Optimizing CRISPR methodology for precise gene editing in the erythroid cell line BEL-A with high efficiency generation of a sickle cell anemia model

CRISPR-Cas9 genome editing technology is a powerful molecular tool for facilitating the study of erythroid cell development in health and disease. It has also opened up innovative approaches for gene therapy for red blood cell diseases, as exemplified by the recently licensed Casgevy for transfusion-dependent β thalassemia and sickle cell disease (SCD). The human erythroid cell line BEL-A recapitulates normal erythroid cell differentiation[1] and provides an ideal founder line, including for introducing mutations to study gene functions in erythroid differentiation or to create cellular models of RBC diseases. Following CRISPR-Cas9 gene editing, clonal cell lines can be produced, providing a sustainable and consistent supply of cells with the desired mutation. However, although CRISPR-mediated gene knockout in BEL-A via the non-homologous end joining (NHEJ) pathway, is highly efficient,[2] introducing specific mutations via homology-directed repair (HDR) is far more challenging and inefficient, often requiring screening of large numbers of clones. In addition, although CRISPR components can be efficiently delivered to cells by lentiviral transduction,[2] the resultant integration of material into the genome is undesirable for many applications. We have therefore optimized the methodology for efficient introduction of specific mutations into BEL-A via nucleofection of ribonucleoprotein (RNP). We demonstrate application of this approach to obtain >70% editing efficiency for the E6V A>T sickle cell mutation, with ~50% of clones homozygous for the mutation, characterizing the resulting lines as model cellular systems for studying sickle cell anemia. The study was performed in compliance with the ethical rules of the United Kingdom.

For the editing optimization experiments, a fluorescent reporter assay (previously described by Richardson *et al.*[3]) was established in BEL-A cells to accurately quantify the efficiency of RNP-based CRISPR-Cas9 editing by HDR *versus* NHEJ pathways, enabling the proportion of cells with precise gene edits (as well as gene knockouts and unedited cells) to be assessed (Figure 1A; gating strategy shown in *Online Supplementary Figure S1A*). The efficiency of precise genome editing (PGE) was determined by the resulting percentage of GFP-positive cells, indicating successful incorporation of a three nucleotide edit that converts BFP to GFP. We used the Amaxa 4D-Nucleofector, which enables multiple parallel transfections in 16-well nucleocuvette strips. Of the programs trialled, DZ100 provided the highest HDR efficiency at 52% while maintaining 88% viability (Figure 1B, C), so was chosen as the default BEL-A nucleofection program going forward.

A systematic examination of parameters - including Cas9 concentration, Cas9 to guide RNA (gRNA) ratio, single-stranded (RBC) DNA oligonucleotide (ssODN) donor concentration and cell number - was then conducted to identify the most effective for PGE of BEL-A by RNP-based CRISPR-Cas9 editing (Figure 1D). While there were relatively minor differences in the parameters trialled, the optimal combination taken forward consisted of 3 μg Cas9, a gRNA:Cas9 ratio of 1:2.5, 100 pmol of ssODN template and $5x10^4$ cells.

To further enhance PGE of BEL-A, several additional strategies were explored, including introduction of previously reported small molecule enhancers (Nedisertib,[4] NU7441 and NU7026, all DNA-PK inhibitors,[5] Alt-R HDR enhancer [Integrated DNA Technologies] and SCR-7,[6] a DNA ligase IV inhibitor), cell cycle synchronization with nocodazole,[7] and cold shock.[8]

Of the small molecule enhancers, Nedisertib gave the greatest improvement of PGE, resulting in a 21% increase compared to the no small molecule control, with NU7441 the second most effective (11% increase compared to control) (Figure 1E). Increasing the concentration of Nedisertib from 1 to 2 μM resulted in no further improvement in PGE efficiency and reduced cell viability by 14%. Alt-R and SCR7 did not increase PGE efficiency compared to the no small molecule control and, in the case of Alt-R, had a negative impact on cell viability (*data not shown*).

Nocodazole induces cell synchronization by inhibiting microtubule polymerization to arrest the cell cycle,[9] enriching cell populations in the G2/M phase. As the HDR pathway is confined to the late S and G2 phases,[10] enrichment of cells during G2 can increase the likelihood of repair by HDR. Cells were cultured in the presence of nocodazole for 18 hours prior to transfection and released from inhibition either immediately after transfection or 6 hours later. However, neither treatment protocol boosted PGE efficiency beyond that seen with Nedisertib or NU7441 alone, instead resulting in a marked reduction in cell viability (Figure 1E).

Finally, a wider range of Nedisertib and NU7441 concentrations was trialled, along with the two small molecule enhancers in combination (*Online Supplementary Figure S1B*). Overall, 0.25 μM Nedisertib was deemed the optimal compromise between editing efficiency (73%) and viability (74%), providing a 24% increase in PGE compared to the optimized parameters with no additional small molecule, with no significant difference in viability observed (Fig-

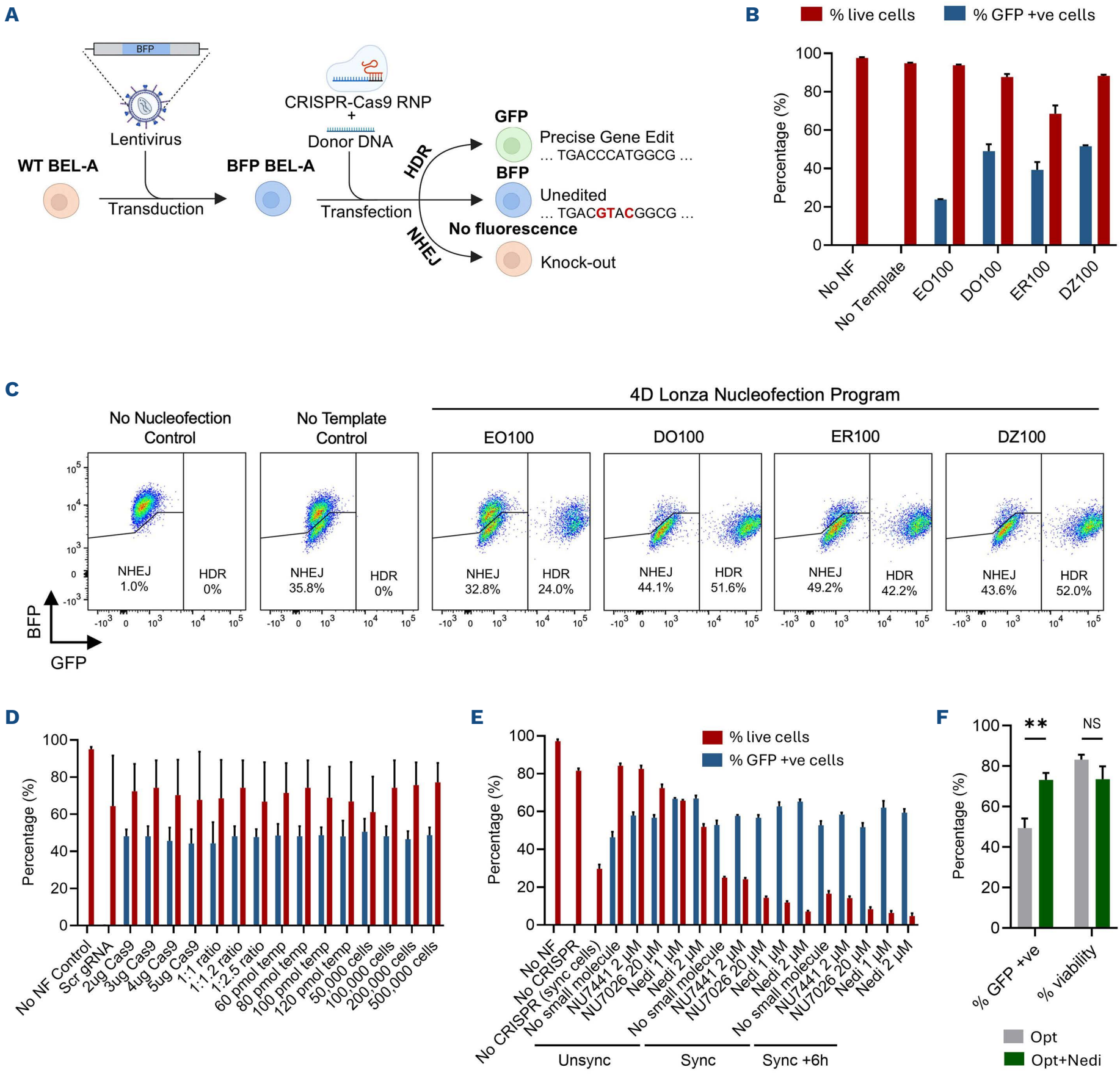

Figure 1. Optimization of precise gene editing strategy for BEL-A cells. BEL-A cells were transduced with lentivirus containing the blue fluorescent protein (BFP) gene to establish a stable BFP-expressing BEL-A cell line. Subsequently, these cells were transfected with CRISPR-Cas9 RNP targeting the *BFP* gene and donor DNA carrying a 3 nucleotide edit which converts BFP to green fluorescent protein (GFP). Flow cytometry was used to analyze the resulting cell populations 48 hours post-transfection. The proportion of cells exhibiting GFP fluorescence corresponds to the editing efficiency via homology-directed repair (HDR), reflecting successful incorporation of the desired edits. Non-fluorescent cells indicate knockout of the *BFP* gene through non-homologous end joining (NHEJ), while BFP-expressing cells signify no editing has occurred. (A) Schematic of BFP to GFP fluorescent conversion assay in BEL-A reporter line to quantify CRISPR-Cas9 editing efficiency. (B) Trialing different Amaxa 4D-Nucleofector programs and (C) representative flow cytometry plots showing gating to determine GFP-positive population. The reporter line was then used to (D) assess different Cas9 quantity, gRNA:Cas9 ratio, single-stranded DNA oligonucleotide (ssODN) template quantity and cell numbers per well of the nucleofection and (E) assess the effect of small molecule HDR enhancers +/- nocodazole-mediated cell cycle synchronization; cells were treated with nocodazole (200 ng/µM) for 16 hours prior to transfection, +/- for an additional 6 hours post-transfection, followed by addition of the small molecule HDR enhancers. Cells were cultured with the small molecules for 24 hours following transfection. Nedi: Nedisertib. (F) Overall precise genome editing efficiency of optimized parameters (Opt) with (N=2) and without (N=5) 0.25 µM Nedi. Statistical significance was determined using Welch's *t* test (**P<0.01). Graphs show percentage live cells quantified using DRAQ7

Continued on following page.

staining and percentage GFP-positive (GFP +ve) cells. Results show mean ± standard deviation, N=3 independent experiments unless otherwise specified. All conditions used 3 μg Cas9, a gRNA to Cas9 ratio of 1:2.5, 100 pmol of ssODN template and 5×10^4 cells in buffer P3 [Lonza] unless otherwise specified. NF: nucleofection; Scr gRNA: scrambled gRNA control; temp: ssODN template; Unsyn: unsynchronised; Sync: synchronized; NS: not significant.

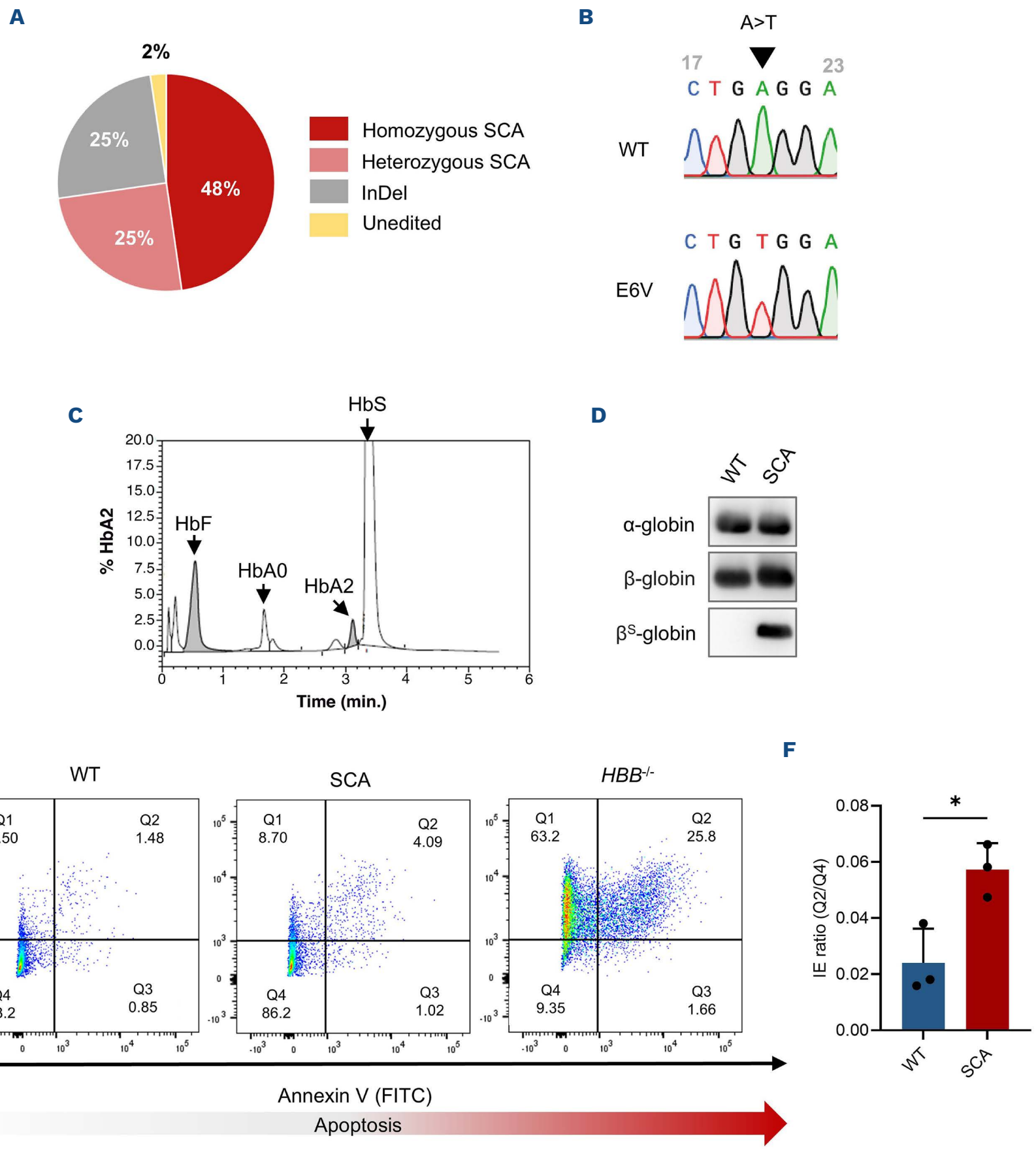

Figure 2. Generation and characterization of sickle cell anemia BEL-A. (A) Summarized sequencing results of 44 single cell-derived BEL-A clones post CRISPR-Cas9 gene editing. (B) Representative sequencing chromatograms from unedited (wild-type [WT]) and homozygous sickle cell anemia (SCA; *HBB*:c.20A>T; E6V) BEL-A lines. (C) HPLC trace from SCA BEL-A cells at day 10 of differentiation with the positions of HbF, HbA0, HBA2 and HbS annotated. Samples were run on the Biorad Variant 2 analyser. (D) Western blot of lysates obtained at day 6 of differentiation of WT and SCA BEL-A, incubated with antibodies to α-, β- (detects both β and β^S) and β^{S-} globin (sc-514378; sc-21756; 200-301-GS5). (E) Representative ineffective erythropoiesis (IE) flow cytometry plots at day 7 of differentiation, and (F) quantification of IE ratio (% Q2 $CD36^{high}AnV^+$/% Q4 $CD36^{low}AnV^-$ cells). *HBB*$^{-/-}$ BEL-A are included as a positive control for the flow cytometry IE assay (as described previously[15]). Results show mean ± standard deviation, N=3 independent experiments. Statistical significance was determined using Welch's *t* test (*P<0.05).

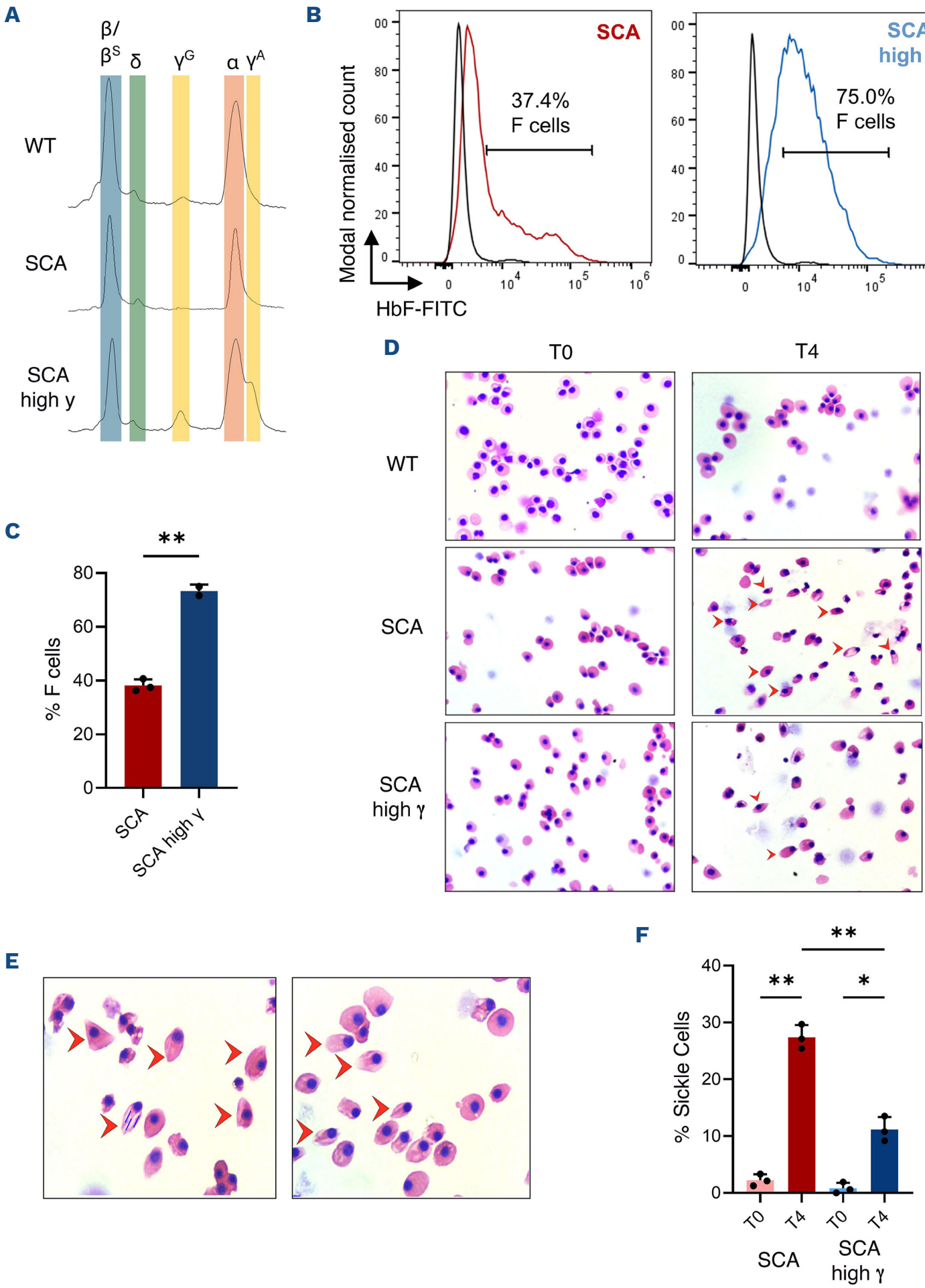

Figure 3. Differential sickling of sickle cell anemia BEL-A cells correlates with γ-globin levels. (A) Reverse phase-high performance liquid chromatography traces for wild-type (WT), sickle cell anemia (SCA) and SCA high γ-globin BEL-A at day 6 of differentiation. Peaks are identified for β-globin or sickle globin (β/β^S), δ-globin (δ), ^{G}γ-globin (^{G}γ), α-globin (α) and ^{A}γ-globin (^{A}γ). (B) Representative flow cytometry plots of fixed and permeabilized SCA and SCA high γ BEL-A at day 6 of differentiation labeled with a HbF-FITC conjugated antibody (blue and red lines - Miltenyi Biotec) alongside IgG control (black lines) to determine % F cells – quantified in (C). (D) Representative images of 10-day differentiated WT, SCA and SCA high γ BEL-A before (T0) and after 4 hours (T4) of hypoxia exposure. Hypoxia was maintained between 1-3% oxygen. Images were taken using the ECHO Rebel Microscope at 20x magnification. Red arrows indicate deformed orthochromatic cells due to hypoxia-induced HbS polymerization identified as sickled for quantification. (E) HbS polymer formation (indicated by blue lines in example cell) in SCA BEL-A. Images taken at 80x magnification. Identified sickle cells indicated by red arrows. (F) Quantification of % sickling in SCA and SCA high γ-globin BEL-A from 150 cells using ImageJ software and cell counter plugin. Results show mean ± standard deviation, N=3 independent experiments. Statistical significance was determined using Welch's ANOVA with Tukey's multiple comparison testing (*P<0.05; **P<0.01).

ure 1F). Other concentrations and combinations did not significantly increase editing efficiency, but rather led to decreased cell viability. Cold shock treatment did not increase PGE efficiency (*data not shown*).

Using the optimal RNP transfection parameters established above (3 μg Cas9, gRNA to Cas9 ratio of 1:2.5, 100 pmol of donor ssODN, 5x10^4 cells), with the addition of Nedisertib (0.25 μM), we proceeded to introduce the E6V A>T sickle cell anemia mutation into BEL-A. A 127 nucleotide (nt) ssODN with homology arms 36 nt from the cut site on the PAM distal side and 91 nt on the PAM proximal side[3] was used alongside a gRNA targeting 1 bp upstream of the E6V A>T loci (*Online Supplementary Figure S2A*). Following transfection, cells were single cell sorted by fluorescence-activated cell sorting (FACS), with 44 clonal cell lines expanded and sequenced. Of these, 32 of 44 clones were positive for the E6V A>T mutation (translating to an overall PGE efficiency of 73%), with 48% biallelic PGE efficiency (21 of 44 clones homozygous for the mutation). Eleven clonal lines contained small insertions or deletions (InDels) on both alleles, indicating editing via NHEJ, with only one (2%) remaining unedited (Figure 2A, B). Differentiation of a clonal homozygous E6V A>T sickle cell anemia BEL-A cell line (SCA BEL-A) confirmed production of both the HbS tetramer and sickle (β^s) globin (Figure 2C, D), while differentiation dynamics and morphology remained unchanged compared to WT BEL-A (*Online Supplementary Figure 2B-G*). SCA BEL-A cells showed a small but significant increase in ineffective erythropoiesis (IE) compared to WT BEL-A (Figure 2E, F), consistent with previous reports of a mild IE phenotype in sickle cell disease,[11] and in line with that of cultured SCA patient-derived erythroblasts.[12]

To phenotypically validate the SCA BEL-A line, differentiated cells were exposed to hypoxia for 4 hours and their morphology compared alongside WT BEL-A and a clonal SCA BEL-A line with high γ-globin production obtained from the same round of editing as confirmed by RP-HPLC (Figure 3A), and HbF flow cytometry (Figure 3B, C). Deformed (sickled) orthochromatic erythroblasts (Figure 3D-F) were observed post-hypoxia, with HbS polymer formation clearly evident (Figure 3C). Sickled reticulocytes were also observed in SCA BEL-A cultures after hypoxia treatment (*Online Supplementary Figure S3*). As expected, the increased γ-globin level resulted in a decrease in the percentage of sickled cells observed after 4 hours of hypoxia compared to SCA BEL-A (11.2%±2.1 *vs*. 27.4%±2.1), while no sickling was observed in WT BEL-A cells (Figure 3D-F).

Overall, this study describes systematic determination of an optimal parameter set for RNP-mediated CRISPR-Cas9 gene editing in BEL-A cells, with PGE efficiency of 48% achieved for introduction of the homozygous E6V A>T SCA mutation. This is a substantial increase compared to the 22% biallelic efficiency reported when introducing the same mutation in a comparable erythroid cell line, HUDEP-2.[13] We also present the generated SCA BEL-A line as a model system that effectively recapitulates sickle cell disease phenotype, with validated decreased sickling characteristics in response to increased γ-globin, in line with the known anti-sickling effect of HbF.[14] The line provides a sustainable supply of disease cells for studying the underlying molecular pathology to identify new therapeutic targets, elucidating the contribution of putative disease modifying mutations, and as a physiologically relevant drug screening platform.

Authors

Deborah E. Daniels,[1*] Joseph Hawksworth,[1*] Sara El Hoss,[2,3] Fatima O. Oyawoye,[1] Ivan Ferrer-Vicens,[1] Marieangela C. Wilson[4] and Jan Frayne[1]

[1]School of Biochemistry, University of Bristol, Bristol, UK; [2]Red Cell Hematology Laboratory, Comprehensive Cancer Center, School of Cancer and Pharmaceutical Sciences, King's College London, UK; [3]Laboratory of Molecular Mechanisms of Hematological Disorders and Therapeutic Implications, Institute IMAGINE, INSERM UMR1163, Paris, France and [4]Proteomics Facility, Faculty of Life Sciences, University of Bristol, Bristol, UK

**DED and JS contributed equally as first authors.*

Correspondence:
J. FRAYNE - Jan.Frayne@bristol.ac.uk

https://doi.org/10.3324/haematol.2024.287221

Received: December 18, 2024.
Accepted: April 2, 2025.
Early view: April 10, 2025.

Disclosures

No conflicts of interest to disclose.

Contributions

JF conceived the study. DED, JH, SEH and IF-V designed experiments. DED and JH conducted the majority of experiments. SEH performed sickling assays. FOO performed western blots. MW performed RP-HPLC analysis. JF, DED and JH analyzed data. DED and JH prepared figures. JF and DED wrote the manuscript. All authors reviewed and edited the manuscript.

Acknowledgments

The authors would like to thank the University of Bristol Proteomics Facility for use of the RP-HPLC system, the University of Bristol Flow Cytometry Facility for use of equipment, and Alexander Macphie of the Hematology Department at the Bristol Royal Infirmary for performing hemoglobin tetramer HPLC.

Funding

This study was supported by MRC grant MR/S021140/1, and the National Institute for Health Research Blood and Transplant Research Unit (NIHR BTRU) in Red Cell Products (IS-BTU-1214-10032). The views expressed are those of the authors and not necessarily those of the National Health Service, NIHR, or the Department of Health and Social Care. SEH received funding from the European Union's Horizon 2020 Research and Innovation Program under the Marie Sklodowska-Curie grant agreement number 101024970.

Data-sharing statement

All data and cell lines presented in this manuscript are available to the academic research community upon request by contacting the corresponding author.

References

1. Trakarnsanga K, Griffiths RE, Wilson MC, et al. An immortalized adult human erythroid line facilitates sustainable and scalable generation of functional red cells. Nat Commun. 2017;8(1):14750.
2. Hawksworth J, Satchwell TJ, Meinders M, et al. Enhancement of red blood cell transfusion compatibility using CRISPR-mediated erythroblast gene editing. EMBO Mol Med. 2018;10(6):e8454.
3. Richardson CD, Ray GJ, DeWitt MA, Curie GL, Corn JE. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. Nat Biotechnol. 2016;34(3):339-344.
4. Fu Y-W, Dai X-Y, Wang W-T, et al. Dynamics and competition of CRISPR-Cas9 ribonucleoproteins and AAV donor-mediated NHEJ, MMEJ and HDR editing. Nucleic Acids Res. 2021;49(2):969-985.
5. Robert F, Barbeau M, Éthier S, Dostie J, Pelletier J. Pharmacological inhibition of DNA-PK stimulates Cas9-mediated genome editing. Genome Med. 2015;7(1):93.
6. Maruyama T, Dougan SK, Truttmann MC, Bilate AM, Ingram JR, Ploegh HL. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. Nat Biotechnol. 2015;33(5):538-542.
7. Lin S, Staahl BT, Alla RK, Doudna JA. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. Elife. 2014;3:e04766.
8. Guo Q, Mintier G, Ma-Edmonds M, et al. "Cold shock" increases the frequency of homology directed repair gene editing in induced pluripotent stem cells. Sci Rep. 2018;8(1):2080.
9. Vasquez RJ, Howell B, Yvon AM, Wadsworth P, Cassimeris L. Nanomolar concentrations of nocodazole alter microtubule dynamic instability in vivo and in vitro. Mol Biol Cell. 1997;8(6):973-985.
10. Heyer W-D, Ehmsen KT, Liu J. Regulation of homologous recombination in eukaryotes. Annu Rev Genet. 2010;44:113-139.
11. Wu CJ, Krishnamurti L, Kutok JL, et al. Evidence for ineffective erythropoiesis in severe sickle cell disease. Blood. 2005;106(10):3639-3645.
12. El Hoss S, Cochet S, Godard A, et al. Fetal hemoglobin rescues ineffective erythropoiesis in sickle cell disease. Haematologica. 2021;106(10):2707-2719.
13. Demirci S, Gudmundsdottir B, Li Q, et al. βT87Q-Globin gene therapy reduces sickle hemoglobin production, allowing for ex vivo anti-sickling activity in human erythroid cells. Mol Ther Methods Clin Dev. 2020;17:912-921.
14. Sewchand LS, Johnson CS, Meiselman HJ. The effect of fetal hemoglobin on the sickling dynamics of SS erythrocytes. Blood Cells. 1983;9(1):147-166.
15. Daniels DE, Ferrer-Vicens I, Hawksworth J, et al. Human cellular model systems of β-thalassemia enable in-depth analysis of disease phenotype. Nat Commun. 2023;14(1):6260.