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# Induction of therapeutic levels of HbF in genome-edited primary $\beta^0$39-thalassaemia haematopoietic stem and progenitor cells

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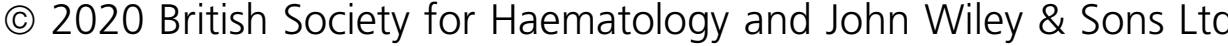
## Summary

Hereditary persistence of fetal haemoglobin (HPFH) is the major modifier of the clinical severity of β-thalassaemia. The homozygous mutation c.-196 C>T in the Aγ-globin (*HBG1*) promoter, which causes Sardinian $\delta\beta^0$-thalassaemia, is able to completely rescue the β-major thalassaemia phenotype caused by the $\beta^0$39-thalassaemia mutation, ensuring high levels of fetal haemoglobin synthesis during adulthood. Here, we describe a CRISPR/Cas9 genome-editing approach, combined with the non-homologous end joining (NHEJ) pathway repair, aimed at reproducing the effects of this naturally occurring HPFH mutation in both *HBG* promoters. After selecting the most efficient guide RNA in K562 cells, we edited the *HBG* promoters in human umbilical cord blood-derived erythroid progenitor 2 cells (HUDEP-2) and in haematopoietic stem and progenitor cells (HSPCs) from $\beta^0$-thalassaemia patients to assess the therapeutic potential of HbF induction. Our results indicate that small deletions targeting the −196-promoter region restore high levels of fetal haemoglobin (HbF) synthesis in all cell types tested. In pools of HSPCs derived from homozygous $\beta^0$39-thalassaemia patients, a 20% editing determined a parallel 20% increase of HbF compared to unedited pools. These results suggest that editing the region of *HBG* promoters around the −196 position has the potential to induce therapeutic levels of HbF in patients with most types of β-thalassaemia irrespective of the β-globin gene (*HBB*) mutations.

**Keywords:** $\beta^0$-thalassemia, genome editing, CRISPR/Cas9, sardinian $\delta\beta^0$-thalassemia, fetal hemoglobin induction.

The switch from fetal (HbF) to adult (HbA) haemoglobin is one of the most important and studied events in haematology. Haemoglobin switching can be thought of as a wave of gene activation that runs along the β-globin gene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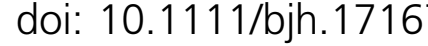
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cluster during development in a 5′ to 3′ direction, leading to the expression of the ε-gene (*HBE*) only in the embryonic stage; of the γ-globin genes (*HBG*s) only in the fetal stage and of the δ- (*HBD*) and β-globin (*HBB*) genes exclusively in postnatal life. In some genetic conditions, *HBG*s continue to be expressed to a variable extent in adult life causing hereditary persistence of fetal haemoglobin (HPFH). People carrying HPFH point mutations in the HBG promoters (non-deletional HPFH) or cluster deletions extending from the *HBB* to the *HBG1* gene (deletional HPFH) do not silence *HBG* expression in adult life, producing HbF at levels that partially or completely compensate the defect in β-globin output. Understanding the switching process and HPFHs has clear implications for the treatment of β-thalassaemia and sickle cell diseases (SCD) as the *HBG* genes can functionally replace the defective *HBB* gene.[1,2] The therapeutic value of this strategy is supported by the frequent co-inheritance of HPFH in patients with non-transfusion-dependent β-thalassaemia.[3-6]

Therefore, finding a way to induce the synthesis of HbF in thalassaemia patients is one of the most pursued approaches for the treatment of β-chain haemoglobinopathies.[7] In recent years, genome-editing techniques based on the CRISPR/Cas9 system have revolutionised the field, allowing scientists to achieve with relative simplicity DNA editing and somatic gene therapy in living cells and/or animals.[8-11] CRISPR/Cas9 modifications of the *HBG* promoters with the aim of reproducing naturally occurring HPFH mutations is predicted to hinder the binding of repressor factors and release the silencing of the *HBG* genes leading to a potentially therapeutic stimulation of HbF production. Hence, editing the *HBG* genes of $CD34^+$ haematopoietic stem and progenitor cells (HSPCs) offers an option for a definitive cure even for patients with haemoglobinopathies that lack an HLA-matched donor.[12] In particular, we focused our interest on the non-deletional HPFH −196 C>T mutation in the *HBG1* promoter, because the co-inheritance of this mutation *in cis* with the common $\beta^0$39-thalassaemia mutation is the molecular basis for the lack of anaemia in the Sardinian $\delta\beta^0$-thalassaemia.[13-16] The best evidence for the importance of the −196 C>T mutation in ameliorating the thalassaemia phenotype comes from an earlier description by Galanello *et al.* of the only known case of homozygous Sardinian $\delta\beta^0$-thalassaemia, accidentally discovered in a symptomless toddler, who had normal haemoglobin levels, consisting of almost 100% HbF.[17]

Considering the complete reversal of the thalassaemic phenotype in the Sardinian homozygous $\delta\beta^0$-thalassaemia patient, we reasoned that reproducing the −196 *HBG* mutation by CRISPR-editing HSPCs derived from $\beta^0$39-thalassaemia patients should rescue the thalassaemia phenotype and lay the basis for clinical application of the autologous transplantation of −196 *HBG*-edited cells in the therapy of thalassaemia and other β-chain haemoglobinopathies.

In this study, we attempted to reproduce the −196 *HBG* mutation by CRISPR/Cas9 genome editing in $CD34^+$ HSPCs from $\beta^0$39-thalassaemia patients and demonstrated that the gene modifications significantly reactivate the developmentally silenced *HBG*s. The benefits shown here for the most severe form of β-thalassaemia well agree with those observed by Weber *et al.*,[18] achieved through editing the same ZBTB7A binding site in SCD HSPCs, suggesting that this approach can be applied to any haemoglobinopathy for which the increase in HbF ameliorates the clinical phenotype.

## Materials and methods

### *SgRNA design and cloning*

Single-guide RNAs (sgRNAs) targeting the HBG promoters in the −196 region were designed using the CRISPR Design Tool (crispr.mit.edu). SgRNA's top and bottom strand oligonucleotides (Table SI) were annealed and ligated into pSpCas9-(BB)-2A-GFP or pLentiCRISPR-V2 (LCV2) plasmids (Addgene, Watertown, MA, USA).

### *CRISPR/Cas9 delivery*

K562 and $\beta^0$39-HSPCs were electroporated by using 5 μg of the pX458-196 plasmid. Ribonucleoprotein delivery in Human Umbilical cord blood-Derived Erythroid Progenitor 2 (HUDEP-2) or $\beta^0$39-HSPCs cells was performed using Lonza-4D-Nucleofector, 200 μM 2′-*O*-methyl-3′-phosphorothioate-modified sgRNA (Synthego, Redwood city, CA, USA) and 8 μg of GeneArt Platinum™Cas9 (Gibco-Thermo Fisher Scientific, Waltham, MA, USA), following manufacturer's instructions.

### *Lentiviral vectors*

HEK-293T cells were transfected with 10 μg of LentiCRISPRV2 (−196 or Cas9), 7·5 μg of psPAX2, 2·5 μg of pMD2G.VSV-G and 1·25 μM of pRSV-REV using PEI.[19] After 72 h, lentiviral vectors (LVs) were collected by ultracentrifugation.

Viral titer was calculated by quantitative polymerase chain reaction (qPCR) evaluating the ratio of *GAG* to the endogenous *Albumin* gene.[20] HUDEP-2 and $\beta^0$39-HSPCs were spinfected using M.O.I. 40.

### *Cell culture*

K562 were grown in RPMI-1640 with 10% fetal bovine serum (FBS; Gibco-Thermo Fisher Scientific, Waltham, MA, USA), 1% L-glutamine and 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA).

HUDEP-2 was cultured as described in Kurita *et al.*[21] $\beta^0$39-HSPCs were isolated from thalassaemia patients participating in the Thalamoss biobank study.[22] HSPCs were sorted through $CD34^+$-MACS (Miltenyi-Biotec, Bergisch Gladbach, Germany). HSPCs were cultured as described previously.[23] Erythroid

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differentiation was monitored by Diff-Quick™ (RAL-diagnostics, Martillac, France) staining of cytospin slides.

*Editing efficiency*

After CRISPR/Cas9 transfection, DNA was extracted, PCR-amplified (Table SI) and sequenced for calculation of the perecentage indels through TIDE (tide.deskgen.com).[24] PCR-amplified DNA of the HSPCs surrounding the target sites, was cloned into pUC19 as described previously.[11]

*Rearrangements and off-targets*

The 4·9 Kb deletion spanning HBG2 was analysed through Multiplex Ligation-dependent Probe Amplification (MLPA; MRC-Holland, Amsterdam, Netherlands). Inversions events were evaluated through PCR screening (primers in Table SI). All HUDEP-2 clones were sequenced for the off-targets listed in Table SII.

*RNA quantification*

The mRNA of globin genes was analysed using the Reverse Transcription Polymerase Chain Reaction (RT-PCR) and quantitative (q)-RT-PCR. For total RNA extraction we used RNeasy (Qiagen, Hilden, Germany) and for cDNA synthesis the SuperScript™ III (Invitrogen-Thermo Fisher Scientific, Waltham, MA, USA). Primers are listed in Table SI. qRT-PCR reactions were performed in the ABI 7700 thermocycler, using SYBR Green detection (Applied-Biosystems-Thermo Fisher Scientific, Waltham, MA, USA). Data were normalised using *HBA*.

*Flow cytometry*

Cells were harvested and processed with Cytofix/Cytoperm™ (BD-Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Permeabilized cells were stained with HbF-APC antibody (Miltenyi-Biotec, Bergisch Gladbach, Germany). Data were recorded using FACSCANTO and analysed with FACSDiva and FlowJo (BD-Biosciences, Franklin Lakes, NJ, USA).

*Statistical analysis*

Data were analysed with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA), expressed as mean ± *SD*. Statistical significance (*P*) was calculated using Student's t-test.

## RESULTS

*Homozygous Sardinian $\delta\beta^0$-thalassaemia is persistently symptomless*

The Sardinian $\delta\beta^0$-thalassaemia subject, originally described at the age of three, is now a grown woman and mother of two children. She steadily maintains close to 140 g/l of haemoglobin (almost 100% HbF) and is still totally symptomless at the age of 24 years, indicating that high levels of HbF production persist into adulthood and are responsible for her normal health. Her haematological follow-up is shown in Table I. Evaluating her genotype analysis (Supplementary results and Table SIII) we can affirm that the *HBG1* −196 C>T homozygous mutation is able to increase the HbF output to the same levels found in fetal life and to completely reverse the $\beta^0$-thalassaemia phenotype.

**Table I.** Haematological follow-up of the symptomless homozygous Sardinian $\delta\beta^0$-thalassaemia patient at different ages. Updated with permission from Galanello *et al.* (2002).[17]

| Age | 18 mo | 3·5 y | 6 y | 24 y |
|---|---|---|---|---|
| RBC, $*10^9$/l (4·2–5·4)§ | 5·6 | 5·5 | 6·0 | 6·08 |
| Hb, g/l (125–160)§ | 112 | 119 | 131 | 139 |
| MCV, fl (78–100)§ | 61·5 | 63·4 | 67·5 | 72·1 |
| MCH, pg (27–31)§ | 20·0 | 21·6 | 22·0 | 22·8 |
| Reticulocytes, % (0·5–1·5)§ | – | 1·0 | 1·8 | 1·02 |
| HbF, % (<2)§ | 99·6 | 99·7 | 99·8 | 99·1 |
| HbA2, % (1·5–3·2)§ | 0·4 | 0·3 | 0·2 | 0·9 |
| Epo, mIU/ml (1–25)§ | – | 23·7 | 12·0 | 26·6 |

Epo, erythropoietin; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; RBC, red blood cells.
§Normal range values for adult females.

*Modification of HBGs at position −196 by CRISPR/Cas9 editing*

With the aim of converting severe $\beta^0$-thalassaemias to the symptomless Sardinian $\delta\beta^0$-thalassaemia phenotype, we used CRISPR/Cas9-mediated genome-editing coupled with NHEJ repair to introduce small indels clustered around the −196 bp position upstream of the *HBG* promoters. These modifications are predicted to affect the binding of the repressor ZBTB7A leading to the release of *HBG* suppression. The most efficient of three different sgRNAs (Fig 1, panels A, B), guide #3 (5′-GCATTGAGATAGTGTGGGGA-3′), which obtained a 23% rate of indels in K562 cells (5–6 edited clones of 26 analysed in three different experiments) through plasmid delivery (pX458; Fig 1, panel C), was selected for subsequent gene-editing studies. However, when this plasmid was delivered to $\beta^0$39-HSPCs, it only determined a 3% indel rate (Fig 1, panel C), hence a percentage too low to reach the 20% threshold of HbF known to be therapeutically relevant.[25] Aiming for a more efficient CRISPR/Cas9 editing, we tested three other delivery strategies using the same #3 sgRNA. As a further optimisation, to better evaluate the increase in HbF output, we shifted from the high-expressing K562 cells to the low HbF-expressing HUDEP-2 cells, an immortalised, cord blood-derived erythroid progenitor cell line. The low level of *HBG* expression in this cell line may facilitate the analysis of the globin product after genome modification. In our hands, among the

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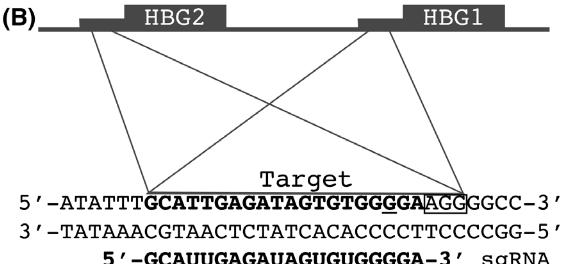
(A)

| Guide | Score | Sequence | PAM |
|---|---|---|---|
| #1 | 23 | ATTGAGATAGTGTGGGGAAG | GGG |
| #2 | 25 | CATTGAGATAGTGTGGGGAA | GGG |
| #3 | 25 | GCATTGAGATAGTGTGGGGA | AGG |

(B)




(C)

| *Cell type* | System of delivery | | | |
|---|---|---|---|---|
| | pX458-EGFP-196 | RNP -196 | IDLV -196 | ICLV -196 |
| *β⁰39-HSPCs* | ~3% ± 0.7 | ~5% ± 0.4 | ND | ~18% ± 2.3 |
| *HUDEP-2* | ND | ~17% ± 2 | ND | ~80% ± 9.2 |
| *K562* | ~23% ± 1.6 | ND | ND | ND |

(D)

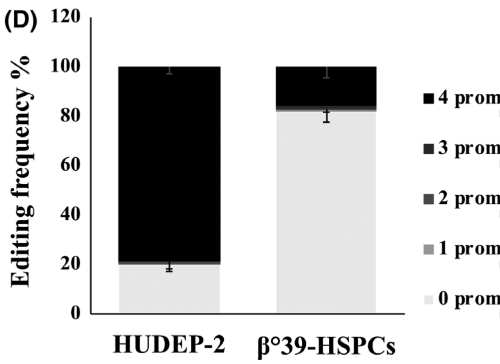



**Fig 1.** Design and efficiency of CRISPR/Cas9-mediated genome editing. (A) Sequences and scores of three single-guide RNAs (sgRNAs) targeted at the −196 position in both *HBG* promoters found by using the MIT CRISPR Design Tool. The score, expressing a prediction for on-target efficiency and off-target potential effects, was quite low in all three sequences (23, 25 and 25 respectively), due to the presence of the same target sequence in both *HBG* promoters. The sgRNA #3 (underlined) was the most efficient sgRNA selected in three independent experiments in K562 cells and was selected for subsequent experiments. (B) Alignment of the sgRNA #3 to its target site in *HBG* promoters: the nucleotide underlined, corresponding to the −196 HPFH mutation, is the predicted site for the double-strand break (DSB) at 2–3 bp upstream of the protospacer adjacent motif (PAM). (C) Efficiency of editing (% ± *SD*) according to the system of delivery used. ND: Not Determined or Not Done. (D) Genome editing efficiency (% ± *SD*) using sgRNA #3 in human umbilical cord blood-derived erythroid progenitor 2 (HUDEP-2) and $\beta^0$39 haematopoietic stem and progenitor cells (HSPCs) (with integrase-competent lentiviral vector, ICLV) calculated by picking and genotyping single clones obtained by limiting dilutions (HUDEP-2) or colony-forming cell assay [single burst-forming unit-erythroid (BFU-E) derived from $\beta^0$39-HSPCs] in three independent experiments. HUDEP-2: 10 edited clones out to 12 analysed. $\beta^0$39-HSPCs: 11 edited colonies out 60 BFU-Es analysed.

three delivery approaches used, only the integrase-competent lentiviral vector (ICLV)-mediated CRISPR/Cas9 delivery achieved an indel rate in HSPCs comparable to that observed in K562 (Fig 1, panel C). In the experiment shown in Figure S2 we found higher ribonucleoprotein (RNP) efficiency of editing in the ZBTB7A knock-out compared to wt HUDEP-2 that might be ascribed to the steric hindrance of ZBTB7A.

### *Genome modification at the predicted target site of the HBG promoters*

To reveal the CRISPR/Cas9-induced genome modification, the regions surrounding the target site in *HBG1* and *HBG2* were sequenced after CRISPR/Cas9 delivery. In HUDEP-2 cells, TIDE analysis indicated that almost 80% of the cells had biallelic indel mutations in both *HBG* promoters (Fig 1, panel D), and the most frequent indel was a 2 bp deletion, occurring at a frequency of 41% and 36% in *HBG1* and *HBG2* respectively (Fig 2, panel A, left). This 2-bp modification is included between positions −197 to −194 of the ZBTB7A binding site present in the upstream regions of both *HBG* genes (Fig 2, panel A, right). The sequencing analysis of the indel mutations at the breakpoint in individual clones of HUDEP-2 also confirmed that the same 2-bp deletion of the target site was the most frequent indel. However, beside the 2-bp deletion, other insertions or deletions of variable length were also found at a lower frequency (Fig 3, panel C). Similarly, edited HSPCs showed 18% of the indels assessed by sequencing individual clones [11 edited clones out 60 burst-forming unit-erythroids (BFU-Es) analysed — Fig 1, panel C,D]. Moreover, the sequencing results from pUC19 subcloned colonies, derived from $CD34^+$ edited DNA, confirmed that the 2-bp deletion, converting the “CCCC” motif into a “CC”, was the predominant Cas9-induced modification even in HSPCs (Fig 2, panel B). Furthermore, we analysed the sequences for the top 10 most homologous off-target regions predicted by the CRISPR Design Tool and did not observe any undesired modifications (Table SII).

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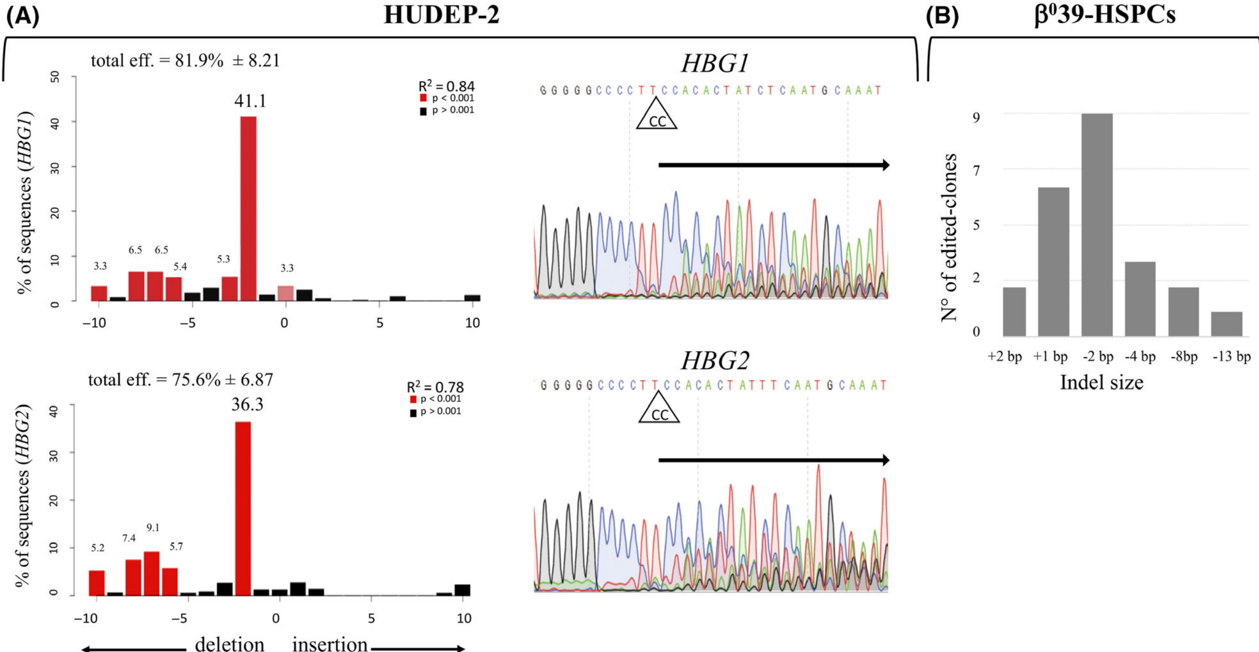


**Fig 2.** Total efficiency and specificity of editing in human umbilical cord blood-derived erythroid progenitor 2 (HUDEP-2) and $\beta^0$39 haematopoietic stem and progenitor cells (HSPCs) . (A) TIDE analysis (left), and relative sequencing histogram (right), reporting % ± *SD* of editing in HUDEP-2 pools (*n* = 3) of both *HBG1* (81·9% ± 8·21, above) and *HBG2* (75·6%± 6·87, below), confirming that the deletion of the two cytidines (triangles) was the most representative indel (arrows show the editing frameshift starting from the DSB point). (B) Distribution of frequency of the specified indels in the *HBG* promoters of $\beta^0$39-HSPCs. The 2 bp deletion was found in nine out of 23 pUC19 clones.

### *Genome editing at the −196 region induces higher HbF synthesis in HUDEP-2 and in HSPCs*

We investigated the effects of genome editing at the transcription and translation level in the HUDEP-2 cell line as well as in primary $\beta^0$39-thalassaemia HSPCs.

To explore whether the modification at position −196 of the *HBG*s could induce γ-globin and HbF expression, we compared pools and individual HUDEP-2 cells treated with or without CRISPR/Cas9. The level of γ-globin transcript, reported in terms of percentage of γ/γ + β mRNAs, was significantly increased, achieving 33% of total globin RNA in the cellular pool and up to 28·9% in the clones (Fig 3, panels A, D). This result is well in agreement with the analysis of HbF levels detected by flow cytometry in edited pools and single clones of HUDEP-2 (Fig 3, panels B, E). Hence, the results at the transcription level were consistent with the results obtained at the translation level and both were highly significant.

To define whether our approach could well mimic the Sardinian $\delta\beta^0$-thalassaemia phenotype in HSPCs, we edited peripheral blood (PB)-derived HSPCs from three different $\beta^0$39-homozygous patients.

We differentiated HSPCs in liquid cultures towards the erythroid lineage and determined the level of HbF expression by flow cytometry in edited, Cas9-treated and untreated pools in three stages of differentiation: (a) the stage of basophilic and polychromatic erythroblasts (Ph2 day3); (b) the stage of orthochromatic erythroblasts (Ph2 day7); and (c) the stage of enucleating-orthochromatic erythroblasts/reticulocytes (Ph3 day6) (Fig 4, panel A). Our results show that only the edited pools significantly increase the synthesis of HbF in the last stage on day 6 of phase 3 (20·4% in edited, 8·3% in Cas9-treated and 3·3% in untreated) (Fig 4, panel B). The cellular increase in HbF content drives the cells of edited pools toward the late stages of differentiation with an increase in the number of orthochromatic and enucleated red blood cells (RBCs) compared to Cas9-treated and untreated samples (Fig 4, panels C, D).

### *Deletion of the HBG2 gene does not affect HbF upregulation*

As predicted by the presence of the target site in both *HBG* promoters, in three out of 10 HUDEP-2 edited clones, we observed biallelic 4·9-Kb deletions that removed the intergenic region, including the whole *HBG2* gene, and left the *HBG1* gene intact (Fig 5, panels A–C). After completion of the fetal to adult globin switch, the *HBG2/HBG1* ratio becomes 2:3. Thus, with the loss of the *HBG2* gene, the total *HBG* expression is predicted to decrease by 2/5ths of the output. However, our results show that both γ-globin expression and HbF production are increased in HUDEP-2 clones that lack the *HBG2* gene (representative clones are shown in

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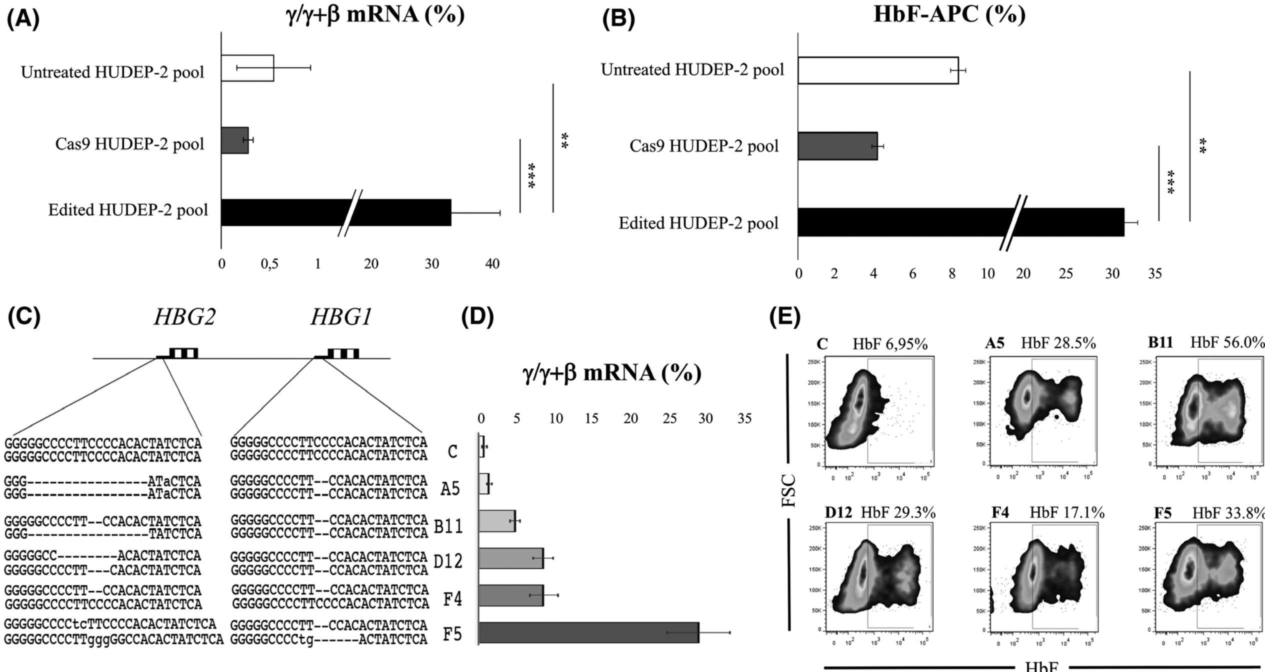


**Fig 3.** γ-globin and HbF expression in human umbilical cord blood-derived erythroid progenitor 2 (HUDEP-2) pools and in single clones. (A) expression of γ-globin as a percentage of γ/γ + β mRNA (± *SD*) in unselected pools of *HBG*-edited, Cas9-treated and untreated HUDEP-2 cells (for each $n = 3$). Statistical significance is indicated by asterisks (**, $P < 0.01$; ***, $P < 0.001$). (B) Flow cytometry analysis of percentage of HbF-APC (± *SD*) in unselected pools of *HBG*-edited, Cas9-treated and untreated HUDEP-2 ($n = 3$ for each). Significance is indicated by asterisks (**, $P < 0.01$; ***, $P < 0.001$). {Untreated: cells without any treatment; Cas9: cells transduced with Cas9 only without single-guide RNA (sgRNA) (LCV2); Edited: edited cells transduced by sgRNA and Cas9 expressing [LCV2 (−196)]}. (C) Sequence of the different indels centred at the target site from representative HUDEP-2 clones edited in the *HBG2* and *HBG1* −200 promoter region after LCV2-196 transduction and isolation by limiting dilutions (dashes indicate nucleotide deletions and lowercase letters nucleotide insertions). (D) γ-globin mRNA expression of the corresponding HUDEP-2 clones on the left. Values are: 0·75% for #C (control: unedited Cas9 clones mix), 1·4% for A5; 4·8% for B11; 8·4% for D12; 8·5% for F4; 29% for F5. Error bars represent standard deviations. (E) Flow cytometry analysis of edited HUDEP-2 clones relative to the unedited control (#C) shows increased levels of fetal haemoglobin synthesis in the edited clones.

Fig 5, panels D, F). This effect suggests that the residual *HBG1* promoter increases its expression at levels higher than its basal promoter activity. The 4·9-Kb deletion was not observed in HSPCs. The occurrence of inversions of the same segment was excluded by PCR screening (Figure S1).

## Discussion

Since their first discovery, HPFHs have intrigued haematologists as they exemplify the ameliorating effect of the persistence of HbF production in β-thalassaemia and sickle cell patients.[26-29] The largest number of non-deletional HPFH clusters around the −196 upstream region of the *HBG* promoter suggests that, after switching, the region is bound by a strong repressor of the *HBG*s. Crossley's group demonstrated that the transcription factor binding the CCCCTTCCCC motif at −200 bp and repressing the *HBG* genes is ZBTB7A (or LRF).[30] The same group showed that the HPFH mutations abolish ZBTB7A binding and release the repression of the *HBG* genes.[30,31] Hence, all the HPFH variants included in this region, with the exception of the −198 T>C variant, which generates a *de novo* binding site for Krüppel-like factor-1 (KLF1),[32,33] act by displacing or hindering the binding of ZBTB7A[30] and preventing the repression of the *HBG* genes.[34-40]

Among the −200 HPFHs, Sardinian $\delta\beta^0$-thalassaemia is one of the best examples of how HPFH mutations modify the severity of $\beta^0$-thalassaemia, as the point mutation causing c.−196 C>T HPFH occurs within the haplotype of the more ancient $\beta^0$39-thalassaemia mutation.[14] The observation that homozygous Sardinian $\delta\beta^0$-thalassaemia is indistinguishable from the phenotype of normal individuals inspired our project as much as it represents a 'natural genome editing' event able to cure $\beta^0$-thalassaemia.[17] Therefore, we used the CRISPR/Cas9 system to introduce indels around the −196 position, hoping that the impairment of the binding of ZBTB7A would by itself relieve γ-globin repression, without the need to reproduce exactly the c. −196 C>T Sardinian mutation. Based on this assumption, we chose to edit the *HBG* promoters using the NHEJ strategy instead of homologous recombination (HR) to achieve much higher editing efficiency in the difficult-to-modify primary HSPCs. Moreover, editing of the *HBG* genes has the advantage that it can be applied to almost any type of β-thalassaemia independent


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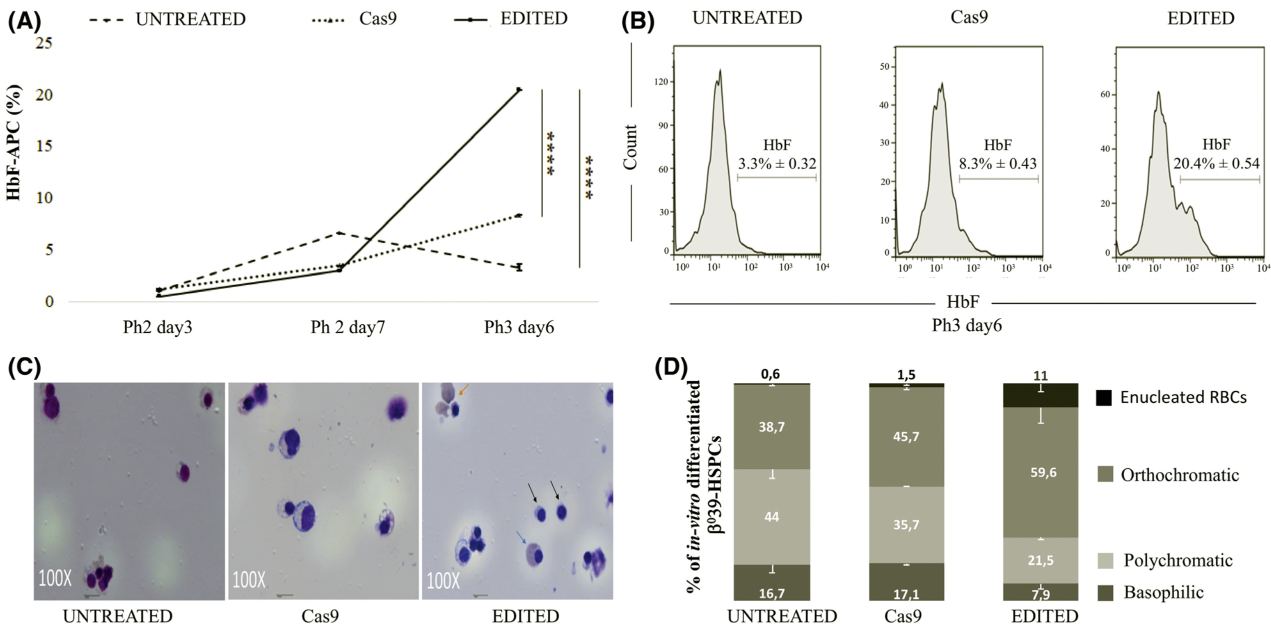


**Fig 4.** HbF expression and *in vitro* differentiation in $\beta^0$39 haematopoietic stem and progenitor cells (HSPCs). (A) HbF expression by flow cytometry analysis at three time-points after *in vitro* differentiation in pools of untreated, Cas9-treated and *HBG*-edited $\beta^0$39-HSPCs (for each $n = 3$). The three different time-points corresponded to a population of prevalent basophilic and polychromatic normoblasts (day 3 of phase 2), orthochromatic normoblasts (day 7 of phase 2) and enucleating-orthochromatic/reticulocytes (day 6 of phase 3). Statistical significance is indicated by asterisks (****, $P < 0.0001$). (B) Flow cytometry histograms showing percentage of HbF-expressing cells in differentiated $\beta^0$39-HSPC pools at the end of differentiation (day 6 of phase 3, $n = 3$). (C) Cell morphology of *in vitro* differentiated $\beta^0$39-HSPCs from untreated, Cas9 and edited cell populations. Enucleating orthochromatic normoblasts are indicated by blue arrows, enucleated RBCs by orange arrows and pyrenocytes (expelled nuclei surrounded by plasma membrane) by black arrows. Pictures were taken on day 7 of Phase 2 of *in vitro* erythroid differentiation, since, at the latter time-point of differentiation, excessive fragility from apoptosis in untreated and Cas9-treated cells precluded the preparation of good cytospin slides. Magnification: 100×. (D) Areas of histograms indicate the relative percentage of the erythroid-differentiated $\beta^0$39-HSPCs (basophilic, polychromatic, orthochromatic and enucleated RBCs) on day 7 of Phase 2 of *in vitro* erythroid differentiation ($n = 3$). Counting was performed using 50× magnification. Error bars represent standard deviations. Untreated, Cas9 and edited refer to pools of cells not treated, treated with Cas9-expressing vector (LCV2) and with Cas9–single-guide RNA (sgRNA)-expressing vector (LCV2(−196)) respectively. [Colour figure can be viewed at wileyonlinelibrary.com]

of the *HBB* mutation. Furthermore, the CRISPR–NHEJ editing strategy allowed us to reach about 20% of the modification of the *HBG* promoters in patient-derived HSPCs and to increase HbF expression to a 20% level, often enabling transfusion independence to be achieved in our cohort of $\delta\beta^0\beta^0$-thalassaemic compound heterozygotes.[41,42]

Recently, other studies have demonstrated that CRISPR/Cas9 editing can be used to boost the output of HbF. In the study by Traxler *et al.*[23] HbF was boosted by editing the −102 to −114 region of the HBG promoters, the region bound by the *HBG* repressor BCL11A.[30,43] Ye *et al.*[44] recreated the 13 Kb deletion of Sicilian $\delta\beta^0$-thalassaemia, and Antoniani *et al.*[45] and Lattanzi *et al.*[46] introduced three different HPFH deletions (3·5 Kb, 7·2 Kb Corfu region and 13·6 Kb, similar to the 12·9 Kb HPFH-5 deletion). Weber *et al.*,[18] similarly to us, identified the ZBTB7A binding site as a potent target for the treatment of SCD.

In our study, we proved that CRISPR-mediated genome editing around the −196 mutation could achieve potentially curative levels of HbF synthesis even in primary HSPCs derived from $\beta^0$39-thalassaemia patients, thus reproducing the molecular genetics of the Sardinian $\delta\beta^0$-thalassaemia.

Our data indicates that, following editing, the most frequent modification even in HSPCs was a 2-bp deletion that included the −196 cytidine nucleotide (40% frequency in HUDEP-2). Besides the 2 bp, other microdeletions in the range of 2–10 bp occurred at a lower frequency. In HSPCs, we did not observe the large 4·9-Kb deletion that included the entire *HBG2* gene, on account of the simultaneous cleavages in the target promoter regions.

We also show that CRISPR editing occurs at a potentially curative frequency in primary HSPC cells derived from $\beta^0$-thalassaemia patients. In these cells, upon differentiation *in vitro*, the editing process is associated with significantly increased levels of HbF, sufficient to overcome the differentiation block typical of β-thalassaemia and to induce the accumulation of more mature and morphologically normal cells *in vitro*.[47] The better differentiation is likely caused by the reduced imbalance of the α/non-α globin chain ratio and the consequent improvement of the ineffective erythropoiesis of β-thalassaemia.[48-50]

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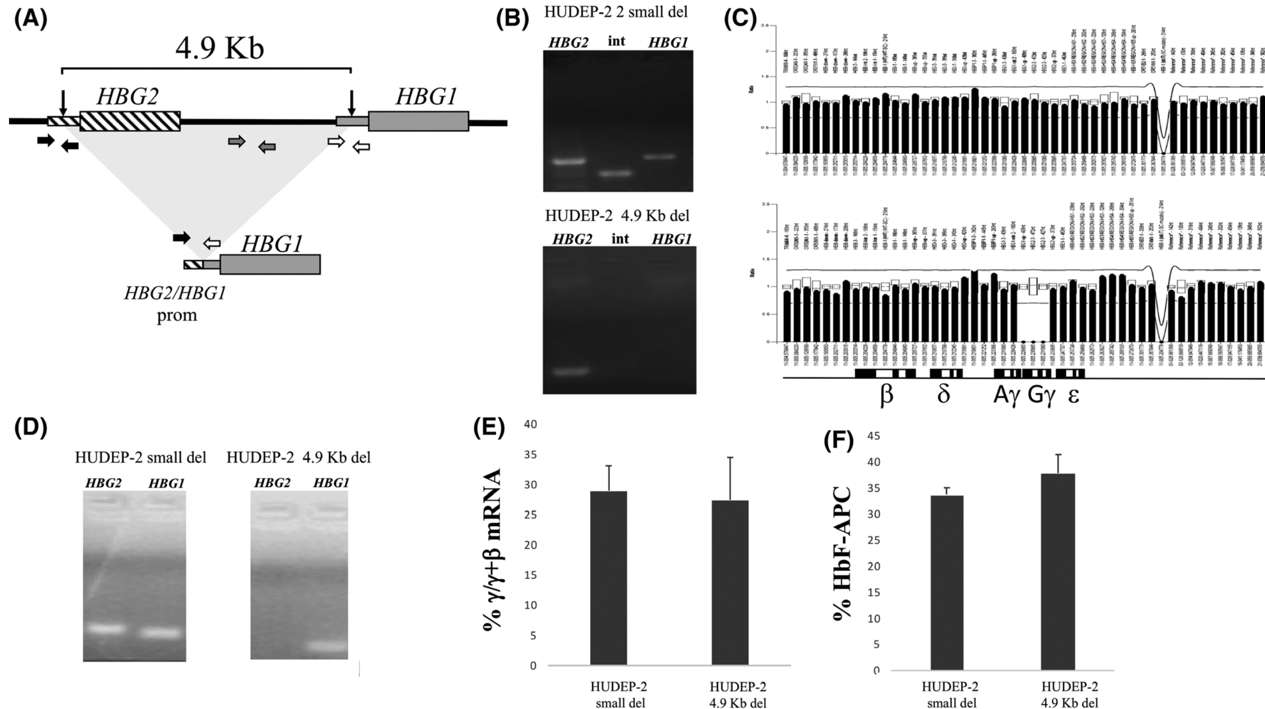


Fig 5. Characterization of the intergenic 4·9 Kb deletion and expression analysis of the Human Umbilical cord blood-Derived Erythroid Progenitor 2 (HUDEP-2) clones with large *versus* small deletions of the ZBTB7A binding site. (A) Schematic representation of the intergenic 4·9 Kb deletion leading to a chimaeric *HBG2/HBG1* gene, made of the proximal promoter of *HBG2* and of the entire residual *HBG1* gene. The presence of the 4·9 Kb deletion produces a single chimaeric amplicon, whereas the intact cluster produces three amplicons. In all clones the residual *HBG1* gene is regulated by the edited *5'HBG2/3'HBG1* chimaeric promoter harbouring biallelic small deletions of 2 to 14 bp that destroy the ZBTB7A binding site. The specific amplification of the *HBG2* or *HBG1* gene is ensured by different forward primers. (B) PCR amplicons of clones bearing small deletions on the top gel and the 4·9 Kb deletion on the bottom gel. (C) Representative Multiplex Ligation-dependent Probe Amplification (MLPA) histograms confirming the gross integrity (top) or the presence of the 4·9 Kb deletion (bottom) in the β-cluster of edited HUDEP-2 clones. (D) Double or single RNA products detected by Reverse Transcription Polymerase Chain Reaction (RT-PCR) amplification of the *HBG2* and *HBG1* mRNAs according to the absence (left) or presence (right) of the 4·9 Kb deletion in HUDEP-2 clones. Expression of γ-globin mRNA (E) and of haemoglobin F (F) in HUDEP-2 clones with small promoter deletions of all four *HBG*s *versus HBG2/1* chimaeric clones (4·9 Kb deletions) carrying small deletions in the edited ZBTB7A binding site ($n$ = 3 for each group). Error bars denote standard deviations.

Exploring safety, we found that the investigated guide directed at the *HBG* regions did not cause any off-target editing in the 10 regions of the genome most closely related to the target site, suggesting that, at least with our guide, off-target editing is absent or negligible.

In this study, we provide a proof of principle of the curative effect of −196 *HBG* editing in HUDEP-2 cells and in patient-derived $\beta^0$-thalassaemia HSPCs. Our results demonstrate that CRISPR-mediated editing of the −196 HPFH is feasible at high frequency in different erythroid cell lines and, at lower but still relevant rates, even in patient-derived HSPCs. In spite of the difference in editing, the edited clones appear to produce similar stimulation of the *HBG* genes and output of HbF. The rate of editing and the extent of HbF induction is in the range that is likely to be therapeutically relevant. Therapeutic efficacy is further supported by the observation that pools of edited patient-derived $\beta^0$39-HSPCs differentiate *in vitro* much better than unedited cells and accumulate a higher number of terminally differentiated erythroid cells. Even though these results were achieved after CRISPR/Cas9 delivery with ICLV vectors, which are unlikely to be approved for clinical trials, the strategy of Weber *et al.* proves that even better results on the same target site can be obtained *in vivo* by engrafting HSPCs with customised RNP.[18]

Our findings suggests that the approach of reactivating HbF in SCD[18] can be applied even to $\beta^0$-thalassaemia patients.

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## Author contributions

MM designed and performed the experiments, analysed the data, and wrote the paper; CAC, IA, MFM, LM, MCS and JW performed the experiments and analysed the data; LY performed data analysis and interpretation and revised the paper; AC performed data analysis and interpretation, RK and YN provided the HUDEP-2 cell line and protocols for culturing; FC and YWK contributed to the design of the experimental strategy and wrote the paper; MGM and PM conceived the study, designed the experiments, analysed the data and wrote the paper. MGM and PM contributed equally to this study.

## Conflicts of interest

The authors declare not to have any conflicts of interest.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table SI**. List of primers used in this study.

**Table SII**. List of potential off-targets for -196 sgRNA-3.

**Table SIII**. Genotype analysis of the homozygous Sardinian $\delta\beta^0$-thalassaemia subject in gene loci known to influence HbF expression (in bold, alleles associated with higher HbF).

**Fig S1**. Evaluation by PCR of inversion events in HUDEP-2 clones treated with LCV2-196.

**Fig S2**. Higher ribonucleoprotein (RNP) −196 editing efficiency in ZBTB7A KO HUDEP-2 cells.

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