UC San Diego
UC San Diego Previously Published Works

Title
Targeted Application of Human Genetic Variation Can Improve Red Blood Cell Production from Stem Cells

Permalink
https://escholarship.org/uc/item/3s39n3ws

Journal
Cell Stem Cell, 18(1)

ISSN
1934-5909

Authors
Giani, Felix C
Fiorini, Claudia
Wakabayashi, Aoi
et al.

Publication Date
2016

DOI
10.1016/j.stem.2015.09.015

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

HHS Public Access

Author manuscript

Cell Stem Cell. Author manuscript; available in PMC 2017 January 07.

Published in final edited form as:

Cell Stem Cell. 2016 January 7; 18(1): 73–78. doi:10.1016/j.stem.2015.09.015.

Targeted Application of Human Genetic Variation Can Improve Red Blood Cell Production from Stem Cells

Felix C. Giani[#1,2,8], **Claudia Fiorini**[#1,2], **Aoi Wakabayashi**[#1,2], **Leif S. Ludwig**[1,2,8,9], **Rany M. Salem**[2,5], **Chintan D. Jobaliya**[3], **Stephanie N. Regan**[2,4], **Jacob C. Ulirsch**[1,2], **Ge Liang**[3], **Orna Steinberg-Shemer**[3], **Michael H. Guo**[2,5], **Tõnu Esko**[2,5], **Wei Tong**[3], **Carlo Brugnara**[7], **Joel N. Hirschhorn**[2,5], **Mitchell J. Weiss**[6], **Leonard I. Zon**[1,2,4], **Stella T. Chou**[3], **Deborah L. French**[3], **Kiran Musunuru**[2,4], and **Vijay G. Sankaran**[1,2,*]

[1]Division of Hematology/Oncology, Boston Children's Hospital and Department of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, USA.

[2]Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA.

[3]Departments of Pathology and Laboratory Medicine and Pediatrics, The Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, PA 19104, USA.

[4]Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138, USA.

[5]Division of Endocrinology and Center for Basic and Translational Obesity Research, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA.

[6]Department of Hematology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA.

[7]Department of Laboratory Medicine, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA.

[8]Department of Neonatology, Charité-Universitätsmedizin Berlin, Berlin 10117, Germany.

[9]Institute for Chemistry and Biochemistry, Freie Universität Berlin, Berlin 14195, Germany.

[#] These authors contributed equally to this work.

SUMMARY

[*]Correspondence: sankaran@broadinstitute.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

AUTHOR CONTRIBUTIONS

Conceptualization, F.C.G., K.M., and V.G.S.; Methodology, F.C.G., C.F., A.W., R.M.S., D.L.F., K.M., and V.G.S.; Investigation, F.C.G., C.F., A.W., L.S.L., R.M.S., C.D.J., S.N.R., J.C.U., G.L., O.S-S., M.H.G., T.E., K.M., and V.G.S.; Data Curation, R.M.S. and J.C.U; Writing – Original Draft, F.C.G., C.F., A.W., M.J.W., L.I.Z., K.M., and V.G.S.; Writing – Review & Editing, F.C.G., C.F., A.W., L.S.L., R.M.S., C.D.J., S.N.R., J.C.U., G.L., O.S-S., M.H.G., T.E., W.T., C.B., M.J.W., L.I.Z., S.T.C., D.L.F., K.M., and V.G.S.; Funding Acquisition, S.T.C., K.M., and V.G.S.; Resources, W.T., C.B., J.N.H., M.J.W., L.I.Z., S.T.C., and D.L.F.; Supervision, D.L.F., K.M., and V.G.S.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online.

Author Manuscript

Author Manuscript

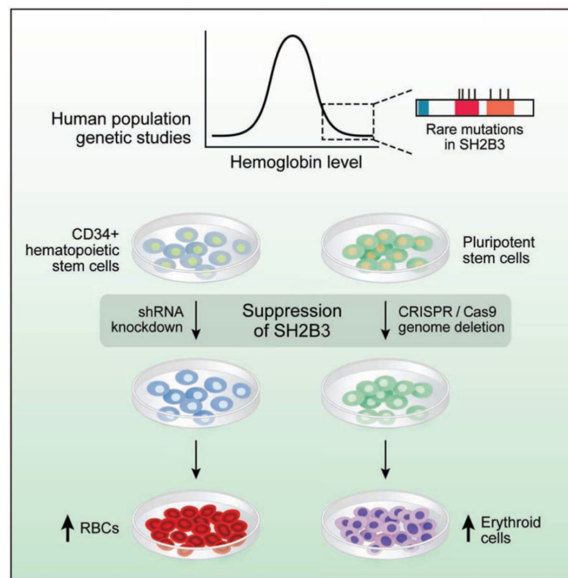
Author Manuscript

Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Multipotent and pluripotent stem cells are potential sources for cell and tissue replacement therapies. For example, stem cell-derived red blood cells (RBCs) are a potential alternative to donated blood, but yield and quality remain a challenge. Here, we show that application of insight from human population genetic studies can enhance RBC production from stem cells. The *SH2B3* gene encodes a negative regulator of cytokine signaling and naturally occurring loss-of-function variants in this gene increase RBC counts *in vivo*. Targeted suppression of SH2B3 in primary human hematopoietic stem and progenitor cells enhanced the maturation and overall yield of *in vitro*-derived RBCs. Moreover, inactivation of *SH2B3* by CRISPR/Cas9 genome editing in human pluripotent stem cells allowed for enhanced erythroid cell expansion with preserved differentiation. Our findings therefore highlight the potential for combining human genome variation studies with genome editing approaches to improve cell and tissue production for regenerative medicine.

Graphical Abstract



There is significant excitement regarding the use of stem cells as a source for cell replacement therapies (Fox et al., 2014). *In vitro* differentiated red blood cells (RBCs) from hematopoietic stem and progenitor cells (HSPCs) have been successfully transfused into recipients in clinical trials (Giarratana et al., 2011; Migliaccio et al., 2012) and similar approaches using pluripotent stem cells (PSCs), including human embryonic stem cells (hESCs) and induced PSCs (iPSCs), are under investigation (Kobari et al., 2012; Slukvin, 2013; Sturgeon et al., 2013). However, utilizing these stem cell sources for RBC production is limited by low yields of fully mature cells, making the process costly and inefficient (Migliaccio et al., 2012; Rousseau et al., 2014). Overcoming these major limitations could enhance our ability to manage the blood supply (Williamson and Devine, 2013).

Numerous ongoing efforts focus on optimizing culture methods to improve RBC production from various stem cell sources (Huang et al., 2015; Rousseau et al., 2014). A complementary approach is to investigate whether human genes that regulate RBC

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

production *in vivo* can be manipulated to enhance this process *in vitro*. Recent genome-wide association studies (GWAS) of red blood cell traits and subsequent fine-mapping identified a common coding single nucleotide polymorphism (SNP) in the *SH2B3* gene (rs3184504), which results in a R262W substitution, that is significantly associated with hemoglobin levels and RBC counts *in vivo* (van der Harst et al., 2012). SH2B3 is an SH2- and PH-domain containing protein that negatively regulates hematopoietic cytokine signaling. Mice with null mutations in *SH2B3* have normal hemoglobin and RBC counts, suggesting human-specific functions in RBC production (erythropoiesis) (Bersenev et al., 2008; Velazquez et al., 2002). The rs3184504 variant associated with increased RBC counts and hemoglobin levels is thought to be a hypomorphic allele (McMullin et al., 2011; van der Harst et al., 2012). Consistent with this possibility, rare loss-of-function (LoF) *SH2B3* alleles are associated with greater elevations in the RBC count and hemoglobin level (erythrocytosis) (Lasho et al., 2010; Spolverini et al., 2013). To extend this observation to a population-based cohort, we examined a group of 4,678 individuals subject to whole-exome sequencing and observed higher hemoglobin/ hematocrit values among individuals with rare putative damaging missense or LoF variants in *SH2B3* (Figure 1A, B, Tables S1, S2).

Given the association between elevated hemoglobin levels and LoF alleles of *SH2B3* in human population genetic studies, we tested whether suppression of SH2B3 can enhance erythropoiesis *in vitro*. We used lentiviral vectors to express short hairpin RNAs (shRNAs) targeting SH2B3 in adult CD34+ HSPCs that were induced to undergo erythroid differentiation (Figure 1C, D). The *SH2B3* LoF cultures differentiated similar to controls, although the erythroid maturation as determined by cell surface phenotyping (loss of CD71, as well as acquisition of CD235a) occurred earlier (Figure 1E, F). Interestingly, we noticed a 1.6 - 2-fold increase in enucleation in SH2B3 LoF erythroblasts (Figure 1G). These observations were confirmed by analysis of cell morphology that demonstrated improved maturation, better hemoglobinization, and greater enucleation (Figure 1H). Gene expression analysis of erythroblasts from the SH2B3 LoF cultures showed a globally similar profile with controls (Figure 1I) with enhanced expression of genes associated with terminal erythroid maturation (Figure 1J). We maintained the cultures following enucleation for several days and did not observe any improvement in maturation in the controls in comparison with the SH2B3 LoF cultures.

Since cells with SH2B3 LoF appeared to undergo erythroid differentiation more readily, we wondered if their proliferative potential was restrained, which could compromise RBC yield. Surprisingly, adult HSPCs with SH2B3 LoF expanded to a significantly greater extent when compared with controls (Figure 2A, Figure S1A). The overall increase in expansion occurred over multiple stages of the differentiation process, suggesting that SH2B3 suppression augments multiple signaling pathways at different stages of differentiation (Figure S1A). The observed effect was not due to suboptimal cytokine concentrations, as the cells were cultured in erythropoietin and other cytokine concentrations where they demonstrated maximal expansion (Figure S1B) (Abkowitz et al., 1991). Overall, the yield of mature RBCs was expanded 3 - 5 fold by suppression of *SH2B3* (Figure 2A). We performed a similar knockdown of *SH2B3* in CD34+ HSPCs from cord blood, which are known to exhibit over 10-fold greater expansion during erythroid differentiation compared to adult

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

HSPCs (Giarratana et al., 2011; Migliaccio et al., 2012; Rousseau et al., 2014). In these cultures, SH2B3 LoF further enhanced enucleated RBC yield by 2 - 4 fold (Figure 2B). In addition, we also utilized an adaptation of a recently described culture approach incorporating a progenitor expansion step with adult CD34+ HSPCs (Lee et al., 2015; Ludwig et al., 2014; Sankaran et al., 2011). In this case, we noted an even greater increase in expansion and overall yield of RBCs of 5 - 7 fold (Figure 2C), demonstrating the generalizability of SH2B3 suppression to augment RBC production from HSPCs.

We then wanted to understand the mechanisms through which suppression of SH2B3 is able to augment erythroid differentiation and expansion. The fraction of apoptotic cells, as assessed by annexin V staining, was not altered by SH2B3 suppression in adult HSPCs (Figure S1C). Cells with SH2B3 LoF underwent 0.5 – 1 additional cell divisions over the 4 day period of differentiation examined (Figures S1D, E) (Sankaran et al., 2012). SH2B3 negatively regulates signaling downstream of multiple cell surface signaling receptors implicated in erythropoiesis, including erythropoietin (EPO), KIT, and integrin receptors (Gery and Koeffler, 2013; McMullin et al., 2011). Accordingly, phosphorylation of STAT5 and KIT receptor, as well as the expression of early EPO responsive genes, were enhanced by SH2B3 suppression (Figures S1F, G) (Moraga et al., 2015). We could verify the importance of both the EPO and KIT/ SCF pathways for the observed augmentation upon SH2B3 suppression by limiting the concentration of both cytokines in the HSPC differentiation cultures (Figure S1H). SH2B3 suppression allowed for erythroid expansion similar to the baseline control with reduced levels of either EPO or SCF, highlighting the role of SH2B3 in both pathways in primary erythroid cells. Thus, SH2B3 LoF appears to facilitate erythroid expansion and maturation by augmenting both the EPO and KIT signaling pathways.

Since production of erythroid cells from human HSPCs appeared to be increased by SH2B3 LoF, we examined whether the RBCs produced *in vitro* were altered from controls. The average volume of RBCs was similar between those arising from control and SH2B3 LoF cultures (average of 108.7 in controls, compared with 110.1 and 104.7 femtoliters in the sh83 and sh84 SH2B3 shRNAs). These values were within the normal range of 103 - 126 femtoliters for reticulocytes (immature RBCs, equivalent to those produced in the culture) found in human adults (d'Onofrio et al., 1995). Moreover, SH2B3 LoF improved hemoglobinization with the mean hemoglobin content being 27 and 26.7 picograms (pg) in sh83 and sh84 treated cells, respectively, compared with 25.4 in controls (human adult reticulocytes generally have concentrations of 25.9 - 30.6 pg (d'Onofrio et al., 1995)). Disorders of the RBC membrane, such as hereditary spherocytosis, are clinically diagnosed by variation in binding of the dye eosin-5-malemide (EMA) (Perrotta et al., 2008). We found that EMA staining was comparable between the SH2B3 LoF RBCs and controls, suggesting normal formation of the RBC cytoskeleton (Figure 2D). In addition, surface expression of RBC antigens, including the Rh blood group (Figure S2A) and CD44, and activity of the cytoplasmic pyruvate kinase enzyme were similar between the SH2B3 LoF RBCs and controls (Figure S2B). RBC ghosts from the SH2B3 LoF and control cells showed identical protein patterns, providing an independent metric for normal RBC maturation (Figure S2C). We also observed normal hemoglobin subunits present in the

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

RBCs with a small increase in fetal hemoglobin (HbF) production with SH2B3 LoF, consistent with the known increase in HbF observed with acceleration at the early stages of erythropoiesis (Figure S2D) (Sankaran and Orkin, 2013). Collectively, these results suggest that RBC physiology was not impaired in cells derived from SH2B3 LoF HSPCs, despite a marked improvement in differentiation and expansion.

Overall, our results suggest that reducing SH2B3 expression could improve *in vitro* production of RBCs for transfusion purposes. While viral mediated shRNA delivery to primary CD34+ HSPCs may be impractical on a large scale, it should be possible to disrupt the *SH2B3* gene in self-renewing cell lines that can be used for RBC production (Rousseau et al., 2014). Despite promising recent advances (Hirose et al., 2013; Huang et al., 2014; Kurita et al., 2013), no immortalized human cell lines allow for consistent differentiation into mature RBCs. However, PSCs can be readily differentiated toward the erythroid lineage with well-established protocols (Huang et al., 2015; Kobari et al., 2012; Mills et al., 2014; Slukvin, 2013; Sturgeon et al., 2014) (Figure 2E). We used CRISPR/Cas9-mediated genome editing to engineer isogenic hESC lines with either homozygous frameshift deletions in *SH2B3* or intact wild-type (WT) alleles (Figure S2E, F) (Ding et al., 2013a; Ding et al., 2013b; Gupta and Musunuru, 2014; Veres et al., 2014). The hESCs were differentiated *in vitro* to generate multipotent hematopoietic progenitor cells (HPCs) with erythroid, megakaryocytic, and myeloid potential (Kennedy et al., 2012; Mills et al., 2014; Sturgeon et al., 2013) (Figure 2E). No differences in the yield of multipotent $CD41a^{+}/CD235a^{+}$ HPCs were noted between differentiating WT and SH2B3 knockout hESCs (Mills et al., 2014). However, in multiple independent isogenic hESC lines studied, loss of SH2B3 augmented erythroid cell production more than 3-fold (Figures 2F, G, S2G). Importantly, maturing WT and SH2B3 knockout erythroblasts exhibited similar morphologies, expression of erythroid cell surface markers, and globin gene expression patterns (Figures 2H, I, S2H). While PSC erythroid differentiation protocols continue to undergo refinement, are not yet optimized for RBC manufacture, and primarily produce the earliest waves of hematopoiesis equivalent to those that developmentally arise from the yolk sac (Kobari et al., 2012; Slukvin, 2013; Sturgeon et al., 2014), the current findings provide a proof-of-principle that stable perturbation of SH2B3 can allow for improved erythroid differentiation and expansion.

It is estimated that a unit of RBCs produced from HSPCs would cost $8,000 – 15,000 using current *in vitro* differentiation protocols, while normal donor-derived units cost less than one tenth of this amount (Migliaccio et al., 2012; Rousseau et al., 2014). While improvements in culture methods and use of systems such as bioreactors may be extremely valuable to improve upon this process, intrinsic alteration of stem cell sources to stimulate erythropoiesis should optimize the process further. To the best of our knowledge this has not been previously tested. Here we show that by utilizing observations from both common and rare human genetic variation, we could perturb the *SH2B3* gene to improve the ability of stem cell-derived hematopoietic progenitors to expand and differentiate into the erythroid lineage. We would note that the improvement of both expansion and differentiation surpasses the maximal expansion that could normally be achieved by simply optimizing cytokine concentrations in such cultures, which illustrates the complementary benefit of targeting intrinsic regulatory pathways. The increase in yields resulting from SH2B3

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

suppression should allow RBCs to be produced from HSPCs at an estimated cost that is less than one-fifth of current approaches and with fewer starting stem cells. Thus, a single RBC unit is estimated to require 25 million CD34+ HSPCs and perturbation of SH2B3 should reduce the required number of starting cells to ~5 million (Migliaccio et al., 2012). With further technological advances it is likely that both cost and efficiency of this process can be improved further. Importantly, our studies suggest that perturbation of *SH2B3* should improve erythroid differentiation from a variety of human stem cell sources, regardless of the differentiation method employed. Nonetheless, hurdles remain to be able to effectively translate these observations to improve and deliver upon the promise of such cell replacement therapies (Fox et al., 2014; Slukvin, 2013). We envision that future studies on the role of human genetic variation of blood cell traits will further contribute to our ability to manipulate this process for therapeutic purposes and manage the ever-increasing demand on the blood supply (Williamson and Devine, 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank D. Nathan, S. Orkin, L. Cheng, R. Kapur, D. Wojchowski, J. Flygare, H. Lodish, C. Dame, and members of the Sankaran lab for valuable comments on this work. We are grateful to L. Gaffney and T. Dicesare for help with illustrations. This work was supported by National Institutes of Health (NIH) grants R21 HL120791 and R01 DK103794 (V.G.S.), K99 HL122515 (R.M.S.), R01 DK100854 (S.T.C.), and R01 GM104464 (K.M.).

REFERENCES

Abkowitz JL, Sabo KM, Nakamoto B, Blau CA, Martin FH, Zsebo KM, Papayannopoulou T. Diamond-blackfan anemia: in vitro response of erythroid progenitors to the ligand for c-kit. Blood. 1991; 78:2198–2202. [PubMed: 1718487]

Bersenev A, Wu C, Balcerek J, Tong W. Lnk controls mouse hematopoietic stem cell self-renewal and quiescence through direct interactions with JAK2. J Clin Invest. 2008; 118:2832–2844. [PubMed: 18618018]

d'Onofrio G, Chirillo R, Zini G, Caenaro G, Tommasi M, Micciulli G. Simultaneous measurement of reticulocyte and red blood cell indices in healthy subjects and patients with microcytic and macrocytic anemia. Blood. 1995; 85:818–823. [PubMed: 7833482]

Ding Q, Lee YK, Schaefer EA, Peters DT, Veres A, Kim K, Kuperwasser N, Motola DL, Meissner TB, Hendriks WT, et al. A TALEN genome-editing system for generating human stem cell-based disease models. Cell Stem Cell. 2013a; 12:238–251. [PubMed: 23246482]

Ding Q, Regan SN, Xia Y, Oostrom LA, Cowan CA, Musunuru K. Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. Cell Stem Cell. 2013b; 12:393–394. [PubMed: 23561441]

Fox IJ, Daley GQ, Goldman SA, Huard J, Kamp TJ, Trucco M. Stem cell therapy. Use of differentiated pluripotent stem cells as replacement therapy for treating disease. Science. 2014; 345:1247391. [PubMed: 25146295]

Gery S, Koeffler HP. Role of the adaptor protein LNK in normal and malignant hematopoiesis. Oncogene. 2013; 32:3111–3118. [PubMed: 23045270]

Giarratana MC, Rouard H, Dumont A, Kiger L, Safeukui I, Le Pennec PY, Francois S, Trugnan G, Peyrard T, Marie T, et al. Proof of principle for transfusion of in vitro-generated red blood cells. Blood. 2011; 118:5071–5079. [PubMed: 21885599]

Gupta RM, Musunuru K. Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. J Clin Invest. 2014; 124:4154–4161. [PubMed: 25271723]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Hirose S, Takayama N, Nakamura S, Nagasawa K, Ochi K, Hirata S, Yamazaki S, Yamaguchi T, Otsu M, Sano S, et al. Immortalization of erythroblasts by c-MYC and BCL-XL enables large-scale erythrocyte production from human pluripotent stem cells. Stem Cell Reports. 2013; 1:499–508. [PubMed: 24371805]

Huang X, Shah S, Wang J, Ye Z, Dowey SN, Tsang KM, Mendelsohn LG, Kato GJ, Kickler TS, Cheng L. Extensive ex vivo expansion of functional human erythroid precursors established from umbilical cord blood cells by defined factors. Mol Ther. 2014; 22:451–463. [PubMed: 24002691]

Huang X, Wang Y, Yan W, Smith C, Ye Z, Wang J, Gao Y, Mendelsohn L, Cheng L. 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. 2015; 33:1470–1479. [PubMed: 25702619]

Kennedy M, Awong G, Sturgeon CM, Ditadi A, LaMotte-Mohs R, Zuniga-Pflucker JC, Keller G. T lymphocyte potential marks the emergence of definitive hematopoietic progenitors in human pluripotent stem cell differentiation cultures. Cell Rep. 2012; 2:1722–1735. [PubMed: 23219550]

Kobari L, Yates F, Oudrhiri N, Francina A, Kiger L, Mazurier C, Rouzbeh S, El-Nemer W, Hebert N, Giarratana MC, et al. Human induced pluripotent stem cells can reach complete terminal maturation: in vivo and in vitro evidence in the erythropoietic differentiation model. Haematologica. 2012; 97:1795–1803. [PubMed: 22733021]

Kurita R, Suda N, Sudo K, Miharada K, Hiroyama T, Miyoshi H, Tani K, Nakamura Y. Establishment of immortalized human erythroid progenitor cell lines able to produce enucleated red blood cells. PLoS One. 2013; 8:e59890. [PubMed: 23533656]

Lasho TL, Pardanani A, Tefferi A. LNK mutations in JAK2 mutation-negative erythrocytosis. N Engl J Med. 2010; 363:1189–1190. [PubMed: 20843259]

Lee HY, Gao X, Barrasa MI, Li H, Elmes RR, Peters LL, Lodish HF. PPAR-alpha and glucocorticoid receptor synergize to promote erythroid progenitor self-renewal. Nature. 2015

Ludwig LS, Gazda HT, Eng JC, Eichhorn SW, Thiru P, Ghazvinian R, George TI, Gotlib JR, Beggs AH, Sieff CA, et al. Altered translation of GATA1 in Diamond-Blackfan anemia. Nat Med. 2014; 20:748–753. [PubMed: 24952648]

McMullin MF, Wu C, Percy MJ, Tong W. A nonsynonymous LNK polymorphism associated with idiopathic erythrocytosis. Am J Hematol. 2011; 86:962–964. [PubMed: 21990094]

Migliaccio AR, Whitsett C, Papayannopoulou T, Sadelain M. The potential of stem cells as an in vitro source of red blood cells for transfusion. Cell Stem Cell. 2012; 10:115–119. [PubMed: 22305561]

Mills JA, Paluru P, Weiss MJ, Gadue P, French DL. Hematopoietic differentiation of pluripotent stem cells in culture. Methods in molecular biology. 2014; 1185:181–194. [PubMed: 25062629]

Moraga I, Wernig G, Wilmes S, Gryshkova V, Richter CP, Hong WJ, Sinha R, Guo F, Fabionar H, Wehrman TS, et al. Tuning cytokine receptor signaling by re-orienting dimer geometry with surrogate ligands. Cell. 2015; 160:1196–1208. [PubMed: 25728669]

Perrotta S, Gallagher PG, Mohandas N. Hereditary spherocytosis. Lancet. 2008; 372:1411–1426. [PubMed: 18940465]

Rousseau GF, Giarratana MC, Douay L. Large-scale production of red blood cells from stem cells: what are the technical challenges ahead? Biotechnol J. 2014; 9:28–38. [PubMed: 24408610]

Sankaran VG, Ludwig LS, Sicinska E, Xu J, Bauer DE, Eng JC, Patterson HC, Metcalf RA, Natkunam Y, Orkin SH, et al. Cyclin D3 coordinates the cell cycle during differentiation to regulate erythrocyte size and number. Genes Dev. 2012; 26:2075–2087. [PubMed: 22929040]

Sankaran VG, Menne TF, Scepanovic D, Vergilio JA, Ji P, Kim J, Thiru P, Orkin SH, Lander ES, Lodish HF. MicroRNA-15a and -16-1 act via MYB to elevate fetal hemoglobin expression in human trisomy 13. Proc Natl Acad Sci U S A. 2011; 108:1519–1524. [PubMed: 21205891]

Sankaran VG, Orkin SH. The switch from fetal to adult hemoglobin. Cold Spring Harb Perspect Med. 2013; 3:a011643. [PubMed: 23209159]

Slukvin. Hematopoietic specification from human pluripotent stem cells: current advances and challenges toward de novo generation of hematopoietic stem cells. Blood. 2013; 122:4035–4046. [PubMed: 24124087]

Spolverini A, Pieri L, Guglielmelli P, Pancrazzi A, Fanelli T, Paoli C, Bosi A, Nichele I, Ruggeri M, Vannucchi AM. Infrequent occurrence of mutations in the PH domain of LNK in patients with

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

JAK2 mutation-negative 'idiopathic' erythrocytosis. Haematologica. 2013; 98:e101–102. [PubMed: 23812944]

Sturgeon CM, Ditadi A, Awong G, Kennedy M, Keller G. Wnt signaling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells. Nat Biotechnol. 2014; 32:554–561. [PubMed: 24837661]

Sturgeon CM, Ditadi A, Clarke RL, Keller G. Defining the path to hematopoietic stem cells. Nat Biotechnol. 2013; 31:416–418. [PubMed: 23657396]

van der Harst P, Zhang W, Mateo Leach I, Rendon A, Verweij N, Sehmi J, Paul DS, Elling U, Allayee H, Li X, et al. Seventy-five genetic loci influencing the human red blood cell. Nature. 2012; 492:369–375. [PubMed: 23222517]

Velazquez L, Cheng AM, Fleming HE, Furlonger C, Vesely S, Bernstein A, Paige CJ, Pawson T. Cytokine signaling and hematopoietic homeostasis are disrupted in Lnk-deficient mice. J Exp Med. 2002; 195:1599–1611. [PubMed: 12070287]

Veres A, Gosis BS, Ding Q, Collins R, Ragavendran A, Brand H, Erdin S, Cowan CA, Talkowski ME, Musunuru K. Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing. Cell Stem Cell. 2014; 15:27–30. [PubMed: 24996167]

Williamson LM, Devine DV. Challenges in the management of the blood supply. Lancet. 2013; 381:1866–1875. [PubMed: 23706803]

Author Manuscript

Highlights

- Genetic mutations in *SH2B3* associate with higher hemoglobin levels in humans
- Suppression of SH2B3 in HSPCs improves erythroid expansion and differentiation
- SH2B3 suppression does not impair the production of fully mature RBCs
- SH2B3 inactivation by genome editing in PSCs increases erythroid expansion

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

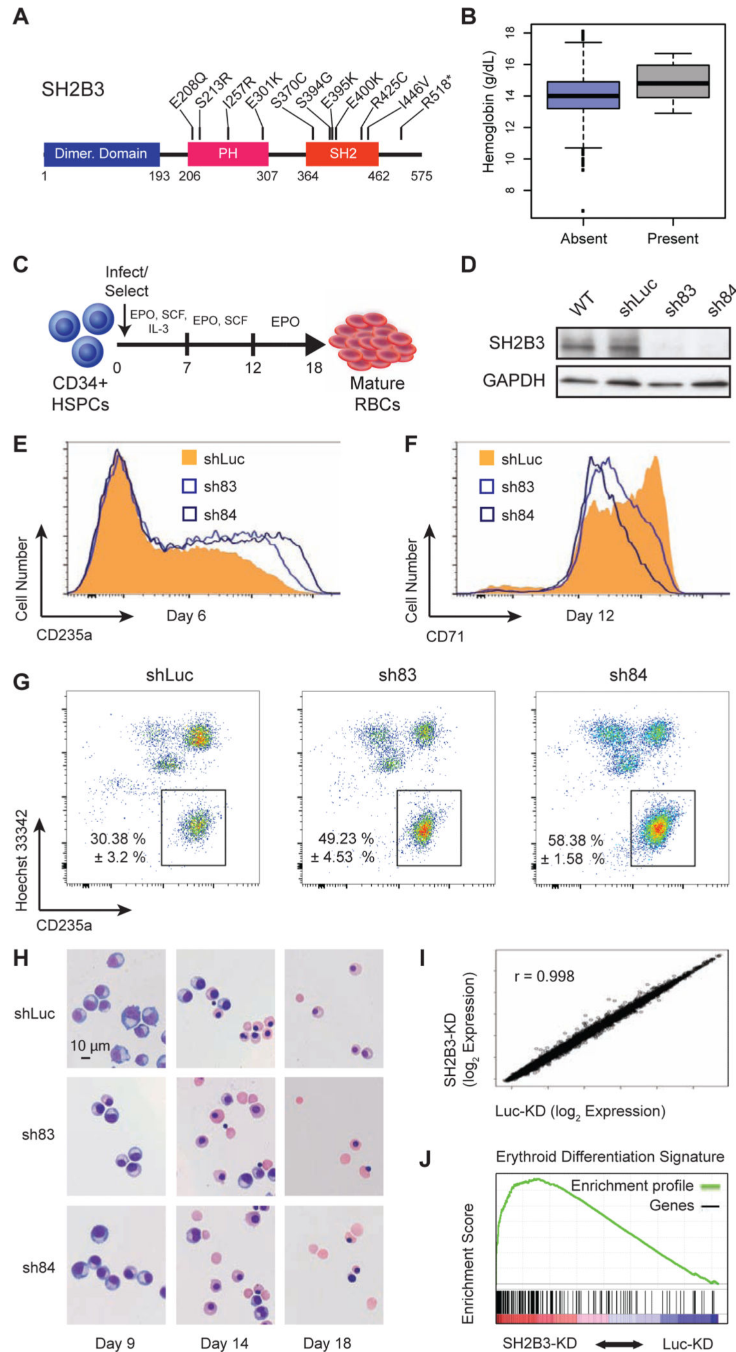


Figure 1. From *in vivo* association of *SH2B3* variants with hemoglobin levels to *in vitro* suppression in primary HSPCs

(A) A schematic of the protein structure of SH2B3, including the dimerization (Dimer. Domain), pleckstrin homology (PH), and Src homology 2 (SH2) domains. The amino acids of these different regions are shown below. The strict damaging missense variants in the PH/SH2 domains and the nonsense variant identified in the European American individuals analyzed are shown.

(B) Box plots for hemoglobin levels in the individuals harboring putative damaging missense and nonsense variants in SH2B3 (n=19) or without such mutations (n = 1995).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Details of the association parameters from gene burden testing of these rare variants are provided in Table S1.

(C) Simplified scheme illustrating differentiation of human CD34+ HSPCs into mature RBCs.

(D) Western blot showing SH2B3 protein levels 8 days following infection.

(E, F) Representative histogram plots of CD235a and CD71, respectively, showing phenotypic surface marker expression of control and SH2B3-KD cells on the indicated day of differentiation.

(G) Representative flow cytometry plots showing cells stained with CD235a and Hoechst 33342 on day 18 of differentiation. Numbers represent the mean percentage of $CD235a^{+}$/Hoechst 33342^{-} cells, indicative of enucleated RBCs, within the depicted gate ± the standard deviation.

(H) Representative cytocentrifuge images of May-Grünwald-Giemsa stained control and SH2B3-KD cells at the indicated days of differentiation. A scale bar is shown in upper left panel.

(I) Scatter plot of mean gene expression values of control (Luc-KD for cells treated with control shLuc) and SH2B3-KD samples (n = 3 independent samples for shLuc, sh83, and sh84). The coefficient of determination (r) is shown.

(J) Enrichment profiles from GSEA comparing the relative expression of genes in SH2B3-KD samples versus control. An enrichment plot showing an erythroid differentiation signature derived by comparing early erythroid progenitors with more differentiated cells is shown ($P < 0.0001$ using a modified Kolmogorov–Smirnov statistical test).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

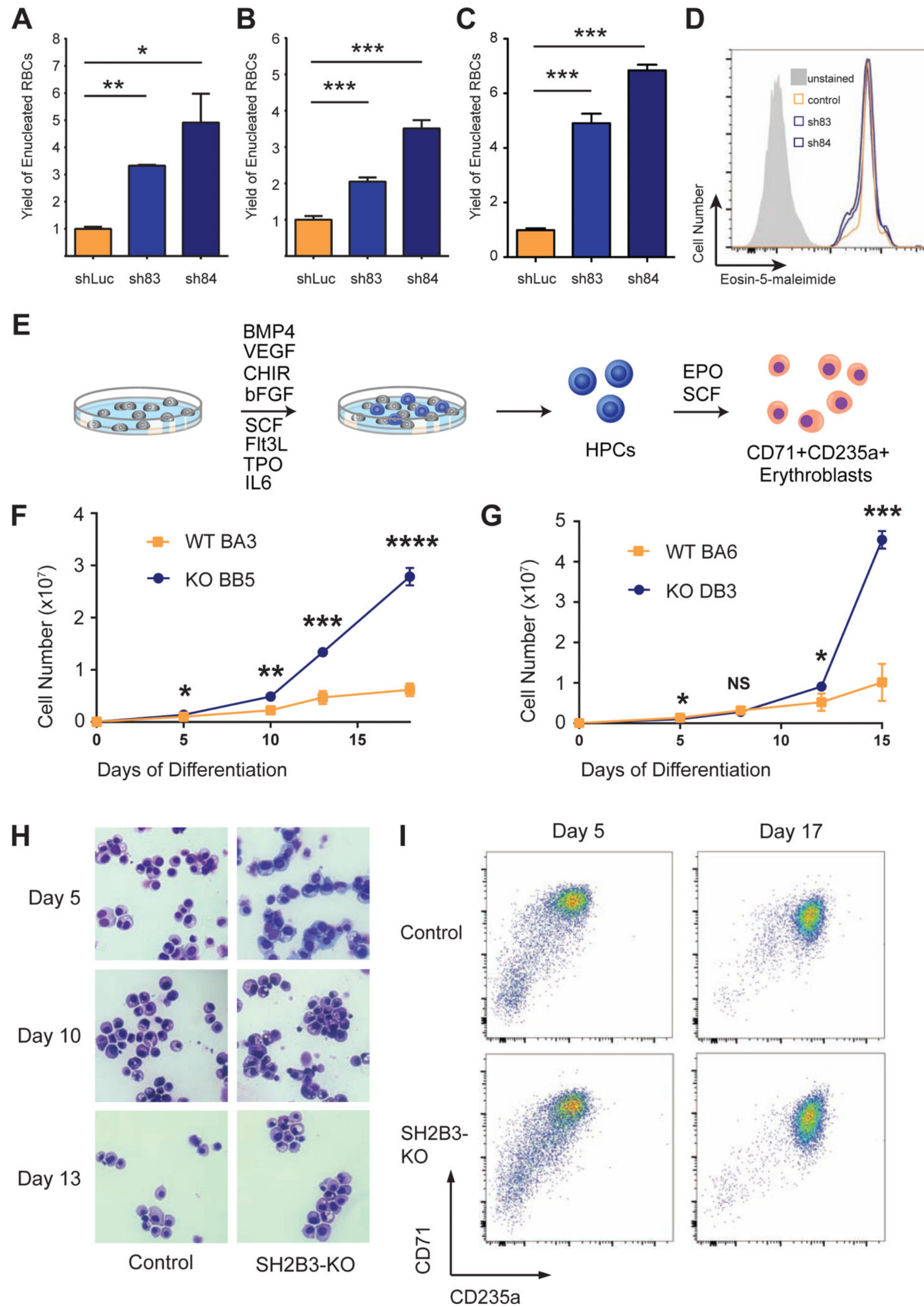


Figure 2. Improved expansion of erythroid cells with SH2B3 suppression in hematopoietic and pluripotent stem cells

(A, B) Mean yield of enucleated RBCs (CD235a$^+$/Hoechst 33342$^-$) observed for control and SH2B3 KD samples derived from adult mobilized peripheral blood (A) or cord blood (B) HSPCs. Values shown are mean ± the standard deviation and were normalized to the control. Comparisons were done by a two-tailed Student's t-test (n = 3 independent experiments; * P < 0.05; ** P < 0.01, *** P < 0.001). See also Figure S1.

(C) Mean yield of enucleated RBCs (CD235a$^+$/Hoechst 33342$^-$) observed for control and SH2B3 KD samples derived from adult mobilized peripheral blood progenitors using a more

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

efficient differentiation protocol involving a CD34+ expansion phase (discussed in Methods). Comparisons were done by a two-tailed Student's t-test ($n = 4$; *** $P < 0.001$).

(D) Representative histogram plots showing eosin-5-maleimide signal on day 18 of mature RBCs derived from peripheral blood-mobilized CD34+ cells. See also Figure S2.

(E) Simplified scheme illustrating differentiation of hESCs into RBCs. The hESCs were cultured in sequential cytokine combinations to induce the production of multipotent hematopoietic progenitor cells (HPCs) that were released into the medium around day 8. The HPCs were collected and cultured further in medium with EPO and SCF to support erythroid differentiation.

(F, G) Erythroid cell expansion of HPCs derived from two independent pairs of isogenic hESC clones (KO, SH2B3-knockout; WT, wild type isogenic controls). The total cell numbers starting with 50,000 cells are shown at various time points as the mean ± the standard deviation. Comparisons were performed by a two-tailed Student's t-test ($n = 3$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$). See also Figure S2.

(H) Representative cytospin images of May Grünwald-Giemsa stained control and SH2B3-KO erythroblasts at the indicated days of culture subsequent to HPC selection.

(I) Representative flow cytometry plots depicting the expression of CD71 and CD235a on erythroblasts derived from SH2B3-KO and isogenic WT hESCs.