Experimental Hematology

Experimental Hematology 2019;74:19–24

Human erythroblasts with c-Kit activating mutations have reduced cell culture costs and remain capable of terminal maturation

Tyler Couch[a,b], Zachary Murphy[b], Michael Getman[b], Ryo Kurita[c], Yukio Nakamura[c], and Laurie A. Steiner[b]

[a]*Department of Biochemistry and Biophysics, University of Rochester, Rochester, NY;* [b]*Center for Pediatric Biomedical Research, Department of Pediatrics, University of Rochester, Rochester, NY;* [c]*Cell Engineering Division, RIKEN BioResource Center, Tsukuba, Ibaraki, Japan*

(Received 21 February 2019; revised 10 April 2019; accepted 13 April 2019)

A major barrier to the in vitro production of red blood cells for transfusion therapy is the cost of culture components, with cytokines making up greater than half of the culture costs. Cell culture cytokines also represent a major expense for in vitro studies of human erythropoiesis. HUDEP-2 cells are an E6/E7 immortalized erythroblast line used for the in vitro study of human erythropoiesis. In contrast to other cell lines used to study human erythropoiesis, such as K562 cells, HUDEP-2 cells are capable of terminal maturation, including hemoglobin accumulation and chromatin condensation. As such, HUDEP-2 cells represent a valuable resource for studies not amenable to primary cell cultures; however, reliance on the cytokines stem cell factor (SCF) and erythropoietin (EPO) make HUDEP-2 cultures very expensive to maintain. To decrease culture costs, we used CRISPR/Cas9 genome editing to introduce a constitutively activating mutation into the SCF receptor gene *KIT*, with the goal of generating human erythroblasts capable of SCF-independent expansion. Three independent HUDEP-2 lines with unique *KIT* receptor genotypes were generated and characterized. All three lines were capable of robust expansion in the absence of SCF, decreasing culture costs by approximately half. Importantly, these lines remained capable of terminal maturation. Together, these data suggest that introduction of c-Kit activating mutations into human erythroblasts may help reduce the cost of erythroblast culture, making the in vitro study of erythropoiesis, and the eventual in vitro production of red blood cells, more economically feasible.

Red blood cells are a lifesaving therapy. Currently, all blood products for transfusion therapy are obtained from blood donors. Reliance on blood donors is associated with safety concerns and contributes to frequent blood product shortages that are particularly severe for rare blood types. Furthermore, red cell alloimmunization is a common complication of inherited anemias that require frequent blood transfusions. The in vitro production of red blood cells (RBCs) would address these problems, especially as the cells can be genetically engineered to prevent alloimmunization [1] or to satisfy the demand for rare blood types.

Multiple attempts have been made to produce red cells in vitro. Initial proof-of-concept experiments were promising, demonstrating that red cells produced ex vivo could survive following transfusion into a human recipient [2]. Since that time, numerous erythroid culture systems for the in vitro production of red blood cells have been developed, including the culture of erythrocytes from adult hematopoietic stem and progenitor cells (HSPCs), umbilical cord blood-derived HSPCs, and pluripotent stem cells. None of these systems has sufficient erythroblast expansion to generate the 2.5 trillion red cells necessary for a unit of blood in a cost-effective manner. Limitations of current in vitro culture systems are also a road block for the study of human erythroid biology. Primary erythroid cultures derived from HSPCs are the gold standard for in

TC designed and performed experiments, analyzed data, and wrote the article. ZM and MG designed and performed experiments. RK and YN provided the HUDEP-2 cells. LAS analyzed data and wrote the article.

Offprint requests to: Laurie Steiner, Department of Pediatrics, University of Rochester, 601 Elmwood Avenue, Box 701, Rochester, NY 14534; E-mail: laurie_steiner@urmc.rochester.edu

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.exphem.2019.04.001.

0301-472X/
https://doi.org/10.1016/j.exphem.2019.04.001

vitro evaluation of human erythropoiesis; however it can be difficult to obtain CD34+ HSPCs, and the number of erythroblasts that can be generated from HSPCs in a cost-effective manner is limited. In addition, primary cells are not always amenable to techniques such as genome editing. Alternative in vitro systems, such as K562 cells, are readily available, cost effective, and amenable to genome editing and other genetic manipulations, but fail to recapitulate key aspects of terminal erythroid maturation, including chromatin condensation and enucleation.

To enhance the expansion capability of cultured erythroblasts, several groups have attempted to immortalize early erythroblasts [3–7]. This approach has shown great promise, successfully enhancing erythroblast expansion, while still permitting terminal maturation and enucleation [3,4]; however, these immortalized erythroblasts are still reliant on two cytokines: stem cell factor (SCF) and erythropoietin (EPO). The cost of these cytokines alone greatly exceeds the cost of obtaining RBCs from donors [8] and is a barrier to generating erythroblasts in vitro for studies of human erythroid biology.

Human umbilical cord-derived erythroid progenitor 2 (HUDEP-2) cells are E6/E7 immortalized basophilic erythroblasts capable of terminal maturation [3] and amenable to CRISPR/Cas9 genome editing [9,10]. Culture medium for these cells is expensive because they are dependent on EPO and SCF for survival. SCF binds to the receptor tyrosine kinase c-Kit to promote cell proliferation and survival. Mutations in exon 17 of the c-Kit proto-oncogene (*KIT*), found in myeloproliferative neoplasms and other malignancies [11], cause the c-Kit protein to spontaneously activate and transduce signal in the absence of SCF [12]. To decrease culture costs, we sought to create erythroblast cell lines capable of SCF-independent expansion by introducing KIT mutations into HUDEP-2 cells using Cas9-sgRNA ribonucleoprotein complexes.

Methods

The single guide RNA was in vitro transcribed as previously described [13]. RNPs containing 2 μg of EnGen Cas9 NLS (New England Biolabs) and 1 μg of sgRNA were electroporated into 1×10^5 HUDEP-2 cells with the Neon Transfection System at 1600 V, 10 msec, 2 pulses. Cells were recovered for 1 week in complete expansion medium after which SCF was withdrawn to select for SCF-independent clones. For additional details, please see Supplementary Methods (online only, available at www.exphem.org).

Results and discussion

We used CRISPR/Cas9 to introduce missense and frameshifting mutations within the vicinity of Asp816 in exon 17 of the KIT gene (Figure 1A). SCF-independent monoclonal lines were subsequently named KITCAT (KIT with constitutively activating transformation). Exon 17 of *KIT* was genotyped by Sanger sequencing in 16 independent cell lines, all of which contained one allele with a D816I mutation (data not shown). To understand the impact of different constitutively activating mutations, three cell lines with unique genotypes were chosen for further characterization (Figure 1B).

All three KITCAT lines characterized were capable of SCF-independent expansion with no difference in doubling time compared with complete medium, and remained dependent on doxycycline for E6/E7 transgene expression (Figure 1C). Unexpectedly, clone B5 was able to bypass the requirement for EPO. Clones A4 and C3 demonstrated decreased EPO dependence. In the absence of EPO, the A4 and C3 cultures had longer doubling times and, at lower EPO concentrations, exhibited a dose-dependent decrease in expansion (Supplementary Figure 1, online only, available at www.exphem.org). Constitutively activating KIT mutations can cause activation of STAT signaling [14], and it is possible that this cross-talk reduces the need for exogenous EPO. To confirm that genome editing resulted in activation of the cKit receptor, we performed cytokine starvation followed by SCF stimulation. As expected, following this treatment, the KITCAT clones showed no increase in c-Kit activation (Figure 1D). Furthermore, the immature and lower-molecular-weight form of c-Kit lacking post-translation glycosylation is phosphorylated in KITCAT cells, but not in HUDEP-2 cells, indicating activation before translocation to the surface membrane, where SCF stimulation would occur [15,16].

To determine the specificity of genome editing on cKit receptor activity, we evaluated two tyrosine kinase inhibitors capable of inhibiting c-Kit, imatinib and dasatinib, on the expansion of HUDEP-2 and KITCAT clones. Dasatinib, but not imatinib, is capable of inhibiting the growth of cells with the c-Kit D816 mutation [14]. Treatment of wild-type HUDEP-2 cells with both inhibitors at a 1 μmol/L concentration significantly reduces viability after 48 hours (Figure 1E). Dasatinib treatment of KITCAT subclones resulted in a reduction in viability similar to that of wild-type HUDEP-2 cells, whereas imatinib had no impact, consistent with previous studies of D816 mutations [16].

While in expansion medium, KITCAT clones maintain an early erythroblast immunophenotype of $CD235a^+$, $CD71^+$, and $CD45^{low/neg}$ (Figure 2A), similar to that of the parenteral HUDEP-2 line. These markers are not helpful in resolving erythroblast maturation stages, so we also assessed KITCAT lines using imaging flow cytometry, which can robustly measure cell and nuclear size, two key morphological features of erythroblast maturation [17,18]. All three KITCAT lines were significantly larger in both cell and nuclear area while in the expansion phase (Figure 2B, C; Supplementary Figure 2, online only, available at www.exphem.org). HUDEP-2 cells are a heterogeneous, polyclonal population [19], making it difficult to determine if the larger cell and nucleus are due to the introduction of the KIT receptor mutation or the more

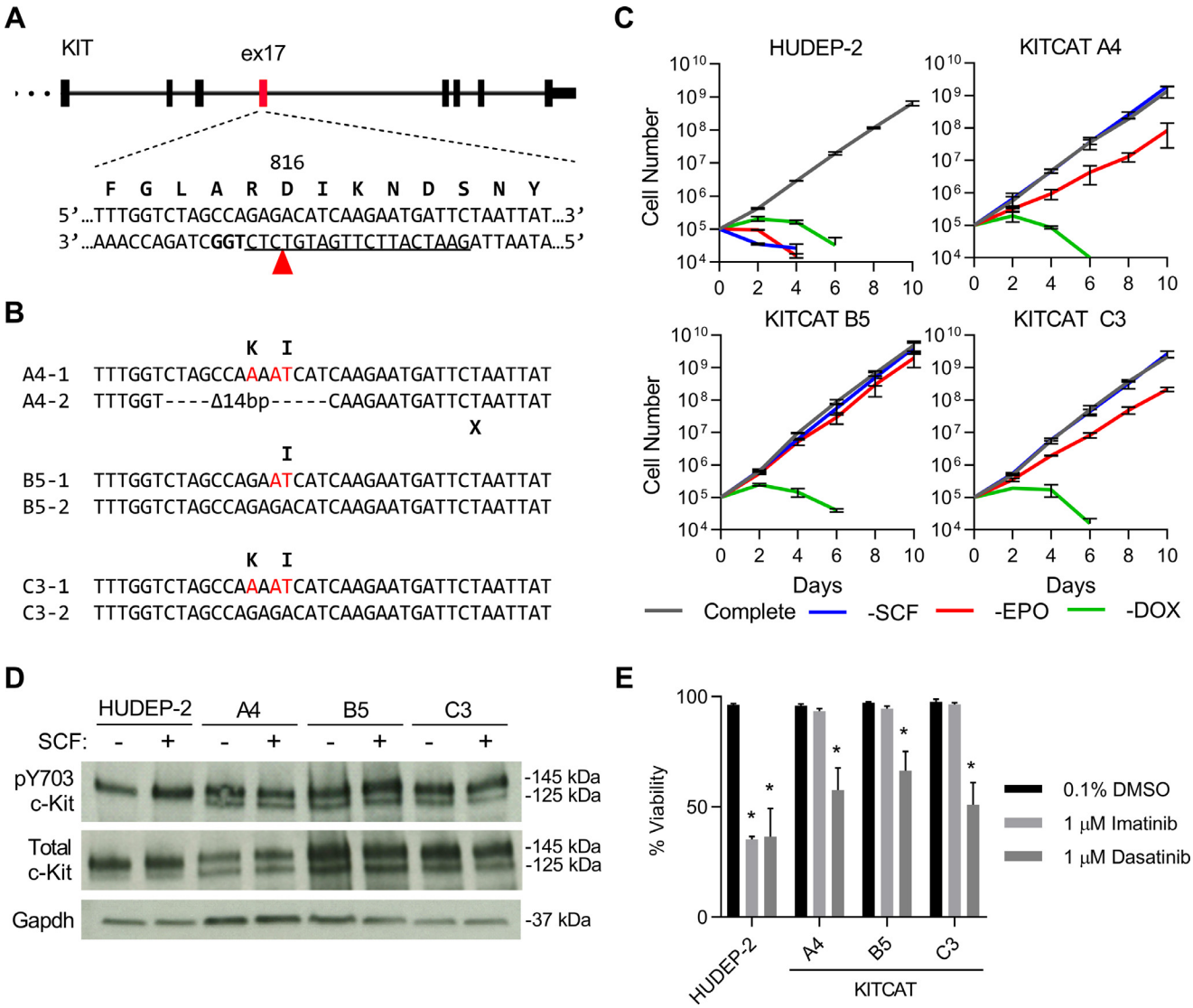


Figure 1. Generation and characterization of KITCAT clonal lines. **(A)** The Cas9 guide sequence (*underlined*) was designed to cut the region coding for D816 (*red arrowhead*) in exon 17 of the KIT gene. Cas9 protospacer adjacent motif (*boldface*). **(B)** Alleles of three unique KITCAT clones chosen for further characterization. Clone A4 carries an activating allele with R815K and D816I point mutations and a 14-bp deletion on the second allele leading to a premature stop codon (X). Both clones B5 and C3 have one wild-type allele and an activating allele, but differ in a R815K mutation. **(C)** Dependence of HUDEP-2 and KITCAT cell lines on SCF, EPO, and doxycycline (DOX) (n = 2). **(D)** Immunoblotting of phosphotyrosine-703 of c-Kit, total c-Kit, and Gapdh (loading control) in cells that were starved of cytokines for 4 hours, then stimulated with 100 ng of SCF for 10 minutes, as indicated. **(E)** Viability of HUDEP-2 and KITCAT cells 48 hours after the addition of tyrosine kinase inhibitors determined by trypan staining ($n = 3$, $^*p < 0.05$).

uniform nature of the KITCAT clones compared with the polyclonal parental HUDEP-2 line.

During erythropoiesis, c-Kit expression turns off between the CFU-E and basophilic erythroblast stages. As HUDEP-2 cells also downregulate *KIT* during maturation (Supplementary Figure 3, online only, available at www.exphem.org), we carefully evaluated the impact of the constitutively active cKit receptor on terminal maturation. HUDEP-2 cells mature more slowly than erythroblasts in vivo, typically taking 7–12 days to become late-stage erythroblasts and reticulocytes. By day 6 of maturation, clones A4 and C3 had a cell area similar to that of HUDEP-2 control cells. In contrast, clone B5 remained significantly larger with a right-skewed distribution in both cell and nuclear size, indicating that a fraction of the cells in this clone are delayed in maturation (Supplementary Figure 2A). By day 12 of maturation, however, the majority of the A4, C3, and B5 cultures were composed of orthochromatic erythroblasts, enucleate reticulocytes, and pyrenocytes, similar to the HUDEP-2 control cells (Figure 3A). Importantly, all three

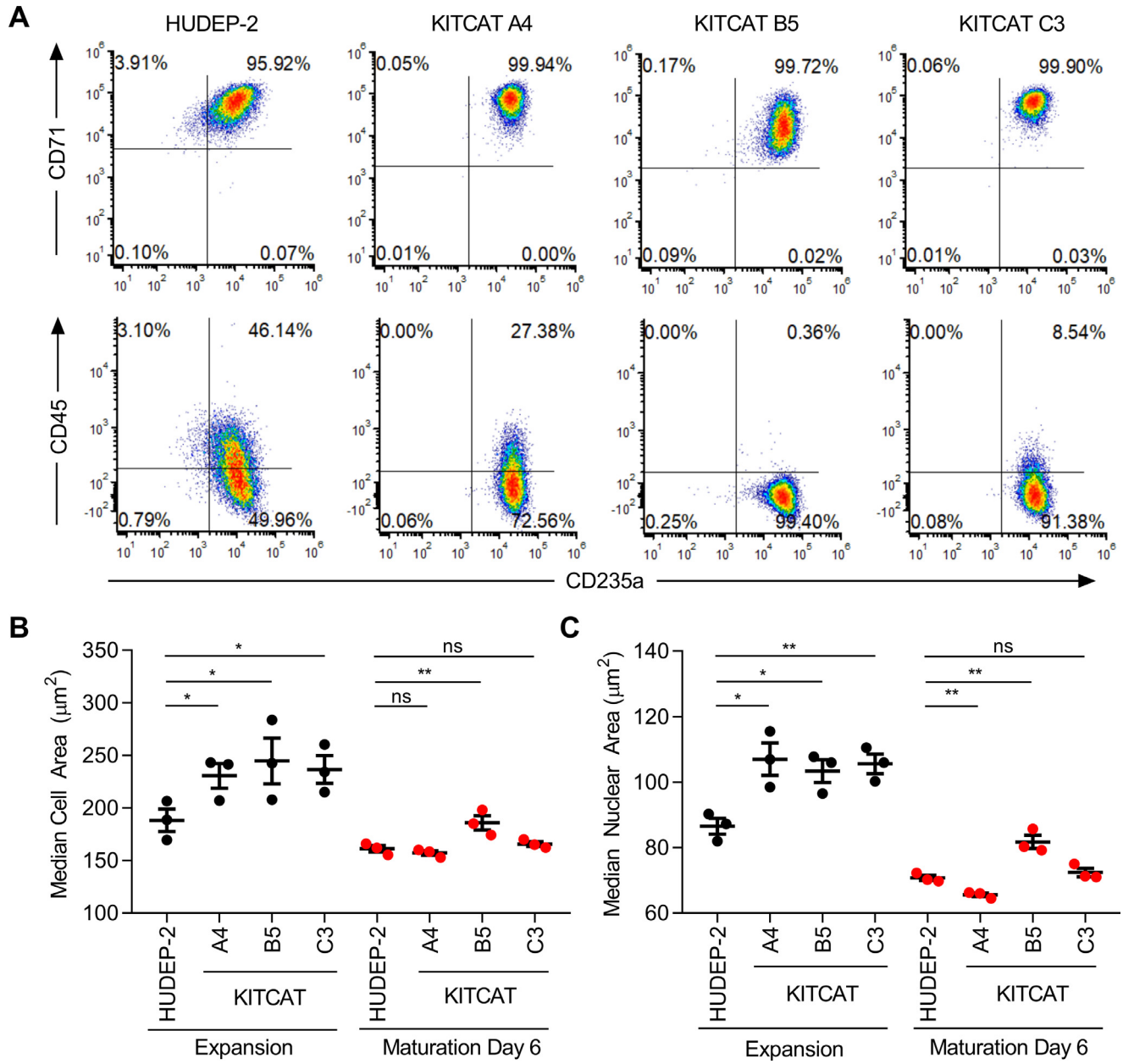


Figure 2. Flow cytometry analysis of HUDEP-2 and KITCAT cells. **(A)** Flow cytometry plots of CD235a, CD71, and CD45 of cells in expansion phase. Quadrant gates set using fluorescence minus one control. **(B)** Median cell and **(C)** nuclear areas determined by imaging flow cytometry of cell lines in expansion and day 6 of maturation. Error bars represent the standard error of the mean of three independent experiments (*$p < 0.05$, **$p < 0.005$).

KITCAT clones displayed greater expansion compared with HUDEP-2 cells during maturation (Figure 3B), yielding 2- to 10-fold more mature erythroblasts than the parental HUDEP-2 line. Interestingly, all three KITCAT clones remained dependent on EPO to maintain robust expansion during maturation (Supplementary Figure 4, online only, available at www.exphem.org).

HUDEP-2 cells are capable of enucleation, but do not enucleate efficiently, with enucleation rates generally <10% [3]. KITCAT cells have a similar enucleation rate (Supplementary Figure 5, online only, available at www.exphem.org), suggesting that introduction of activating KIT mutations does not prevent enucleation. HUDEP-2 cells are used as a model system to study hemoglobin switching because they express primarily beta globin. Intriguingly, the globin profile of maturing KITCAT cells varied by clone, with B5 expressing primarily gamma globin, A4 expressing similar levels of beta and gamma globin, and C3 expressing primarily beta globin (Figure 3C),

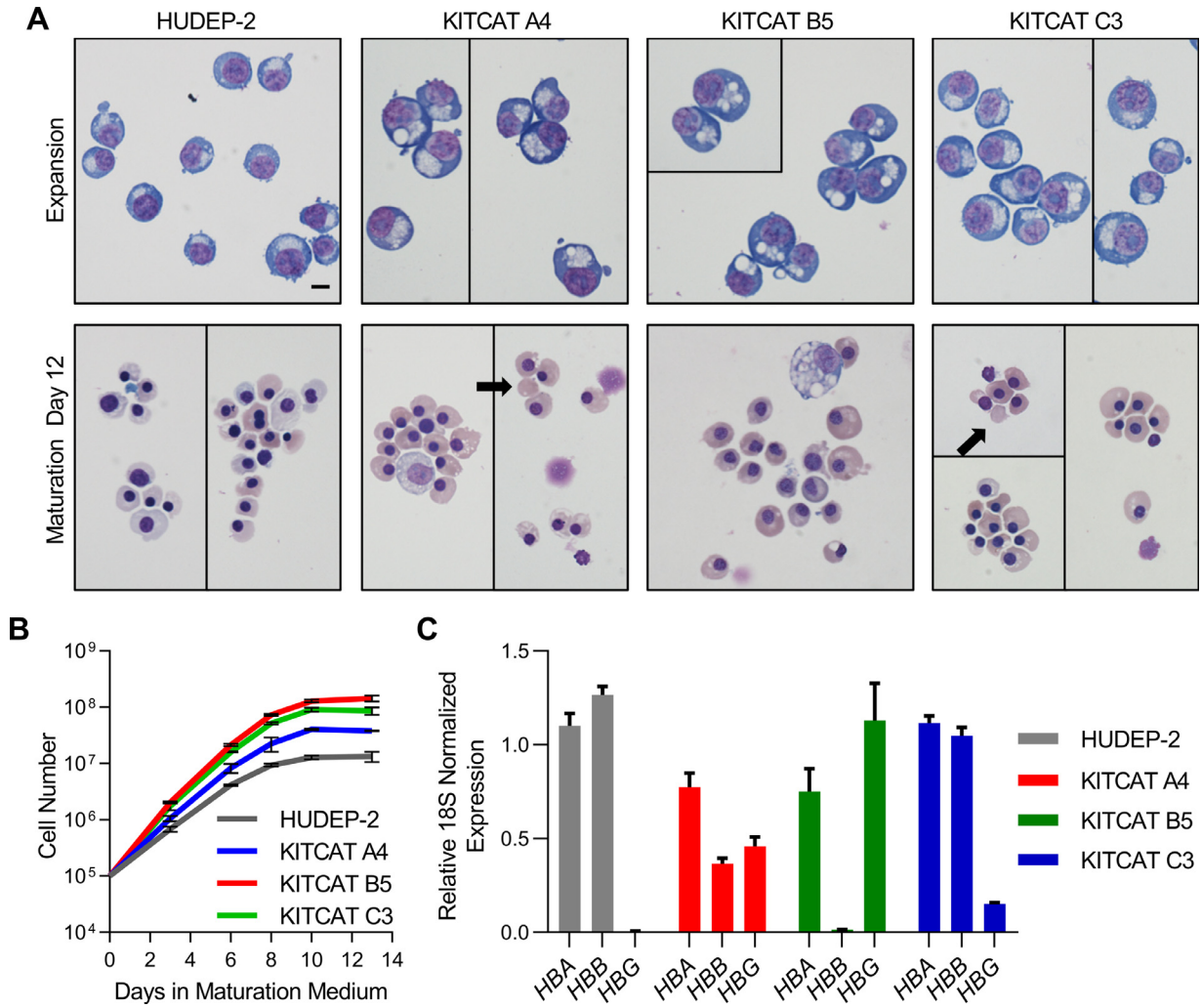


Figure 3. KITCAT clones are capable of terminal maturation and enucleation. **(A)** Wright–Giemsa staining of cells in expansion and day 12 of maturation. *Arrows* indicating enucleate cells. **(B)** Cell expansion in maturation measured by trypan exclusion. SCF was present throughout HUDEP-2 maturation, but excluded from KITCAT maturation medium. **(C)** Alpha globin (HBA), beta globin (HBB), and gamma globin (HBG) expression measured on day 3 of maturation by real-time quantitative reverse transcription polymerase chain reaction. All images collected at the same magnification and size bar indicates 10μm.

demonstrating that constitutive activation of cKit signaling does not necessarily result in loss of adult globin expression.

Together, these studies demonstrate that the introduction of constitutively activated c-Kit in immortalized human erythroblasts allows for SCF-independent expansion, drastically reducing the costs of culture and improving the culture yield of mature erythroblasts. The expression of constitutively activated c-Kit did not prevent terminal maturation but, in one clone, delayed it, suggesting specific KIT receptor genotypes may be more amenable to promoting erythroblast expansion while still allowing for efficient terminal maturation. As such, KITCAT cells represent a novel tool for the study of human erythropoiesis. Although KITCAT cells themselves are not appropriate for transfusion therapy, many strategies for generating RBCs for transfusion purposes utilize genetically modified erythroblasts [3–5]. Our results suggest that genetically modifying the KIT receptor, in combination with ongoing efforts to optimize the in vitro maturation and enucleation of human erythroblasts, may make the large-scale production of RBCs more economically feasible.

Acknowledgments

This work was supported by National Institutes of Health, NIDDK, United States Grant R01DK104920 (LAS) and a National Science Foundation, United States graduate research fellowship (TC).

Conflict of interest disclosure

The authors declare no competing financial interests.

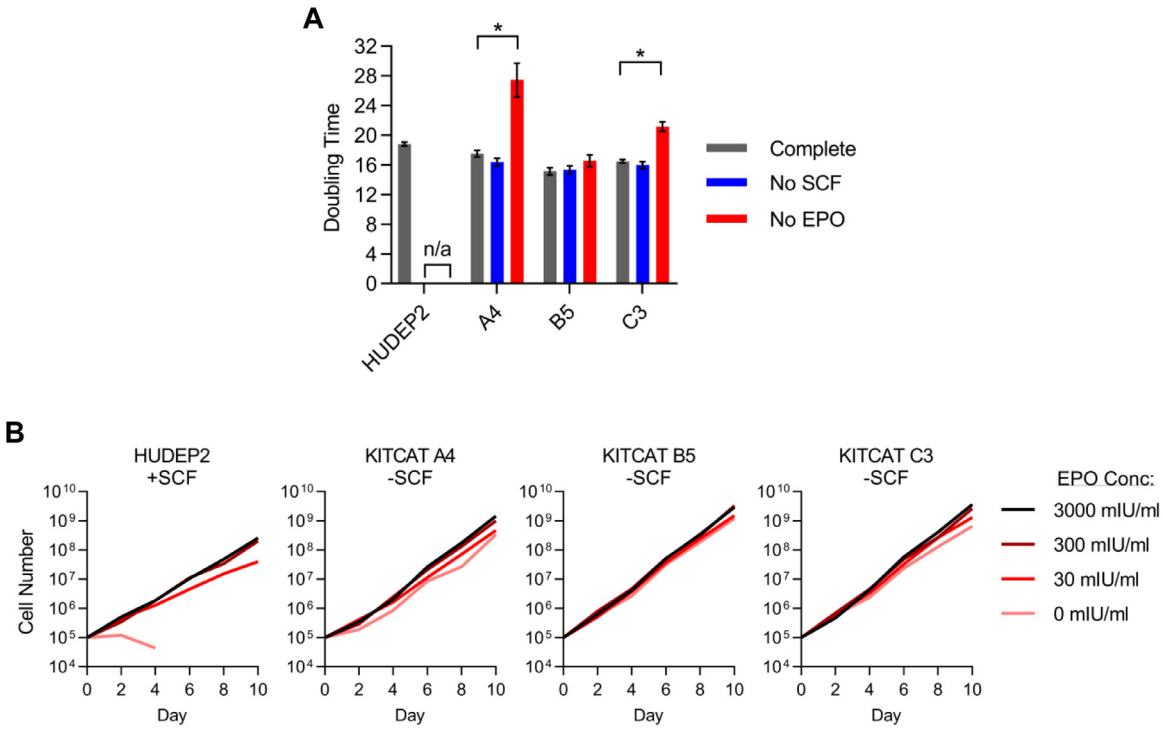
References

1. Hawksworth J, Satchwell TJ, Meinders M, et al. Enhancement of red blood cell transfusion compatibility using CRISPR-mediated erythroblast gene editing. EMBO Mol Med. 2018;10:e8454.
2. Giarratana MC, Rouard H, Dumont A, et al. Proof of principle for transfusion of in vitro-generated red blood cells. Blood. 2011;118:5071–5079.
3. 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:e59890.
4. 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.
5. Huang X, Shah S, Wang J, et al. Extensive ex vivo expansion of functional human erythroid precursors established from umbilical cord blood cells by defined factors. Mol Ther. 2014;22:451–463.
6. Wong S, Keyvanfar K, Wan Z, Kajigaya S, Young NS, Zhi N. Establishment of an erythroid cell line from primary CD36+ erythroid progenitor cells. Exp Hematol. 2010;38:994–1005.e1001–1002.
7. 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.
8. Timmins NE, Nielsen LK. Blood cell manufacture: Current methods and future challenges. Trends Biotechnol. 2009;27:415–422.
9. Vinjamur DS, Bauer DE. Growing and genetically manipulating human umbilical cord blood-derived erythroid progenitor (HUDEP) cell lines. Methods Mol Biol. 2018;1698:275–284.
10. Moir-Meyer G, Cheong PL, Olijnik A-A, et al. Robust CRISPR/Cas9 genome editing of the HUDEP-2 erythroid precursor line using plasmids and single-stranded oligonucleotide donors. Methods Protocols. 2018:1.
11. Liang J, Wu YL, Chen BJ, Zhang W, Tanaka Y, Sugiyama H. The C-kit receptor-mediated signal transduction and tumor-related diseases. Int J Biol Sci. 2013;9:435–443.
12. Kitayama H, Kanakura Y, Furitsu T, et al. Constitutively activating mutations of c-kit receptor tyrosine kinase confer factor-independent growth and tumorigenicity of factor-dependent hematopoietic cell lines. Blood. 1995;85:790–798.
13. Gundry MC, Brunetti L, Lin A, et al. Highly efficient genome editing of murine and human hematopoietic progenitor cells by CRISPR/Cas9. Cell Rep. 2016;17:1453–1461.
14. Yang Y, Letard S, Borge L, et al. Pediatric mastocytosis-associated KIT extracellular domain mutations exhibit different functional and signaling properties compared with KIT-phosphotransferase domain mutations. Blood. 2010;116:1114–1123.
15. Tabone-Eglinger S, Subra F, El Sayadi H, et al. KIT mutations induce intracellular retention and activation of an immature form of the KIT protein in gastrointestinal stromal tumors. Clin Cancer Res. 2008;14:2285–2294.
16. Shi X, Sousa LP, Mandel-Bausch EM, et al. Distinct cellular properties of oncogenic KIT receptor tyrosine kinase mutants enable alternative courses of cancer cell inhibition. Proc Natl Acad Sci US Robust CRISPR/Cas9 genome editing of the HUDEP-2 erythroid precursor line using plasmids and single-stranded oligonucleotide donors. A. 2016;113:E4784–E4793.
17. Palis J. Primitive and definitive erythropoiesis in mammals. Front Physiol. 2014;5:1–9.
18. McGrath KE, Catherman SC, Palis J. Delineating stages of erythropoiesis using imaging flow cytometry. Methods. 2017;112:68–74.
19. Chung JE, Magis W, Vu J, et al. CRISPR-Cas9 interrogation of a putative fetal globin repressor in human erythroid cells. PLoS One. 2019;14:e0208237.

SUPPLEMENTAL METHODS

Cell Culture

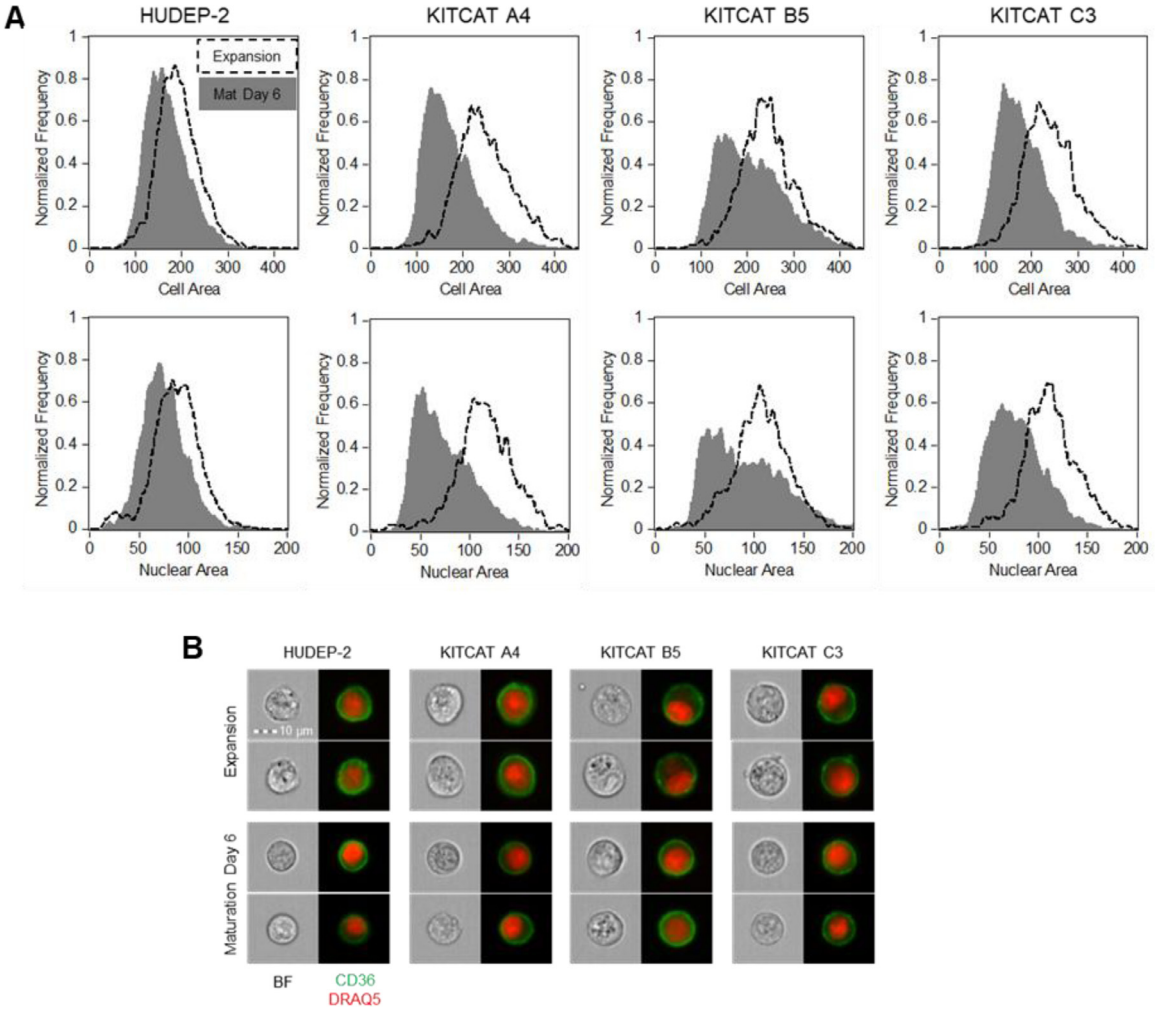
HUDEP-2 cells expansion medium was comprised of StemSpan SFEM (Stemcell Technologies) supplemented with 50 ng/ml human SCF (PeproTech), 3 IU/ml erythropoietin (Procrit, Janssen Pharmaceutica), 2 μM dexamethasone, and 1 ug/ml doxycycline (Sigma). Cells were differentiated in Iscove's Modified Dulbecco's Medium containing 1% penicillin/streptomycin, 330 μg/ml holo-transferrin, 2 IU/ml heparin (Sigma), 10 μg/ml insulin (Tocris Bioscience), 5% human peripheral blood plasma (Stemcell Technologies), 3 IU/ml EPO (PROCRIT, Janssen Pharmaceutica), 1 μg/ml doxycycline (Sigma), 1% GlutaMAX (Gibco), and 100 ng/ml SCF.



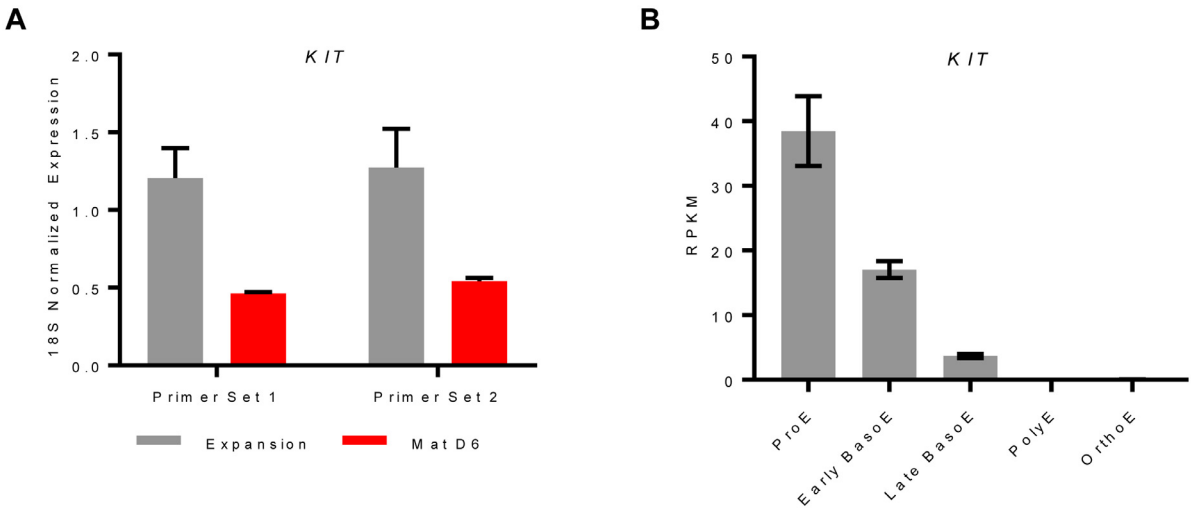
Supplemental Figure. 1: Additional cell count measurements. (A) Doubling time of HUDEP-2 and KITCAT subclones (n = 2, *p < 0.05). (B) Growth curves of HUDEP-2 and KITCAT subclones grown in variable concentrations of EPO. KITCAT cultures were grown without SCF.

Supplemental Table. Oligonucleotide sequences

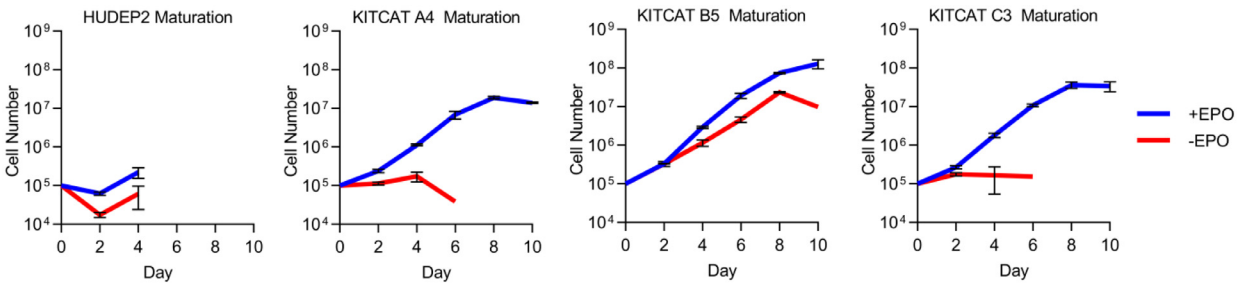
Oligonucleotide	Purpose	Sequence (5' to 3')
KIT ex17 sgRNA	sgRNA synthesis	taatacgactcactataGGAATCATTCTTGATGTCTCgttttagagctagaa
sgRNA common reverse primer (previously reported[3])	sgRNA synthesis	AGCACCGACTCGGTGCCACT
KIT ex17 Fwd	PCR	CTTCCGTGTGTCCTTGGGAGAT
KIT ex17 Rev	PCR	TCTGTTTCCTTCACATGCCCCA
Harvard PrimerBank ID 148005048c1 Fwd	*KIT* RT-qPCR	CGTTCTGCTCCTACTGCTTCG
Harvard PrimerBank ID 148005048c1 Rev	*KIT* RT-qPCR	CCCACGCGGACTATTAAGTCT
Harvard PrimerBank ID 148005048c2 Fwd	*KIT* RT-qPCR	CGTGGGCGACGAGATTAGG
Harvard PrimerBank ID 148005048c2 Rev	*KIT* RT-qPCR	CTTCTTTCCCATACAAGGAGCG
Human α-globin Fwd (previously reported[4])	*HBA* RT-qPCR	GCACGCTGGCGAGTATGG
Human α-globin Rev (previously reported[4])	*HBA* RT-qPCR	AAGTGCGGGAAGTAGGTCTTGGT
Human β-globin Fwd (previously reported[4])	*HBB* RT-qPCR	AACTGTGTTCACTAGCAACCTCAA
Human β-globin Rev (previously reported[4])	*HBB* RT-qPCR	GAGTGGACAGATCCCCAAAGGA
Human γ-globin Fwd (previously reported[4])	*HBG* RT-qPCR	GATGCCATAAAGCACCTGGATG
Human γ-globin Rev (previously reported[4])	*HBG* RT-qPCR	TTGCAGAAGAAAGCCTATCCTTGA
18S Fwd	RT-qPCR normalization	TTGACGGAAGGGCACCACCAG
18S Rev	RT-qPCR normalization	GCACCACCACCCACGGAATCG



Supplemental Figure. 2: Additional Imaging Flow Cytometry measurements. (A) Representative histogram distributions of the cell (top) and nuclear (bottom) area from expansion and after 6 days of maturation. (B) Representative images of cells with cell areas at the median of the population.



Supplemental Figure. 3: *KIT* is downregulated with erythroblast maturation. (A) *KIT* transcript levels were measured by RT-qPCR in HUDEP-2 cells in expansion and following 6 days of maturation. (B) *KIT* expression in CD34+ derived human erythroblasts quantified by previously published RNA-seq[5].



Supplemental Figure. 4: KITCAT clones expand with EPO. HUDEP-2 and KITCAT clones were placed into maturation medium lacking SCF, with or without EPO (n = 2).

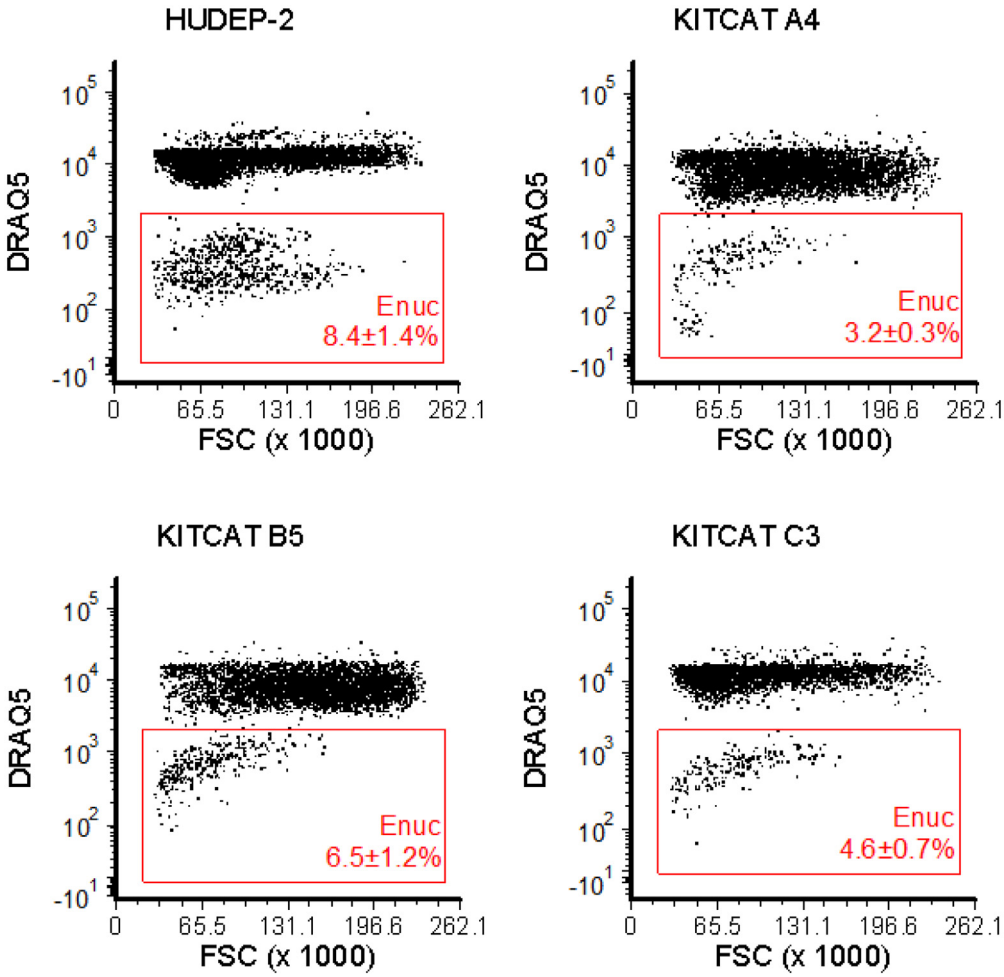
On day 8 of maturation, doxycycline was removed to promote enucleation. Unless otherwise indicated, KITCAT expansion and maturation medium was the same except SCF was excluded.

Cloning and Sequencing of Alleles

Exon 17 was PCR amplified with the KIT ex17 primers using Q5 polymerase (New England Biolabs) and A-tailing performed using Taq Polymerase (New England Biolabs). PCR products were cloned into the pCR2.1 vector using the TOPO-TA cloning kit and transformed into chemically competent *Escherichia coli* XL-1 Blue cells by standard heat shock. Individual colonies were minipreped and Sanger sequenced using the forward KIT ex17 primer.

Flow Cytometry

Cells were surface stained with CD235a-PECy7 (BD Biosciences, clone GA-R2), CD71-FITC (BD Biosciences, clone



Supplemental Figure. 5: Enucleation of HUDEP-2 and KITCAT cells. Enucleation was determined by loss of DRAQ5 staining intensity on day 13 of maturation. Error represents one standard deviation (n = 3).

M-A712), and CD45-APCCy7 (Biolegend, clone 2D1) in 10% normal mouse serum in PB2[1] for 20 minutes. After washing, cells were run on a LSR II (BD Biosciences) cytometer and analyzed with FCS Express 6 (DeNovo Software, Glendale, CA). To measure enucleation, cells were washed and resuspended in PB2 containing 2.5 μM DRAQ5 (eBioscience) and 1 μg/ml 4',6-diamidino-2-phenylindole for viability (DAPI; Sigma).

Imaging Flow Cytometry

Cells were surface stained with CD36-FITC (BD Bioscience, clone CB38), and DNA stained with 2.5 μM DRAQ5 (eBioscience) and 1 μg/ml DAPI (Sigma). A minimum of 20,000 cell events were collected for each sample on the ImageStream (Amnis/EMD Millipore) using INSPIRE (v4.1) and analyzed with the IDEAS software (v6.2, Amnis/EMD Millipore). Cytoplasmic area was determined using a combined mask of Morphology(M02,CD36) OR AdaptiveErode(M01, BF,81) and nuclear area using the Morphology(M11, DRAQ5) mask as previously described[1].

Western Blot

For cytokine starvation, cells were washed into IMDM medium containing 1 μg/ml doxycycline and cultured for 4 hours. Cultures were then stimulated with 100 ng/ml SCF for 10 minutes, then collected by centrifugation, and resuspended in RIPA buffer (Cell Signaling Technology). Lysates resolved, transferred, and blotted as previously described[2]. Antibodies for c-Kit (#37805) and pY703-c-Kit (#3073) were purchased from Cell Signaling Technology. Antibody for Gapdh loading control (sc-32233) was purchased from Santa Cruz Biotechnology.

Tyrosine Kinase Inhibitor Treatment

Dasatinib and Imatinib were purchased from Sigma and resuspended in DMSO. HUDEP-2 and KITCAT clones were treated with 1 μM of inhibitor or 0.1% DMSO for 48 hours in expansion phase, then viability assessed by Trypan staining.

Reverse transcription quantitative PCR

RNA was isolated from HUDEP-2 cells using the Qiagen RNeasy kit utilizing on-column DNase digestion. Expression was measured using Luna Universal One-Step RT-qPCR kit (New England Biolabs) and normalized to 18S.

SUPPLEMENTAL REFERENCES

1. McGrath KE, Catherman SC, Palis J. Delineating stages of erythropoiesis using imaging flow cytometry. *Methods*. 2017;112:68-74.
2. Malik J, Lillis JA, Couch T, Getman M, Steiner LA. The Methyltransferase Setd8 Is Essential for Erythroblast Survival and Maturation. *Cell Reports*. 2017;21(9):2376-2383.
3. Gundry MC, Brunetti L, Lin A, et al. Highly Efficient Genome Editing of Murine and Human Hematopoietic Progenitor Cells by CRISPR/Cas9. *Cell Rep*. 2016;17(5):1453-1461.
4. Campbell AD, Cui S, Shi L, et al. Forced TR2/TR4 expression in sickle cell disease mice confers enhanced fetal hemoglobin synthesis and alleviated disease phenotypes. *Proc Natl Acad Sci USA*. 2011;108(46):18808-18813.
5. An X, Schulz VP, Li J, et al. Global transcriptome analyses of human and murine terminal erythroid differentiation. *Blood*. 2014;123(22):3466-3477.