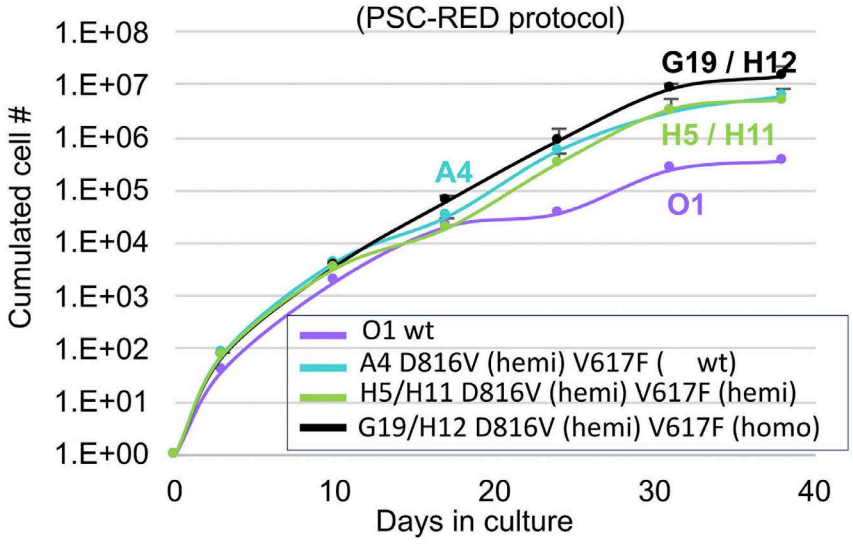
B



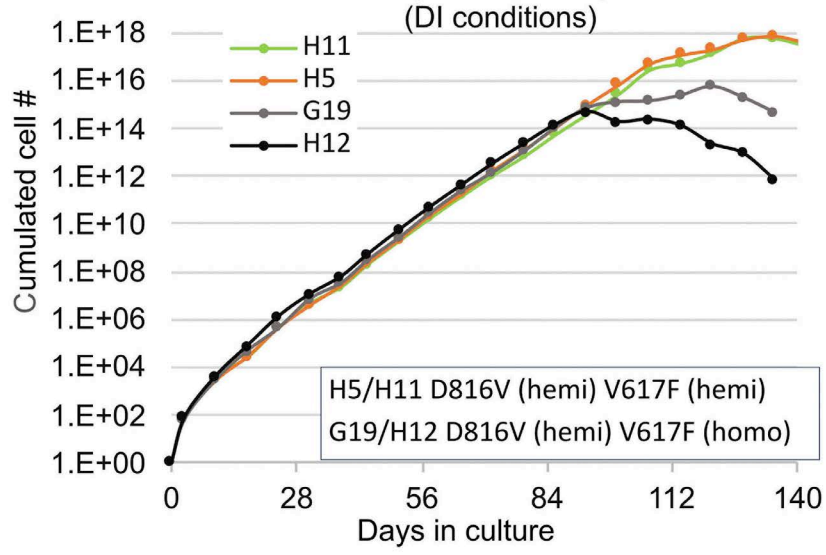
C



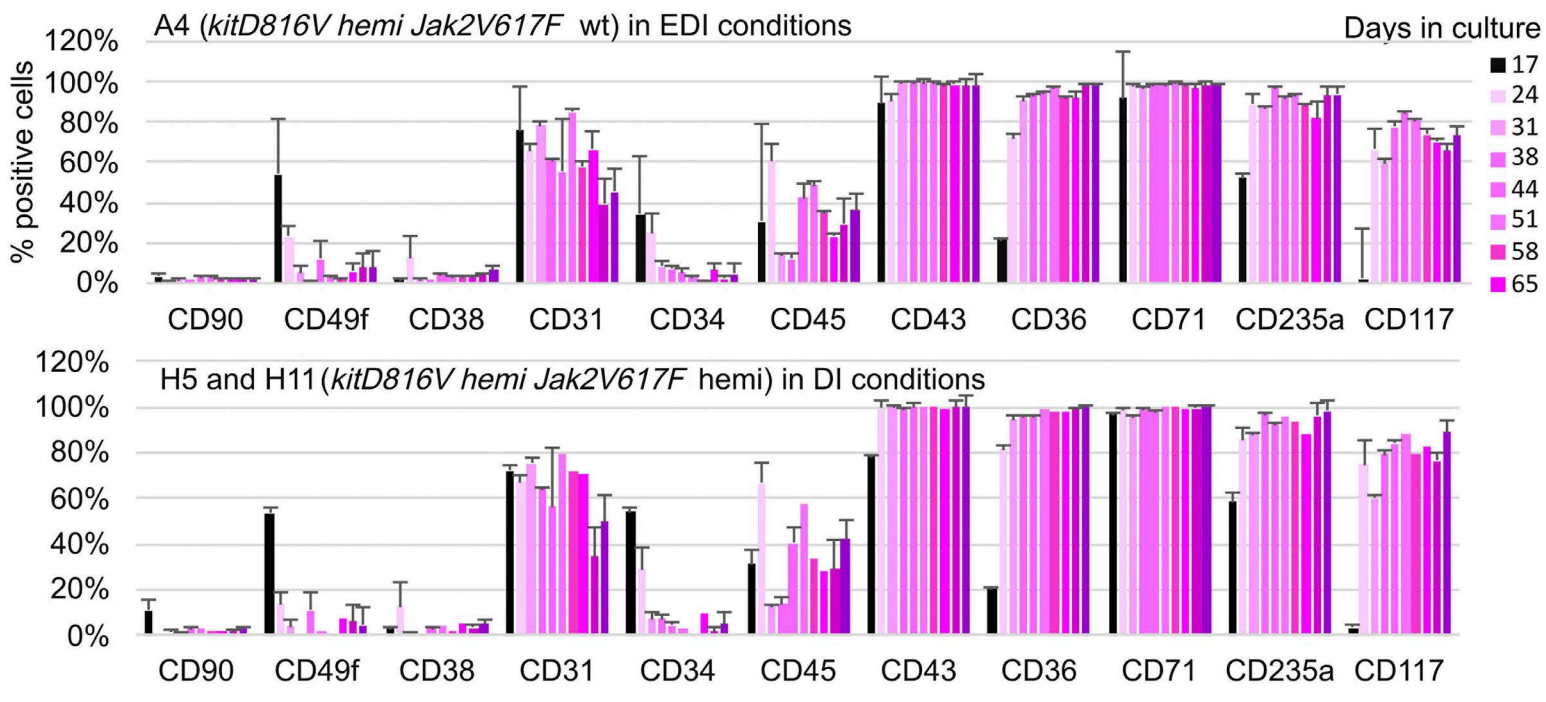
D



E



F



Continued on following page.

Figure 4. Production of self-renewing erythroblasts. (A) Diagram illustrating the differentiation conditions. The * indicates the absence of stem cell factor (SCF) in supplement S2 and S3. See Figure 1A for details. (B) Growth curves illustrating that day-17 hematopoietic progenitor cells (HPC) derived from the A4 and B34 cells can differentiate into erythroblasts that can self-renew for about 45 to 55 days in the presence of dexamethasone (Dex) and 3-isobutyl-1-methylxanthine (IBMX) but in the absence of any cytokines, yield a more than 1,000-fold amplification. Averages (± standard deviation [SD]) of 2 experiments are shown. (C) Growth curve illustrating that day-17 HPC derived from the A4 cells can differentiate into erythroblasts that can self-renew for >140 days in the presence of Dex and IBMX and 1 unit/mL of erythropoietin, yielding more than a 10^{20}-fold amplification. Averages (± SD) of 2 experiments are shown. (D) Growth curves illustrating the differentiation of 4 lines of induced pluripotent stem cells (iPSC) hemizygous (hemi) for the *kit*D816V mutation and either hemi (H5 and H11) or homozygous (homo) for the *Jak2*V617F mutations. All four iPSC lines proliferate and differentiate in the absence of SCF and erythropoietin (Epo) at rate that are greater than that of control cells (O1) differentiated in the presence of SCF and Epo. Averages (± SD) of 2 experiments are shown. (E) Growth curves illustrating that day-17 HPC derived from the H5/H11 and G18/H12 iPSC lines can differentiate into erythroblasts that can respectively self-renew for about 120 and 100 days in the presence of Dex and IBMX but in the absence of any cytokines, yielding >10^{18}-fold amplification in the case of the H5 and H11 lines and >10^{16}-fold amplification for the G19 and H12 lines. Average (± SD) of 2 SRE are shown for each genotypes. (F) Bargraph illustrating the expression of 11 markers in the A4 *kit*D816V SRE (cultured in erythropoieitin, Dex and IBMX [EDI] and in the kitjak2 line H5 and H11 (cultured in Dex and IBMX [DI]). Bars represent the average (± SD) expression measured in 2 independent experiments for the A4 *kit*D816V SRE and the average (± SD) expression of the H5 and H11 SRE measured weekly from day 17 to 93. The day-17 HPC (black bars) differentiate into SRE between days 17 and 31. After day 31, expression of the markers is very stable over time. SRE resemble iPSC-derived late colony-forming unit-erythrocytes (CFU-E) or pro-erythroblasts.

5B; *Online Supplementary Figures S11A, S12*). Viability, assessed by propidium iodide and acridine orange staining on a Luna FL cell counter, was above 95% between day 3 and day 9 (Figure 5B). Viability on day 11 was lower but varied between experiments. Together, these results suggest that the cells divided at least 5 times during the differentiation period.

FACS analysis revealed that expression of the CD235a antigen, which was expressed in almost all cells throughout the differentiation period, increased as the cells progressed toward terminal maturation (Figure 5C; *Online Supplementary Figure S11A*). In contrast, CD43 expression, an antigen recently identified as useful to assess erythroid differentiation in iPSC,[35] was present on almost all cells at days 3 and 6 but was nearly completely silenced by day 9. Similarly, CD36 expression decreased over time but its silencing occurred later than that of CD43. Finally, CD71, one of the last markers to be silenced during erythroid differentiation, also followed the expected pattern of expression, though its silencing was not as complete as that of the other markers, possibly because our culture conditions are not optimal for reticulocyte maturation. Romanovsky staining confirm the FACS analysis, with pro- and basophilic erythroblasts abundant during the initial days of differentiation, and polychromatophilic, orthochromatophilic erythroblasts and reticulocytes dominating during the final days (Figure 5D; *Online Supplementary Figure S11B*). The overall patterns of differentiation were very similar for the A4 SRE and for the H5 kitJak2 SRE (*Online Supplementary Figure S11C-E*).

In order to further examine the viability, we stained A4 and H5 kitJak2 SRE with Annexin V-FITC and DAPI and monitored apoptosis by FACS from days 3 to 11 (Figures 5E; *Online Supplementary Figures S11D, S12*). This analysis revealed that the proportion of Annexin V-positive apoptotic cells ranged from 11% to 16% between days 3 and 9, confirming the cells' high viability during differentiation. At day 11, the percentages of apoptotic cells were significantly higher and more variable across experiments, aligning with the results obtained with propidium iodide staining.

Staining with Draq5 and FACS analysis showed that the rate of enucleation varied from 30% to 55% averaging about 30% for the A4 lines and more than 50% for the H5 SRE (Figure 5F). On average, cell amplification during terminal differentiation, based on more than ten experiments, exceeded a 30-fold increase for the A4 line and reached about 28-fold for the H5 line. This resulted in a yield of more than ten reticulocytes per A4 or H5 SRE plated on day 0 (Figure 5F). Determining the optimal day for harvesting the cRetic proved challenging, because the rate of enucleation tended to increase between day 9 and 11, but in some experiments the viability of the cells decreased dramatically at either day 10 or 11. Additional experiments revealed that both the A4 and all kitjak2 SRE that we generated could differentiate into cRetic up to the point of senescence (*Online Supplementary Figure S13*).

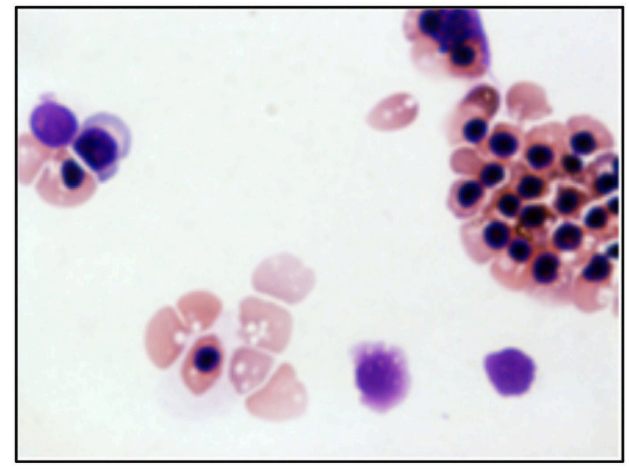
Characterization of the cultured reticulocytes derived from the A4 and kitjak2 cells

In order to evaluate the quality of the RBC originating from the A4 and the kitjak2 SRE, we purified enucleated cells using PALL Acrodisc filters. We then generated HPLC globin profiles and gathered morphological data via light microscopy, and red cell indices with the Advia 2120 blood count analyzer. The purified RBC expressed 78-82% fetal (Aγ and Gγ), 12-17% embryonic (ε) and 5-7% adult (β) β-like globins. They also expressed trace amounts of embryonic (ζ) α-like globins (Figure 5G). Morphologically the cells were larger than standard adult RBC (mean corpuscular volume [MCV] of about 125 fL *vs*. 85 fL, and diameter of about 10.5 um *vs*. 8.3 um) and were well hemoglobinized since they exhibited hemoglobin concentrations similar to control cells (about 28 g/dL) (Figure 5H).

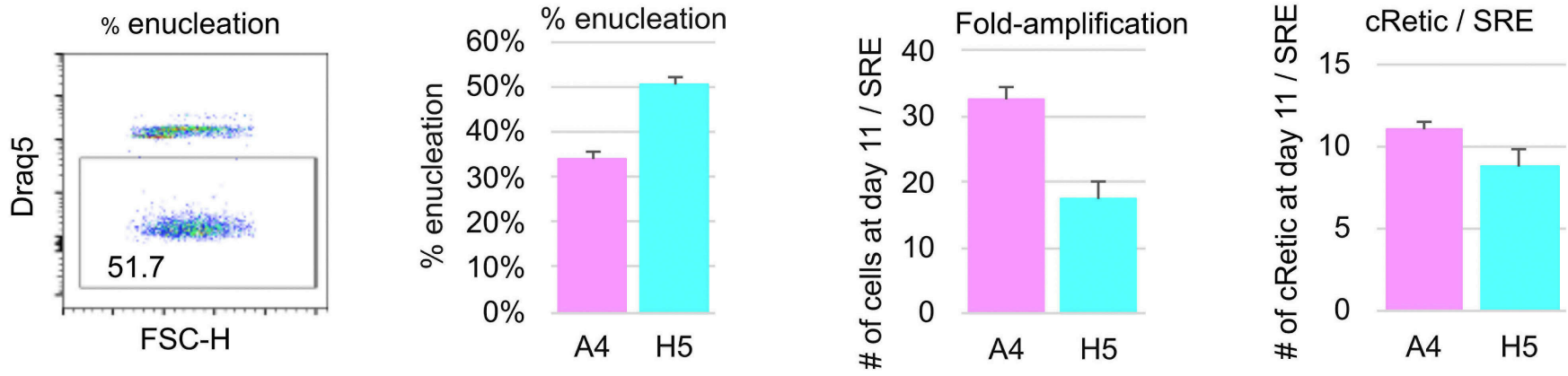
A

Cells	Self-renewing conditions	Day 0 - 5	Day 5 – 11
KitD816V (A4)	Dex Epo, IBMX	R5 medium Epo 4U/mL (day 0) RU486, 5% human plasma	Pure RPMI
Kit (H5/H11, G19/H12)	Dex IBMX		

B



C



D

E

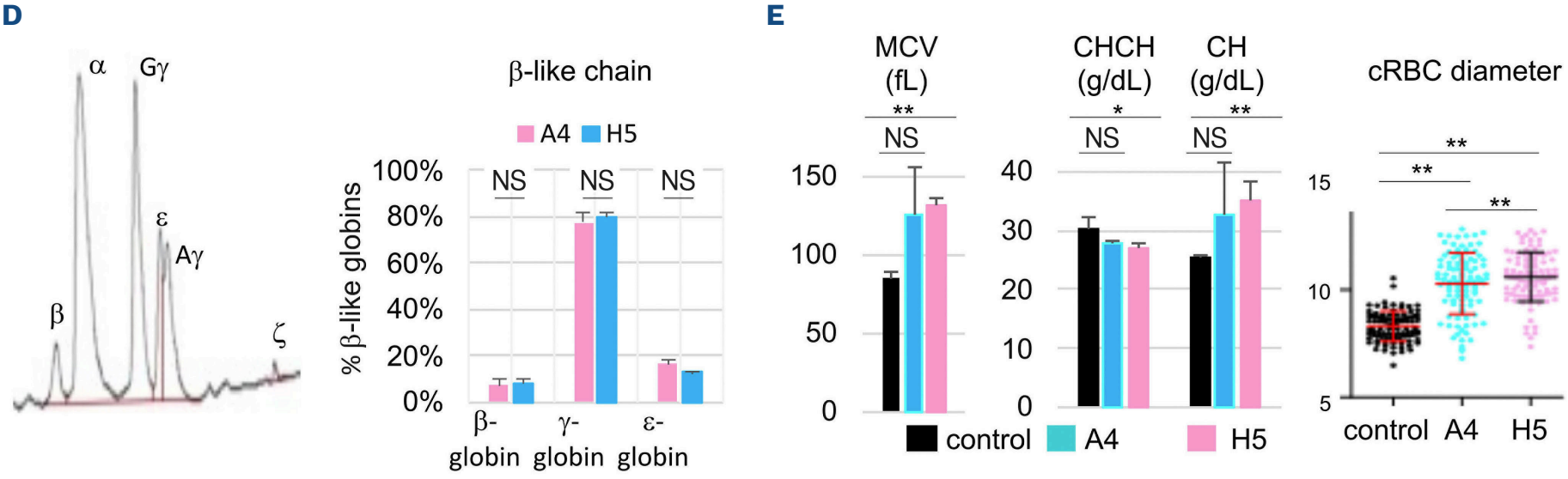


Figure 5. Differentiation of the self-renewing erythroblasts. (A) Differentiation conditions. (B) Growth curve illustrating the average cell growth and viability ± standard deviation (SD); (N=3) dur-ing H5 (define) self-renewing erythroblast (SRE) differentiation. (C) Dotplots illustrating the expres-sion of CD235a, CD43, CD36 and CD71 during H5 *kit*Jak2 SRE differentiation. Expression of CD235a increases overtime, while CD43, CD36 and CD71 are sequentially silenced. Uns.: unstained. (D) Micrographs illustrating Romanovsky staining of cells generated from the H5 SRE after 10 days of differentiation. Most cells are reticulocytes or orthochromatic erythroblasts. Inset: cell pellet obtained at day 9 illustrates the strong hemoglobinization of the cells. (E) Differentiating H5 SRE were analyzed by fluorescence activated cell sorting (FACS) after staining with DAPI and Annexin V-FITC. Bar graph illustrates the average percentage ± SD; (N=3) of necrotic, late and early apoptotic cells at days 3, 6, 9 and 11. (F, left) Dotplots illustrating a flow cytometry analysis of H5 cells stained with Draq5 at day 10 of differentiation. Right: the 3 bar graphs respectively illustrate the percentage enucleation, the cumulated fold-amplification at day 10 or 11 (calculated as the product of the fold-amplification observed after each feeding between days 0 and 10 or 11), and the number of cultured reticolocytes (cRetic)/SRE (calculated by multiplying the rate of enucleation by the cumulated fold-amplification at day 10 or 11). The averages (± SD) of 5 experiments are plotted. (G, right) Chromatogram illustrating a reverse phase high pressure liquid chromatograhpy (HPLC) analysis of globin chain expression of cRetic obtained by differentiation of A4 and H5 SRE. Bar graphs illustrate the averages (± SD) of 2 experiments. (E, left) Red blood cell (RBC) indices of cells generated from the A4 and H5 lines obtained using an Advia blood count analyzer; (N=3); CHCM: cellular hemoglobin concentration mean of intact RBC (optically measured MCHC); CH: mean optical hemoglobin con-tent of intact RBC. (E, right) RBCl diameter assessed by microscopy; 100 cells /cell type were analyzed.

Karyotypic stability

In order to examine if the *kit*D816V and *jak2*V617F mutations induce karyotypic instability or aneuploidy, we compared chromosome copy numbers in SRE at passage 20 to 22 and in iPSC at passage 38-40, before and after CRSPR/cas9 editing through low-pass whole-genome sequencing, using O1 and O2 donor-derived PB mononuclear cells as controls. Analysis with the CNVKit software[28] showed no detectable copy numbers variations in any cell line studied while confirming the marked aneuploidy of control transformed cells sequenced at an equivalent depth (Figure 6; *Online Supplementary Figure S14*). We concluded that cells carrying both the *kit*D816V and *jak2*V617F can be propagated in culture for long periods of time without acquiring karyotypic abnormalities.

Reagent red blood cells

One application of cRetic is the production of reagent RBC for identifying allo-antibodies in chronic transfusion recipients,[36] minimizing transfusion reactions. Reagent cells typically come from volunteer blood, but there are shortages of cells to detect allo-antibodies in sickle cell anemia and myelodysplastic patients who constitute over 80% of all allo-immunized individuals at most blood banks. Investigate whether cRetic could fill this role, we tested whether we could detect Rhesus C, c, E, and e antigens on the surface of A4 cRetic using a solid phase red cell adherence assay.[37] This indicated that 5 million RBC per test could be used to identify all four antibodies (Figure 7), demonstrating that cRetic produced without cytokines can be used as reagent RBC.

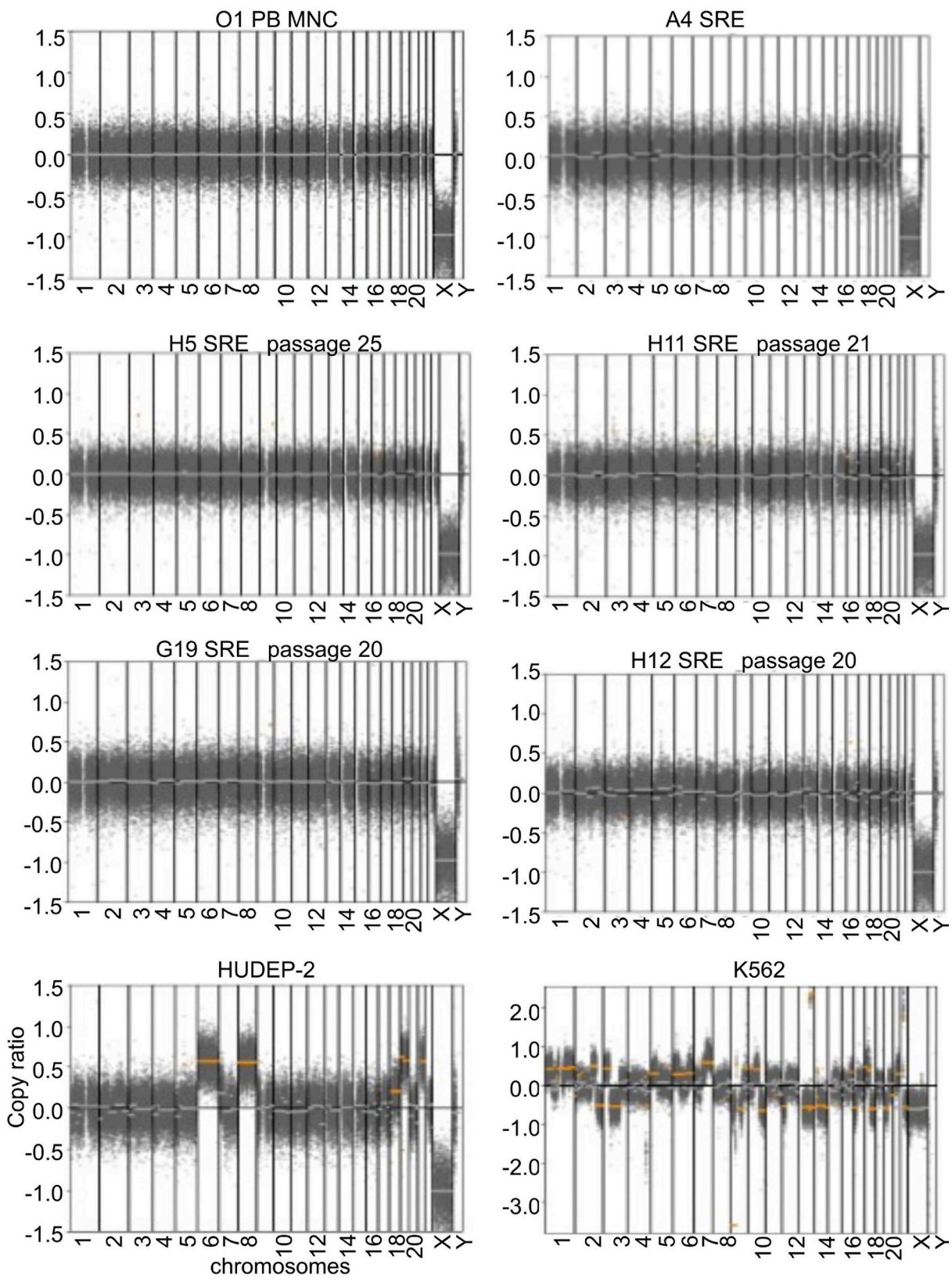
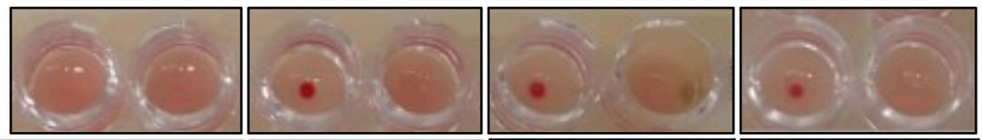


Figure 6. Copy number analysis by low-pass sequencing. Genomic DNA was sequenced at a depth of 1.5 Gb and analyzed using the CNVkit software. Graphs illustrate that peripheral blood mononucelar cells (PB MNC) O1, and passage 20 to 25 A4, H5, H11, G19 and H12 self-renewing erythroblasts (SRE) exhibit no detectable copy number variants greater than 1 mb (the limit of detection for this read depth). By contrast, transformed cells, HUDEP-2 and K562 cells, cultured for a long period of time exhibit a high level of aneuploidy.



	NYBC1	NYBC2	NYBC3		A4 reticulocytes		A4 reticulocytes	
Phenotype	Ce	cE	ce	ce	ce	ce	c e	c e
Test Ab	e	E	C	c	E	e	C	c
Result	pos	pos	neg	pos	neg	pos	neg	pos

Figure 7. Cultured red blood cells as reagent red blood cells in a solid-phase red cell adherence assay. Treated capture strips were coated as suggested by the manufacturer (Immucor) with about 5 million A4 cultured red blood cells (cRBC) or with control adult RBC of known (NYBC samples) RhCcE phenotypes. The phenotypes of the coated cells were then determined by solid phase red cell adherence assay using test antibodies and immunoglobulin (Ig)G-coated indicator RBC also as recommended by the manufacturer. In this system, a visible red pellet at the bottom of the well indicates absence of the antigen on the tested RBC, while the absence of a pellet indicates presence of the antigen. The phenotypes determined with this capture assay agreed in all cases with the expected (known) phenotypes, demonstrating that cRBC can be used as reagent RBC in this system. NYBC3 are control adult RBC samples.

Discussion

Our findings highlight the profound influence of constitutive alleles of the *kit* and *jak2* genes on the erythroid differentiation of iPSC. The *kit*D816V mutation completely alleviated the need for SCF and Epo, as all iPSC lines that we generated, regardless of genotype, were able to proliferate and differentiate into erythroblasts in the absence of these cytokines at about the same rate as control cells in the presence of cytokines. Homozygosity for the *kit*D816V partially hindered terminal erythroid differentiation, but hemizygous and heterozygous variants underwent terminal differentiation and showed high enucleation rates. iPSC with both the *kit*D816V and j*ak2*V671F mutations also thrived and differentiated without any cytokines, often surpassing control cells supplemented with SCF and Epo.

The *kit*D816V and *jak2*V671F allowed iPSC to differentiate into erythroid cells in the absence of any cytokines starting at day 17 (Figure 4D). In additional experiments (*data not shown*), we also observed that day-10 HPC cultured according to the short version of the PSC-RED protocol in which the expansion step from day 10 to 17 is omitted (Figure 1A), could also differentiate into mature erythroid cells in the complete absence of cytokine. These data show that the signaling pathways activated by the *kit*D816V SCF receptor, which is known to interact with the Epo receptor[25] are necessary and sufficient to specify the erythroid differentiation of day-10 HPC, at least in the presence of IBMX and Dex.

Using flow cytometry, we identified eight distinct cell populations through dimension reduction tools, flowSOM and UMAP. These groupings align reasonably well with the known sequence of antigen profiles during iPSC differentiation into hematopoietic and erythroid lineages. However, iPSC-derived erythropoiesis is complex because developmental hematopoiesis occurs in successive primitive, erythro-myeloid, and definitive waves, each linked to the production of specific globins.[16] As such, our defined populations likely encompass significant developmental heterogeneity because the markers we employed don't sharply distinguish between primitive and definitive erythroid cells. Consequently, the cRetic that we generated are probably a blend of primitive erythroid cells, which express embryonic and fetal globins, and more advanced fetal-like cells that exhibit both fetal and adult globins.

The key discovery presented here is that SRE can reproducibly be generated from iPSC carrying the *kit*D816V and *jak2*V671F mutations. While the single-mutant *kit*D816V cells exhibited modest self-renewal - lasting approximately 50 days without cytokines - their proliferation potential surged to 140 days in the presence of Epo. Most remarkably, double-mutant kitjak2 cells thrived for about 120 days without cytokines. All of the SRE cultures that we developed, proliferated with a high viability, little signs of differentiation, retained the ability to enucleate at a high rate until senescence, and could be consistently passaged at ratios from 1:10 to 1:15 every 3-4 days, utilizing a cost-effective, chemically-defined, albumin-free medium supplemented with a minimal amount of recombinant transferrin.

The self-renewal of kitjak2 cells hinges on the inclusion of two specific small molecules, Dex and IBMX. Dex is essential for the self-renewal of the A4 and kitjak2 SRE since withdrawal of this molecule leads to rapid differentiation. Dex has long been known to control erythroblast self-renewal, but its mechanism of action in erythropoiesis remains only partially understood.[38] Ashley *et al*. have recently shown that erythroid cultures of adult but not CB human CD34$^+$ cells are sensitive to Dex.[39] Furthermore, Dex treatment was found to result in the expansion of immature CFU-E populations, likely through the upregulation of p57Kip2. The observed lack of Dex sensitivity of human CB progenitors reported by Ashley, contrasts with findings in mice, where both fetal liver BFU-E and CFU-E have been shown to respond to Dex.[40,41] Further studies will be necessary to determine if p57Kip2 plays a role in the self-renewal of the kitjak2 cells or if other mechanisms are involved.

IBMX, a non-specific inhibitor of cAMP and cGMP phos-

pho-diesterases, elevates intracellular cAMP levels.[42] We incorporated IBMX in the PSC-RED protocol because high cAMP concentrations facilitate early HPC specification.[43,44] IBMX also regulates erythropoiesis. The EpoR doesn't directly control cAMP, but agents such as forskolin and prostaglandins which modify cAMP concentration, do influence Epo-mediated erythropoiesis.[45] Notably, cAMP-inducing agents have been shown to amplify the proliferation of colony-forming erythroid progenitors.[46,47] We found that IBMX is essential for the self-renewal of A4 and kitjak2 erythroblasts, as its omission markedly reduced their proliferation and viability (*data not shown*). Understanding how *kit*D816V, *jak2*V617F, Dex and IBMX jointly extend the self-renewal of kitjak2 cells will require additional studies.

The generation of kitjak2 cells is reproducible, as we successfully produced multiple lines from two distinct donors. SRE differentiate from iPSC-derived day-17 HPC in less than 2 weeks. This contrasts with immortalization using the HPV E6/E7 proteins which demands several months of transduced cell cultivation before cell lines emerge.[5] This reproducibility, paired with the rapid differentiation, indicates that no additional genetic or epigenetic alterations, beyond our engineered modifications, are necessary for the erythroblasts to achieve self-renewal without cytokines.

The cell lines that we generated are diploid and appear karyotypically stable likely because mutations in cytokine receptors and signaling adaptors don't directly drive chromosomal instability. Cultured RBC derived from the kitjak2 lines should be safe for transfusion applications, despite the association between several malignancies and the *kit*D816V and *jak2*V617F alleles, because RBC lack nuclei, and because any residual nucleated cells can be killed by irradiation.[48]

Expression of *jak2*V617F in mouse fetal liver triggers activation of Stat5, the STAT normally activated by Epo, but also activates Stat1 and Stat3.[49] In humans, the *JAK2*V617F allele is associated with PV but can also cause essential thrombocythemia. By contrast, mutations in *JAK2* exon 12, which are much rarer than the V617F mutation, are more specifically associated with PV. Experiments in which both mutations have been introduced in iPSC have shown that, compared with *JAK2*V617F-iPSC, *JAK2* exon 12-iPSC produced a greater number of erythroid cells that displayed more mature morphology and expressed more adult hemoglobin.[50] Importantly, exon12 mutations led to significantly higher levels of phospho-STAT1 but lower phospho-STAT3 compared with *JAK2*V617F-iPSC in response to erythropoietin. These studies suggest that ectopic STAT1 activation plays a role in PV in humans. Whether stat1 and stat3 are activated in humans kitjak2 SRE is currently unknown. Additionally, these data suggest that combining a *Jak2* exon 12 and the *Kit*D816V mutations might offer an alternative method for producing cytokine-independent SRE.

Differentiating a single iPSC using the PSC-RED protocol generates about 10^3 day-17 HPC. Each of these HPC can produce about 10^{18} kitjak2 self-renewing erythroblasts, which each yield up to ten RBC. Thus, theoretically, a single iPSC is sufficient to produce the number of RBC present in $>10^9$ liters of blood (10^{21} cells), surpassing the global annual transfusion volume. Cytokine-independent SRE, such as those from the O1 and O2 donors, who have blood group 0 and test negative for RhD and other clinically significant RBC antigens, therefore have ample proliferation capacity to meet the needs for the foreseeable future.

Although the culture of iPSC and the generation of day-17 HPC remain costly, it is important to recognize that over 99% of the cell expansion needed for cRetic production from kitjak2 cells occurs at the SRE stage, using a cost-effective culture medium. Consequently, the cost of producing day-17 HPC constitutes only a minor fraction of the overall expense incurred during cRetic production. Contrary to cells immortalized with HPV16 E6/E7 protein overexpression, kitjak2 SRE do not grow indefinitely. However, this isn't a significant concern since new, genetically identical kitjak2 SRE can be readily produced from iPSC. More importantly, the approval of cRetic for clinical use by regulatory authorities, when derived from late passage cells after extensive proliferation, remains uncertain because prolonged cell division inevitably leads to the accumulation of harmful mutations. The capacity of kitjak2 SRE to undergo over 70 population doublings is adequate to produce large quantities of cRetic. Enhancing their proliferative capacity beyond this point may not be beneficial for their clinical application.

Kim *et al.*[51] in mice, and Liu *et al.*[52] in humans demonstrated that overexpressing the transcription factor BmiI, part of the polycomb group complex 1, in HPC can produce adult SRE. These SRE can expand up to 10^{12} times in the presence of SCF, Epo, and Dex, while still retaining the capacity to differentiate into cRetic. This overexpression of BmiI presents a promising alternative for cRetic production. An advantage of this method is the potential for BmiI cRetic to express higher levels of adult globins compared to kitjak2 cRetic, although the HPLC globin profile of BmiI cRetic is not yet reported. In contrast, kitjak2 cRetic have the benefit of growing without cytokines and originating from immortal iPSC.

A limitation of the kitjak2 cells is their fetal/embryonic characteristics, including their larger size compared to adult cells and predominant expression of hemoglobin F (Hb F). However, Hb F expression does not pose a concern for certain uses of cRetic. For instance, for drug delivery and reagent RBC applications, where oxygen transport isn't the primary function of the cRetic, the type of hemoglobin expressed is not a major factor. Additionally, in transfusions for premature infants, an increasingly common procedure, Hb F expression can be beneficial, because it might prevent

retinopathies.[53] Yet, for adult transfusion, Hb A is preferable. Therefore, different cell sources for cRetics production may be better suited for specific translational applications.
A detailed characterization of the kitjak2 cRetic will be necessary before testing these cells in clinical trials. Like all enucleated erythroid cells created *in vitro*, kitjak2-derived enucleated cells are reticulocytes, not mature RBC. While transfused cRetic have been shown to mature into RBC *in vivo,*[1] it would be interesting to explore if cRetic can mature into RBC *in vitro*, considering the lack of established storage methods for cRetic.
In summary, we have developed a novel approach to produce cRetic from iPSC which is more practical and most cost effective than previous approaches. The ability of kitjak2 iPSC to differentiated into SRE that are karyotypically stable, can proliferate for up to 4 months in a cost-effective, chemically-defined, albumin- and cytokine-free cell culture medium, and that retain the ability to enucleate at a high rate should facilitate the production of cRetic for translational applications.

Disclosures
No conflicts of interest to disclose.

Contributions
SZ and ZY performed experiments. EO performed, designed and interpreted experiments. EEB performed, designed and interpreted experiments, wrote the manuscript and supervised the study.

Data-sharing statement
Cells generated in these studies are available upon request.

References

1. Kupzig S, Parsons SF, Curnow E, Anstee DJ, Blair A. Superior survival of ex vivo cultured human reticulocytes following transfusion into mice. Haematologica. 2017;102(3):476-483.
2. Villa CH, Cines DB, Siegel DL, Muzykantov V. Erythrocytes as carriers for drug delivery in blood trans-fusion and beyond. Transfus Med Rev. 2017;31(1):26-35.
3. Giarratana MC, Kobari L, Lapillonne H, et al. Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. Nat Biotechnol. 2005;23(1):69-74.
4. Migliaccio G, Sanchez M, Masiello F, et al. Humanized culture medium for clinical expansion of human erythroblasts. Cell Transplant. 2010;19(4):453-469.
5. Kurita R, Funato K, Abe T, et al. Establishment and characterization of immortalized erythroid progenitor cell lines derived from a common cell source. Exp Hematol. 2019;69:11-16.
6. Trakarnsanga K, Griffiths RE, Wilson MC, et al. An immortalized adult human erythroid line facilitates sustainable and scalable generation of functional red cells. Nat Commun. 2017;8:14750.
7. Cervellera CF, Mazziotta C, Di Mauro G, et al. Immortalized erythroid cells as a novel frontier for in vitro blood production: current approaches and potential clinical application. Stem Cell Res Ther. 2023;14(1):139.
8. Akimov SS, Ramezani A, Hawley TS, Hawley RG. Bypass of senescence, immortalization, and transformation of human hematopoietic progenitor cells. Stem Cells. 2005;23(9):1423-1433.
9. Malagutti N, Rotondo JC, Cerritelli L, et al. High human papillomavirus DNA loads in inflammatory middle ear diseases. Pathogens. 2020;9(3):224.
10. Carbone M, Gazdar A, Butel JS. SV40 and human mesothelioma. Transl Lung Cancer Res. 2020;9(S1):S47-S59.
11. Hirose S, Takayama N, Nakamura S, et al. Immortalization of erythroblasts by c-MYC and BCL-XL enables large-scale erythrocyte production from human pluripotent stem cells. Stem Cell Rep. 2013;1(6):499-508.
12. Lee E, Lim ZR, Chen HY, et al. Defined serum-free medium for bioreactor culture of an immortalized human erythroblast cell line. Biotechnol J. 2018;13(4):e1700567.
13. Kurita R, Suda N, Sudo K, et al. Establishment of immortalized human erythroid progenitor cell lines able to produce enucleated red blood cells. PLoS One. 2013;8(3):e59890.
14. Olivier E, Qiu C, Bouhassira EE. Novel, High-yield red blood cell production methods from CD34-positive cells derived from human embryonic stem, yolk sac, fetal liver, cord blood, and peripheral blood. Stem Cells Transl Med. 2012;1(8):604-614.
15. Ferguson DCJ, MacInnes KA, Daniels DE, Frayne J. Chapter 1 - iPSC-derived erythroid cells. In: Birbrair A, ed. Recent advances in iPSC-derived cell types: Academic Press; 2021. p. 1-30.
16. Qiu C, Olivier EN, Velho M, Bouhassira EE. Globin switches in yolk sac-like primitive and fetal-like definitive red blood cells produced from human embryonic stem cells. Blood. 2008;111(4):2400-2408.
17. Olivier EN, Zhang SP, Yan Z, et al. PSC-RED and MNC-RED: albumin-free and low-transferrin robust erythroid differentiation protocols to produce human enucleated red blood cells. Exp Hematol. 2019;75:31-52.
18. Griffiths RE, Kupzig S, Cogan N, et al. Maturing reticulocytes internalize plasma membrane in glycophorin A-containing vesicles that fuse with autophagosomes before exocytosis. Blood. 2012;119(26):6296-6306.
19. Bayley R, Ahmed F, Glen K, Mccall M, Stacey A, Thomas R. The productivity limit of manufacturing blood cell therapy in scalable stirred bioreactors. J Tissue Eng Regen Med. 2018;12(1):e368-e378.
20. Sivalingam J, Su EY, Lim ZR, et al. A scalable suspension platform for generating high-density cultures of universal red blood cells from human induced pluripotent stem cells. Stem Cell Reports. 2021;16(1):182-197.
21. Kweon S, Kim S, Baek EJ. Current status of red blood cell manufacturing in 3D culture and bioreactors. Blood Res. 2023;58(Suppl 1):S46-S51.
22. Worobec AS, Semere T, Nagata H, Metcalfe DD. Clinical correlates of the presence of the asp816Val c-kit mutation in the peripheral blood mononuclear cells of patients with mastocytosis. Cancer. 1998;83(10):2120-2129.
23. James C, Ugo V, Le Couédic J-P, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature. 2005;434(7037):1144-1148.
24. Couch T, Murphy Z, Getman M, Kurita R, Nakamura Y, Steiner LA. Human erythroblasts with c-Kit activating mutations have reduced cell culture costs and remain capable of terminal

maturation. Exp Hematol. 2019;74:19-24.
25. Wu H, Klingmüller U, Besmer P, Lodish HF. Interaction of the erythropoietin and stem-cell-factor receptors. Nature. 1995;377(6546):242-246.
26. Van Gassen S, Callebaut B, Van Helden MJ, et al. FlowSOM: using self-organizing maps for visualization and interpretation of cytometry data. Cytometry A. 2015;87(7):636-645.
27. Stolarek I, Samelak-Czajka A, Figlerowicz M, Jackowiak P. Dimensionality reduction by UMAP for visualizing and aiding in classification of imaging flow cytometry data. IScience. 2022;25(10):105142.
28. Talevich E, Shain AH, Botton T, Bastian BC. CNVkit: genome-wide copy number detection and visualization from targeted DNA sequencing. PLOS Computational Biology. 2016;12(4):e1004873.
29. Quintelier K, Couckuyt A, Emmaneel A, Aerts J, Saeys Y, Van Gassen S. Analyzing high-dimensional cytometry data using FlowSOM. Nat Protoc. 2021;16(8):3775-3801.
30. Mcinnes L, Healy J, Saul N, Großberger L. UMAP: uniform manifold approximation and projection. J Open Souce Softw. 2018;3(29):861.
31. Akin C, Arock M, Valent P. Tyrosine kinase inhibitors for the treatment of indolent systemic mastocytosis: are we there yet? J Allergy Clin Immunol. 2022;149(6):1912-1918.
32. Wessely O, Deiner EM, Beug H, Von Lindern M. The glucocorticoid receptor is a key regulator of the decision between self-renewal and differentiation in erythroid progenitors. EMBO J. 1997;16(2):267-280.
33. England SJ, McGrath KE, Frame JM, Palis J. Immature erythroblasts with extensive ex vivo self-renewal capacity emerge from the early mammalian fetus. Blood. 2011;117(9):2708-2717.
34. Lacombe C, Mayeux P. Biology of erythropoietin. Haematologica. 1998;83(8):724-732.
35. Bai J, Fan F, Gao C, et al. CD169-CD43 interaction is involved in erythroblastic island formation and erythroid differentiation. Haematologica. 2023;108(8):2205-2217.
36. Storry JR, Olsson ML, Reid ME. Application of DNA analysis to the quality assurance of reagent red blood cells. Transfusion. 2007;47(Suppl 1):S73-S78.
37. Ching E. Solid phase red cell adherence assay: a tubeless method for pretransfusion testing and other applications in transfusion science. Transfus Apher Sci. 2012;46(3):287-291.
38. Zingariello M, Bardelli C, Sancillo L, et al. Dexamethasone predisposes human erythroblasts toward impaired lipid metabolism and renders their ex vivo expansion highly dependent on plasma lipoproteins. Front Physiol. 2019;10:281.
39. Ashley RJ, Yan H, Wang N, et al. Steroid resistance in Diamond Blackfan anemia associates with p57Kip2 dysregulation in erythroid progenitors. J Clin Invest. 2020;130(4):2097-2110.
40. Flygare J, Rayon Estrada V, Shin C, Gupta S, Lodish HF. HIF1alpha synergizes with glucocorticoids to promote BFU-E progenitor self-renewal. Blood. 2011;117(12):3435-3444.
41. Hwang Y, Futran M, Hidalgo D, et al. Global increase in replication fork speed during a p57(KIP2)-regulated erythroid cell fate switch. Sci Adv. 2017;3(5):e1700298.
42. Ahmad F, Murata T, Shimizu K, Degerman E, Maurice D, Manganiello V. Cyclic nucleotide phosphodiesterases: important signaling modulators and therapeutic targets. Oral Dis. 2015;21(1):e25-e50.
43. Olivier EN, Marenah L, McCahill A, Condie A, Cowan S, Mountford JC. High-efficiency serum-free feeder-free erythroid differentiation of human pluripotent stem cells using small molecules. Stem Cells Transl Med. 2016;5(10):1394-1405.
44. Diaz MF, Li N, Lee HJ, et al. Biomechanical forces promote blood development through prostaglandin E2 and the cAMP-PKA signaling axis. J Exp Med. 2015;212(5):665-680.
45. Boer AK, Drayer AL, Vellenga E. cAMP/PKA-mediated regulation of erythropoiesis. Leuk Lymphoma. 2003;44(11):1893-1901.
46. Belegu M, Beckman B, Fisher JW. beta-adrenergic blockade of prostaglandin E2- and D2-induced erythroid colony formation. Am J Physiol. 1983;245(5 Pt 1):C322-327.
47. Fisher JW, Radtke HW, Jubiz W, Nelson PK, Burdowski A. Prostaglandins activation of erythropoietin production and erythroid progenitor cells. Exp Hematol. 1980;8(Suppl 8):65-89.
48. Francis RO. Chapter 42 - Irradiation of blood products. In: Shaz BH, Hillyer CD, Reyes Gil M, eds. Transfusion medicine and hemostasis (Third Edition): Elsevier; 2019. p. 261-265.
49. Shi J, Yuan B, Hu W, Lodish H. JAK2 V617F stimulates proliferation of erythropoietin-dependent erythroid progenitors and delays their differentiation by activating Stat1 and other nonerythroid signaling pathways. Exp Hematol. 2016;44(11):1044-1058.
50. Nilsri N, Jangprasert P, Pawinwongchai J, Israsena N, Rojnuckarin P. Distinct effects of V617F and exon12-mutated JAK2 expressions on erythropoiesis in a human induced pluripotent stem cell (iPSC)-based model. Sci Rep. 2021;11(1):5255.
51. Kim R, Ah, Olsen L, Jayme, England J, Samantha, et al. Bmi-1 regulates extensive erythroid self-renewal. Stem Cell Rep. 2015;4(6):995-1003.
52. Liu S, Wu M, Lancelot M, et al. BMI1 enables extensive expansion of functional erythroblasts from human peripheral blood mononuclear cells. Mol Ther. 2021;29(5):1918-1932.
53. Stutchfield CJ, Jain A, Odd D, Williams C, Markham R. Foetal haemoglobin, blood transfusion, and retinopathy of prematurity in very preterm infants: a pilot prospective cohort study. Eye. 2017;31(10):1451-1455.