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BMI1 enables extensive expansion of functional erythroblasts from human peripheral blood mononuclear cells

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Transfusion of red blood cells (RBCs) from ABO-matched but genetically unrelated donors is commonly used for treating anemia and acute blood loss. Increasing demand and insufficient supply for donor RBCs, especially those of universal blood types or free of known and unknown pathogens, has called for *ex vivo* generation of functional RBCs by large-scale cell culture. However, generating physiological numbers of transfusable cultured RBCs (cRBCs) *ex vivo* remains challenging, due to our inability to either extensively expand primary RBC precursors (erythroblasts) or achieve efficient enucleation once erythroblasts have been expanded and induced to differentiation and maturation. Here, we report that ectopic expression of the human *BMI1* gene confers extensive expansion of human erythroblasts, which can be derived readily from adult peripheral blood mononuclear cells of either healthy donors or sickle cell patients. These extensively expanded erythroblasts (E3s) are able to proliferate exponentially (>1 trillion-fold in 2 months) in a defined culture medium. Expanded E3 cells are karyotypically normal and capable of terminal maturation with approximately 50% enucleation. Additionally, E3-derived cRBCs can circulate in a mouse model following transfusion similar to primary human RBCs. Therefore, we provide a facile approach of generating physiological numbers of human functional erythroblasts *ex vivo*.

INTRODUCTION

Transfusion of red blood cells (RBCs) is a standard and indispensable cellular therapy in current clinical practice. In 2017, over 300 million RBC units (2×10^{12} RBCs per unit) were needed globally in elective and emergency surgery and for treating anemic patients who need frequent transfusion, while the current donor-based supply is often insufficient to meet this need.[1] Due to limited donor availability, risks of pathogen contaminations, and potential allo-immunization to donor RBCs even after ABO blood-type matching, *ex vivo* production of functional erythrocytes in a laboratory setting is highly desirable and has been pursued in the past decades.[2,3]

Mature RBCs that lack nuclei and cell proliferative capacity can be generated from primary hematopoietic stem and progenitor cells (HSPCs) residing naturally in bone marrow of adults, via committed erythroid proliferating progenitors called erythroblasts. Various sources of stem cells, which have extensive self-renewal and proliferation capacity, have been explored for *ex vivo* generation of erythroblasts that are able to divide ~10 times before being differentiated to mature and enucleated erythrocytes.[4–6] For example, significant progress has been made in the generation of mature human erythrocytes from $CD34^+$ HSPCs.[7] However, because of limitations in the efficiency of differentiation *in vitro*, production of large quantities of erythrocytes from a relatively small number of primary $CD34^+$ HSPCs remains a challenge.[2,4,8]

The advent of human pluripotent stem cells (PSCs), which can be expanded infinitely in culture, provides an unprecedented hope that we may have a renewable or unlimited cell source for massive production of RBCs and other types of hematopoietic cells.[9,10] Over the past 20 years, we and others gradually improved the efficiency of human PSC hematopoietic differentiation in generating erythroid cells.[11–19] However, the efficiency of generating terminally differentiated, enucleated erythrocytes from human PSCs remains low (~10%).[20] Even if the obstacle could be overcome in the near future, lengthy processes of generating induced PSCs and then producing re-differentiated hematopoietic cells in multiple steps are significantly

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disadvantageous for efficacious and cost-effective production of mature erythrocytes in large quantities.

An alternative approach that has been also investigated in recent years is to find a way to directly and extensively expand or immortalize erythroid precursors (erythroblasts) from a postnatal source of individual patients or desirable human donors, without the need of generating induced PSCs and lengthy differentiation from expanded stem cells.[6,21] A study with mouse early erythroid progenitors also reported their immortalization by a c-*myc* vector, although it is unclear whether it is applicable to human counterparts.[22] Although human erythroblasts can be immortalized by defined genetic factors, full maturation and enucleation of these genetically engineered erythroblasts to produce functional RBCs are usually blocked by the presence of potent pro-growth genes or induced genetic or epigenetic alterations.[21,23,24] For example, we previously used a lentiviral vector expressing a set of reprogramming factors and immortalized postnatal human umbilical cord blood (UCB)-derived erythroblasts.[21] Around the same time, others used lentiviral vectors to transduce human $CD34^+$ cells and inducibly express one or two genes in proliferating erythroblasts.[23,24] Kurita et al.[25,26] showed that inducible expression of the *HPV16-E6/E7* oncogene is able to immortalize human erythroblasts derived from either human PSCs or UCB $CD34^+$ cells, generating erythroblast-like cell lines such as HUDEP2. The method was later used with human adult bone marrow $CD34^+$ HSPCs to establish an erythroid cell line, BEL-A.[24,27] Both HUDEP2 cell line and BEL-A could proliferate for years while retaining the basic capability to differentiate into erythrocytes. However, the *HPV16-E6/E7* transduced erythroblasts after expansion carry abnormal karyotypes.[28] More critically, significant cell death was observed shortly after withdrawal of the induced *HPV16-E6/E7* gene expression, in order to achieve terminal differentiation.[24,25,28]

Recently, the mouse *Bmi1* (also called *Bmi-1* or *Pcgf4*) gene was reported to confer extensive self-renewal capacity upon mouse bone-marrow-derived erythroblasts (ESREs).[29] Notably, the *Bmi-1* transduction did not interfere with the ability of mouse ESREs to terminally mature to erythrocytes *in vitro* and *in vivo*.[29] The BMI1 protein encoded by mouse *Bmi1* or human *BMI1* gene is a component of the polycomb repressive complex 1 (PRC1) that catalyzes ubiquitination and degradation of the repressive form of histone H2A and is involved in cell-fate determination and maintenance/self-renewal of many cell types.[30–32] It is widely known that mouse *Bmi1* gene is required for maintenance of adult stem cells in many organs; inactivation of *Bmi1* leads to impaired stem cell self-renewal.[33–36] These studies showed that *Bmi1* acts at least partially by repressing the *Ink4a/Arf* locus, which encodes a cyclin-dependent kinase inhibitor $p16^{Ink4a}$ and a tumor suppressor $p19^{Arf}$.[37–39] Increasing evidence demonstrates that BMI1 regulates HSPC activity both intrinsically and extrinsically.[39–45] The mouse *Bmi1* gene is highly expressed in mouse erythroid progenitor cells, and its deficiency impairs erythroid differentiation.[46] BMI1 is also important for human erythroid development from human $CD34^+$ HSPCs.[46] In contrast, little is known about the role of BMI1 in cell proliferation and differentiation of human erythroblasts.

Encouraged by a 2015 report that *Bmi1* overexpression enhances mouse erythroblast self-renewal *ex vivo* and the fact that BMI1 is often associated with self-renewal of human hematopoietic cells,[29,46] we started investigating several years ago if BMI enables extensive proliferation of functional human erythroblasts. We mainly focused on erythroblast cultures established from human peripheral blood mononuclear cells (PBMCs). Here, we report that the enforced *BMI1* gene expression in proliferating human erythroblasts effectively represses spontaneous differentiation, conferring more than 1 trillion (10^{12})-fold expansion of human erythroblasts in 2 months. Moreover, these extensively expanded erythroblast (E3) cells are karyotypically normal and capable of efficient terminal maturation, yielding ~50% enucleated erythrocytes after induction toward terminal differentiation. Additionally, E3 cells are amenable to genetic manipulations including gene knockout and overexpression. Most importantly, E3-derived erythrocytes are able to circulate in immune-deficient mice following transfusion, similar to human primary RBCs.

RESULTS

Human PBMC-derived erythroblasts depend on BMI1 for expansion in culture

Although human erythroblast cultures could be differentiated from a small number of $CD34^+$ HSPCs isolated from UCB, adult marrow, or cytokine-immobilized PBMCs,[47–52] they can also be established directly from unsorted adult PBMCs in a defined medium that selectively supports erythroblast commitment and expansion. We and others have used this system to establish human erythroblast culture from adult PBMCs and UCB mononuclear cells (MNCs), without the need to first purify $CD34^+$ HSPCs.[11,21,53–55] In this cell culture system, the committed or emerging erythroblasts differentiated from $CD34^+$ HSPCs continuously proliferate, while lymphocytes and other mature myeloid cells, which represent the majority of PBMC or UBC MNCs die out. Approximately at day 8, the total cell number recovers and reaches the input level after the initial reduction (Figure 1A). Nearly all the cells at day 8 and onward show erythroblast phenotypes (Figures 1B and 1C). The total number of erythroblasts reaches a peak at approximately 18 days after culturing of PBMCs, before a gradual decline over the next 2 weeks. In this study, we examined the cell properties at day 32 of erythroblast culture when there are still enough cells available for analysis, in comparison with erythroblasts at an earlier stage (day 12) using established. Differentiation of erythroblasts was monitored at the indicated days by flow cytometric analysis based on the expression of band 3 and alpha integrin (CD49d).[56,57] It appears that the established erythroblasts spontaneously and gradually differentiated into more mature erythrocytes (Figures 1C and 1D). This conclusion is further supported by evidence of reduced cell proliferation, terminal erythrocyte differentiation, and cell apoptosis (Figures 1E–1H).

To address possible roles of BMI1 in human erythroblast proliferation, we performed an analysis of the *BMI1* gene expression during the course of the erythroblast culture established from PBMCs. As shown in Figure 2A, The *BMI1* mRNA was expressed significantly lower in late-stage (day 32) than in early-stage erythroblasts (days

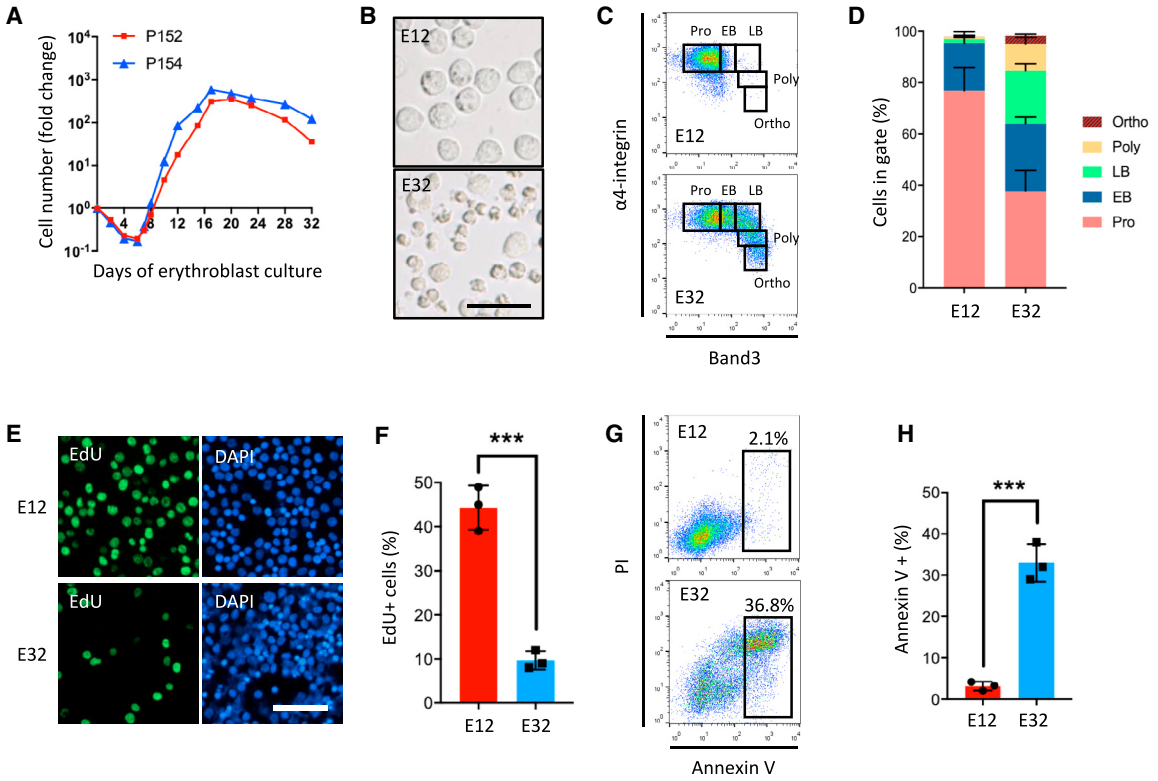


Figure 1. Erythroblast enrichment and expansion from human peripheral blood mononuclear cells (PBMCs)

(A) PBMCs were cultured toward the formation and expansion of erythroblasts in a serum-free medium supplemented with growth factors. Numbers of viable cells were counted at various days, normalized by the input PBMC number (as 1 or 10^0) at the start of culture (day 0). Data of PBMC cultures from two adult normal donors P152 and P154 were presented. (B) The morphology of erythroblasts from early stage (E12) and late stage (E32) of erythroblast cultures. Scale bar, 20 μm. (C) Flow cytometric analysis of erythroblast cultures at the early stage (E12; upper) or later stage (E32; lower) days after culture. Nearly all the cells in the cultures are positive for CD235a (glycophorin A). Based on the expression of band 3 and α4-integrin (CD49d), erythroblasts are separated into 5 populations showing different stages of erythroid differentiation: pro-erythroblasts (pros), early basophilic (EB) erythroblasts, late basophilic (LB) erythroblasts, polychromatic (poly) erythroblasts, and orthochromatic (ortho) erythroblasts. (D) Quantitative results of flow cytometric analysis of day 12 and 32 erythroblast cultures from 3 independent experiments. (E) Representative images of new DNA synthesis in proliferating erythroblasts after incubation with EdU nucleotide analog on the indicated days. DAPI staining after cell permeabilization is used to mark nuclei. Scale bar, 50 μm. EdU, 5-ethynyl-2′-deoxyuridine; DAPI, 4′-6-diamidino-2-phenylindole. (F) Percentages of EdU^+ (proliferating) cells at indicated days after erythroblast culture. Data represent the mean ± SEM from 3 independent experiments. ***$p < 0.001$. (G) Representative of flow cytometric analysis of viable or apoptotic cells in erythroblast cultures. PI staining here is used to identify cells with leaking membrane, and annexin V staining is to identify cells with phosphorylated serine expressed on the surface of apoptotic cells and/or mature RBCs. PI, propidium iodide. (H) Quantification of annexin V-positive cells at indicated days from triplicate experiments. Data represent the mean ± SEM. ***$p < 0.001$.

8–16). This is consistent with the *BMI1* gene expression pattern using sorted human primary erythroid cells at various stages in marrow, in comparison with human $CD34^+$ HSPCs that expresses *BMI1* at a high level.[58] To test the hypothesis that BMI1 is critical to erythroblast growth, we used short hairpin RNAs (shRNAs)-mediated knockdown approach to reduce the *BMI1* gene expression. We designed two separate shRNAs targeting either BMI1 coding cDNA sequence (CDS) or a 3′ untranslated region (UTR), respectively (Figure 2B). Lentiviral vectors expressing an shRNA were constructed (Figure 2C) and used to transduce early-stage erythroblasts (day 8 of erythroblast cultures) for BMI1 loss-of-function investigation. Reduction of BMI1 protein levels by shRNA-mediated knockdown in erythroblasts by either shRNA was validated (Figure 2D). We found that the knockdown of BMI1 by either BMI1 shRNA substantially inhibited the proliferation of erythroblasts from day 8 and onward, compared to the shGFP shRNA control (Figure 2E). To further confirm the specificity of BMI1 knockdown by shRNA, we constructed a lentiviral vector expressing human *BMI1* CDS as a transgene (Figure 2F), in conjunction with the use of a shRNA targeting either BMI1 CDS or UTR region. As shown in Figure 2G, *BMI1* transgene only rescued the growth of shBMI1-UTR transduced erythroblasts as expected, but not that transduced by shBMI1-CDS that should also block the *BMI1* transgene expression. Together, these results suggest the *BMI1* gene expression is essential to maintain a proliferative state of human erythroblasts established in culture.

BMI1 induces extensive expansion of erythroblasts from adult healthy donors

We next tested whether the ectopic expression of the *BMI1* transgene can prolong the expansion of human erythroblasts in culture, which

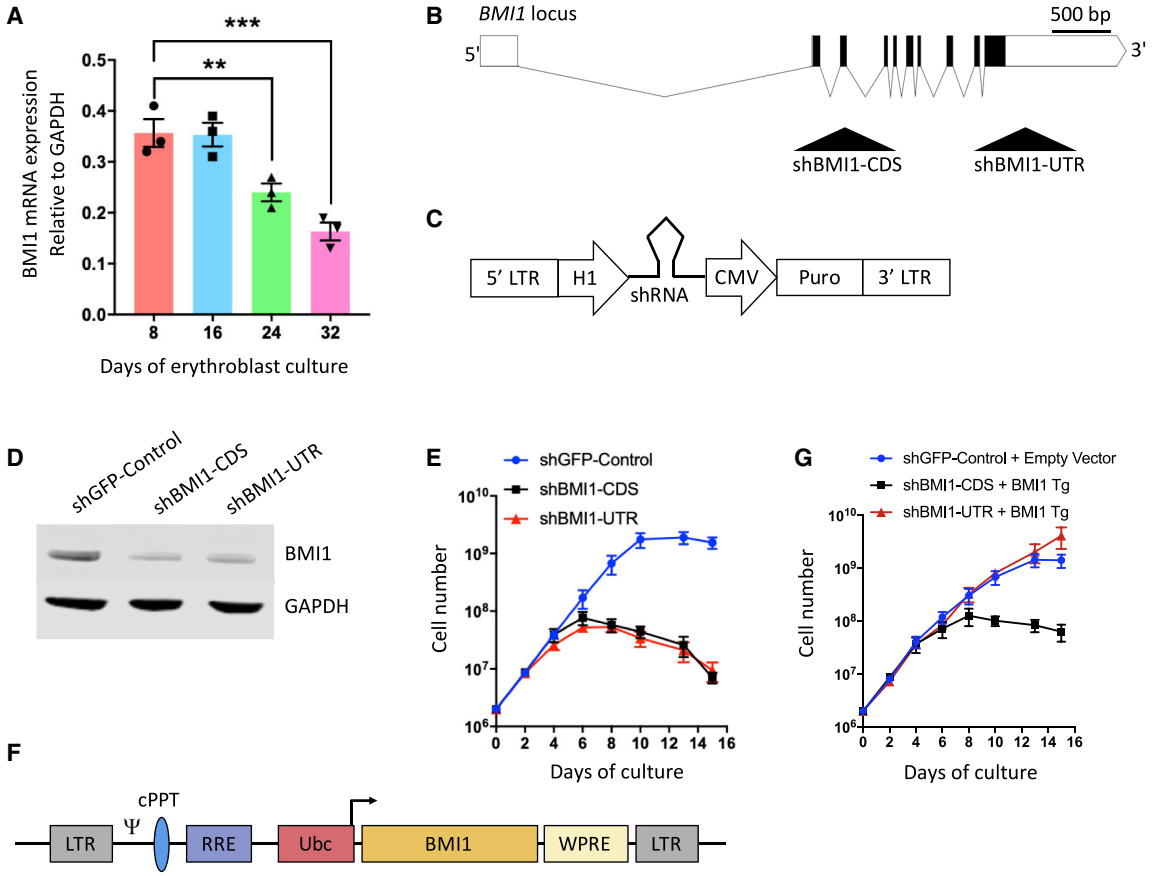
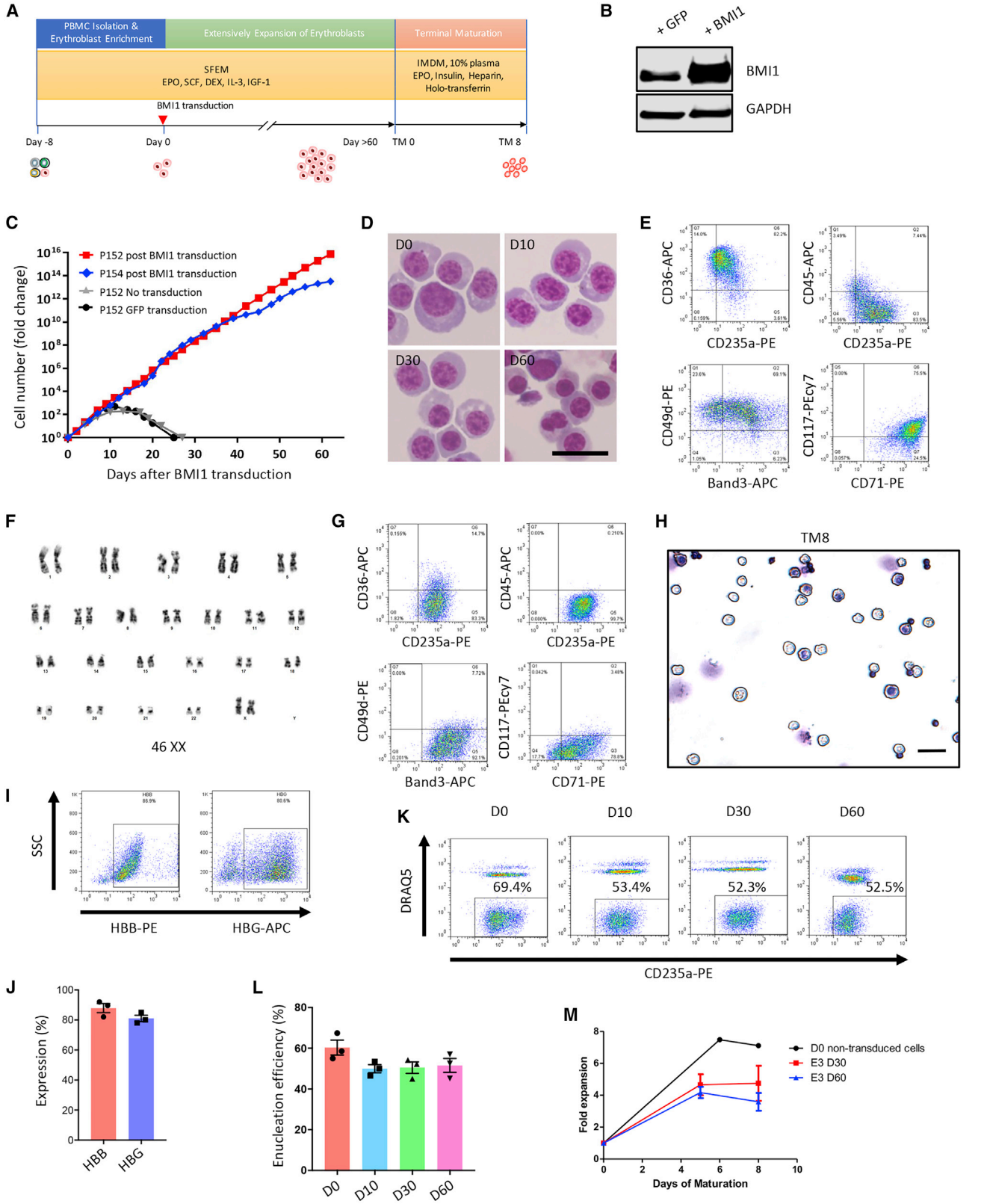


Figure 2. BMI1 is essential for erythroblast expansion in culture

(A) qRT-PCR analysis of human *BMI1* mRNA expression levels in erythroblasts at the indicated days. Transcript levels were normalized to GAPDH mRNA. Data were plotted as mean with SEM. **p < 0.01; ***p < 0.001. (B) Overview of the human *BMI1* gene and targeting locations of two short hairpin RNAs (shRNAs). CDS, coding cDNA sequence; UTR, untranslated region. (C) Schematic of a polycistronic lentiviral vector encoding shRNA targeting the endogenous human *BMI1*gene. LTR, long terminal repeat; CMV, cytomegalovirus promoter; puro, puromycin resistant gene. (D) Western blot to validate the efficiency of lentivirus-mediated knockdown of BMI1 proteins in cultured erythroblasts. (E) The expansion curve of erythroblasts after transduction of shRNA vectors for BMI1 knockdown (mean ± SEM, n = 3 biological replicates). (F) Schematic of a lentiviral vector overexpressing BMI1 CDS. LTR, long terminal repeat; Ψ, psi, RNA target site for packaging by nucleocapsid; cPPT, central polypurine tract; RRE, rev response element; Ubc, human ubiquitin c promoter; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element. (G) Expansion of erythroblasts after co-transduction of two lentiviral vectors, one expressing a shRNA and one expressing BMI1 cDNA. Mean ± SEM, n = 3 biological replicates).

normally only lasts for 3–4 weeks (Figure 1A). We used the lentiviral vector expressing human BMI1 CDS (Figure 2F) to transduce established erythroblasts at 8 days after PBMC culture (defined as day 0 for erythroblast expansion), as shown in Figure 3A. We compared the levels of BMI1 proteins in expanded erythroblasts from a GFP control and *BMI1*-transduced cultures. As shown in Figure 3B, the BMI1 protein level was much higher in BMI1-transduced erythroblasts than in the control GFP-transduced cells. Similar to untransduced erythroblasts, the GFP-transduced cells ceased proliferation within 3 weeks post-transduction (Figure 3C). In contrast, BMI1-transduced erythroblasts continued to proliferate for 60 days and then gradually slowed down in proliferation, resulting in a $\geq 10^{14}$-fold expansion of erythroblasts established from PBMCs of two adult donors (Figure 3C). To simplify the nomenclature, we named these cells "extensively expanded erythroblasts," or E3 cells. E3 cells at different time points (up to 60 days post-BMI1-transduction) displayed mainly a blastic morphology and a basophilic cytoplasm (Figure 3D), similar to those of early-stage erythroblasts (days 8–24 after PBMC cultures). Even after 60 days after BMI transduction, the E3 cells continued expressing high levels of CD36, CD71, and CD49d, and intermediate levels of CD235a and CD117. Band3 expression was also noted, indicating that spontaneous differentiation co-existed with E3 cell proliferation in these cultures (Figures 3E, S1A–S1C, and S1E). In addition, the E3 cells maintained a normal diploid karyotype even at day 60 after BMI1 transduction (Figures 3F and S1D). In additional to several PBMC cultures established from diverse genetic backgrounds, similar E3 cell cultures were obtained with UCB MNCs, including a frozen stock that has been stored in liquid nitrogen for 22 years (Figure S2).



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To investigate the capacity of BMI1-E3 cells to terminally differentiate into reticulocytes, a maturation medium was applied to replace the expansion medium at various time points after transduction and expansion (Figure 3A). Previous data showed that the transgene expression from this lentiviral vector using the Ubc promoter is significantly lower in differentiated cells as compared to proliferating erythroblasts (data not shown). Similar to normal erythroblasts after maturation induction, BMI1-E3 cells after differentiation progressively downregulated the cell-surface expression of CD117, CD36, CD71, and CD49d (Figures 3G and S1F). By day 8 of terminal differentiation, the majority of cells expressed Band3, an erythrocyte maturation marker (Figure 3G). Consistent with flow cytometric analysis, histochemical staining showed that the majority of cells after terminal differentiation are enucleated mature erythrocytes (Figure 3H). We also examined the expression levels of hemoglobin HBB and HBG in differentiated erythrocytes by flow cytometry, indicating a high percentage of adult hemoglobin (HBB) as well as HBG present after terminal maturation (Figure 3I-3J). Finally, levels of nucleated versus enucleated erythroid cells were assessed by staining with DRAQ5, a DNA- or nuclei-illuminating dye. Figure 3K showed representative enucleation profiles of terminally differentiated E3 cells cultured up to 60 days after BMI1 transduction. Quantitative analysis demonstrated that BMI1 transduction to human erythroblasts did not interfere with erythroid differentiation, once the culture was switched to a terminal maturation condition (Figures 3L and 3M). Taken together, these data indicate that BMI1 can induce extensive expansion of erythroblasts from healthy donors and BMI1-E3 cells maintain the potential to terminally mature into erythrocytes *ex vivo*.

BMI1 induces extensive expansion of erythroblasts from sickle cell disease (SCD) patients

Next, we examined whether the action of BMI1 transduction is reproducible in erythroblasts derived from PBMCs of two adult patients with SCD. PBMCs from SCD patients were similarly cultured for erythroblast enrichment and expansion, followed by BMI1 transduction and further expansion (Figure 4). Similar to what we observed with normal adult PBMCs, BMI1 enabled extensive expansion of erythroblasts from SCD patients' PBMCs, resulting in more than 1 trillion (10^{12})-fold expansion of erythroblasts after 60 days (Figure 4A). Surface marker presentations and cell morphology (Figures 4B-4D and S3) of SCD-derived E3 cells also followed the same pattern as E3 cells from normal donors. We then evaluated the enucleation levels of erythroblasts at different time points after BMI1 transduction and expansion. Similar results were seen to those with non-sickle-cell erythroid cells (Figures 4E-4H), although a slight decrease of enucleation efficiency was noted for the SCD E3 cells that have been expanded for 60 days after BMI transduction (Figures 4I and 4Ji). Collectively, these findings imply that BMI1 also confers extensive expansion of functional erythroblasts derived from PMBCs of SCD patients.

Uses of BMI1-E3 cells for genetic modification

BMI1-induced E3 cells could be a valuable resource for clinical applications of cell and gene therapies. To broaden and enhance the utility of these culture-expanded erythroblasts, we tested the feasibility of genetic manipulation by inserting a transgene or deleting an endogenous gene such as CD55 that encodes a cell-surface protein on erythrocytes (Cromer blood group antigen). It was reported that CD55 serves as a receptor for *Plasmodium falciparum*, a deadly malaria parasite that infects human erythrocytes.[59] It was reported that CD55 null erythrocytes were refractory to invasion by all isolates of *P. falciparum* because parasites failed to attach properly to the erythrocyte surface. Thus, CD55 null erythrocytes may be a better source of RBC transfusion to anemia patients in malaria-prevalent areas, especially for those who became resistant to malaria drugs. To delete the CD55 gene in the human genome, two guide RNAs (gRNAs) were used to target its exon 1 and make an 85-bp deletion including the ATG start codon and part of the coding region, as described in our previous study.[60] The chemically modified sgRNAs and spCas9 nuclease were assembled into a ribonucleoprotein (RNP) complex and then delivered into BMI1-E3 cells by electroporation (Figure 5A). CRISPR/Cas9 RNP-mediated gene knockout led to the absence of the CD55 protein on the cell surface of BMI1-E3 cells (Figures 5B and 5C) and differentiated erythrocytes (Figures 5D and 5E). The efficiency of gene knockout by this method reached nearly 100% with minimal toxicity to transduced erythroblasts (Figure 5C). The CD55 depletion did not interfere with the BMI1-E3 cell expansion and subsequent terminal differentiation (Figure 5E). Our data provide proof of principle that the E3 cells are highly amenable to CRISPR/Cas9-mediated gene editing to generate genetically modified erythrocytes.

We also tested if overexpression of an exogenous protein could be also achieved efficiently in the BMI1-E3 cells and derived erythrocytes. We used a cell-surface marker encoded by a truncated nerve growth factor receptor (tNGFR) that has been used widely for selecting and tracking of genetically engineered cells.[61] More than 99% of

Figure 3. BMI1 induces extensive expansion of erythroblasts from healthy donor PBMCs

(A) A scheme for BMI1 transduction of PBMC-derived erythroblasts, extensive expansion, and terminal differentiation in culture. Erythroblasts established at an early stage (E8) were used for BMI transduction (counted as day 0). Erythroblasts were expanded continuously for at least 60 days and tested for their potential for terminal maturation. SFEM, serum-free expansion medium. (B) Western blot to assess BMI1 protein levels after *GFP* or *BMI1* transgene transduction. (C) Expansion curves of erythroblasts after *BMI1* gene transduction. Established early erythroblasts from health donors P152 and 154 were used. (D) Histochemical (Gimesa) staining of BMI1-transduced erythroblasts at different time points. Scale bar, 20 μm. (E) Flow cytometric analysis of surface markers on the BMI1-induced extensively expanded erythroblasts (BMI1-E3) 60 days after BMI1 transduction. (F) A representative normal karyotype of the BMI1-E3 cells 60 days after expansion from donor P152. (G) Flow analysis of surface markers after terminal maturation. (H) Histochemical (Gimesa) staining of erythrocytes after terminal maturation. Scale bar, 20 μm. (I) Flow cytometric analysis of HBB and HBG in erythrocytes after terminal maturation. (J) Quantitative analyses of differentiated erythrocytes expressing HBB and HBG. (K) The levels of enucleation of BMI1-E3 cells at different time points, all after terminal differentiation for 8 days. (L) Quantification of enucleation rate at different time points. (M) Expansion fold of differentiating E3 cells in terminal maturation.

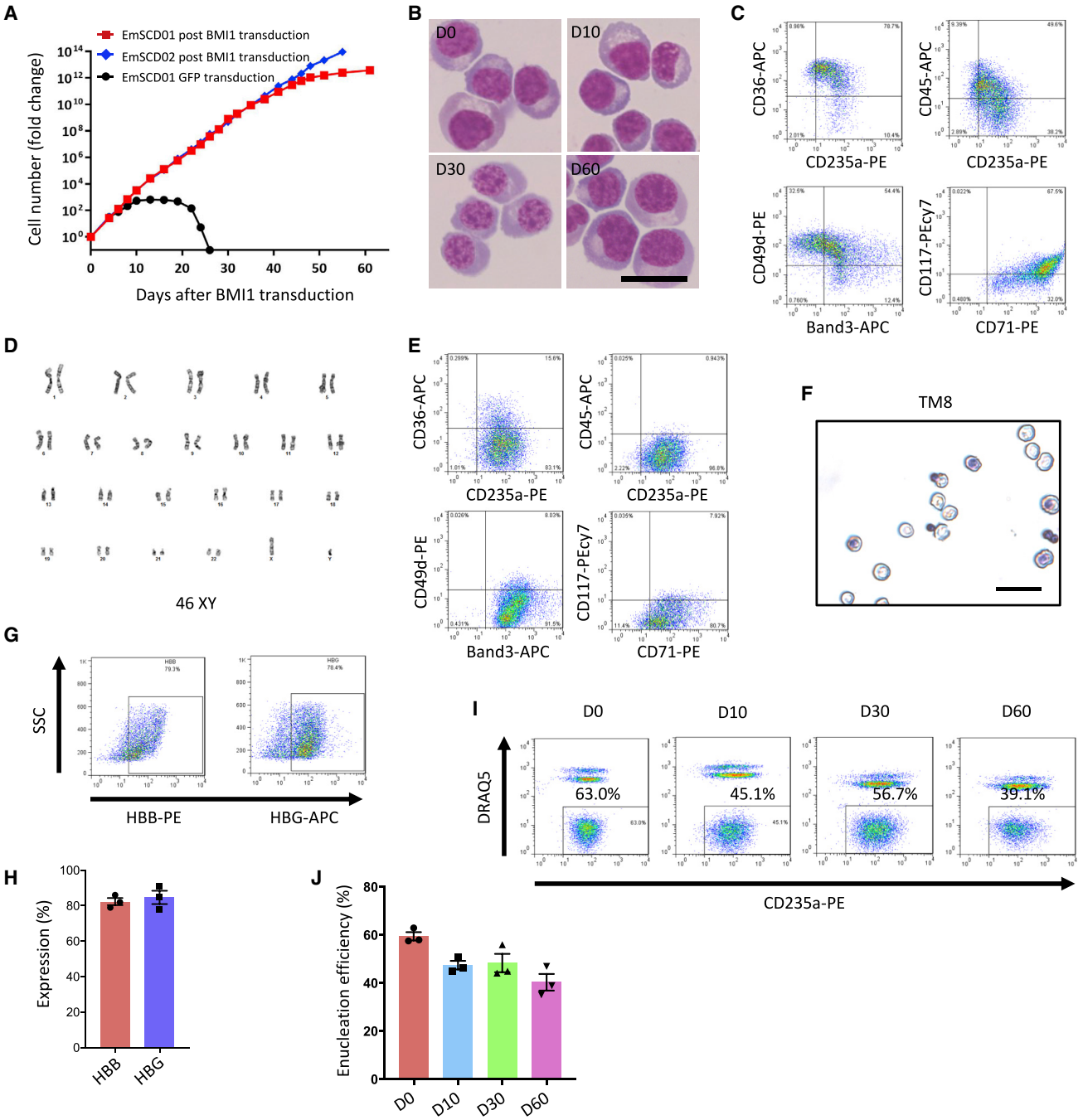

Figure 4. BMI1 induces extensive expansion of erythroblasts from PBMCs of sickle cell disease (SCD) patients

(A) Expansion curve of transduced erythroblasts from two SCD patients (EmSCD01 and EmSCD02) by the BMI1 or a control (GFP) lentiviral vector. (B) Histochemical (Gimesa) staining of BMI1 transduced SCD erythroblasts at different time points. Scale bar, 20 μm. (C) Flow cytometric analysis of surface markers on SCD erythroblasts 60 days after BMI1 transduction. (D) A representative normal karyotype of the BMI1-E3 cells 60 days after expansion from EmSCD01. (E) Flow cytometric analysis of surface markers after terminal maturation. (F) Histochemical (Gimesa) staining of erythrocytes after terminal maturation. Scale bar, 20 μm. (G) Flow cytometric analysis of HBB and HBG on erythrocytes after terminal maturation. (H) Quantitative analyses of differentiated erythrocytes expressing HBB and HBG. (I) Flow cytometric analysis of enucleation rates of BMI1-transduced SCD erythroblasts at different time points. (J) Quantification of enucleation rate of BMI1 transduced SCD erythroblasts at different time points.

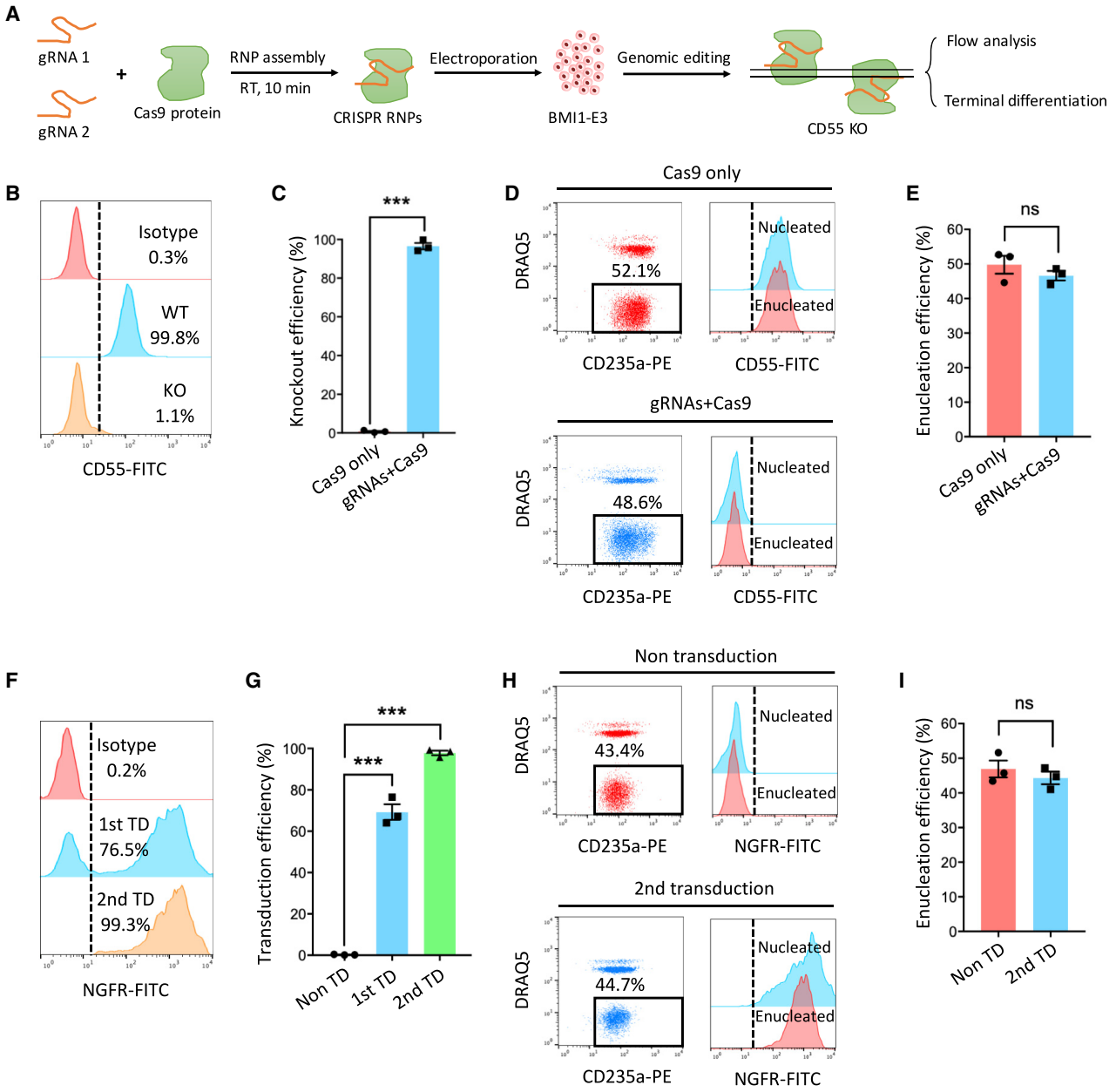

Figure 5. Amenability of BMI1-E3 cells to genetic manipulation

(A) Schematic diagram of the experimental approach for genome editing. The CD55-targeting guide (g)RNAs were assembled with recombinant Cas9 protein as an active CRISPR/Cas9-gRNA RNP complex. BMI1-E3 cells at day 30 were electroporated by the RNP complex. 3 days after electroporation, some of the cells were used for terminal differentiation, and the rest were used for flow analysis. CRISPR, clustered regularly interspaced short palindromic repeats; RNP, ribonucleoprotein; KO, knockout. (B) Representative flow cytometric analysis showing the efficiency of CD55 knockout on BMI1-E3 cells 3 days after CRISPR RNP electroporation. (C) Quantitative analyses of results shown in (B) from 3 independent experiments. Data represent the mean ± SEM. ***$p < 0.001$. (D) The enucleation efficiency assessed by flow cytometric analysis on BMI1-E3 electroporated with either Cas9 protein only or gRNAs+Cas9 complex at day 8 after terminal differentiation. $CD235a^+DRAQ5^+$ nucleated cells and $CD235a^+DRAQ5^-$ mature enucleated erythrocytes were gated respectively for CD55 expression analysis. (E) Quantitative analyses of results shown in (D) from 3 independent experiments. Data represent the mean ± SEM; ns, not significant. (F) Representative flow cytometry histograms showing the transduction efficiency of a polycistronic lentivirus on BMI1-E3 cells, expressing a truncated NGFR on cell surface. Twice successive transductions were performed in order to achieve high enough percentage of $NGFR^+$ erythroblasts. TD, transduction. (G) Quantitative analyses of results shown in (F) from 3 independent experiments. Data represent the mean ± SEM. ***$p < 0.001$. (H) The enucleation efficiency measured by flow cytometry on BMI1-E3 without transduction at day 8 after terminal differentiation. $CD235a^+DRAQ5^+$ nucleated cells and $CD235a^+DRAQ5^-$ mature enucleated erythrocytes were gated separately for NGFR expression analysis. (I) Quantitative analyses of results shown in (H) from 3 independent experiments. Data represent the mean ± SEM; ns, not significant.

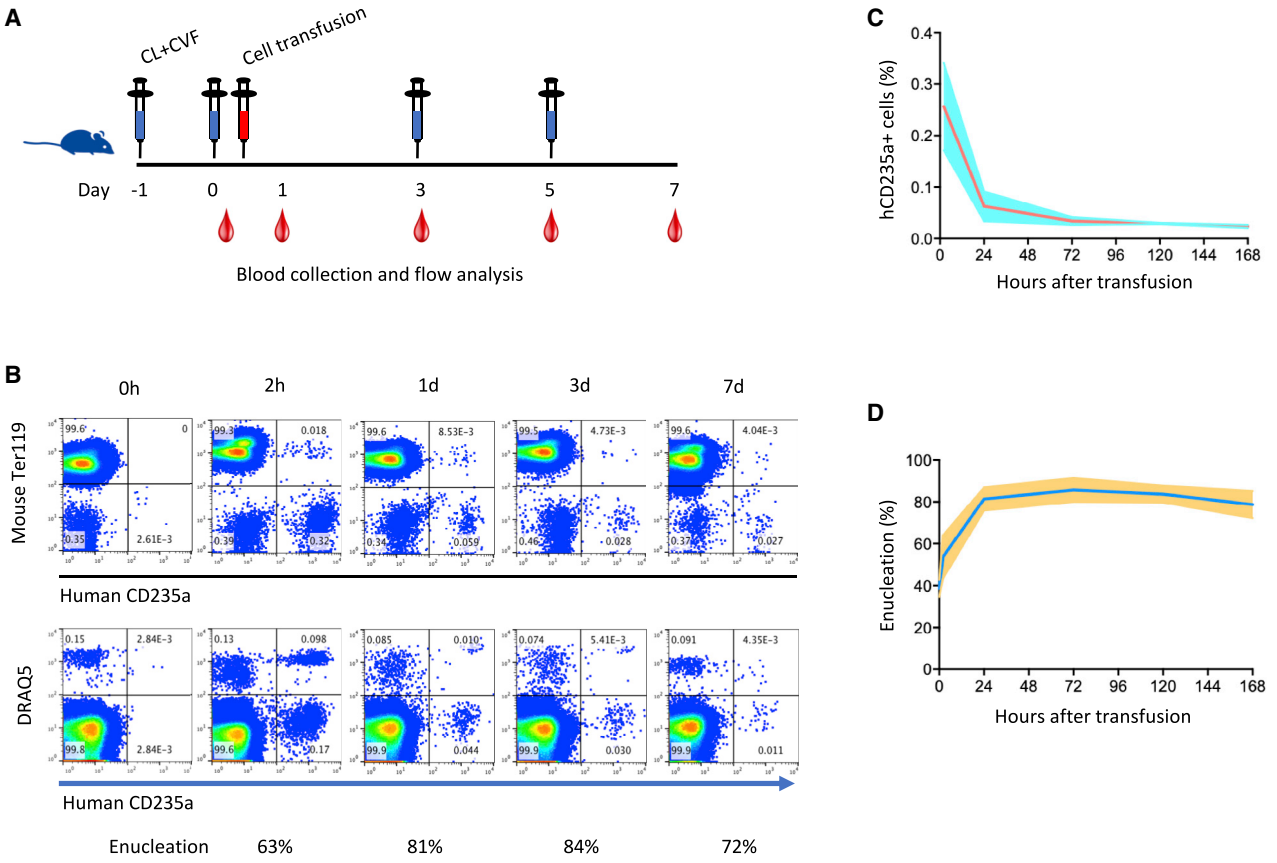

Figure 6. *In vivo* circulation of erythrocytes differentiated from BMI1-E3 in a mouse model

(A) Experimental schematic. Differentiated cells after erythroid terminal maturation were injected intravenously into NSG mice (4×10^7 cells per mouse) that were pre-treated with clodronate liposomes (CLs, 100 μL at day −1 and 50 μL at day 0) and cobra venom factor (CVF, 20 μg at day −1 and 10 μg at day 0 before transfusion). At days 3 and 5, mice were treated with 30 μL CL and 10 μg CVF. To determine the kinetics of human erythrocyte clearance, 5 μL blood samples were collected into heparinized tubes at various time points after transfusion and stained with anti-human CD235a, and the levels of surviving transfused human erythrocytes were measured by flow cytometric analysis. (B) Flow analysis of human erythrocytes in circulation at different time points after transfusion. Mouse Ter119 was used to label mouse erythrocytes. (C) Percentages of human (h)CD235$^+$ cells in mouse blood. The red line is the mean from 3 independent experiments and shaded zone (blue) shows the range of values of mean ± SEM. (D) Enucleation rate of human erythroblasts *in vivo*. The blue line is the mean from 3 independent experiments and shaded zone (yellow) shows the range of values of mean ± SEM.

erythroblasts expressed tNGFR on the cell surface after two rounds of lentivirus transduction (Figures 5F and 5G). Moreover, we demonstrate that the forced transgene expression in E3 cell erythroblasts did not alter their differentiation potential, including maturation and enucleation shown in Figures 5H and 5I.

In vivo circulation of erythrocytes differentiated from BMI1-E3 in a mouse model

We next examined whether human erythrocytes differentiated from BMI1-E3 cells have the capacity to circulate (and possibly further mature) *in vivo*, using an improved mouse model.[62,63] In this transfusion model using NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice, innate immunological rejection to human erythrocytes was further reduced through both macrophage depletion using clodronate liposomes (CLs) and complement inhibition by cobra venom factor (CVF). Following this conditioning, we treated NSG mice with CLs and CVF as shown in Figure 6A. For each treated mouse, we injected intravenously 4×10^7 differentiated cells from the BMI1-E3 cells or equal numbers of primary RBCs from healthy donors as a control. Flow cytometric analysis showed that human erythrocytes expressing human erythrocyte CD235a marker (but not mouse erythrocyte marker Ter119) were present in mouse blood for at least 1 week after injection (Figure 6B). The levels and kinetics of differentiated erythrocytes from the BMI1-E3 cells were similar to human primary RBCs shown in Figure S5. Similar to previous studies with primary human RBCs,[63] we noted that the numbers of human erythrocytes were detected at the highest levels 2 h post-transfusion and then declined very rapidly (Figures 6C and S5). However, they could be detected in mouse blood for at least 1 week. Among the cells expressing the human erythrocyte marker CD235a, we also examined the levels of nucleated and enucleated cells based on DRAQ5 DNA staining. Interestingly, percentages of enucleated erythrocytes derived from the

BMI1-E3 maturation products increased in the circulation in mice, to reach from ~57% at transfusion to approximately 72%–84% after transfusion (Figure 6D). Taken together, these data indicate that BMI1-E3 cells can differentiate into enucleated reticulocytes that are able to circulate *in vivo*, as tested in a mouse model for transfusion of human RBCs.

DISCUSSION

In this study, we investigated the role of BMI1 in primary erythroblast expansion and maturation from human PBMCs or UCB MNCs. Our results revealed for the first time that BMI1 is critical to human erythroblast expansion; its deficiency leads to impaired erythroblast expansion. Additionally, we found that ectopic expression of BMI1 transgene represses the spontaneous differentiation found in the current standard erythroblast culture system and confers the sustained proliferative potential upon erythroblasts from PBMCs of either healthy donors or SCD patients. As a result, BMI1-transduced human erythroblasts can expand for at least 2 months with more than one trillion (10^{12})-fold amplification. Importantly, these E3s retained the capability of terminal differentiation and maturation, achieving 50% enucleated efficiency after induction in culture. Moreover, BMI1-E3 cells are amenable to genetic manipulations, including CRISPR/Cas9-mediated gene ablation and overexpression of an exogenous protein with high efficiency. At last, we presented the evidence that terminally differentiated erythrocytes from the BMI-E3 cell culture can circulate *in vivo*, as tested in a mouse model for human RBC transfusion, similar to primary human RBCs.

Although the importance of generating human RBCs by cell culture in a laboratory setting for various of applications in transfusion and other forms of cell/gene therapy has been widely recognized, producing physiological numbers of cultured RBCs (cRBCs) has been challenging. In the past decade, we and others have been using human stem cells a renewal source, because stem cells have tremendous proliferative capacity while in principle retaining potential to differentiate into functional progeny such as erythrocytes.[4–6,20,61] However, several approaches that have been tested so far for the purpose of generating physiological numbers of cRBCs or their immediate precursors (erythroblasts) were found to be unsatisfactory.[6,20] One example is the generation of erythroblasts from culture-expanded human $CD34^+$ HSPCs,[7] where their extensive expansion required for producing physiological numbers of erythroblasts (and then erythrocytes) has remained elusive. Alternatively, efforts to generate erythrocytes from human PSCs that can be expanded infinitely have also proven suboptimal, because there appears to be a block in the differentiation program to produce enucleated and functional erythrocytes from PSC-derived $CD34^+$ HSPCs.[6,20]

Because of these issues, we and others have been focusing on a third approach, which is to genetically manipulate directly erythroblasts derived from adult PBMCs or purified $CD34^+$ HSPCs such that they gain the ability to proliferate much more extensively than primary cells.[21,23–26] The basic concept is to suppress spontaneous differentiation of erythroblasts cultured *ex vivo* once they are established from human $CD34^+$ HSPCs or PBMCs directly, in order to allow the undifferentiated erythroblasts to proliferate extensively. In the absence of genetic manipulation, culture-expanded primary human erythroblasts can proliferate 100- to 500-fold *ex vivo* under the currently optimized culture conditions, before they cease cell proliferation and produce short-lived cells resembling mature erythrocytes. To augment this process and produce significantly more extensive proliferation *ex vivo*, the role of various regulatory genes has been investigated in these cells over the years. Many single transgenes, such as the human telomere elongation reverse transcriptase (*TERT*) gene, have proven insufficient to extend human erythroblast proliferation for >100-fold (data not shown). In other cases, the addition of other transgenes singly or in combination, such as HPV16-E6/E7 or c-MYC together with BCL-xL, support extensive proliferation of transgenic erythroblasts, although the resulting extensively expanded or even immortalized human erythroblasts show defects in terminal maturation; however, the immortalized erythroblasts do not efficiently produce enucleated erythrocytes that can survive for an extended time *ex vivo* and *in vivo* (at least in a mouse transfusion model). Although the mechanisms of how HPV16-E6/E7 and c-MYC stimulate cell proliferation and tumor transformation have been extensively studied, a mechanism of how these "oncogenes" block erythroblast differentiation via genetic or epigenetic means remains unclear.

We envisioned that an important breakthrough could be made by identification of a "goldilocks" transgene that when expressed in erythroblasts is strong enough to suppress spontaneous erythroblast differentiation but insufficient to block terminal maturation once the cultured erythroblasts are induced to terminal differentiation. To identify such a gene, we focused on the genes that are expressed at a high level in early-stage human erythroblasts that are highly proliferative, but not in late-stage cultured erythroblasts with undergoing differentiation. We identified the human *BMI1* gene as an interesting candidate (Figure 1A), which is further supported by the previous data showing that BMI enables stem cell self-renewal or cell-fate maintenance,[31–36,64] as well as a promising result with adult mouse marrow-derived erythroblasts.[29] To further validate this target, we attempted to achieve ectopic expression of the human *BMI1* gene by a lentiviral vector to transduce and express human erythroblasts at a high level but reduce transgene expression once erythroblasts are induced to terminal differentiation to mature erythrocytes. Therefore, a chemical inducer such as tetracycline is not required during erythroblast proliferation or differentiation. Our data presented here demonstrate that ectopic expression of human *BMI1* gene confers an extensive expansion of human primary erythroblasts from diverse postnatal origins, under a chemically defined culture condition. The vastly expanded E3 cells (> 1 trillion-fold in 2 months) remain karyotypically normal and functional in generating enucleated erythrocytes. To the best of our knowledge, our approach represents the greatest yield of human enucleated cRBCs after extensive expansion of a postnatal cell source. The ability to generate physiological numbers of cRBCs is of importance for transfusion medicine. Considering that a typical unit of packed RBCs for transfusion contains

about 2.5×10^{12} enucleated RBCs (5×10^5 μL of whole blood is collected for each RBC unit, at a concentration of 5×10^6 RBCs per μL of whole blood), a yield of 10×10^{18} enucleated RBCs from E3 cultures is the equivalent of 4×10^6 packed RBC units for transfusion (Figure S6). Of course, this calculation reflects the theoretical upper limit of biological potential, not the practical RBC numbers or volumes one could generate from one batch.

We noticed that as erythroid culture goes, proerythroblasts (day 0 to day 10 after BMI1 transduction) started showing the sign of low-level differentiation into early basophilic erythroblasts, late basophilic erythroblasts, and even polychromatic erythroblasts. Furthermore, over several batches of BMI1-transduced E3 cells from more than 7 distinct donors or patients, cell proliferation declined, and levels of differentiated erythrocytes significantly increased after continuous cultures for 2 months. At this time, we have not explored the exact underlying mechanism(s). There are at least two possibilities responsible to the observation. The first possibility is that the transgene expression from the lentiviral vector in proliferating erythroblasts is gradually silenced, a phenomenon that is also observed in other proliferating cell types such as PSCs in culture. The second possibility is that proliferating erythroblasts reach the cellular life-span limit of a somatic cell, normally due to intrinsic restrictions such as the shortening of telomeres at the end of chromosomes. Our preliminary data suggest that the E3 cells continue expressing the *TERT* gene at a moderate level, and it is likely that silencing of the *BMI1* transgene expression from the integrated exogenous lentiviral vector is the most likely explanation (although both possibilities are not mutually exclusive). Future investigations will address the issue to extend high-level expansion of a transgene in erythroblasts and therefore sustained E3 cell proliferation. Nonetheless, the current level of E3 expansion is sufficient to produce a physiologic number of cRBCs under a cGMP-compliant culture condition, because it is easy to obtain a large number of PBMCs from an adult donor (there are >100 million PBMCs from 10 mL of peripheral blood) as starting materials. Therefore, we will be able to produce in principle millions of units of RBCs, based on the current expansion rate ($\geq 10^{12}$ fold). Moreover, leukocyte filtering and irradiation could be used to remove the nucleated cells that contain a lentiviral vector encoding BMI1 transgene to reduce the risk of tumorigenesis. The remaining challenges to be addressed in the future investigation include how to produce a very large number of cRBCs efficaciously and cost effectively for clinical applications, how to ensure the functionality of enucleated erythrocytes *in vivo* after freeze and thaw, and what circumstances the product will be first used safely in clinic.

The approach we report here provides a novel source of *ex vivo* generated functional cRBCs to meet the growing and unmet needs for blood transfusion, either for individual patients who need chronic transfusion of best matched donor RBCs or for a broad patient population receiving a truly universal RBC blood type after genetic enhancement. In addition, the cRBCs with genetic enhancement provide novel types of long-lasting cellular vesicles that produce therapeutic proteins or RNAs in cRBCs and then deliver *in vivo* after transfusion. For the latter applications of cell and gene therapy, we may need fewer cRBCs as required for standard RBC transfusion. The approach we described here may provide a solution to overcome a shortcoming of the current approaches that use UCB or adult $CD34^+$ HSPC-derived cRBCs and are limited by the industrial production of cRBCs.[65] Furthermore, our data presented in this report show that the BMI1-transduced E3 cells can be genetically modified efficiently by gene knockout or overexpression, without evidence that the genetic modification may adversely affect the potential to generate mature and enucleated erythrocytes.

MATERIALS AND METHODS

Approvals of using primary human cells from anonymous donors for laboratory research

Use of anonymous human blood cells for laboratory research was approved by the Institutional Review Board of Johns Hopkins University. Human PBMCs from two SCD patients (coded as EmSCD01 and EmSCD02) were collected at Emory University Hospital (Atlanta, GA, USA) after informed consent and approval of the Emory University IRB.

Isolation of MNCs and enrichment of primary erythroblasts

Newborn cord blood and adult peripheral blood of healthy donors or sickle cell patients were 1:1 diluted with 0.6% ACD(A)-PBS, then MNCs were isolated via Ficoll-Paque premium (GE Healthcare Life Sciences, Uppsala, Sweden) as we previously published.[10] The MNCs in the interphase layer were harvested and frozen in liquid nitrogen. The thawed MNCs (4×10^6 per mL) were cultured in a serum-free expansion medium (SFEM, STEMCELL Technologies, Vancouver, BC, Canada) supplemented with erythropoietin (EPO; 3 U/mL; R&D Systems, Minneapolis, MN, USA), human stem cell factor (SCF; 100 ng/mL; R&D Systems), dexamethasone (DEX; 1 μmol/L; Sigma-Aldrich, St. Louis, MO, USA), interleukin (IL)-3 (10 ng/mL; PeproTech, Rocky Hill, NJ, USA), and insulin-like growth factor (IGF)-1 (40 ng/mL, Peprotech) as previously described.[10,11] This culture is termed "erythroblast expansion medium." The cell number of wells that reached 70% confluence was counted, and fresh medium was added to obtain a final concentration at $3–5 \times 10^5$ cells/mL. Total cell counts and cell viability were assessed with the trypan blue exclusion method.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from expanded primary erythroblasts using an RNeasy kit (QIAGEN, Valencia, CA, USA) and treated with a DNA-free kit (Ambion, Austin, TX, USA) to get rid of residual DNA contamination. Total RNA (1 μg) was reverse transcribed into cDNA by using oligo-dT and Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA), followed by RNase H digestion. The quantitative PCR (qPCR) assays were performed using SYBR green PCR master mix (Life Technologies) in StepOnePlus quantitative PCR instrument (Life Technologies). The abundance of human GAPDH mRNA was used as the normalization control. All experiments were performed in triplicate, and a negative control (lacking a cDNA template) was included in each assay. Sequences

of primers used for BMI1 quantification were as follows: forward, 5′-CAGGTGGGGATTTAGCTCAG-3′; and reverse, 5′-CTTTCATTGTCTTTTCCGCC-3′.

Constructions of lentivirus vectors and production of lentivirus particles

The cDNA for the human *BMI1* gene was obtained by PCR and cloned by inserting into the FUGW plasmid (Addgene, catalog no. 14883, Cambridge, MA, USA). Lentivirus vectors encoding shRNA targeting against the human *BMI1* gene were constructed by subcloning synthetic DNA sequence encoding a shRNA that targets either a coding region in exon 2 (5′-CCAGACCACTACTGAATATAA-3′) or a 3′ UTR region (5′-GATACTCCTATGGACGTTAAT-3′) of the *BMI1* endogenous sequence into pGLV3-GFP-PURO backbone (abbreviated as shBMI1-CDS and shBMI1-UTR).

Lentivirus particles were produced by co-transfecting 293T cells with the packaging plasmid psPAX2 (Addgene, catalog no. 12260) and the envelope plasmid pMD2.G (Addgene, catalog no. 12259) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in Opti-MEM medium (Gibco, Grand Island, NY, USA). Supernatants containing recombinant lentiviruses were collected at 48 and 72 h post-transfection, followed by centrifugation at 500 × *g* for 5 min to remove any residual human 293T cells. Virus was concentrated by an Amicon Ultra-15 centrifugal filter device (100,000 Da; Merck Millipore, Billerica, MA, USA) and stored at −80°C.

Lentivirus transduction and extensive expansion of erythroid precursors

0.2×10^6 of erythroblasts were re-suspended in 300 μL of erythroblast expansion medium supplemented with polybrene (4 μg/mL, Sigma-Aldrich), then lentivirus particles were added into the medium at a multiplicity of infection (MOI; number of virus per cell) of 2. This was followed by the spin-infection procedure: the mixture was centrifuged at 1,500 × *g* for 2 h at room temperature (RT) to allow close contact between virus and the cells. Overnight transduced cells were washed and cultured into ultra-low attachment plate with fresh expansion medium. The cells were counted, and fresh medium was changed every 2 or 3 days to obtain a final cell concentration at $0.5–2 \times 10^6$ cells/mL.

Terminal maturation (TM) of human erythroblasts

TM of erythroblasts were performed as we previously published[21] with brief modification. Erythroblasts were seeded in erythroid TM medium by mixing with Iscove's modified Dulbecco's medium (IMDM, Invitrogen) plus 10% pooled normal adult human peripheral blood plasma (Innovative Research, Novi, MI, USA), 3 U/mL EPO, 500 μg/mL human holo-transferrin (R&D Systems), 4 U/mL heparin (Sigma), and 10 μg/mL insulin (Sigma). Fresh medium was changed every 2 or 3 days to maintain the cell density below 3×10^6 cells/mL until day 8.

Flow cytometric analysis

Approximately 5×10^4 cells were washed in PBS and resuspended in 50 μL of PBS containing 1% of fetal bovine serum (FBS; Gibco) and then stained with the appropriate dilution of the antibody and incubated for 30 min at 4°C in the dark. Quadrant/gating in dot plots was determined, based on the isotype staining. Antibodies used in this study were as follows: CD235a-PE (catalog no. 349106, Biolegend, San Diego, CA, USA), CD36-APC (catalog no. 550956, BD, Franklin Lakes, NJ, USA), CD71-PE (catalog no. 334106, Biolegend), CD49d-PE (α4-integrin, catalog no. 304304, Biolegend), CD45-APC (catalog no. 368512, Biolegend), CD117-PEcy7 (catalog no. 313211, Biolegend), DRAQ5 (catalog no. 4084L, Cell Signaling Technology, Danvers, MA, USA), CD55-fluorescein isothiocyanate (FITC) (catalog no. 311306, Biolegend), CD271-FITC (NGFR, catalog no. 345103, Biolegend), Ter119-APC (catalog no. 116212, Biolegend). Band3-APC was kindly provided by Dr. Xiuli An at New York Blood Center. For intracellular staining, we used the cell fixation and permeabilization kit (Invitrogen) to do fixation and permeabilization before cell staining with HBB-PE (catalog no. sc-21757; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or HBG-FITC (catalog no. MHFH01; Invitrogen). Stained cells were monitored by BD FACSCalibur cytometer (BD Biosciences, San Diego, CA, USA) and analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Wright-Giemsa staining

Cells were spun onto glass slides (Superfrost white microscope slides; Fisher Scientific, Pittsburgh, PA, USA) using Shandon cytospin 4 (Thermo Scientific, Waltham, MA, USA), stained with a Wright-Giemsa solution (Hema 3, Fisher Scientific) as previously described. Bright-field images were taken using Nikon Eclipse TE2000-U inverted microscope (Nikon, Melville, NY, USA).

Karyotyping

Karyotyping of human erythroblasts by G-banding (400–500 bands) was determined by the method we previously described.[21] Based on the criteria used in clinical practice, 20 cells were selected for chromosome counting and gross examination, and at least 5 karyotyping images with high-resolution banding were further examined.

Western blot assay

Human erythroblasts were lysed in RIPA buffer (Thermo Fisher Scientific, San Jose, CA, USA) supplemented with protease and phosphatase inhibitor cocktails (Roche, Nutley, NJ, USA), followed by centrifugation at 16,000 × *g* for 20 min at 4°C. Protein concentrations of cell lysates were measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific). Proteins (20 μg per sample) were separated by NuPAGE 4%–12% Bis-Tris gel (Invitrogen) followed by transfer to nitrocellulose membrane (GE Healthcare) at 300 mA for 1 h. The membranes were blocked in 5% (w/v) skim milk in TBS-T (Tris-buffered saline containing 0.1% Tween 20; Bio-Rad, Hercules, CA, USA) and incubated overnight at 4°C with 1:1,000 dilution of anti-BMI1 (catalog no. ab126783, Abcam, Cambridge, MA, USA) and 1:1,000 dilution of anti-GAPDH (catalog no. 5174, Cell Signaling Technology, Danvers, MA, USA). The membrane filters were then incubated with 1:10,000 dilution of goat anti-rabbit immunoglobulin G (IgG) (catalog no. 926-32211; LI-COR Biosciences, Lincoln, NE, USA) secondary antibody for 1 h at room temperature. Detected

protein bands were visualized by Odyssey Imaging Systems as we previously published.[66]

EdU assay for cell proliferation

A cell proliferation assay based on DNA synthesis was performed with Click-iT plus EdU Alexa Fluor 488 imaging kits (Thermo Fisher Scientific) according to the manufacturer's instruction. In brief, cells were incubated with 10 μM EdU solution for 4 h at 37°C. Cells were fixed in 3.7% formaldehyde in PBS for 15 min and permeabilized with 0.5% Triton X-100 for 20 min. Afterward, freshly prepared reaction cocktail was added, and cells were incubated at room temperature away from light for 30 min. Lastly, cellular DNA was stained with 5 μg/mL DAPI (4′, 6-diamidino-2-phenylindole, dihydrochloride; Vector Laboratories, Burlingame, CA, USA) solution and incubated for 30 min at room temperature in the dark. Washing steps were performed with PBS except during fixation and permeabilization steps, when 3% bovine serum albumin (BSA) in PBS was used. Proliferation index was then determined by quantifying the fraction of EdU-labeled cells and DNA-dye-labeled cells using a Celigo high-throughput microwell image cytometer (Nexcelom Bioscience, Lawrence, MA, USA).

Viable and apoptotic cell analysis

For cell apoptosis analysis, cells were collected and stained with a dead cell apoptosis kit (Thermo Fisher Scientific) containing both annexin V conjugated with the Alexa Fluor 488 dye and propidium iodide (PI). Apoptotic-like cells were analyzed using a BD FACSCalibur machine. Data analysis was performed with FlowJo software.

Genome editing by CRISPR-RNP in human erythroblasts

Chemically modified gRNA oligomers were synthesized by Synthego (Redwood City, CA, USA). The 20-nt-long specific sequences for targeting the CD55 gene were as follows: 5′-GGCGCGCCATGACCGTCGCG-3′ and 5′- GTTGTGCCTGCCGGCCGTGT-3′. 40 pmol of the Cas9 2NLS nuclease (Synthego) was complexed with 2 gRNAs (50 pmol each) to form RNPs for 10 min at room temperature prior to electroporation. After that, 0.2×10^6 BMI1-induced erythroblasts were reconstituted in P3 primary cell electroporation solution, according to the manufacturer's instructions (Lonza, Basel, Switzerland) and mixed with RNP complexes. The supplemented cell solution was transferred into the Lonza 4D-Nucleofector with 20 μL Nucleocuvette strips and electroporated using the EO-100 program. Recovered cells were cultured for 72–96 h prior to flow cytometry analysis and erythroid terminal differentiation.

Transgene expression of a marker gene tNGFR on expanded erythroblasts

The pEhfflucmCNsin lentiviral vector expressing both tNGFR and humanized firefly luciferase genes was kindly provided by Dr. Xianzheng Zhou.[61] Lentivirus production using 293T cells and transduction on expanded erythroblasts were performed as described above.

A mouse model for human RBC transfusion

The use of immunodeficient mice for the erythrocyte transfusion assay was approved by the Animal Care and Use Committee at Johns Hopkins University. 8-week-old female NSG mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained under specific pathogen-free conditions. All animal experiments were conducted in accordance with approved protocols.

Macrophage and complement depletion *in vivo* were performed by intravenous injection of liposome-encapsulated CL2MP (CLs; Liposoma, Amsterdam, the Netherlands) and CVF (Quidel, San Diego, CA, USA) respectively. CLs were given at 100 and 50 μL per mouse at day −1 and day 0, and 30 μL per mouse, with an interval of 3 days for long-term studies. CVF was given at 20 μg at day −1 and 10 μg at day 0 before transfusion, and 10 μg per mouse, with an interval of 3 days for long-term studies.

After pre-conditioning of mice at days −1 and 0 (time of transfusion), human erythrocytes differentiated from BMI1-induced E3 cells or primary human RBCs were injected intravenously into NSG mice (4×10^7 cells per mouse). To determine the kinetics of human erythrocyte clearance, 5 μL blood samples were collected into heparinized tubes at various time points post-transfusion and stained with an anti-human CD235a monoclonal antibody. The levels of viable transfused human erythrocytes were measured by flow cytometric analysis.

Statistical analysis

Statistical analysis was carried out by GraphPad Prism version 8 (La Jolla, CA, USA). Statistical significance was performed by unpaired two-tailed Student's t tests. All experiments were replicated at least three times. All data are presented as mean ± SEM; p values <0.05 were considered significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$); $p > 0.05$, non-significant (ns).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2021.01.022.

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AUTHOR CONTRIBUTIONS

S.L., M.W., T.C., and L.C. designed the concepts and experiments; S.L., M.W., M.L., and Y.G. performed the experiments and the data analysis; S.L., M.W., and L.C. interpreted the results and wrote the manuscript. J.D. and J.D.R. optimized the mouse transfusion model and collected the SCD blood samples. J.D.R. and T.C. also revised the manuscript. All authors critically read and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Roberts, N., James, S., Delaney, M., and Fitzmaurice, C. (2019). The global need and availability of blood products: a modelling study. Lancet Haematol. *6*, e606–e615.
2. Migliaccio, A.R., Masselli, E., Varricchio, L., and Whitsett, C. (2012). Ex-vivo expansion of red blood cells: how real for transfusion in humans? Blood Rev. *26*, 81–95.
3. Lee, E., Sivalingam, J., Lim, Z.R., Chia, G., Shi, L.G., Roberts, M., Loh, Y.H., Reuveny, S., and Oh, S.K. (2018). Review: In vitro generation of red blood cells for transfusion medicine: Progress, prospects and challenges. Biotechnol. Adv. *36*, 2118–2128.
4. Migliaccio, A.R., Whitsett, C., Papayannopoulou, T., and Sadelain, M. (2012). The potential of stem cells as an in vitro source of red blood cells for transfusion. Cell Stem Cell *10*, 115–119.
5. Bouhassira, E.E. (2012). Concise review: production of cultured red blood cells from stem cells. Stem Cells Transl. Med. *1*, 927–933.
6. Shah, S., Huang, X., and Cheng, L. (2014). Concise review: stem cell-based approaches to red blood cell production for transfusion. Stem Cells Transl. Med. *3*, 346–355.
7. Giarratana, M.C., Rouard, H., Dumont, A., Kiger, L., Safeukui, I., Le Pennec, P.Y., François, S., Trugnan, G., Peyrard, T., Marie, T., et al. (2011). Proof of principle for transfusion of in vitro-generated red blood cells. Blood *118*, 5071–5079.
8. Seo, Y., Shin, K.H., Kim, H.H., and Kim, H.S. (2019). Current Advances in Red Blood Cell Generation Using Stem Cells from Diverse Sources. Stem Cells Int. *2019*, 9281329.
9. Chou, B.K., Mali, P., Huang, X., Ye, Z., Dowey, S.N., Resar, L.M., Zou, C., Zhang, Y.A., Tong, J., and Cheng, L. (2011). Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. Cell Res. *21*, 518–529.
10. Dowey, S.N., Huang, X., Chou, B.K., Ye, Z., and Cheng, L. (2012). Generation of integration-free human induced pluripotent stem cells from postnatal blood mononuclear cells by plasmid vector expression. Nat. Protoc. *7*, 2013–2021.
11. Huang, X., Wang, Y., Yan, W., Smith, C., Ye, Z., Wang, J., Gao, Y., Mendelsohn, L., and Cheng, L. (2015). Production of Gene-Corrected Adult Beta Globin Protein in Human Erythrocytes Differentiated from Patient iPSCs After Genome Editing of the Sickle Point Mutation. Stem Cells *33*, 1470–1479.
12. Ye, Z., Liu, C.F., Lanikova, L., Dowey, S.N., He, C., Huang, X., Brodsky, R.A., Spivak, J.L., Prchal, J.T., and Cheng, L. (2014). Differential sensitivity to JAK inhibitory drugs by isogenic human erythroblasts and hematopoietic progenitors generated from patient-specific induced pluripotent stem cells. Stem Cells *32*, 269–278.
13. Xie, Y., Bai, H., Liu, Y., Hoyle, D.L., Cheng, T., and Wang, Z.Z. (2015). Cooperative Effect of Erythropoietin and TGF-β Inhibition on Erythroid Development in Human Pluripotent Stem Cells. J. Cell. Biochem. *116*, 2735–2743.
14. Fujita, A., Uchida, N., Haro-Mora, J.J., Winkler, T., and Tisdale, J. (2016). β-Globin-Expressing Definitive Erythroid Progenitor Cells Generated from Embryonic and Induced Pluripotent Stem Cell-Derived Sacs. Stem Cells *34*, 1541–1552.
15. Ruiz, J.P., Chen, G., Haro Mora, J.J., Keyvanfar, K., Liu, C., Zou, J., Beers, J., Bloomer, H., Qanash, H., Uchida, N., et al. (2019). Robust generation of erythroid and multilineage hematopoietic progenitors from human iPSCs using a scalable monolayer culture system. Stem Cell Res. (Amst.) *41*, 101600.
16. Yang, C.T., Ma, R., Axton, R.A., Jackson, M., Taylor, A.H., Fidanza, A., Marenah, L., Frayne, J., Mountford, J.C., and Forrester, L.M. (2017). Activation of KLF1 Enhances the Differentiation and Maturation of Red Blood Cells from Human Pluripotent Stem Cells. Stem Cells *35*, 886–897.
17. Bernecker, C., Ackermann, M., Lachmann, N., Rohrhofer, L., Zaehres, H., Araúzo-Bravo, M.J., van den Akker, E., Schlenke, P., and Dorn, I. (2019). Enhanced Ex Vivo Generation of Erythroid Cells from Human Induced Pluripotent Stem Cells in a Simplified Cell Culture System with Low Cytokine Support. Stem Cells Dev. *28*, 1540–1551.
18. Olivier, E.N., Zhang, S., Yan, Z., Suzuka, S., Roberts, K., Wang, K., and Bouhassira, E.E. (2019). PSC-RED and MNC-RED: Albumin-free and low-transferrin robust erythroid differentiation protocols to produce human enucleated red blood cells. Exp. Hematol. *75*, 31–52.e15.
19. Shen, J., Zhu, Y., Lyu, C., Feng, Z., Lyu, S., Zhao, Y., Hoyle, D.L., Ji, G., Miao, W., Zhang, X., et al. (2020). Sequential cellular niches control the generation of enucleated erythrocytes from human pluripotent stem cells. Haematologica *105*, e48–e51.
20. Hansen, M., von Lindern, M., van den Akker, E., and Varga, E. (2019). Human-induced pluripotent stem cell-derived blood products: state of the art and future directions. FEBS Lett. *593*, 3288–3303.
21. Huang, X., Shah, S., Wang, J., Ye, Z., Dowey, S.N., Tsang, K.M., Mendelsohn, L.G., Kato, G.J., Kickler, T.S., and Cheng, L. (2014). Extensive ex vivo expansion of functional human erythroid precursors established from umbilical cord blood cells by defined factors. Mol. Ther. *22*, 451–463.
22. Mayers, S., Moço, P.D., Maqbool, T., Silva, P.N., Kilkenny, D.M., and Audet, J. (2019). Establishment of an erythroid progenitor cell line capable of enucleation achieved with an inducible c-Myc vector. BMC Biotechnol. *19*, 21.
23. Hirose, S., Takayama, N., Nakamura, S., Nagasawa, K., Ochi, K., Hirata, S., Yamazaki, S., Yamaguchi, T., Otsu, M., Sano, S., et al. (2013). Immortalization of erythroblasts by c-MYC and BCL-XL enables large-scale erythrocyte production from human pluripotent stem cells. Stem Cell Reports *1*, 499–508.
24. Trakarnsanga, K., Griffiths, R.E., Wilson, M.C., Blair, A., Satchwell, T.J., Meinders, M., Cogan, N., Kupzig, S., Kurita, R., Nakamura, Y., et al. (2017). An immortalized adult human erythroid line facilitates sustainable and scalable generation of functional red cells. Nat. Commun. *8*, 14750.
25. Kurita, R., Suda, N., Sudo, K., Miharada, K., Hiroyama, T., Miyoshi, H., Tani, K., and Nakamura, Y. (2013). Establishment of immortalized human erythroid progenitor cell lines able to produce enucleated red blood cells. PLoS ONE *8*, e59890.
26. Kurita, R., Funato, K., Abe, T., Watanabe, Y., Shiba, M., Tadokoro, K., Nakamura, Y., Nagai, T., and Satake, M. (2019). Establishment and characterization of immortalized erythroid progenitor cell lines derived from a common cell source. Exp. Hematol. *69*, 11–16.
27. Hawksworth, J., Satchwell, T.J., Meinders, M., Daniels, D.E., Regan, F., Thornton, N.M., Wilson, M.C., Dobbe, J.G., Streekstra, G.J., Trakarnsanga, K., et al. (2018). Enhancement of red blood cell transfusion compatibility using CRISPR-mediated erythroblast gene editing. EMBO Mol. Med. *10*, e8454.
28. Daniels, D.E., Downes, D.J., Ferrer-Vicens, I., Ferguson, D.C.J., Singleton, B.K., Wilson, M.C., Trakarnsanga, K., Kurita, R., Nakamura, Y., Anstee, D.J., and Frayne, J. (2019). Comparing the two leading erythroid lines BEL-A and HUDEP-2. Haematologica *105*, e389–e394.
29. Kim, A.R., Olsen, J.L., England, S.J., Huang, Y.S., Fegan, K.H., Delgadillo, L.F., McGrath, K.E., Kingsley, P.D., Waugh, R.E., and Palis, J. (2015). Bmi-1 Regulates Extensive Erythroid Self-Renewal. Stem Cell Reports *4*, 995–1003.
30. Schuettengruber, B., Bourbon, H.M., Di Croce, L., and Cavalli, G. (2017). Genome Regulation by Polycomb and Trithorax: 70 Years and Counting. Cell *171*, 34–57.
31. Park, I.K., Qian, D., Kiel, M., Becker, M.W., Pihalja, M., Weissman, I.L., Morrison, S.J., and Clarke, M.F. (2003). Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature *423*, 302–305.
32. Molofsky, A.V., Pardal, R., Iwashita, T., Park, I.K., Clarke, M.F., and Morrison, S.J. (2003). Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature *425*, 962–967.
33. Rizo, A., Olthof, S., Han, L., Vellenga, E., de Haan, G., and Schuringa, J.J. (2009). Repression of BMI1 in normal and leukemic human CD34(+) cells impairs self-renewal and induces apoptosis. Blood *114*, 1498–1505.

34. Biehs, B., Hu, J.K., Strauli, N.B., Sangiorgi, E., Jung, H., Heber, R.P., Ho, S., Goodwin, A.F., Dasen, J.S., Capecchi, M.R., and Klein, O.D. (2013). BMI1 represses Ink4a/Arf and Hox genes to regulate stem cells in the rodent incisor. Nat. Cell Biol. *15*, 846–852.

35. Chiba, T., Seki, A., Aoki, R., Ichikawa, H., Negishi, M., Miyagi, S., Oguro, H., Saraya, A., Kamiya, A., Nakauchi, H., et al. (2010). Bmi1 promotes hepatic stem cell expansion and tumorigenicity in both Ink4a/Arf-dependent and -independent manners in mice. Hepatology *52*, 1111–1123.

36. Zacharek, S.J., Fillmore, C.M., Lau, A.N., Gludish, D.W., Chou, A., Ho, J.W., Zamponi, R., Gazit, R., Bock, C., Jäger, N., et al. (2011). Lung stem cell self-renewal relies on BMI1-dependent control of expression at imprinted loci. Cell Stem Cell *9*, 272–281.

37. Bruggeman, S.W., Valk-Lingbeek, M.E., van der Stoop, P.P., Jacobs, J.J., Kieboom, K., Tanger, E., Hulsman, D., Leung, C., Arsenijevic, Y., Marino, S., and van Lohuizen, M. (2005). Ink4a and Arf differentially affect cell proliferation and neural stem cell self-renewal in Bmi1-deficient mice. Genes Dev. *19*, 1438–1443.

38. Molofsky, A.V., He, S., Bydon, M., Morrison, S.J., and Pardal, R. (2005). Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. Genes Dev. *19*, 1432–1437.

39. Oguro, H., Yuan, J., Ichikawa, H., Ikawa, T., Yamazaki, S., Kawamoto, H., Nakauchi, H., and Iwama, A. (2010). Poised lineage specification in multipotential hematopoietic stem and progenitor cells by the polycomb protein Bmi1. Cell Stem Cell *6*, 279–286.

40. Iwama, A., Oguro, H., Negishi, M., Kato, Y., Morita, Y., Tsukui, H., Ema, H., Kamijo, T., Katoh-Fukui, Y., Koseki, H., et al. (2004). Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. Immunity *21*, 843–851.

41. Rizo, A., Dontje, B., Vellenga, E., de Haan, G., and Schuringa, J.J. (2008). Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1. Blood *111*, 2621–2630.

42. Bordeleau, M.E., Aucagne, R., Chagraoui, J., Girard, S., Mayotte, N., Bonneil, E., Thibault, P., Pabst, C., Bergeron, A., Barabé, F., et al. (2014). UBAP2L is a novel BMI1-interacting protein essential for hematopoietic stem cell activity. Blood *124*, 2362–2369.

43. Hu, T., Kitano, A., Luu, V., Dawson, B., Hoegenauer, K.A., Lee, B.H., and Nakada, D. (2019). Bmi1 Suppresses Adipogenesis in the Hematopoietic Stem Cell Niche. Stem Cell Reports *13*, 545–558.

44. Kato, Y., Hou, L.B., Miyagi, S., Nitta, E., Aoyama, K., Shinoda, D., Yamazaki, S., Kuribayashi, W., Isshiki, Y., Koide, S., et al. (2019). Bmi1 restricts the adipogenic differentiation of bone marrow stromal cells to maintain the integrity of the hematopoietic stem cell niche. Exp. Hematol. *76*, 24–37.

45. Chen, G., Zhang, Y., Yu, S., Sun, W., and Miao, D. (2019). Bmi1 Overexpression in Mesenchymal Stem Cells Exerts Antiaging and Antiosteoporosis Effects by Inactivating p16/p19 Signaling and Inhibiting Oxidative Stress. Stem Cells *37*, 1200–1211.

46. Gao, R., Chen, S., Kobayashi, M., Yu, H., Zhang, Y., Wan, Y., Young, S.K., Soltis, A., Yu, M., Vemula, S., et al. (2015). Bmi1 promotes erythroid development through regulating ribosome biogenesis. Stem Cells *33*, 925–938.

47. Giarratana, M.C., Kobari, L., Lapillonne, H., Chalmers, D., Kiger, L., Cynober, T., Marden, M.C., Wajcman, H., and Douay, L. (2005). Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. Nat. Biotechnol. *23*, 69–74.

48. Miharada, K., Hiroyama, T., Sudo, K., Nagasawa, T., and Nakamura, Y. (2006). Efficient enucleation of erythroblasts differentiated in vitro from hematopoietic stem and progenitor cells. Nat. Biotechnol. *24*, 1255–1256.

49. Mettananda, S., Clark, K., Fisher, C.A., Sloane-Stanley, J.A., Gibbons, R.J., and Higgs, D.R. (2018). Phenotypic and molecular characterization of a serum-free miniature erythroid differentiation system suitable for high-throughput screening and single-cell assays. Exp. Hematol. *60*, 10–20.

50. Chaurasia, P., Berenzon, D., and Hoffman, R. (2011). Chromatin-modifying agents promote the ex vivo production of functional human erythroid progenitor cells. Blood *117*, 4632–4641.

51. Boehm, D., Murphy, W.G., and Al-Rubeai, M. (2009). The potential of human peripheral blood derived CD34+ cells for ex vivo red blood cell production. J. Biotechnol. *144*, 127–134.

52. Zhang, Y., Wang, C., Wang, L., Shen, B., Guan, X., Tian, J., Ren, Z., Ding, X., Ma, Y., Dai, W., and Jiang, Y. (2017). Large-Scale Ex Vivo Generation of Human Red Blood Cells from Cord Blood $CD34^+$ Cells. Stem Cells Transl. Med. *6*, 1698–1709.

53. van den Akker, E., Satchwell, T.J., Pellegrin, S., Daniels, G., and Toye, A.M. (2010). The majority of the in vitro erythroid expansion potential resides in CD34(-) cells, outweighing the contribution of CD34(+) cells and significantly increasing the erythroblast yield from peripheral blood samples. Haematologica *95*, 1594–1598.

54. Heideveld, E., Masiello, F., Marra, M., Esteghamat, F., Yağcı, N., von Lindern, M., Migliaccio, A.R., and van den Akker, E. (2015). CD14+ cells from peripheral blood positively regulate hematopoietic stem and progenitor cell survival resulting in increased erythroid yield. Haematologica *100*, 1396–1406.

55. Heshusius, S., Heideveld, E., Burger, P., Thiel-Valkhof, M., Sellink, E., Varga, E., Ovchynnikova, E., Visser, A., Martens, J.H.A., von Lindern, M., and van den Akker, E. (2019). Large-scale in vitro production of red blood cells from human peripheral blood mononuclear cells. Blood Adv. *3*, 3337–3350.

56. Hu, J., Liu, J., Xue, F., Halverson, G., Reid, M., Guo, A., Chen, L., Raza, A., Galili, N., Jaffray, J., et al. (2013). Isolation and functional characterization of human erythroblasts at distinct stages: implications for understanding of normal and disordered erythropoiesis in vivo. Blood *121*, 3246–3253.

57. Yan, H., Wang, Y., Qu, X., Li, J., Hale, J., Huang, Y., An, C., Papoin, J., Guo, X., Chen, L., et al. (2017). Distinct roles for TET family proteins in regulating human erythropoiesis. Blood *129*, 2002–2012.

58. Novershtern, N., Subramanian, A., Lawton, L.N., Mak, R.H., Haining, W.N., McConkey, M.E., Habib, N., Yosef, N., Chang, C.Y., Shay, T., et al. (2011). Densely interconnected transcriptional circuits control cell states in human hematopoiesis. Cell *144*, 296–309.

59. Egan, E.S., Jiang, R.H., Moechtar, M.A., Barteneva, N.S., Weekes, M.P., Nobre, L.V., Gygi, S.P., Paulo, J.A., Frantzreb, C., Tani, Y., et al. (2015). Malaria. A forward genetic screen identifies erythrocyte CD55 as essential for Plasmodium falciparum invasion. Science *348*, 711–714.

60. Wu, M., Liu, S., Gao, Y., Bai, H., Machairaki, V., Li, G., Chen, T., and Cheng, L. (2018). Conditional gene knockout and reconstitution in human iPSCs with an inducible Cas9 system. Stem Cell Res. (Amst.) *29*, 6–14.

61. Huang, X., Guo, H., Kang, J., Choi, S., Zhou, T.C., Tammana, S., Lees, C.J., Li, Z.Z., Milone, M., Levine, B.L., et al. (2008). Sleeping Beauty transposon-mediated engineering of human primary T cells for therapy of CD19+ lymphoid malignancies. Mol. Ther. *16*, 580–589.

62. Hu, Z., Van Rooijen, N., and Yang, Y.G. (2011). Macrophages prevent human red blood cell reconstitution in immunodeficient mice. Blood *118*, 5938–5946.

63. Chen, B., Fan, W., Zou, J., Zhang, S., He, J., Shu, C., Zhao, G., Sun, T., Hu, Z., and Yang, Y.G. (2017). Complement Depletion Improves Human Red Blood Cell Reconstitution in Immunodeficient Mice. Stem Cell Reports *9*, 1034–1042.

64. Oguro, H., Iwama, A., Morita, Y., Kamijo, T., van Lohuizen, M., and Nakauchi, H. (2006). Differential impact of Ink4a and Arf on hematopoietic stem cells and their bone marrow microenvironment in Bmi1-deficient mice. J. Exp. Med. *203*, 2247–2253.

65. Huang, N.J., Pishesha, N., Mukherjee, J., Zhang, S., Deshycka, R., Sudaryo, V., Dong, M., Shoemaker, C.B., and Lodish, H.F. (2017). Genetically engineered red cells expressing single domain camelid antibodies confer long-term protection against botulinum neurotoxin. Nat. Commun. *8*, 423.

66. Liu, S., Mahairaki, V., Bai, H., Ding, Z., Li, J., Witwer, K.W., and Cheng, L. (2019). Highly Purified Human Extracellular Vesicles Produced by Stem Cells Alleviate Aging Cellular Phenotypes of Senescent Human Cells. Stem Cells *37*, 779–790.