Gene therapy approaches to promote fetal hemoglobin production for the treatment of β-hemoglobinopathies

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Abbreviations

AAV: Adeno-associated virus

BaEV: Baboon envelope proteins

BCL11A: B-cell lymphoma/leukemia 11A

Cas9: CRISPR associated protein 9

CRISPR: Clustered regulatory interspaced short palindromic repeats

CsH: Cyclosporin H

DSB: Double-strand break

FDA: Food and Drug Administration

GvHD: Graft-versus-host disease

HbA: Adult hemoglobin

HBB: β-globin gene

HbF: Fetal hemoglobin

HBG1: Hemoglobin subunit gamma 1

HBG2: Hemoglobin subunit gamma 2

HDR: Homology direct repair

HPFH: Hereditary persistence of fetal hemoglobin

HSCT: Hematopoietic stem cell transplantation

HSPCs: Hematopoietic stem and progenitor cells

IGF2BP1: Insulin-like growth factor 2 mRNA binding protein-1

KLF1: Kruppel-like Factor 1

LVs: Lentiviral vectors

MOI: Multiplicity of infection

NHEJ: Non-homologous end joining

SCD: Sickle cell disease

sgRNA: Single guide ribonucleic acid

shmiR: MicroRNA-embedded shRNA

ssODN: Single-stranded-oligodeoxynucleotide

TALENs: Transcription activator-like effector nucleases

VCN: Vector copy number

VSV-G: Vesicular-stomatitis-virus glycoprotein

ZFNs: Zinc-finger nucleases

1. Summary

Different mutations in the *β-globin* gene cause one of the most frequent single-gene disorders worldwide known as β-hemoglobinopathies. Initial therapeutic approaches consisted of the introduction of a wild-type copy of the *β-globin* gene by vectors (LVs). However, the induction of fetal hemoglobin (HbF, α2γ2) by LVs and other molecular approaches, reproducing the benign condition referred to as hereditary persistence of fetal hemoglobin (HPFH), has become a promising alternative treatment for these blood disorders.

There is a big disparity of opinions to determine the most efficient and reliable gene therapy for inherited diseases such as β-hemoglobinopathies. While generally LVs seem to be the strategy of choice for disorders where high expression of the transgene is needed to ensure therapeutic effects, CRISPR/Cas9 looks more favorable for diseases where controlled gene expression is essential. However, both techniques present disadvantages, including insertional mutagenesis for LVs, and possible off-target cutting for CRISPR/Cas9 gene editing.

In this study, a head-to-head comparative analysis of different gene therapy approaches was performed utilizing LVs and CRISPR/Cas9 to promote HbF production and determined their limitations, efficacy, and safety profile for the treatment of β-hemoglobinopathies. LVs encoding for 1) *γ-globin* gene, 2) IGF2BP1, and 3) miRNA-embedded shRNA for BCL11A, were tested. To enhance viral transduction efficiency in HSCs, two novel baboon envelope proteins (BaEV) were compared to the highly used vesicular-stomatitis-virus-G protein (VSV-G). On the other hand, the CRISPR/Cas9 system was employed to knock-down KLF1 and BCL11A genes involved in HbF repression, and to disrupt the binding site of different transcription factors in the *γ-globin* gene (HBG1/2) promoter.

Two tested VSV-G lentiviral strategies (IGF2BP1 and miRNA-embedded shRNA for BCL11A) yielded the highest HbF levels among all approaches (~50%) with clinically relevant VCNs (<2) and no impaired differentiation. VSV-G γ-globin lentivirus treatment showed 20% HbF induction and relevant VCN, whereas all BaEV-LVs induced weaker HbF resurgence. Alternatively, BCL11A and HBG1/2 CRISPR/Cas9 gene disruption approaches also generated therapeutic HbF levels (~40%), showed low off-targets effect, weak transcript impairment, and no adverse

effect during hematopoiesis. KLF1 gene editing showed stronger gene dysregulation and lower HbF resurgence.

Based on our findings, we endorse BCL11A and HBG1/2 gene disruption approaches as the most promising strategies to be applied in the clinic due to their safety profile and high efficacy. On the other hand, VSV-G envelope protein and the *γ-globin* construct might be the safest LV strategy for β-hemoglobinopathies to be implemented in clinical studies. Nonetheless, *γ-globin* gene addition depends on efficient transgene expression, and therefore, alternative strategies to reverse the fetal-to-adult hemoglobin switch, such as BCL11A knockdown or IGF2BP1 overexpression, need to be examined and enhanced as more efficient LV treatment options.

2. Zusammenfassung

Verschiedene Mutationen im β-Globin-Gen sind die Ursache für eine der weltweit häufigsten Einzelgenstörungen, die als β-Hämoglobinopathien bekannt sind. Anfängliche therapeutische Ansätze bestanden in der Einführung einer gesunden Kopie des β-Globin-Gens mittels lentiviraler Vektoren (LVs). Die Induktion von fötalem Hämoglobin (HbF, α2γ2) durch LVs und andere molekulare Ansätze, die die gutartige erbliche Persistenz von fötalem Hämoglobin (HPFH) reproduzieren, ist jedoch eine vielversprechende alternative Behandlung für diese Bluterkrankungen geworden.

Es gibt große Meinungsverschiedenheiten, um die effizienteste und zuverlässigste Gentherapie für Erbkrankheiten wie β-Hämoglobinopathien zu bestimmen. Während LVs im Allgemeinen die Strategie der Wahl für Krankheiten zu sein scheinen, bei denen eine hohe Transgenexpression für therapeutische Wirkungen erforderlich ist, sieht CRISPR/Cas9 für Erkrankungen, bei denen eine kontrollierte Genexpression wesentlich ist, günstiger aus. Beide Techniken weisen jedoch Nachteile auf, einschließlich Insertionsmutagenese für LVs und mögliches Schneiden außerhalb des Ziels für die CRISPR/Cas9-Geneditierung.

In dieser Studie führten wir eine Kopf-an-Kopf-Vergleichsanalyse verschiedener gentherapeutischer Ansätze unter Verwendung von LVs und CRISPR/Cas9 durch, um die HbF-Produktion zu fördern, und bestimmten deren Grenzen, Wirksamkeit und Sicherheitsprofil für die Behandlung von β-Hämoglobinopathien. LVs Kodifizierung für 1) γ-Globin-Gen, 2) IGF2BP1 und 3) in miRNA eingebettete shRNA für BCL11A, wurden getestet. Um die Effizienz der Virusübertragung in HSCs zu verbessern, wurden zwei neuartige Pavianhüllproteine (BaEV) mit dem häufig verwendeten vesikulären Stomatitis-Virus-G-Protein (VSV-G) verglichen. Andererseits wurde das CRISPR/Cas9-System eingesetzt, um KLF1- und BCL11A-Gene, die an der HbF-Repression beteiligt sind, abzubauen und die Bindungsstelle mehrerer Transkriptionsfaktoren im Promotor des γ-Globin-Gens (HBG1 / 2) zu stören.

Zwei getestete lentivirale VSV-G-Strategien (IGF2BP1 und miRNA-eingebettete shRNA für BCL11A) ergaben die höchsten HbF-Spiegel unter allen Ansätzen (~ 50%) mit klinisch relevanten VCNs (<2) und keine beeinträchtigte Differenzierung. Die Behandlung mit VSV-G-γ-Globin-Lentivirus zeigte eine 20% ige HbF-Induktion und relevante VCN, während alle BaEV-LVs ein schwächeres HbF-Wiederaufleben induzierten. Andererseits erzeugten BCL11A- und HBG1/2-CRISPR/Cas9-Genstörungsansätze auch therapeutische HbF-Spiegel (~40%), zeigten einen geringen Off-Target-Effekt, eine schwache Transkriptbeeinträchtigung und keinen nachteiligen Effekt während der Hämatopoese. Alternativ zeigte die KLF1-Geneditierung eine stärkere Gendysregulation und ein geringeres Wiederaufleben von HbF.

Basierend auf unseren Erkenntnissen unterstützen wir BCL11A- und HBG1/2-Genstörungsansätze als die vielversprechendsten Strategien, die aufgrund ihres Sicherheitsprofils und ihrer hohen Wirksamkeit in der Klinik angewendet werden können. Andererseits könnten das VSV-G-Hüllprotein und das γ-Globin-Konstrukt die sicherste LV-Strategie für β-Hämoglobinopathien sein, die in klinischen Studien implementiert werden können. Nichtsdestotrotz leidet die Zugabe von γ-Globin-Genen unter einer effizienten Transgenexpression, und daher können alternative Strategien zur Umkehrung des Hämoglobin-Wechsels von Fötus zu Erwachsenem, z. BCL11A Knockdown, IGF2BP1-Überexpression, untersucht und verbessert werden und können somit als effizientere LV-Behandlungsoptionen angewendet werden.

3. Publications

3.1. Original Publications

1. **Daniel-Moreno A***, Lamsfus-Calle A*, Wilber A, Chambers C, Johnston I, Antony JS, Epting T, Handgretinger R, Mezger M. Comparative analysis of lentiviral gene transfer approaches designed to promote fetal hemoglobin production for the treatment of β-hemoglobinopathies. ***Blood cells, molecules, and diseases***. 2020.

2. Lamsfus-Calle A*, **Daniel-Moreno A***, Antony JS, Epting T, Heumos L, Baskaran P, Casadei N, Latifi N, Siegmund D, Kormann MSD, Handgretinger R, Mezger M. Comparative gene editing analysis targeting BCL11A, KLF1 and HBG1/2 in $CD34^+$ HSPCs cells by CRISPR/Cas9 for the induction of fetal hemoglobin. ***Nature Scientific Reports***. 2020.

3. **Daniel-Moreno A***, Lamsfus-Calle A*, Antony JS, Handgretinger R, Mezger M. CRISPR/Cas9-modified Hematopoietic Stem Cells – The future for Stem Cell Transplantation. ***Bone Marrow Transplantation***. 2018.

4. Antony JS, Latifi N, Haque AKMA, Lamsfus-Calle A, **Daniel-Moreno A**, Graeter S, Baskaran P, Weinmann P, Mezger M, Handgretinger R, Kormann MSD. Gene correction of HBB mutations in $CD34^+$ hematopoietic stem cells using Cas9 mRNA and ssODN donors. ***Molecular and Cellular Pediatrics***. 2018.

5. Lamsfus-Calle A*, **Daniel-Moreno A***, Ureña-Bailén G, Raju J, Antony JS, Handgretinger R, Mezger M. Review. Hematopoietic stem cell gene therapy: The optimal use of lentivirus and gene editing approaches. ***Blood Reviews***. 2019.

6. Ureña-Bailén G, Lamsfus-Calle A, **Daniel-Moreno A**, Raju J, Schlegel P, Seitz C, Antony JS, Handgretinger R, Mezger M. CRISPR/Cas9 technology: towards a new generation of improved CAR-T cells for anticancer therapies. ***Briefings in Functional Genomics***. 2019.

7. Antony JS, Haque AKMA, Lamsfus-Calle A, **Daniel-Moreno A**, Mezger M, Kormann MSD. CRISPR/Cas9 system: A promising technology for the

treatment of inherited and neoplastic hematological diseases. ***Advances in Cell and Gene Therapy*****.** 2018.

3.2. Submitted Manuscripts

1. Lamsfus-Calle A*, **Daniel-Moreno A***, Ureña-Bailén G, Rottenberger J, Raju J, Epting T, Marciano S, Heumos L, Baskaran P, Antony JS, Handgretinger R, Mezger M. Universal gene correction approaches for β-hemoglobinopathies using CRISPR/Cas9 and AAV6 donor templates. ***The CRISPR Journal****.* Under review.

3.3. Poster Presentations

1. **Forschungskolloquium (2018), Tübingen**. Daniel-Moreno A*, Lamsfus-Calle A*, Wilber A, Chambers C, et al. Comparative analysis of lentiviral gene transfer approaches designed to promote fetal hemoglobin production for the treatment of β-hemoglobinopathies.

2. **XXIVth Annual Meeting DG-GT (2018), Freiburg**. Lamsfus-Calle A*, Daniel-Moreno A*, Antony JS, Epting T, Latifi N, Kaftancioglu M, Siegmund D, Kormann MSD, Handgretinger R, Mezger M. Comparative gene editing analysis targeting BCL11A, KLF1 and HBG1/2 in $CD34^+$ cells by CRISPR/Cas9 as a treatment for β-hemoglobinopathies.

3. **XXIVth Annual Meeting DG-GT (2018), Freiburg**. Antony JS, Kaftancioglu M, Böhringer J, Lamsfus-Calle A, Daniel-Moreno A, et al. CRISPR/Cas9-mediated hematopoietic stem cell gene therapy for metachromatic leukodystrophy (MLD).

4. **Reisensburg Retreat (2019), Günzburg**. Lamsfus-Calle A*, Daniel-Moreno A*, Antony JS, Epting T, Latifi N, et al. Gene editing of hematopoietic stem cells from patients with β-hemoglobinopathies by CRISPR/Cas9.

3.4. Oral Presentations

1. Presentation on “Comparative analysis of lentiviral gene transfer approaches designed to promote fetal hemoglobin production for the treatment of β-hemoglobinopathies” at Reisensburg Retreat, Günzburg. 2019.

3.5. Contribution to the publications

1. Comparative analysis of lentiviral gene transfer approaches designed to promote fetal hemoglobin production for the treatment of β-hemoglobinopathies.

For this project, I contributed to establish, improve, and further develop our lentiviral production protocol, including pseudotyping with non-human lentiviral envelope proteins. I designed and performed a comparative study of different therapeutic lentiviral constructs in HSPCs for the treatment of β-hemoglobinopathies, analyzed and plotted the data, and wrote the manuscript.

2. Comparative targeting analysis of KLF1, BCL11A and HBG1/2 in $CD34^+$ HSPCs by CRISPR/Cas9 for the induction of fetal hemoglobin.

During this study, I contributed to establish a CRISPR/Cas9 gene therapy platform required for this publication and executed the experiments. We performed a comparative gene disruption study to promote HbF and evaluated the safety profile of each approach by RNA-seq and GUIDE-seq. We also performed these approaches in a GMP-grade device at a small scale. I also provided support for data analysis, graph plotting, and writing the manuscript.

3. CRISPR/Cas9-modified Hematopoietic Stem Cells –The future for Stem Cell Transplantation.

For this scientific review, I performed a thorough literature search, and elaborated a table summarizing all gene therapy preclinical studies performed in malignant and non-malignant diseases. I also designed and draw illustrations, and wrote the manuscript.

4. Introduction

4.1. β-hemoglobinopathies

The most common inherited monogenic diseases are β-hemoglobinopathies, encompassing β-thalassemia and Sickle cell disease (SCD), with an estimated incidence of 275,000 newborns with SCD and 56,000 with β-thalassemia major every year.[1] Approximately 7% of the global population is a carrier of the disease, mainly in Mediterranean countries, Africa, the Middle East, Southeast Asia, and India.[2] Also, β-hemoglobinopathies have become much more common in North America and Europe in the past decades due to immigration.[3]

These diseases are characterized by a defective (β^+) or absent (β^0) production of the beta chain of hemoglobin. This can be triggered by more than 300 possible mutations identified up to now in the human *β-globin* gene (*HBB*) on chromosome 11,[4] which consists of three exons and two introns with a total length of 1,600 bp (Figure 1). One specific mutation in *HBB* at codon 6 (glutamine > valine) results in a structurally altered protein that undergoes polymerization, leading to a sickle form of the red blood cell causing SCD.[5]

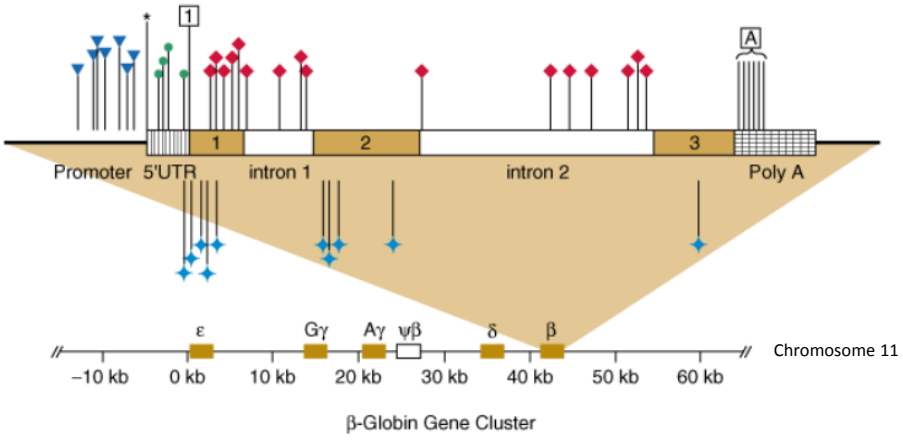

Figure 1. Representation of the globin gene cluster and the most common point mutations in the *HBB* gene causing β-hemoglobinopathies. Point mutations are localized in the promoter (▾), the CAP site (*), the 5'-untranslated region (•), the initiation codon (1), each of the three exons (✦), defective RNA splicing (♦), or the polyadenylation signal (A). Modified from Jameson and Kopp (2019).[6]

β-thalassemia is an autosomal recessive disorder, and therefore, only homozygous carriers exhibit mild (thalassemia intermedia, $\beta^+\beta^+/\beta^+\beta^0$) or severe

(thalassemia major, $\beta^0\beta^0$) clinical symptoms. Mutations induce erythropoiesis perturbations, high apoptosis of red blood cells, and subsequent anemia.[7] The disturbed expression of *β-globin* is compensated by *α-globin* up-regulation that generates intracellular aggregations of hemoglobin in erythroid precursor cells.[8] Symptoms of disease include fatigue, headaches, and dizziness at early stages, which further develop to skeletal deformities and hepatosplenomegaly.[9] Consequently, patients rely on life-long blood transfusions with accompanying infections and iron overload.[10] Blood transfusions are necessary every 3-4 weeks and the life expectancy of patients with major thalassemia is significantly reduced, where most patients die at the age of 30 to 40 due to organ dysfunction.

Even though allogeneic hematopoietic stem cell transplantation (HSCT) is a healing approach for β-hemoglobinopathies, its success depends on several factors including the recipient's disease, graft selection, graft-versus-host disease (GvHD), and complications resulting from immune suppression.[11,12] Most importantly, the majority of patients do not find a suitable HLA-matched donor, whilst the lack of therapy access and high costs make it especially hard for patients in the most affected areas to get the treatment. Only an estimated 10% of patients with β-hemoglobinopathies get treated by allogenic HSCT, and therefore, it becomes highly important to develop efficient universal treatment alternatives to cure this type of diseases.

In the last decades, a gene therapy lentivirus approach in combination with autologous HSCT has become a great alternative to allogeneic HSCT to treat patients with β-hemoglobinopathies.[13] This novel therapeutic approach consisted in the introduction of a wild-type *β-globin* gene by means of lentiviral vectors.[14] As a matter of fact, treated patients with β-hemoglobinopathies showed clinical benefit during the first clinical trials after HBB-encoding lentiviral gene therapy (NCT01639690, NCT02151526, and NCT02453477).[15]

Instead, different studies have demonstrated that fetal hemoglobin (HbF; 2α2γ) can bind oxygen with superior affinity than adult hemoglobin (HbA; 2α2β) and it is functional when reactivated in adults.[10,16,17] Individuals with the benign condition called hereditary persistence of fetal hemoglobin (HPFH) are characterized by increased HbF caused by mutations in the promoter of the hemoglobin gamma gene

(*HBG*) or large deletions in *HBB*.[18] In β-thalassemia patients with HPFH, it has been observed that >20% of HbF expression could reduce the disease severity of β-hemoglobinopathies.[19] Thus, bearing in mind this 20% as the minimum therapeutic percentage of HbF expression, the most relevant gene therapy approaches to overexpress HbF have been compared and evaluated for this thesis as possible treatments for β-hemoglobinopathies.

4.2. Gene therapy approaches to promote fetal hemoglobin production

The expression of the different globin genes is regulated by the locus control region (LCR) and a number of repressors and enhancers. The most important regulators are KLF1 and BCL11A, which are involved in the γ- to β-globin switching process, directly increasing *β-globin* expression and down-regulating *γ-globin* (Figure 2).[20] The great success showed by earlier *β-globin*-encoding lentiviral clinical trials has sparked novel alternative strategies to overexpress *γ-globin*. Numerous research groups have developed different approaches in the last decade, either by means of lentivirus vectors [21-25] or by targeting regulators involved in the Fetal to Adult hemoglobin switching utilizing the CRISPR/Cas9 system.[20,26,27]

4.2.1. Lentiviral gene transfer for HbF resurgence

In the mid-80s, gene transfer through retroviruses arose as a suitable treatment for blood disorders,[28] where lentiviruses derived from the human immunodeficiency virus (HIV) were selected because of their capacity to transduce dividing and non-dividing cells, lower genotoxicity, and up to 9kb transgene-carrying capacity.[29-31] Also advantageous is the possibility to design lentiviral vector carrying particular regulatory elements from a specific cell type to control the transgene expression.[4] Important to note is the number of integration events per cell or vector copy number (VCN) to achieve gene expression levels comparable to normality and reduce the likelihood to have insertional mutagenesis. As stipulated by the U.S. Food and Drug Administration (FDA), the VCN should be lower than five integration events per cell.[32] Nonetheless, gene therapy by lentivirus gene transfer imposed some risks that have been reduced with the development of self-inactivating (SIN) vectors by deleting the enhancer/promoter regions of the long terminal repeat (LTR).[33,34]

A number of different lentiviral vectors and constructs have been tested in recent years to overexpress HbF as a possible treatment option for β-hemoglobinopathies.[21-25] For our study, we have compared the most relevant HbF-inducing LV vectors encoding for: 1) IGF2BP1 (insulin-like growth factor 2 mRNA-binding protein-1), a recently discovered fetal-to-adult hemoglobin switching factor, 2) miRNA-embedded shRNA specific for the *γ-globin* repressor protein BCL11A, and 3) *γ-globin* gene under the influence of the *β-globin* gene promoter (Figure 2).

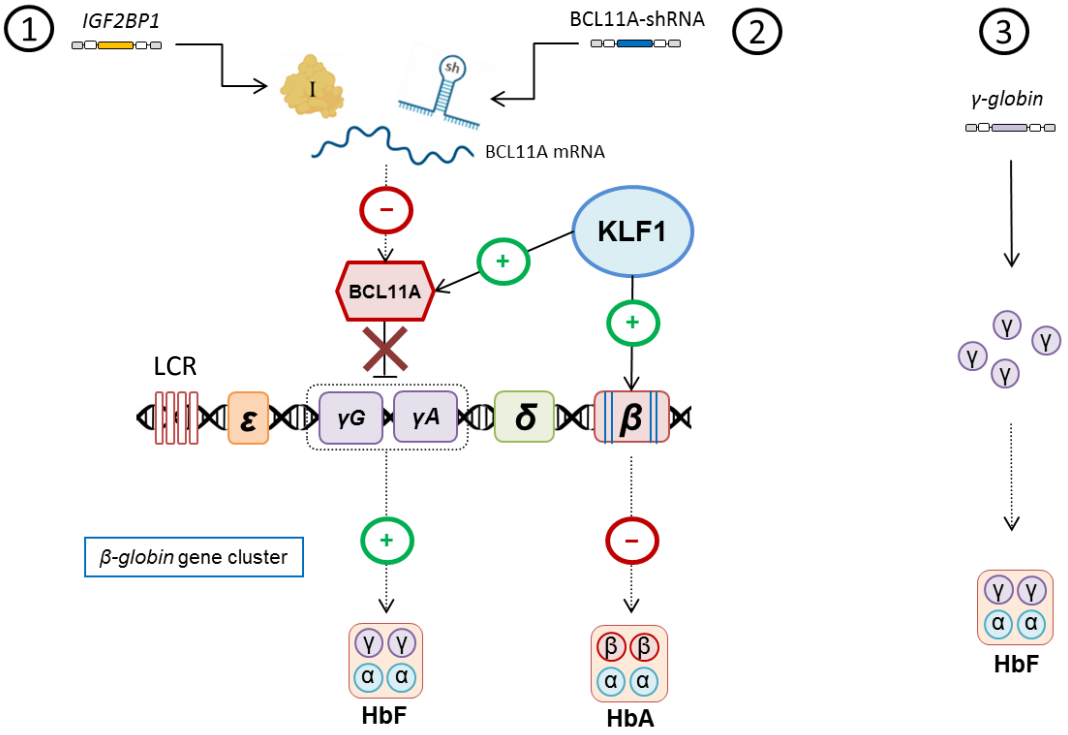

Figure 2. Different lentiviral treatment options used in this investigation to promote fetal hemoglobin production for the treatment of β-hemoglobinopathies, including lentivectors encoding for: 1) IGF2BP1, a fetal-to-adult hemoglobin switching factor; 2) miRNA-embedded shRNA specific for the *γ-globin* repressor BCL11A, and 3) the *γ-globin* gene under the influence of the *β-globin* promoter. KLF1 is a transcription factor that positively regulates BCL11A and the expression of the *β-globin* gene. IGF2BP1 and the BCL11A-miRNA-embedded shRNA silence the expression of BCL11A, and therefore, *γ-globin* is expressed. The mutated *β-globin* gene is represented with blue stripes. Modified from Antony et al. (2018).[13]

Furthermore, an innovative baboon envelope protein (BaEV) has been compared to the more frequently used vesicular-stomatitis-virus-G protein (VSV-G)-LVs, since the former has shown several transduction advantages in $CD34^+$ HSCs. Baboon pseudotyped vectors utilized two viral entry receptors (ASCT-1 and ASCT-2) that are vastly expressed in HSCs, boosting their capacity to transduce quiescent

HSCs. Therefore, fewer viral particles are required, resulting in reduced cytotoxicity and genotoxicity.[35-37] A BaEV mutant version lacking the R peptide which inhibits viral entry (BaEV-RLess) was also included in our study due to its superior transduction potential when compared to the wild-type BaEV.[35] With our study we aimed to finalize the most promising therapeutic strategy for β-hemoglobinopathies by performing a head-to-head comparison of LV vectors with the capacity to increase HbF in HSCs.

4.2.2. CRISPR/Cas9 gene disruption for HbF resurgence

Although novel constructs have improved the safety of lentiviral gene therapy, the risk of insertional mutagenesis is still present. As a matter of fact, one β-thalassemia patient developed a myeloid-biased cell dominant clone expansion because the therapeutic HBB construct integrated adjacently to an oncogene. Nonetheless, this patient did not develop leukemia during the next years.[38]

Gene editing is a seemingly advantageous alternative to lentiviral gene transfer which consists in the introduction of double-strand breaks (DSBs) in the DNA, which activates two different types of cell repair mechanisms: 1) non-homologous end-joining (NHEJ) and 2) homology-directed repair (HDR). NHEJ takes place more frequently and leads to insertion and deletions (InDels) of nucleotides due to prompt DNA repair, hence changing the gene reading frame and hindering gene transcription and/or translation.[39] On the contrary, HDR events occur at a low rate and results in the correction of the gene by homologous recombination with the sister chromatid.[40] Therefore, NHEJ can be applied to disrupt target genes after DSBs. The most utilized tools to induce DSBs are: 1) transcription activator-like effector nucleases (TALENs), 2) zinc finger nucleases (ZFNs) and 3) the CRISPR/Cas9 system. TALENs and ZFNs have high specificity and fidelity; however, they require intricate protein manufacturing, making their production time-consuming and costly.[41] In our recent study, we carried out a comparison of these three techniques and observed that CRISPR/Cas9 offered a more efficient on-target gene editing.[42] The CRISPR/Cas9 system comprises an endonuclease (Cas9) and a single guide RNA (sgRNA), and is at the moment the most popular gene-editing tool. The complete CRISPR system can be delivered by a single electroporation of the mRNA or ribonucleoprotein (RNP) to sufficiently attain high gene editing efficiencies and low cytotoxicity.[43] On the other hand, this system also presents the risk of undesirable

DSBs in other *loci*, or what is known as off-targets. Therefore, assessing the safety profile of the CRISPR system should always be required for every gene editing therapeutic approach.[44] In the last years, several techniques have been developed to determine the number of off-targets, including ChIP-seq, Digenome-seq, BLISS, BLESS, CIRCLE-seq, and GUIDE-seq.[45-51]

Recent gene-editing clinical trials are very effective in ameliorating the symptoms of β-hemoglobinopathies by inducing the production of HbF and mimic individuals with HPFH (NCT03655678, NCT03745287; Figure 3). The CRISPR/Cas9 approach utilized in these clinical trials consists in HSCT of β-thalassemia HSCs after disrupting the *BCL11A* enhancer. In our study, we have evaluated the efficacy and safety profile of this approach and two other promising CRISPR/Cas9-gene disruption approaches to promote HbF resurgence for the treatment of β-hemoglobinopathies. In brief, these strategies consist in gene disruption of: 1) *BCL11A*, a *γ-globin* gene repressor; 2) *KLF1,* a transcription factor that positively regulates BCL11A, and 3) the binding site of BCL11A located in the *HBG1/2* promoter (Figure 3).

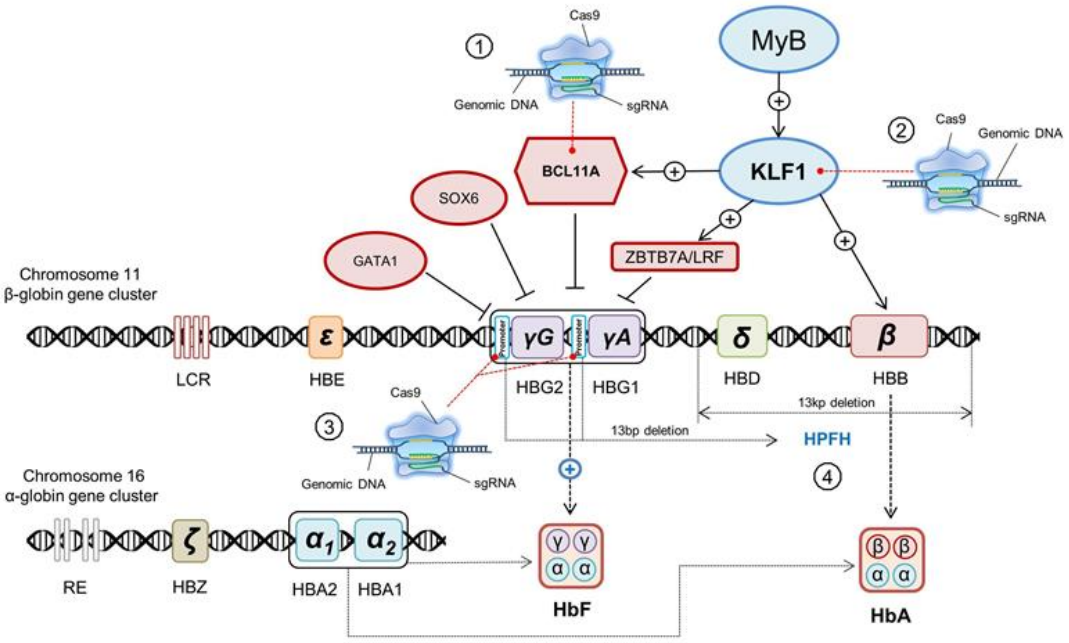

Figure 3. Molecular regulators involved in the fetal (HbF) to adult (HbA) hemoglobin switch and CRISPR/Cas9 gene disruption strategies carried out in our study to promote HbF production as a remedy for β-thalassemia and SCD. The *β-globin* gene cluster, located in chromosome 11, consists in: locus control region (LCR), epsilon globin (HBE), 2 subunits of gamma globin (HBG1/2), delta globin (HBD), and beta globin (HBB). The *α-globin* gene cluster, located in chromosome 16, comprises zeta

globin (HBZ) and two subunits of alpha globin (HBA). In our study we utilized the CRISPR/Cas9 system to gene disrupt: (1) the gamma globin repressor *BCL11A*, (2) *KLF1*, and (3) the binding site of BCL11A in the promoter of *HBG1/2*. (4) Representation of hereditary persistence of HbF (HPFH) benign condition, which can be caused by a 13bp deletion in the *γ-globin* promoter or by a large 13kb deletion of the δ- and β-globin genes.[13]

5. Aim of the study

β-hemoglobinopathies are the most common inherited monogenic diseases, hence it is of high importance to determine the safest and most efficient gene therapy for the treatment of these diseases. For this study, the main objective was to perform a side-by-side comparison of different gene therapy approaches to promote fetal hemoglobin production and evaluate their efficiency and safety to determine the most propitious strategy.

Nevertheless, there were several other side objectives in this investigation that were also achieved. Firstly, a broad lentiviral platform was established to effectively produce high viral titers and attain adequate transduction efficiencies in different cell lines and primary cells. Additionally, pseudotyping of several lentiviral envelope proteins was evaluated to enhance transduction efficiencies depending on the cell type. This lentivirus platform is now being used in our research group to study and develop treatments for other inherited disorders or for immunotherapy.

Just as important, we have established a gene-editing GMP-based CRISPR/Cas9 platform in our facilities, which has allowed the development of several new gene therapy investigations within our group, including very sophisticated approaches such as gene correction and gene addition for the treatment of not only β-hemoglobinopathies, but also neurometabolic diseases like metachromatic leukosdistrophy (MLD) and other genetic disorders.

6. Results and Discussion

6.1. Lentiviral gene transfer: Comparative analysis of lentiviral gene transfer approaches designed to promote fetal hemoglobin production for the treatment of β-hemoglobinopathies (paper 1)

The resurgence of HbF as a treatment for β-hemoglobinopathies is a suitable alternative to *β-globin* gene transfer because β-thalassemia patients presenting the benign condition HPFH show minor symptoms of disease when they have at least 20% of HbF in blood, confirming the therapeutic potential of this approach. However, gene transfer efficiency and transgene expression are big challenges that need to be amended for *β-globin and γ-globin* gene addition. Thus, alternative strategies to reverse the fetal-to-adult hemoglobin switch, need to be explored and further optimized as future and more efficient treatment options. For this reason, *BCL11A* knockdown and *IGF2BP1* overexpression have been included in this study. Also, previous studies showed several benefits of baboon envelope proteins over the generally employed VSV-G, such as higher tropism in HSCs and lower genotoxicity, which could aid in improving lentiviral gene transfer for the treatment of these blood disorders.[35,36,52]

Up to date, no one-to-one comparative analysis of different HbF-inducing LV constructs has been carried out in HSCs to determine their therapeutic potential for β-hemoglobinopathies, nor pseudotyping of these LV constructs with different envelope proteins has been performed in order to assess efficacy and suitability towards clinical translation. In our study, we have conducted a thorough comparative analysis of different HbF-inducing LV constructs and envelope proteins, while we have suggested the most efficient and safest therapeutic approach for β-hemoglobinopathies based on our results.

6.1.1. Lentivirus production

First, we optimized our virus production platform using three different envelope proteins (VSV-G, BaEV-RLess, BaEV) and a GFP-encoding construct under the influence of a PGK promoter. The most efficient lentivirus production procedure we established, based on the protocol developed by Girard-Gagnepain,[35] was selected in accordance with the titer. Our results demonstrated that titers measured by cell

culture titration were on average an 86 and 56 fold higher for VSV-G over BaEV-RLess and BaEV envelope proteins, respectively. However, viral titers calculated by p24 ELISA kit and qPCR did not show such differences as before, but <4-fold higher infectious units (IFU) for VSV-G over both BaEV envelopes measured by ELISA, and <5-fold higher by qPCR.

Cell culture titration was selected as the most consistent titration method because only the functional viral particles are taken into account. ELISA and qPCR methods overestimated baboon-pseudotyped viral titer probably due to the presence of non-functional or empty baboon lentiviral particles. Similar incomplete assembly has been reported earlier for baboon pseudotyped lentiviruses.[53] We also noticed a clear effect of insert size on the transduction efficiency of baboon-pseudotyped lentiviral vectors. Large constructs (pLVX-IGF2BP1; 7kb) hamper virus production in comparison to smaller constructs (pCL20-BCL11A-siRNA; 4kb), hence determining the number of functional particles. This finding implies a lower cargo capacity of baboon-pseudotyped lentiviruses; however, further studies to identify limitations and improve baboon envelopes are needed since its positive tropism in HSCs makes them promising candidates to substitute VSV-G in future clinical trials. In a nutshell, our results showed superior performance of VSV-G compared to baboon counterparts.

6.1.2. Transduction efficiency

We assessed the transduction efficiency of these three envelope proteins with a GFP-encoding LV construct in Lenti-X 293T cells and found that VSV-G envelope showed very high transduction efficiency (99 ± 0.05% GFP^+ cells), while BaEV-RLess (77 ± 1.1%) and BaEV (66 ± 2.2%) resulted in lower but adequate level of transduction. We extended our transduction analysis to other cell types including K-562 human erythroleukemia cells and $CD34^+$ HSPCs, where high transduction efficiency (78-94%) with baboon envelopes in K-562 cells was detected. However, in the clinically relevant $CD34^+$ HSPCs, lower transduction efficiency (40-58%) was observed with BaEV envelopes. Therefore, a transduction enhancer (RetroNectine) and a concentrator were employed to improve transduction efficiencies. However, there was no significant improvement in comparison to ultracentrifugation. On the other hand, transduction of HSPCs in the presence of Cyclosporine H (CsH)

increased efficiencies with VSV-G (18.6%), BaEV-RLess (25.3%), and BaEV (7.5%), and therefore, CsH was employed in all following experiments.

Consequently, this already established transduction procedure was used with the therapeutic constructs pLVX-IGF2BP1, pCL20-BCL11A-shRNA and pCL20-γ-globin to assess their transduction efficiency. As explained above, the transgene size has an effect on virus production, and therefore, significant differences in transduction efficiencies for different constructs can be expected. All three constructs comprise erythroid-specific promoters; ergo, K562 cell culture titration was employed to determine the viral titers, whilst transduction for the constructs incorporating a GFP reporter gene was determined by flow cytometry on day 7 post-transduction. Interestingly, pCL20-BCL11A-shRNA lentiviral particles showed high transduction for VSV-G (92 ± 1.1%), adequate level for BaEV-RLess (30 ± 0.7%) and low level for BaEV (14 ± 0.6%). On the contrary, pLVX-IGF2BP1 showed minor transduction efficiency for baboon envelopes except for VSV-G (75.6 ± 0.3%).

According to previous studies, baboon envelope proteins exhibit higher tropism in HSPCs, hence lead to higher transduction with lower amount of infectious units (IFU), which reduces the risk of multicopy integration and insertional mutagenesis.[35-37,52] Our results verified this observation, since the RLess baboon envelope exhibited, at multiplicity of infection (MOI) 20, >60% transduction efficiency in HSPCs, while using VSV-G higher MOIs were needed to reach the same efficiency. BaEV-RLess, characterized by the deletion of the R peptide, outperformed BaEV in all types of cells and treatments as stated before in earlier investigations.[35] Finally, the VCN was clinically relevant as a maximum of ~2 provirus copies per cell were detected for all treatments, reducing the risk of insertional mutagenesis.

6.1.3. *γ-Globin* up-regulation and HbF expression

We determined the functional outcome of our transgene overexpression through qPCR and hemoglobin electrophoresis. The gene expression analysis through qPCR results exhibited high level of *γ-globin* transcripts in all treatments with VSV-G envelope, where a 3-fold increase was detected for pLVX-IGF2BP1 and pCL20-BCL11A-siRNA, whilst pCL20-*γ-globin* led to ~1.8-fold increment. As the VSV-G-pCL20-BCL11A-shRNA treatment employs shRNA to repress the expression of BCL11A transcripts, the qPCR data confirmed >2-fold decrease in BCL11A levels.

Transcript analysis for IGF2BP1 also indicated remarkable upregulation of IGF2BP1 (~5*10^3 fold) in the VSV-G-pLVX-IGF2BP1 treatment since this fetal-specific gene is expressed at very low levels in adult cells. However, in the baboon-pseudotyped treatments, no apparent *γ-globin* up-regulation was detected at mRNA level.

At protein level, we assessed HbF expression by HPLC and found a considerable rise in HbF for all transduced treatments when compared to untreated $CD34^+$ HSCs (6 ± 1.8%). Remarkably, the highest HbF expression was detected in VSV-G treatments for pLVX-IGF2BP1 (50 ± 1.6%) and pCL20-BCL11A-siRNA (50 ± 6.5%), whereas pCL20-γ-globin strategy yielded moderate but still therapeutic levels of HbF (20 ± 2.6%). Considering HbF expression per VCN, IGF2BP1 transgene induced the highest HbF production per provirus copy. As observed at RNA level, baboon treatments expressed lower levels of HbF, being BaEV-RLess superior in all treatment groups. Therefore, our comparative study showed clinically relevant VCNs and therapeutic levels of HbF (>20%) attained using VSV-G envelope, while BaEV-RLess and BaEV induced weaker HbF resurgence.

Erythroid differentiation, maturation and proliferation were not hampered in the different LV treatments. Flow cytometry analysis found no substantial differences between treated samples and the control, where all treatments presented >90% $CD235^+$, indicating that all constructs had no apparent effect during our *in vitro* differentiation. However, it is important to mention that each transgene might have its own limitations. For instance, the pCL20-BCL11A-siRNA construct includes short hairpin RNAs (shRNAs) which were previously associated with endogenous dysregulation and cytotoxic effects.[54,55] Importantly, BCL11A is involved in hematopoiesis and brain development, and thereby, a solid knockdown with shRNA could impair erythropoiesis among other negative effects[19,56,57]. Nonetheless, for the time being, no negative effects have been observed after BCL11A knockdown in mouse and human HSCs.[58] On the other hand, overexpression of IGF2BP1, though very effective for HbF resurgence even with low VCN indicating that SPTA1 is a very efficient erythroid-specific promoter, could also modulate cell metabolism and oncogenesis, and therefore, animal transplantation studies are necessary.[59] The VSV-G-enveloped pCL20-γ-globin construct presents none of the above explained limitations, since there no implication in signaling pathways as with BCL11A and

IGF2BP1, but direct up-regulation of HbF. As a matter of fact, this strategy has been recently implemented in a clinical trial (NCT02186418).

In a nutshell, our lentivirus study indicated that VSV-G-pseudotyped LVs have superior transduction efficiency in HSCs in comparison to BaEVRLess- and BaEV-pseudotyped LVs. Out of the three molecular approaches of this investigation, LV-mediated overexpression of γ-globin stood out over the others due to the associated safety concerns. Thus, the LV encoding for γ-globin, together with the VSV-G envelope, might be the safest strategy to be implemented in the clinics. However, as mentioned above, γ-globin gene addition presents several challenges such as gene transfer efficiency and transgene expression. Therefore, BCL11A knockdown or IGF2BP1 overexpression, need to be further investigated as alternative and highly efficient treatment options.

6.2. CRISPR/Cas9 gene disruption: Comparative targeting analysis of KLF1, BCL11A and HBG1/2 in CD34^{+} HSPCs by CRISPR/Cas9 for the induction of fetal hemoglobin (paper 2)

Lentiviral gene therapy is succeeding in current clinical trials for β-hemoglobinopathies (NCT02140554, NCT01639690, NCT02453477). Nevertheless, as explained above, there is a safety concern due to insertional mutagenesis and uncontrolled expression. For that reason, other gene therapy techniques such as gene disruption by CRISPR/Cas9 are taking over, mimicking the benign HPFH by knocking down genes involved in the HbF-to-HbA globin switch.

In our study, we have completed a one-to-one comparison of the three most promising CRISPR/Cas9 approaches for HbF resurgence as a treatment for β-hemoglobinopathies, which consists of disrupting three genes: *KLF1*, *BCL11A*, and *HBG1/2* promoters. Furthermore, due to several concerns on the CRISPR/Cas9 safety, we have also analyzed the off-target effect and transcript dysregulation of each approach to determine which strategy has the best safety profile to be applied in the clinic.

6.2.1. Gene editing

Our study started optimizing the electroporation protocol necessary to transfect the CRISPR/Cas9 components in K-562 cells and $CD34^+$ HSCs. For this purpose, DsRed mRNA was transfected under different electroporation settings to identify the most efficient setup, achieving >90% of transfection efficiency and viability in both cell types. The CRISPR/Cas9 system was next transfected as plasmid and two different sgRNAs (T1 and T2) for each target gene were screened. Insertions and deletions (InDels) were determined by T7 endonuclease-I (T7E1) assay for KLF1 (T1: 36 ± 6.5%; T2: 35 ± 5.1%), BCL11A (T1: 22 ± 2.2%; T2: 17 ± 1.4%), and HBG1/2 (T1: 31 ± 14.4%; T2: 21 ± 6.0%), demonstrating the functionality of the system.

Besides, all three gene disruption strategies were tested in $CD34^+$ HSPCs, this time using Cas9 as RNP and chemically modified sgRNAs as opposed to the CRISPR plasmid explained above. Firstly, a variety of sgRNA:Cas9 molar ratios were examined and a molar ratio of 2:1 was identified as the most effective in terms of on-target indels. Secondly, the sgRNAs were tested in HSCs at this ratio and high levels of gene editing were observed (63-91%) with the exception of HBG1/2 T1 (55±10.1%). Finally, gene-edited HSCs were *in vitro*-differentiated to the erythroid lineage for 21 days, whilst no impaired differentiation and proliferation were found.

6.2.2. *γ-Globin* up-regulation and HbF expression

To assess the inverse switch from HbA to HbF after gene disruption, we carried out a transcript analysis through qRT-PCR, and observed a >4-fold *γ-globin* up-regulation in all treated samples, especially in *HBG1/2* gene-disrupted treatment, where >6.5-fold *γ-globin* up-regulation was attained. Furthermore, transcripts for *KLF1* and *BCL11A* were determined after *KLF1* disruption, detecting an evident decrease of *KLF1* expression (KLF1 T1: 4-fold, KLF1 T2: 2-fold) and consequent BCL11A down-regulation (2-fold). Likewise, in BCL11A gene-disrupted samples, BCL11A transcript expression decreased 2-fold when compared to the control treatment.

Most importantly, after 21 days of erythroid differentiation of gene-disrupted $CD34^+$ HSCs, the HbF expression was analyzed by intracellular staining and HPLC-mediated hemoglobin electrophoresis. Remarkably, we detected by HPLC that all treated samples induced significant levels of HbF over the controls, particularly

BCL11A T2 and HBG1/2 T2 yielded HbF levels of 39.5 and 41.9%, respectively. Moreover, intracellular staining showed a pronounced increase of HbF^+ cells in all treated samples, where a strong correlation with HPLC results was observed.

In individuals with HPFH, several mutations in *KLF1* have been observed to generate different levels of HbF ranging from 3 to 31%.[60] This study is the earliest attempt to disrupt *KLF1* using CRISPR/Cas9 system, attaining similar HbF levels (~25%) as those observed in HPFH patients carrying the mutations K288X or S270X.[60,61]

Knocking-out *BCL11A* would impair erythropoiesis and limit the engraftment potential, and therefore, in this study, we have carried out a mild knock-down of *BCL11A* by targeting its enhancer (GATAA box).[27,62,63] This moderate *BCL11A* knock-down yielded therapeutic level of HbF up to 40% for sgRNA T2 with no apparent adverse effect in hematopoiesis.

Gene disruption of the BCL11A biding site in the *HBG1/2* promoters was initially executed by Traxler et al.[26] The idea is to mimic the 13-bp natural-occurring deletion described in a proportion of individuals with HPFH. After gene disruption with the CRISPR/Cas9 system, we observed a 13-bp deletion which might probably take place through microhomology-mediated end-joining (MMEJ) since this target site is flanked by short homology sequences.[26,64-66] This approach induced higher HbF level than the other strategies, showing an undeniable potential for disease treatment.

6.2.3. Safety profile

As transcription factors, KLF1 and BLC11A are implicated in numerous signaling pathways, hence a detailed transcriptome analysis becomes necessary to determine the safety profile of these approaches. All three gene disruption strategies exhibited high overall similarity to the control (92% to 99%). However, gene disruption of KLF1 and HBG1/2 led to 2327 and 2129 genes with impaired transcription, respectively, in contrast to BCL11A, where 1017 genes were dysregulated. Furthermore, several important genes involved in cell cycle, apoptosis, and different immune pathways were disturbed in KLF1- and HBG1/2-treated samples, whilst no oncogenes or tumor suppressor genes were dysregulated for BCL11A.

As previously explained, the main concern of utilizing the CRISPR/Cas9 system is the possibility of unwanted off-target sites. For this reason, we have performed an *in vitro* detection of off-targets through GUIDE-seq, after which no off-targets were detected, with the exception of HBG1/2 sgRNA, where one off-target was identified.

When taking a closer look at our CRISPR/Cas9 gene disruption strategies to promote HbF, some important aspects have to be discussed to pinpoint the most promising approach to treat β-hemoglobinopathies. Despite *KLF1* gene disruption secured therapeutic HbF levels with no detected off-targets after GUIDE-seq analysis, the enormous dysregulation at RNA level could raise a red flag prior to clinical translation. As a matter of fact, there are several previous studies demonstrating that disturbances in *KLF1* expression have consequences in genes involved in cell-cell interaction, microcytosis and cancer.[60,67] On the contrary, *BCL11A* gene disruption exhibited a more secure safety profile, where no off-targets and a lower gene dysregulation were observed. This approach is currently in a clinical trial for the treatment of β-thalassemia (NCT03655678) and SCD (NCT03745287) with no negative outcome so far, corroborating our results. Lastly, the *HBG1/2* gene disruption approach revealed an intermediate safety profile and outstanding HbF resurgence, hence this strategy could also be considered as a treatment. A recent study has conducted this method in primates, where no toxicity was found in blood cells at least during the next 1.5-years.[68]

6.2.4. Clinical translation

Another major point for these strategies to be applied to the clinic is to test them in a GMP device, in particular the GMP-grade CliniMACS Prodigy (Miltenyi). After screening for the most efficacious electroporation setting for HSPCs in the Prodigy electroporator using DsRed mRNA, KLF1 T1, BCL11A T2 and HBG1/2 T2 sgRNAs were electroporated with this device, attaining high indel rates (54-86%). Also, HbF resurgence reached therapeutic levels for all strategies (KLF1 T1: 20 ± 2.8%, BCL11A T2: 41 ± 7.8%; HBG1/2 T2: 42 ± 3.6%). These promising results reinsure once more the possibility for clinical translation of these proposed approaches.

6.3. CRISPR/Cas9-modified Hematopoietic Stem Cells – The future for Stem Cell Transplantation (paper 3)

In this review article, we discuss the therapeutic potential of autologous hematopoietic stem cell transplantation (HSCT) after CRISPR/Cas9 gene editing as a well-supported alternative to autologous HSCT, since the latter presents several limitations e.g. absence of appropriate donor, GvHD, and complications resulting from immune suppression. Moreover, detailed information about the most relevant gene-editing studies utilizing CRISPR/Cas9 for HSC-related diseases is provided, with the purpose to enlighten future gene therapy studies and clinical trials.

In the last section of this review, possible clinical-grade improvements of CRISPR/Cas9-mediated autologous HSCT are examined, while future clinical perspectives are presented with the intention to give an overview of how this promising technology will develop during the next few years.

7. Concluding remarks

There is a big disparity of opinions to determine the most efficient, reliable, and accessible gene therapy for inherited diseases such as β-hemoglobinopathies. While lentiviral vectors seem to generally be the strategy of choice for diseases where high transgene expression is needed for therapeutic effects, CRISPR/Cas9 looks more propitious for disorders where controlled gene expression is essential. Nonetheless, lentiviruses can be harmful due to insertional mutagenesis and uncontrolled expression of the transgene, whereas CRISPR/Cas9 presents unspecific cutting in other *loci* that could generate undesirable effects.

In this study, we have compared several lentiviral and CRISPR/Cas9 approaches to promote the production of HbF to treat β-hemoglobinopathies in order to have a deeper understanding of their limitations, efficacy, and benefits, with the hope to bring some interesting insights that could help to find the most appropriate strategy for these blood disorders. On the one hand, we have ascertained that two of the tested lentivirus strategies (IGF2BP1 and shmiR for *BCL11A*) yielded the highest HbF levels of all approaches with clinically relevant VCN and no impaired differentiation. On the other hand, *BCL11A* and *HBG1/2* CRISPR/Cas9 gene disruption generated therapeutic HbF levels, showed low off-target effect, weak transcript impairment, and no adverse effect during hematopoiesis.

Based on our findings, we suggest that *BCL11A* and *HBG1/2* gene disruption approaches are the most promising strategies to be applied into the clinics due to their safety profile and high efficacy. As a matter of fact, *BCL11A* gene disruption is being studied in clinical trials, where initial data shows successful treatment of disease with no reported side effects. Also, an *HBG1/2* gene disruption approach is under pre-clinical investigation by a leading genome editing company, which is expected to start a clinical trial in the near future. Alternatively, it is important to mention that γ-globin lentiviral gene transfer strategy is also currently in clinical trial, demonstrating its therapeutic potential for the treatment of these diseases.

During the next years, the outcome of ongoing clinical trials will certainly unravel which strategy involved in the resurgence of HbF for the treatment of β-hemoglobinopathies has a finer safety profile and higher efficacy. However, the gist of the matter is for these therapeutic treatments to be accessible in the most affected

areas, where the lack of therapy availability and high costs make it especially hard for patients to get proper medical care.

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10. Appendix

PAPER 1

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Comparative analysis of lentiviral gene transfer approaches designed to promote fetal hemoglobin production for the treatment of β-hemoglobinopathies

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ABSTRACT

β-Hemoglobinopathies are among the most common single-gene disorders and are caused by different mutations in the *β-globin* gene. Recent curative therapeutic approaches for these disorders utilize lentiviral vectors (LVs) to introduce a functional copy of the *β-globin* gene into the patient's hematopoietic stem cells. Alternatively, fetal hemoglobin (HbF) can reduce or even prevent the symptoms of disease when expressed in adults. Thus, induction of HbF by means of LVs and other molecular approaches has become an alternative treatment of β-hemoglobinopathies. Here, we performed a head-to-head comparative analysis of HbF-inducing LVs encoding for: 1) IGF2BP1, 2) miRNA-embedded shRNA (shmiR) sequences specific for the γ-*globin* repressor protein BCL11A, and 3) γ-*globin* gene. Furthermore, two novel baboon envelope proteins (BaEV)-LVs were compared to the commonly used vesicular-stomatitis-virus glycoprotein (VSV-G)-LVs. Therapeutic levels of HbF were achieved for all VSV-G-LV approaches, from a therapeutic level of 20% using γ-*globin* LVs to 50% for both IGF2BP1 and BCL11A-shmiR LVs. Contrarily, BaEV-LVs conferred lower HbF expression with a peak level of 13%, however, this could still ameliorate symptoms of disease. From this thorough comparative analysis of independent HbF-inducing LV strategies, we conclude that HbF-inducing VSV-G-LVs represent a promising alternative to *β-globin* gene addition for patients with β-hemoglobinopathies.

1. Introduction

The β-hemoglobinopathies, sickle cell disease (SCD) and β-thalassemia, are the most common monogenic diseases [1]. A few hundred disease-causing mutations have been described in the human *β-globin* gene (*HBB*), which can cause pronounced anemia [2,3]. Patients with severe β-hemoglobinopathies are dependent on regular blood transfusions and their life expectancy is significantly reduced due to iron overload and organ dysfunction [4]. Currently, the most utilized curative therapy for β-hemoglobinopathies is bone marrow transplantation (BMT) from a histocompatible donor [5], but this option is only available to a certain extent depending on the racial and ethnic background of the patient [6,7]. In the case of an incomplete match, the risk of graft-versus-host disease (GvHD) reactions is elevated, resulting in life-threatening complications [8].

To overcome the limitations of allogeneic transplantation, autologous transplantation of gene-manipulated hematopoietic stem cells (HSCs; $CD34^+$) has gained great attention in the last two decades [6]. Promising clinical trials have been performed for β-hemoglobinopathies with autologous HSCs which have been modified by lentivirus-mediated delivery of a functional human *β-globin* (*HBB*) gene. The first multicenter clinical trials with *HBB*-encoding lentiviral gene therapy for β-hemoglobinopathies (NCT01639690, NCT02151526, and NCT02453477) exhibited clinical benefit in treated patients (http://clinicaltrials.gov/). Likewise, recent genome editing clinical trials (NCT03655678, NCT03745287) based on the induction of fetal hemoglobin (HbF; $\alpha^2\gamma^2$), mimicking individuals with hereditary persistence of fetal hemoglobin (HPFH), are very promising in ameliorating

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the manifestations of β-hemoglobinopathies [9–11].

As an alternative to LV-mediated *β-globin* gene addition, the clinical benefits of HbF prompted strategies to introduce an exogenous γ-*globin* gene or to reactivate the endogenous γ-*globin* genes [6,12]. For the latter, a number of positive and negative regulators of the fetal γ-*globin* gene can be downregulated or overexpressed to induce HbF [13–16]. In addition, several optimizations were employed with different LV envelope proteins and culture conditions to enhance the viral transduction into HSCs [17,18]. However, no head-to-head comparison of LV vectors with the capacity to increase HbF has been performed in $CD34^+$ HSCs to assess the therapeutic potential for β-hemoglobinopathies.

In the present study, we compared lentiviral delivery for three different molecular targets and envelope proteins to identify a suitable strategy to be used in the clinic for the treatment of β-hemoglobinopathies. LV constructs were: 1) insulin-like growth factor 2 mRNA binding protein-1 (IGF2BP1), a newly discovered posttranscriptional negative regulator of the fetal hemoglobin repressor BCL11A [16,19,20]; 2) microRNA-embedded shRNA (shmiR) sequences specific for the γ-*globin* repressor protein BCL11A [15]; and 3) γ-*globin* gene, under the influence of *β-globin* promoter [14]. Notably, the transduction advantages of baboon envelope proteins (BaEV) were reported over commonly utilized vesicular-stomatitis-virus-G envelope protein (VSV-G) in $CD34^+$ HSCs since BaEV-LVs use the neutral amino acid transporters 1 and 2 (ASCT1 and ASCT2), which are expressed in HSCs [13,17,21]. Moreover, the mutant version of the baboon envelope without the R peptide which hiders viral entry (BaEV-RLess), was found to exhibit superior transduction compared to the endogenous baboon envelope (BaEV) [22]. Therefore, in this study, we utilized these three different envelope proteins to deliver the HbF-inducing LV constructs specified above and aimed to identify the optimal therapeutic combination.

2. Materials and methods

2.1. Cell culture

All cell types were cultured at 37 °C with 5% CO_2. BM-derived $CD34^+$ HSCs from independent adult donors were obtained using protocols approved by the local ethics committee/institutional review board (IRB) and after informed written consent (829/2016BO2), as well as leukapheresis products purchased from Key Biologics (Memphis, TN). Immunomagnetic enrichment of HSCs was performed using a magnetic-activated cell sorting system (CliniMACS System, Miltenyi Biotec), according to the manufacturer's instructions. Flow cytometry analysis demonstrated that cell purity and viability were 97.2% and 99.9%, respectively. $CD34^+$ HSCs were cultured in StemMACS™ HSC Expansion Media (Miltenyi Biotec) supplemented with human cytokines (Miltenyi Biotec): SCF (100 ng/ml), TPO (20 ng/ml), and Flt3-L (100 ng/ml).

Lenti-X 293T cells were purchased from Clontech and cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS (Gibco), 1% L-glutamine (Biochrom), and 1% Penicillin/Streptomycin (Biochrom). K562 cells were acquired from Sigma-Aldrich and cultured in RPMI (Thermo Fisher Scientific) supplemented with 10% FBS, 1% L-glutamine, and 1% Penicillin/Streptomycin.

2.2. In vitro differentiation of $CD34^+$ HSCs into erythrocyte precursors

$CD34^+$ HSCs were cultured according to the three-phase differentiation protocol [23]. In brief, the first phase (days 0–7) consisted of StemMACS™ HSC Expansion Media containing 1% Penicillin/Streptomycin, 1% L-glutamine, 1% lipids (Miltenyi), 100 ng/ml of SCF (Miltenyi), 10 ng/ml IL-3 (Miltenyi), 0.5 U/ml erythropoietin (PeproTech) and 200 μg/ml transferrin (Sigma Aldrich). In the second phase (days 7–11), erythropoietin was increased to 3 U/ml, whereas in the third phase (days 11–21), transferrin was augmented to 1 mg/ml. Erythroid differentiation and maturation were monitored by flow cytometry (BD FACSCalibur) using PE-conjugated anti-CD34 (Miltenyi), PE-conjugated anti-CD235a (Miltenyi), PerCP-conjugated anti-CD45 (Miltenyi) and APC-conjugated anti-CD71 (Miltenyi) at two different time points: day 0 and day 21.

2.3. Production and concentration of lentiviral vectors

Lenti-X 293T cells (5.8×10^6) were seeded in a T175 culture flask (Corning) without antibiotics. After 48 h, DMEM was replaced by 10 ml of Opti-MEM (Thermo Fisher Scientific). The cells were then transfected with 25 μg of packaging plasmid, 8 μg of VSV-G (St. Jude Children's Research Hospital, Memphis, TN, USA) or 20 μg for BaEV plasmids (Miltenyi), and 25 μg of the transfer vector (St. Jude Children's Research Hospital, Memphis, TN, USA), utilizing JetPEI (Polyplus transfection) as a transfection reagent. After 6 h, 10% FBS was added to the cells and 16 h later the supernatant was replaced by 10 ml of Opti-MEM with 4% FBS. The viral supernatant was harvested 72 h post-transfection and 100-fold concentrated by centrifugation (4 °C/4 h/20,000*g*) in 100 μl of StemMACS™ HSC Expansion Media.

The following plasmids were utilized in our study: 1) pRRL ppt-PGK-GFP-wpre-sin18 (Miltenyi) encoding for GFP (PGK-GFP) under control of the human phosphoglycerate kinase (PGK) promoter that is constitutively active, 2) pLVX-SPTA IGF2BP1 P2A ZsGreen (pLVX-IGF2BP1) encoding for IGF2BP1 under control of the α-spectrin erythroid promoter, 3) pCL20c ANK-GFP-miREsh49 BCL11A PRE-O (pCL20-BCL11A-shRNA) with the miRNA-embedded shRNA (shmiR) sequences specific for BCL11A under control of the erythroid-specific Ankyrin-1 (ANK) promoter, and 4) pCL20c Ins-400 mLAR βV5 Δγ m3 (pCL20-γ-*globin*) encoding for γ-*globin* genomic sequences under control of the β-globin promoter and portions of the locus control region (LCR). Furthermore, our collaborator from Miltenyi Biotec GmbH provided us the plasmids pLTG1413 and pLTG1414 encoding for the baboon envelope proteins BaEV-RLess and BaEV, respectively, which were originally designed by Girard-Gagnepain et al. [17]. To confirm that insert sequences were correct, plasmids were verified by Sanger sequencing (data not shown). Additional details about the LVs used in this study are summarized in Table 1.

2.4. LV titration

To determine the number of infectious units (IFU) per milliliter and for calculating multiplicity of infection (MOI), cell culture titration by serial dilutions was selected as the most reliable. Nonetheless, titer calculations by p24 Assay and qRT-PCR were also performed to unveil difficulties in the production of baboon pseudotyped lentiviruses.

2.5. Titration by cell culture and flow cytometry analysis

For cell culture-based titration, 1×10^5 Lenti-X 293T (for the constitutive-promoter construct pRRL-PGK) or K562 cells (for the erythroid-specific constructs pLVX-IGF2BP1-SPTA1 and pCL20-BCL11A-shRNA-ANK) were seeded in a 12-well plate (Corning). 2-fold serial dilutions from a fraction of the concentrated viral supernatant were performed to transduce the cells. After 72 h, transduction efficiencies were determined by flow cytometry for vectors encoding for expression of GFP to determine the number of IFU per milliliter for further calculation of MOIs.

2.6. Titration by p24 assay

Viral titer was also determined by measuring p24 antigen using a standard ELISA method in a microtiter plate coated with an anti-HIV-1 p24 capture antibody, following the manufacturer's instructions (Takara Bio).

Table 1
Lentiviral vectors utilized in this study.

Vector name	Short name	Transgene	Vector size (bp)	Proviral sequence size (bp)	Promoter	Reference
pRRL_ppt_PGK_GFP_wpre_sin18	pRRL-GFP	eGFP	7689	4042	PGK	Addgene (#12252)
pLVX-SPTA IGF2BP1 P2A ZsGreen	pLVX-IGF2BP1	IGF2BP1	9745	7097	SPTA1	de Vasconcellos, Tumburu, Byrnes, Lee, Xu, Li, Rabel, Clarke, Guydosh, Proia and Miller [16]
pCL20c ANK-GFP-miRE-sh49 BCL11A PRE-O	pCL20-BCL11A-shRNA	BCL11A shmiR	7291	3967	ANK	Guda, Brendel, Renella, Du, Bauer, Canver, Grenier, Grimson, Kamran, Thornton, de Boer, Root, Milsom, Orkin, Gregory and Williams [15]
pCL20c Ins-400 mLAR βV5 Δγ m3	pCL20-γ-globin	γ-Globin	10,925	7542	β-Globin	Wilber, Hargrove, Kim, Riberdy, Sankaran, Papanikolaou, Georgomanoli, Anagnou, Orkin, Nienhuis and Persons [14]

PGK: murine phosphoglycerokinase; SPTA1: spectrin alpha, erythrocytic 1; ANK: pol II-based erythroid-specific Ankyrin-1.

2.7. Titration by qRT-PCR

A qRT-PCR based lentiviral titer assay utilizing primers for the 5′-LTR region was carried out following the manufacturer's instructions (Mellgen Laboratories Inc).

2.8. Cell transduction

In preliminary experiments with the aim to gradually establish viral transduction in different cell types, 1×10^4 Lenti-X 293T, K562, and $CD34^+$ HSCs were transduced in 96-well plates (Corning). Next, different culture conditions were tested to enhance lentiviral transduction in HSCs. Thereby, plates coated with recombinant RetroNectin at a concentration of 16 μg/ml were tested in HSCs following the manufacturer's instructions (Takara/Clontech). Also, Cyclosporin H (CsH), a lentiviral transduction enhancer, was added to the viral supernatant at a concentration of 8 μM as indicated by Petrillo [18].

In the comparative study for the different HbF-inducing LVs, Cyclosporin H (CsH) was used as a transduction enhancer. Subsequently, for each construct-envelope combination, 3 technical replicates of 1×10^4 HSCs each were transduced in a 96-well plate (Corning). Transduction efficiencies were determined by flow cytometry for vectors encoding GFP using cells collected 7 days after transduction.

2.9. Provirus quantitation

Vector copy number (VCN) was determined 18 days after transduction utilizing the 'Lentiviral Copies in Cells' Kit following the manufacturer's instructions (Mellgen Laboratories Inc.).

2.10. RNA isolation, cDNA synthesis, and qRT-PCR assays

For the purpose of determining the expression level of the different globin proteins and other genes of interest, transduced $CD34^+$ HSCs from each construct-envelope combination were harvested on culture day 14. Total RNA was isolated using the RNeasy Mini kit and QiaShredder spin columns (Qiagen), in accordance with the manufacturer's protocol. RNA at a concentration of 500 ng was used for cDNA synthesis with the QuantiTect reverse transcription kit (Qiagen).

Amplification and quantification of cDNA were performed with the CFX96 TM Real-Time PCR Detection System (Bio-Rad Laboratories). PCR was run utilizing KAPA SYBR FAST 2× MasterMix (KAPA Biosystems). Results were normalized against the expression of the housekeeping gene *β2-microglobulin* (*β2M*). The cycle quantitation (Cq) values for the unknown samples were evaluated with the equation $2^{(Cq\ \beta 2M\ -\ Cq\ target\ gene)}$ [24]. All primer sequences are presented in Table 2.

Table 2
Primer sequences utilized for qRT-PCR in the comparative study.

Gene	For/rev	Primer sequence	Reference
B2M	For	GATGAGTATGCCTGCCGTGT	[45]
	Rev	AATTCATCCAATCCAAATGAG	[45]
α-Globin	For	CTGGCGAGTATGGTGCG	[46]
	Rev	GAAGTGCGGGAAGTAGGTC	[46]
β-Globin	For	TGCACGTGGATCCTGAGAACT	[46]
	Rev	AATTCTTTGCCAAAGTGATGGG	[46]
γ-Globin	For	TGGCAAGAAGGTGCTGACTTC	[47]
	Rev	TCACTCAGCTGGGCAAAGG	[47]
BCL11A	For	AACCCCAGCACTTAAGCAAA	Own design
	Rev	GGAGGTCATGATCCCCTTCT	Own design
IGF2BP1	For	AGACCTTACCCTTTACAACCC	Own design
	Rev	GAAAAGACCTACAGCAGCC	Own design

2.11. HbF quantification by HPLC

HbF resurgence was assessed by high-performance liquid chromatography (HPLC). Frozen cell pellets were lysed in 200 μl deionized sterile water and ultrasonicated for 5 min. Cell debris was removed by centrifugation at 13.000*g*. The supernatant was then concentrated to a final volume of 30 μl using a Nanosep molecular filter (PALL Corporation) with a 10 kDa membrane by centrifugation at 13.000*g*. Hemoglobin species from cell lysates were separated using a PolyCAT A cation exchanger column (PolyLC Inc., USA). The analysis was performed on an elite-LaChrom HPLC-system (Merck-Hitachi) using a gradient elution mode with a bis-tris buffer system (buffer A: bis-tris 20 mM, NH_4-acetate 13 mM, KCN 1 mM and buffer B: bis-tris 20 mM, Na-acetate 38 mM, KCN 1 mM, NaCl 200 mM). Hemoglobin proteins were detected by absorbance measurements at 415 nm.

2.12. Statistics

Student's *t*-test was applied wherever appropriate to determine significant differences between mean values using GraphPad Prism version 8.1.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Virus production

We optimized lentivirus production for the three envelope proteins (VSV-G, BaEV-RLess, BaEV) using a PGK-regulated GFP reporter construct (Table 1). The most efficient protocol, modified from the procedure proposed by Girard-Gagnepain, was selected based on titer determinations from three independent methods. After one freeze-thaw cycle, viral titer was reduced by 12–17% with no significant difference observed among the three envelope proteins (Fig. 1A). Thus, we used freshly produced viral particles in all our experiments to avoid titer loss. Interestingly, mean virus titer calculated by flow cytometry analysis for GFP expression in transduced cells was 86- and 56-fold higher for VSV-G over BaEV-RLess and BaEV envelope proteins, respectively (Fig. 1B). These pronounced differences were, however, not observed when viral titers were determined using p24 ELISA kit or qRT-PCR, where VSV-G titer was < 4-fold higher over baboon envelopes by ELISA (Fig. 1C), and < 5-fold higher by qRT-PCR (Fig. 1D). Consequently, cell-mediated titration, based on functional viral particles, was selected as the most reliable method and utilized in following experiments.

3.2. Transduction efficiency

Transduction efficiencies of the three envelope proteins were assessed in Lenti-X 293T cells using the PGK-GFP-encoding LV construct. Flow cytometry analysis on day 7 post-transduction found that VSV-G envelope resulted in very high transduction efficiency (99 ± 0.05% GFP^+ cells), while BaEV-RLess (77 ± 1.1%) and BaEV (66 ± 2.2%) showed lower but decent efficiencies (Fig. 1E). Transduction analysis was extended to K562 human erythroleukemia cells and $CD34^+$ HSCs. Remarkably, we observed a relatively high level of transduction efficiency (range 78–94%) with baboon envelopes in K562 cells (Fig. 1E). However, this trend was not reproduced in $CD34^+$ HSCs, as lower transduction efficiencies (range 40–58%) were attained with baboon envelopes (Fig. 1E). Interestingly, MOI for VSV-G pseudotyped LVs were pronouncedly higher (MOI 100) than Baboon lentiviruses, where lower MOIs (BaEV-RLess: 20, BaEV: 10) were required to achieve decent transduction rates. Furthermore, no significant improvement in transduction efficiency of $CD34^+$ HSCs was observed using a concentration step to increase viral titer without or with the transduction enhancer RetroNectin (Fig. 1F). Transduction of HSCs was, however, generally improved by addition of Cyclosporine H (CsH) to the medium with percentages of GFP^+ cells significantly increased for VSV-G (18.6%; $p < 0.0001$, $n = 3$), BaEV-RLess (25.26%; $p = 0.0088$, $n = 3$), and BaEV (7.45%; $p = 0.3220$, $n = 3$) (Fig. 1G), and therefore, CsH was used in all subsequent experiments.

We next performed the same virus production protocol for the pLVX-IGF2BP1, pCL20-BCL11A-shRNA, and pCL20-γ-*globin* constructs. Since these constructs contain erythroid-specific promoters, viral titers were determined by qRT-PCR (Fig. 2A) as well as by human erythroleukemia K562 cell culture titration, where the latter was the method of choice to calculate MOIs shown in Fig. 2B. Subsequently, HSCs were transduced with the maximum amount of fresh virus particles in order to achieve the highest transduction efficiency and HbF resurgence, avoiding freeze-thaw cycle viral loss. Transduction efficiency was assessed for pLVX-IGF2BP1 and pCL20-BCL11A-shRNA by flow cytometry on day 7 post-transduction as both constructs possessed a GFP reporter gene (Fig. 2B; Table 1). A similar experiment was not conducted for pCL20-γ-*globin* construct because it lacked a fluorescent reporter. Lentiviral particles from pCL20-BCL11A-shRNA showed the highest transduction score for VSV-G (92.2 ± 1.1%), moderate level for BaEV-RLess (29.5 ± 0.7%) and lower level for BaEV (14.1 ± 0.6%; Fig. 2B). Transduction efficiency for pLVX-IGF2BP1 was similarly high for VSV-G (75.6 ± 0.3%) but declined appreciably for both baboon envelopes with mean levels of 1.9% and 1.7%, respectively (Fig. 2B). In these experiments, baboon MOIs were significantly lower than VSV-G when using the pLVX-IGF2BP1 construct (VSV-G: 35; BaEVRLess: 10; BaEV: 5), and for pCL20-BCL11A-shRNA (VSV-G: 150; BaEVRLess: 70; BaEV: 50). Finally, average vector copy number (VCN) ranged from 0.1–2.2 provirus copies per cell for all treatments (Fig. 2C), which is acceptable in terms of clinical relevance as the risk of insertional mutagenesis is likely lower if fewer vector copies are inserted per cellular genome. Results are summarized in Table 3. Also, the percentage of GFP^+ cells in correlation with the VCN showed that pLVX-IGF2BP1 construct generally exhibits higher GFP expression level per transgene copy in comparison to pCL20-BCL11A-shRNA (Fig. 2D).

3.3. γ-Globin up-regulation and HbF expression

Functional outcome of LV-mediated expression of the three molecular targets was evaluated using qRT-PCR and HPLC quantification of hemoglobin tetramers. Quantitative gene expression analysis on day 14 post-transduction showed elevated levels of γ-*globin* transcripts relative to total (γ-*globin* + *β-globin*) for all treatments using VSV-G envelope, with the greatest mean fold increase noted for pLVX-IGF2BP1 (3-fold) and pCL20-BCL11A-shRNA (3.1-fold) compared to pCL20-γ-*globin* where levels were ~1.8-fold over background (Fig. 3A). qRT-PCR also confirmed a > 2-fold decrease in BCL11A transcripts due to the activity of the shmiR with mean levels of only 25% of the control (Fig. 3B). A similar analysis for IGF2BP1 showed a magnitude of IGF2BP1 expression (5×10^3 fold), which is inherent to LV treatment since adult cells express very low levels of this fetal-specific gene (Fig. 3C). While IGF2BP1 binds to BCL11A mRNA, it does not cause a reduction in BCL11A transcript level as observed in our study (data not shown). On the other hand, none of the baboon-pseudotyped LVs conferred a marked positive effect on γ-*globin* mRNA levels (Fig. 3D, E). Next, we assessed the HbF expression, at protein level, using HPLC and noted a significant increase in HbF for most transduced treatments compared to untreated $CD34^+$ HSCs (5.97 ± 1.8%) (Fig. 4A–C). VSV-G envelope treatments showed the highest HbF expression (Fig. 4A, D), for both pLVX-IGF2BP1 (50 ± 1.6%) and pCL20-BCL11A-shRNA (50 ± 6.5%) treatments, while pCL20-γ-*globin* led to moderate level of HbF resurgence (20 ± 2.6%; Fig. 4A). Similar to the abovementioned qRT-PCR results, baboon pseudotyped LVs showed lower HbF expression, where BaEV-RLess outperformed BaEV in all treatment groups (Fig. 4B, C). Results are summarized in Table 3. In this context, it is important to mention that, when analyzing the HbF% per VCN for all VSV-G treatments, IGF2BP1 transgene exhibited the highest HbF expression level per transgene copy in comparison to the other constructs (Fig. 4E).

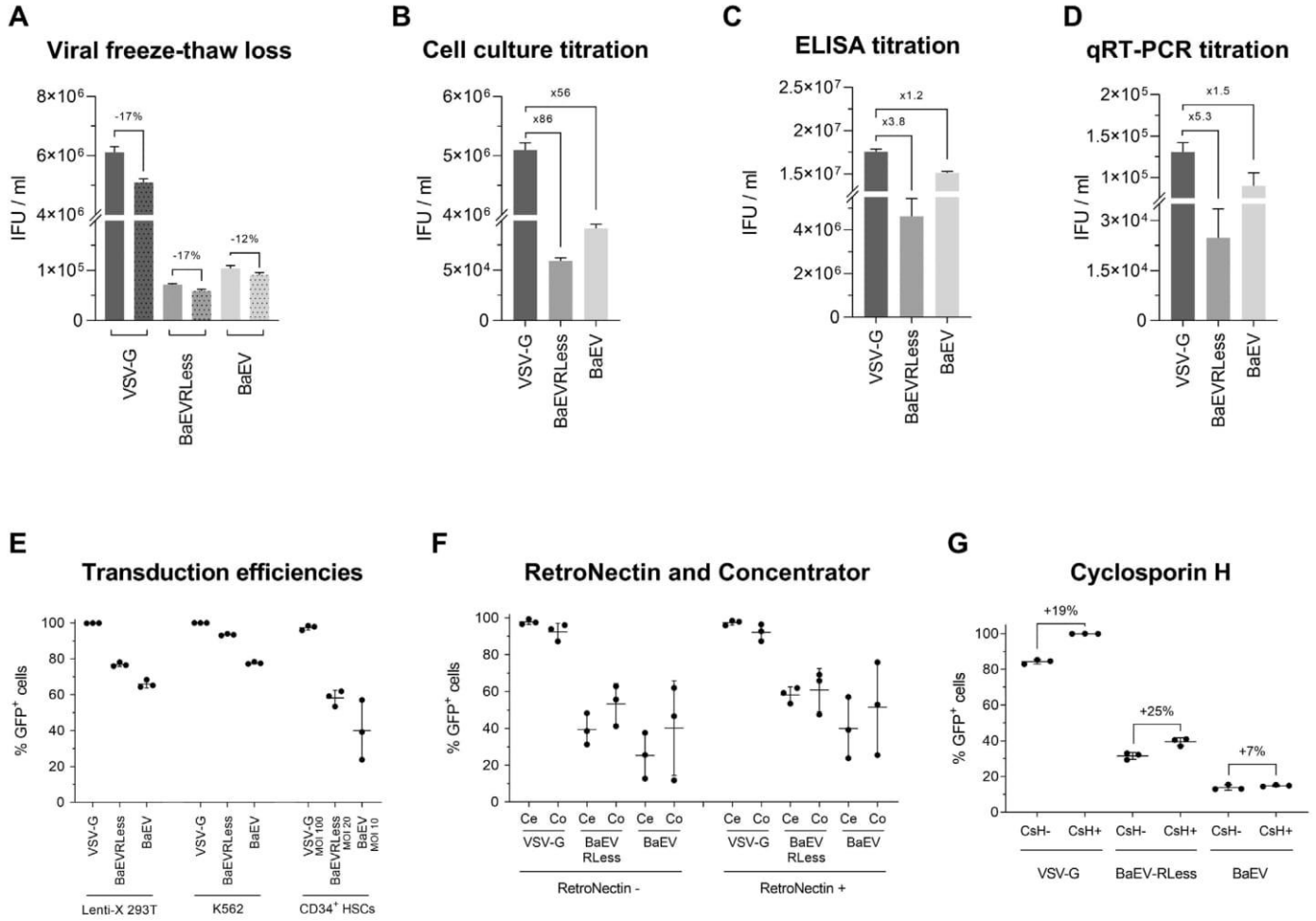


Fig. 1. Lentivirus production and transduction efficiency for alternative envelope proteins. Lentiviral vector particles encoding for a PGK-regulated GFP cassette and pseudotyped with VSV-G, BaEV-RLess, or full-length BaEV were produced by transient transfection as described in the materials and methods section. (A) Reduction in viral titer after one freeze-thaw cycle determined by flow cytometry titration analysis of transduced Lenti-X 293T cells for expression of GFP. Freeze and thaw titers are shown with a dot pattern. (B–D) Number of infectious particles per ml (IFU/ml) for the three studied envelope proteins determined by: (B) cell culture and flow cytometry, (C) p24 ELISA assay, (D) and qRT-PCR. (E) Maximum transduction efficiencies achieved in Lenti-X 293T, K562, and human CD34$^+$ HSCs. MOIs for HSCs were determined by Lenti-X 293T cell culture titration. (F) Percentages of transduced GFP$^+$ HSCs for the indicated envelope proteins when utilizing different methods such as RetroNectin transduction enhancer, Concentrator (Co) or ultracentrifugation (Ce). (G) Percentage enhancement of Cyclosporine H (CsH) on HSC transduction for LV pseudotyped with the three independent envelope proteins. All experiments were performed in triplicates.

Erythroid differentiation and maturation, monitored by flow cytometry on day 21, indicated > 90% CD235$^+$ cells in all treatments with no significant differences to the control (Fig. 4F; Table 3). Likewise, proliferation rates were not significantly different among treatments and control (Table 3).

4. Discussion

Individuals with β-thalassemia who co-inherit HPFH mutations have reduced complications or no symptoms of disease due to continued expression of HbF in adulthood [3,4,14,25–27]. Moreover, HbF levels above 20% in the circulation were observed to have therapeutic benefits for patients with β-hemoglobinopathies [28,29]. Therefore, attempts are being made to induce HbF either by LVs [14–16] or CRISPR/Cas9 system [6,9–11,30,31]. Here, we conducted a comparative analysis of three independent lentiviral strategies to reactivate HbF as a treatment option for β-hemoglobinopathies and assessed efficacy and suitability of each for clinical translation.

Earlier studies identified advantages of baboon envelope proteins over VSV-G as the former exhibited greater tropism in HSCs and led to higher transduction using lower quantities of infectious virus (i.e., lower MOI) [13,17,21,32–34]. Our results confirmed this finding as BaEV-RLess at an MOI 20 yielded ~60% mean transduction efficiency in HSCs, where higher MOIs are needed to achieve a similar result with VSV-G. Also, average VCN per cell for pCL20-BCL11A-shRNA was comparable for LVs pseudotyped with VSV-G and the baboon envelope proteins even though the MOI was considerably higher for VSV-G. Notably, BaEV-RLess, which lacks the R peptide sequence that hinders viral fusion activity [22], outperformed the full-length BaEV in all cell types and treatments, supporting previous outcomes [17].

In spite of positive results for the PGK-GFP reporter construct, our comparative study showed that therapeutic levels of HbF (≥20%) were only attained utilizing LV pseudotyped with the VSV-G envelope. We noticed that, during virus production with Lenti-X 293T cells, fewer viral particles were generated for baboon envelopes compared to VSV-G. Interestingly, we observed substantial variation in the assessment of infectious viral particles when using ELISA-based, qPCR-based, and cell-based titration methods, where results from cell-mediated titration were the most reliable since only functional viral particles are considered. We surmise that overestimated results using ELISA and qPCR methods might be due to the presence of numerous empty or non-functional baboon lentiviral particles as incomplete assembly of baboon viruses was reported earlier [35]. In addition, we observed a strong effect of insert size on the transduction efficiency of baboon

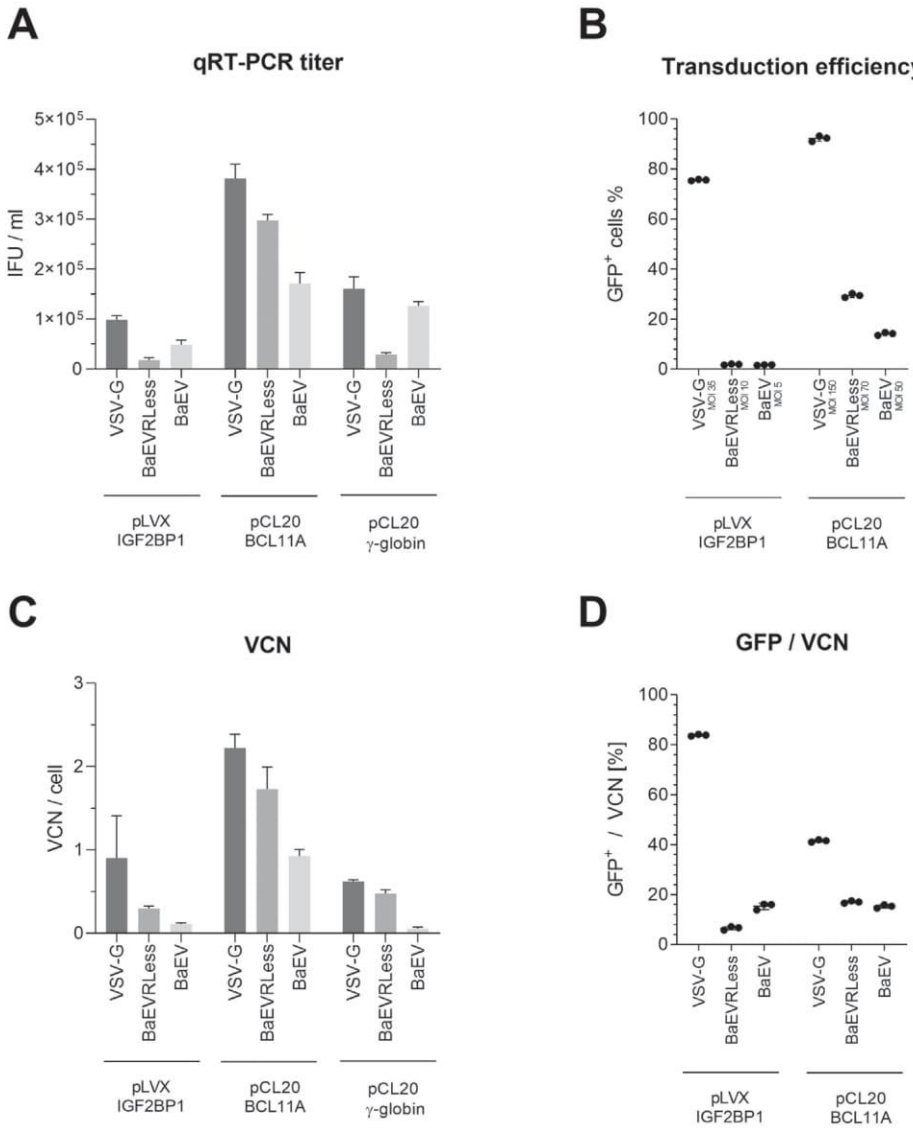


Fig. 2. Impact of alternative envelope proteins on lentivirus-mediated gene transfer of HbF inducing factors in human CD34$^+$ cells. Lentiviral vector particles encoding for expression of IGF2BP1 or BCL11A shmiR under control of an erythroid-specific promoter and coupled to expression of GFP and pseudotyped with VSV-G, BaEV-RLess, or full-length BaEV were produced by transient transfection as described in the materials and methods. (A) Viral titer determined by qRT-PCR for the three different envelope-construct combinations. (B) Transduction efficiencies achieved in HSCs for constructs pLVX-IGF2BP1 and pCL20-BCL11A-shRNA with envelope proteins VSV-G, BaEV-RLess, and BaEV at the indicated MOI determined by K562 cell culture titration. (C) Clinically relevant VCNs for all VSV-G, BaEV-RLess, and BaEV treatments. (D) Percentage of GFP$^+$ cells in correlation with the VCN. All experiments were performed in triplicates.

pseudotyped lentiviruses. This suggests that cargo capacity for LV with baboon envelopes could be inferior, hence larger constructs such as pLVX-IGF2BP1 (7 kb) would have limited virus production when compared to smaller constructs as pCL20-BCL11A-shRNA (4 kb). Thus, the size of the insert region in the transfer plasmid would ultimately determine the number of functional particles. Further studies are needed to overcome the limitations of baboon envelopes as they have potential clinical usage due to positive tropism for HSCs, which was also evident in our investigation. While it is possible that baboon pseudotyped lentivirus with smaller insert sizes could substitute for

Table 3
Summary of collective results in CD34$^+$-derived erythroblasts. Data is indicated as mean ± SD.

Envelope	Construct	Cell proliferation[a]	VCN[b]	γ/(γ + β)[c]	%HbF[d]	%CD235$^+$/CD71$^+$[e]
–	Control	1128.33 ± 214.26	–	0.23 ± 0.20	5.97 ± 1.76	91.80 ± 0.17
VSV-G	IGF2BP1	1115.00 ± 10.00	0.90 ± 0.51	0.67 ± 0.03	50.50 ± 1.55	92.00 ± 1.30
	shBCL11A	940.00 ± 63.84	2.22 ± 0.16	0.70 ± 0.02	49.90 ± 6.51	94.47 ± 0.67
	γ-Globin	970.67 ± 42.16	0.62 ± 0.02	0.40 ± 0.09	19.97 ± 2.59	93.13 ± 2.19
BaEVRLess	IGF2BP1	930.00 ± 56.35	0.29 ± 0.03	0.30 ± 0.03	11.37 ± 1.10	91.30 ± 0.57
	shBCL11A	930.00 ± 124.90	1.73 ± 0.26	0.32 ± 0.01	11.80 ± 1.65	91.45 ± 1.06
	γ-Globin	948.33 ± 93.05	0.48 ± 0.04	0.26 ± 0.05	8.40 ± 0.98	89.65 ± 0.07
BaEV	IGF2BP1	1008.33 ± 35.12	0.11 ± 0.01	0.32 ± 0.02	8.13 ± 1.50	91.60 ± 1.13
	shBCL11A	951.67 ± 137.96	0.93 ± 0.08	0.30 ± 0.08	9.57 ± 1.25	92.60 ± 0.34
	γ-Globin	1040.00 ± 111.69	0.05 ± 0.02	0.28 ± 0.07	7.67 ± 2.25	82.97 ± 0.81

[a] Fold-increase in viable cells from day of transduction to culture day 14.
[b] Genomic DNA was isolated from the bulk cell population on day 18 of culture to determine vector copy number (VCN).
[c] Levels of γ-*globin* transcripts relative to total (γ-*globin* + *β-globin*) determined by qRT-PCR on culture day 14.
[d] Percentage of fetal hemoglobin (HbF) quantified by HPLC on culture day 21.
[e] Erythroid differentiation was determined by flow cytometry analysis for co-expression of CD235$^+$/CD71$^+$ on culture day 21.

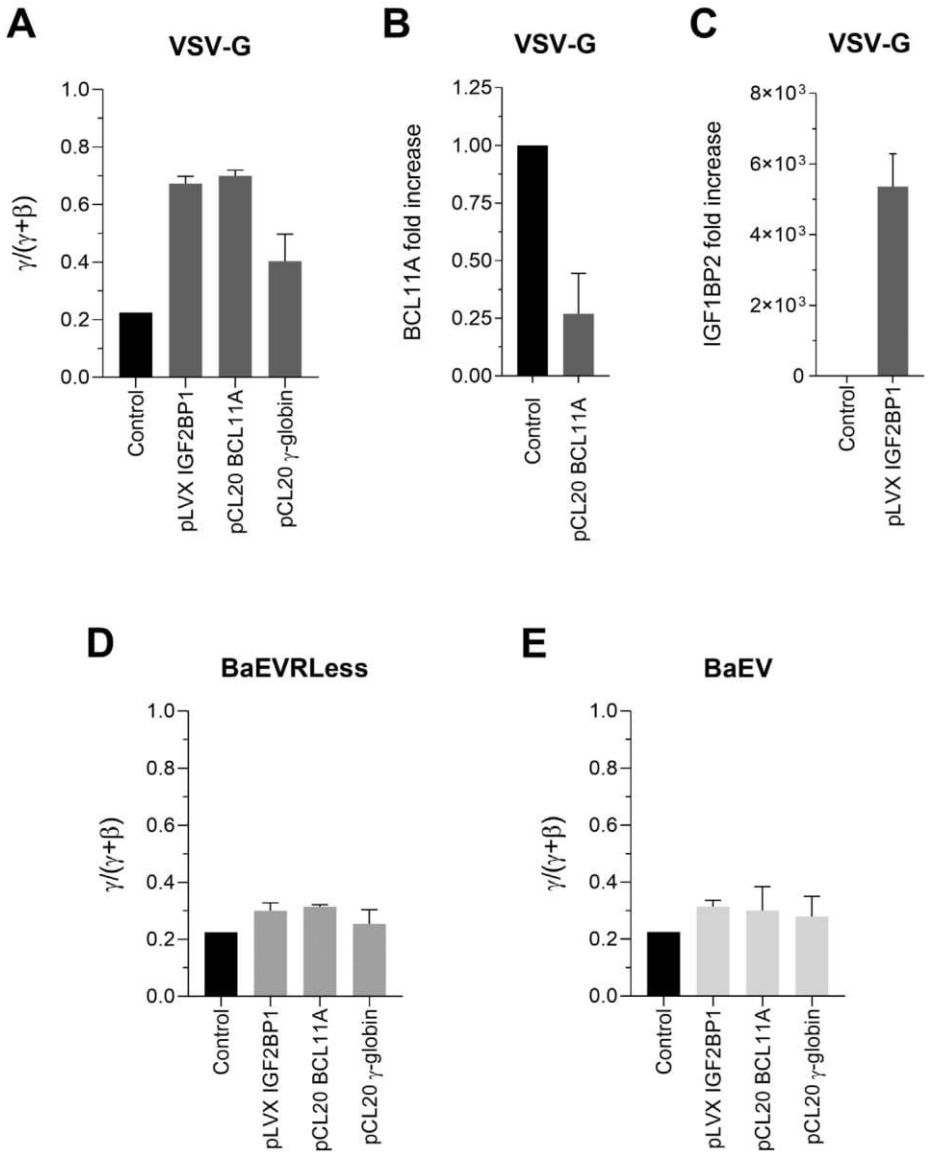
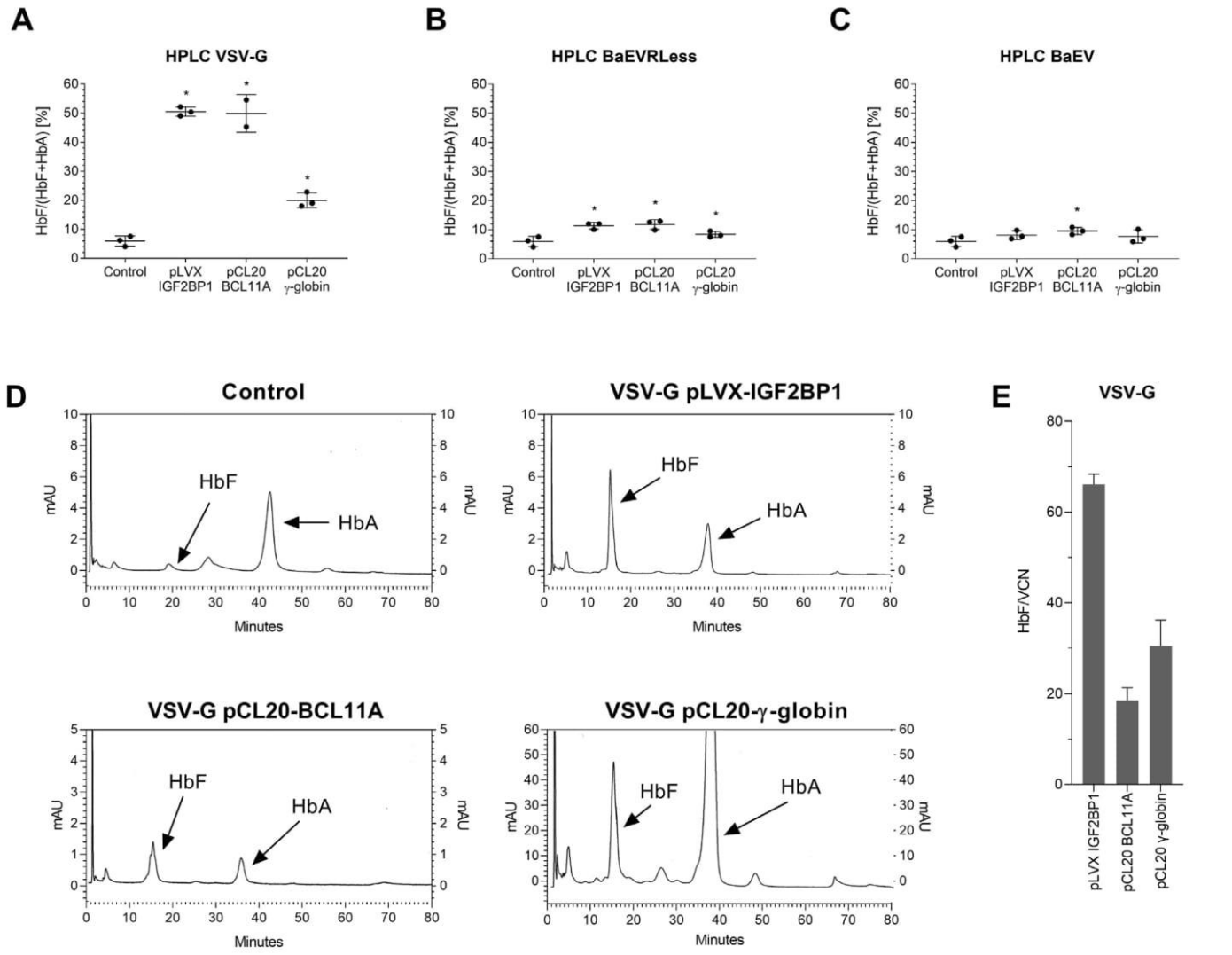


Fig. 3. Functional gene expression analysis of HbF inducing factors in culture-differentiated erythroblasts. Human CD34$^+$ cells left untreated (control) or transduced with lentiviral vectors encoding for erythroid-specific expression of γ-*globin* genomic sequences, shmiR sequences targeting *BCL11A*, or *IGF2BP1* cDNA, and pseudotyped with either VSV-G, BaEVRLess, or full-length BaEV envelope proteins are shown. Culture-differentiated erythroblasts were extracted of total RNA on day 14 and quantitative qRT-PCR was used to determine transcript levels of (A) γ-*globin* transcripts relative to total (γ-*globin* + β-*globin*), (B) *BCL11A*, and (C) *IGF2BP1* with VSV-G LVs; (D–E) γ-*globin* expression relative to total (γ-*globin* + β-*globin*) analysis for (D) BaEV-RLess and (E) BaEV treatments. All experiments were performed in triplicates.

VSV-G in HSC gene delivery and other cell types, our results showed that VSV-G envelope protein is superior compared to baboon derivatives.

Our comparison of three different constructs (pLVX-IGF2BP1, pCL20-BCL11A-shRNA, and pCL20-γ-*globin*) revealed that therapeutic levels of HbF (> 20%) were achieved with the VSV-G envelope for all constructs, which could be applicable to the treatment of β-hemoglobinopathies since the VCNs were also clinically relevant. However, each construct was observed to have possible limitations and different HbF induction levels depending on their promoter and regulators involved. For example, pCL20-BCL11A-shRNA, which yielded strong HbF resurgence for all three tested envelopes (up to 50%) and presented the highest VCN, employs RNA interference (RNAi) technology using short hairpin RNAs (shRNAs) that has been associated with cytotoxic effects due to endogenous dysregulation [36,37]. In addition, BCL11A is predominantly involved in hematopoiesis and brain development, and a strong knockdown with shRNA could lead to impaired erythropoiesis [28,38,39]. Nevertheless, no negative effects have been reported in mice and human HSCs upon pronounced lineage-specific BCL11A knockdown [40], while the HbF levels yielded in our study are comparable to the HbF persistence observed in BCL11A haploinsufficient patients [41,42]. Interestingly, lentiviral-mediated overexpression of IGF2BP1 yielded also exceptionally high HbF reactivation (~50%) with lower VCN, which could indicate that SPTA1 is a highly efficient erythroid-specific promoter. Despite the promising results, overexpression of proteins involved in anabolism such as IGF2BP1 might modulate cell metabolism and oncogenesis [43], but this possibility can only be determined by animal transplantation studies. On the contrary, the aforementioned limitations are not associated with VSV-G-enveloped pCL20-γ-*globin* construct since addition of the γ-*globin* gene has no apparent impact on signaling pathways, and rather results in direct upregulation of HbF. This strategy, which has been in fact already initiated (NCT02186418), led to clinically meaningful HbF induction (> 20%) with reduced VCN (< 0.75) that limits the possibility of random insertional events.

Due to the fact that this comparative study includes different lentiviral constructs and envelope proteins with varying tropism in HSCs, some details which could facilitate the interpretation of the results must be emphasized. Lentivirus production certainly depends on construct size and envelope protein pseudotyped, which leads to variable viral titers. Also, even though it would be optimal to attain a similar VCN to equitably compare all treatments, the VCN cannot be accurately predicted prior to cell transduction since it is influenced by multiple variables such as target cell type, donor variability, cell activation, and lentiviral infectious ability. Moreover, as explained above, each construct incorporates a different erythroid-specific promoter, hence



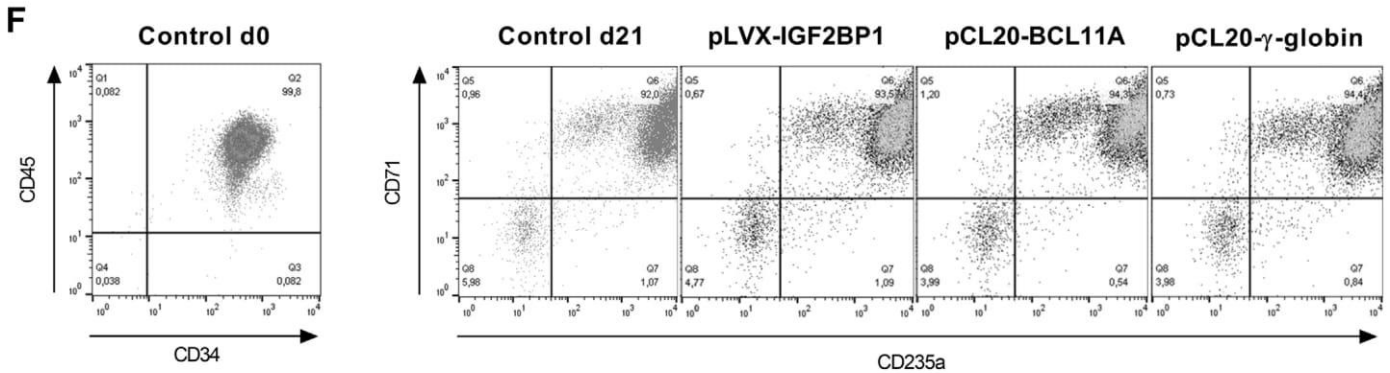


Fig. 4. Fetal hemoglobin levels for terminally differentiated erythroblasts. Untreated human CD34$^+$ cells (control) or transduced with lentiviral vectors encoding for erythroid-specific expression of γ-*globin*, *BCL11A* shmiR, or *IGF2BP1* and pseudotyped with either VSV-G, BaEV-RLess, or BaEV envelope proteins were culture-differentiated as described in materials and methods. Hemolysates prepared from erythroblasts at terminal stage of culture (day 21) were subjected to HPLC analysis to quantify hemoglobin tetramers. (A) VSV-G, (B) BaEV-RLess, and (C) BaEV. *: $p \leq 0.05$. (D) Representative HPLC histograms for control, VSV-G pLVX-IGF2BP1, VSV-G pCL20-BCL11A, and VSV-G pCL20-γ-*globin*. Milli-Absorbance Units (mAU). (E) HbF/VCN correlation for VSV-G-LV constructs. (F) Erythroid differentiation of HSCs determined by flow cytometry analysis for co-expression of CD235$^+$/CD71$^+$ on day 21 for all VSV-G-pseudotyped constructs. The experiments were performed in triplicates.

similar VCN would not ultimately yield similar HbF resurgence. It is also worth mentioning the importance of incorporating early-expressed promoters which might help to induce a strong HbF resurgence by driving the expression of the gene earlier in hematopoiesis.

Another alternative lentiviral approach that has demonstrated clinically relevant outcome for the treatment of β-hemoglobinopathies is LIN28B overexpression [44]. LIN28B regulates the fetal phenotype by suppressing BCL11A, enabling the expression of the γ-*globin* genes. This promising study showed strong therapeutic HbF resurgence (> 30%) with no detected negative implications in cell maturation, a similar outcome to what we have observed for the different lentiviral strategies we performed in our study.

In conclusion, our results showed that LVs pseudotyped with VSV-G yielded improved transduction efficiency in HSCs compared to baboon counterparts. Clinically relevant levels of HbF were achieved using three independent molecular approaches to modulate levels of IGF2BP1, BCL11A, or γ-globin. Current clinical success using *β-globin* addition vectors suggests that γ-*globin* gene addition represents the most suitable alternative for clinical application. That said, γ-*globin* gene addition suffers from the same challenges as *β-globin* including gene transfer efficiency and transgene expression. Thus, alternative strategies with the potential to reverse the developmental switch from fetal-to-adult hemoglobin, e.g. BCL11A knockdown, IGF2BP1 or LIN28B overexpression, also need to be explored and optimized as future treatment options.

CRediT authorship contribution statement

Alberto Daniel-Moreno: Investigation, Formal analysis, Writing - original draft. **Andrés Lamsfus-Calle:** Investigation, Formal analysis, Writing - original draft. **Andrew Wilber:** Resources, Writing - original draft. **Christopher B. Chambers:** Resources. **Ian Johnston:** Resources. **Justin S. Antony:** Resources, Writing - original draft. **Thomas Epting:** Investigation. **Rupert Handgretinger:** Resources. **Markus Mezger:** Resources, Writing - original draft, Conceptualization, Methodology, Supervision.

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Declaration of competing interest

None of the authors state any conflicts of interest.

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PAPER 2

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Comparative targeting analysis of *KLF1*, *BCL11A*, and *HBG1/2* in CD34$^+$ HSPCs by CRISPR/Cas9 for the induction of fetal hemoglobin

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β-hemoglobinopathies are caused by abnormal or absent production of hemoglobin in the blood due to mutations in the *β-globin* gene (*HBB*). Imbalanced expression of adult hemoglobin (HbA) induces strong anemia in patients suffering from the disease. However, individuals with natural-occurring mutations in the *HBB* cluster or related genes, compensate this disparity through *γ-globin* expression and subsequent fetal hemoglobin (HbF) production. Several preclinical and clinical studies have been performed in order to induce HbF by knocking-down genes involved in HbF repression (*KLF1* and *BCL11A*) or disrupting the binding sites of several transcription factors in the *γ-globin* gene (*HBG1/2*). In this study, we thoroughly compared the different CRISPR/Cas9 gene-disruption strategies by gene editing analysis and assessed their safety profile by RNA-seq and GUIDE-seq. All approaches reached therapeutic levels of HbF after gene editing and showed similar gene expression to the control sample, while no significant off-targets were detected by GUIDE-seq. Likewise, all three gene editing platforms were established in the GMP-grade CliniMACS Prodigy, achieving similar outcome to preclinical devices. Based on this gene editing comparative analysis, we concluded that *BCL11A* is the most clinically relevant approach while *HBG1/2* could represent a promising alternative for the treatment of β-hemoglobinopathies.

Sickle cell disease (SCD) and β-thalassemia, commonly known as β-hemoglobinopathies, are inherited blood disorders caused by mutations in the human *β-globin* gene (*HBB*)[1–4]. In healthy condition, adult human hemoglobin (HbA) consists of 2 α and 2 β chains, whereas fetal hemoglobin (HbF) expressed in early gestation comprises 2 α chains and 2 γ chains. Notably, HbF was observed to bind oxygen with greater affinity than HbA, being functional when reactivated in adults[3,5,6].

Recent studies have generated substantial experimental evidence that HbF reactivation by gene disruption of specific transcription factors and regulators could provide a therapeutic benefit for β-hemoglobinopathies[7]. It has long been appreciated that *KLF1* and *BCL11A* are key regulators involved in the process of *γ-* to *β-globin* switching and the repression of these genes leads to HbF resurgence[6–11]. Interestingly, healthy individuals with a benign genetic condition namely hereditary persistence of fetal hemoglobin (HPFH) were observed to exhibit persistent production of functional HbF[4,10,12,13]. HPFH is caused by large deletions in the *δ-* and *β-globin* genes, or point mutations in the *γ-globin* promoter and *γ-globin* repressors, such as *KLF1* and *BCL11A*[5]. Importantly,

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co-inheritance of HPFH with β-thalassemia was noticed to alleviate the clinical manifestations of the latter[14]. Therefore, to attain a clinical profit for β-hemoglobinopathies, several attempts were made to re-establish the expression of HbF either by lentiviral transfer of the *γ-globin* gene or by CRISPR/Cas9-mediated gene disruption of specific regulators[14–19].

Though lentiviral gene transfer of *β-globin* exhibited positive effects in treated β-thalassemia patients[20], the high volume of semi-random integration sites by lentivirus and the transactivation of the proto-oncogene *HMGA2* raised major safety concerns for this approach[21,22]. Due to the afore-mentioned reasons, CRISPR/Cas9-mediated gene disruption of specific regulators to re-express HbF is a promising alternative[7]. Thus, several studies have targeted various genetic regulators by CRISPR/Cas9 to reactivate HbF expression, resulting in a profound effect after genetic interference of *BCL11A*, *KLF1*, and *HBG1/2* promoters[14,17,23]. Nevertheless, no head-to-head comparison has been performed earlier in $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) for these three targets to assess their therapeutic potential for β-hemoglobinopathies by up-regulating HbF without raising safety issues. Therefore, in the present study, we compared all these targets in parallel for their impact on HbF resurgence and performed safety measurements by molecular analyses in order to select the best candidate for clinical translation.

Results

Gene editing. First, we established the optimal electroporation parameters to transfect exogenous mRNA in K-562 cells and $CD34^+$ HSPCs utilizing a DsRed reporter construct. Best electroporation settings were chosen for both K-562 cells (1450 V, 10 ms, 3 pulses) and $CD34^+$ HSPCs (1650 V, 10 ms, 3 pulses) where high transfection efficiency and viability were achieved (>90%; Supplementary Fig. S1a). Further, to validate sgRNAs we electroporated K-562 cells with recombinant pX-330 vector targeting *KLF1*, *BCL11A*, and *HBG1/2* genomic regions. Each locus was targeted with two different sgRNAs (Fig. 1a) and gene-targeting efficacy was assessed by T7 endonuclease-I (T7E1) assay. Varying levels of mean indel frequencies were observed for *KLF1* (T1: 36.2 ± 6.5%; T2: 34.9 ± 5.1%), *BCL11A* (T1: 22.2 ± 2.2%; T2: 17.0 ± 1.4%), and *HBG1/2* (T1: 30.9 ± 14.4%; T2: 21.1 ± 6.0%; Supplementary Fig. S1b).

Next, to assess CRISPR/Cas9-mediated HbF up-regulation strategy with bone marrow-derived $CD34^+$ HSPCs, we utilized Cas9 RNP with chemically modified sgRNAs instead of pX-330 vectors as the latter was shown to be less effective. We tested several molar ratios of sgRNA:Cas9 and found that a molar ratio of 2:1 was more effective in generating on-target indels (data not shown). Interestingly, elevated levels of gene editing were noticed in ICE analysis for all the tested sgRNAs (range of 63–91%; Fig. 1b), except for *HBG1/2* T1 where lower indels (54.7 ± 10.1%) were spotted. Later, gene-edited $CD34^+$ HSPCs were differentiated towards erythroid lineage for 21 days, confirmed with specific erythroid markers expression (CD71 and CD235a), and molecularly analyzed for HbF expression. None of the treated samples showed proliferation or impaired erythroid differentiation (Fig. 1c).

Transcript analysis of *γ-globin*, *KLF1* and *BCL11A*. Our qRT-PCR analyses showed that, compared to control samples, HbF up-regulation was noted in *KLF1*-edited samples (>5 fold) and *BCL11A* (>4 fold) for both tested targets. Notably, elevated *γ-globin* transcripts were observed in *HBG1/2* gene-targeted samples (>6.5 fold; Fig. 2a). Also, *KLF1* and *BCL11A* transcripts were quantitatively determined by qRT-PCR, showing a marked down-regulation of KLF1 transcripts (*KLF1* T1: 4 fold, *KLF1* T2: 2 fold; Fig. 2b) with a characterized subsequent *BCL11A* down-regulation (~2 fold; Fig. 2c) after *KLF1* gene disruption. Following the same pattern, a 2-fold down-regulation of *BCL11A* transcripts was observed only in *BCL11A* T2 when the enhancer of this gene was genetically disrupted (Fig. 2c).

HbF quantification by intracellular staining and HPLC. In order to assess HbF expression at protein level in gene-edited $CD34^+$ HSPCs, cells were analyzed by HPLC-mediated hemoglobin electrophoresis and intracellular staining. Notably, hemoglobin electrophoresis revealed that all the edited samples induced higher HbF levels in comparison to the controls (Fig. 2d), while *BCL11A* T2 and *HBG1/2* T2 achieved the most pronounced HbF levels up to 39.5 and 41.9%, respectively (Fig. 2e). Moreover, *in vitro* differentiation of non-edited $CD34^+$ HSPCs into erythrocyte precursors produced similar amounts of HbF as the standard human controls (Fig. 2e). After flow cytometry analysis, we found elevated numbers of HbF^+ $CD34^+$ HSPCs for all the tested target genes (range 50.8–91.7%) where the strongest effect was noted for *HBG1/2* T2 (Fig. 2f and Supplementary Fig. S1c). Of note, hemoglobin electrophoresis results strongly correlated with HbF intracellular staining (Spearman's rho coefficient: $\rho = 0.799$, $p < 0.0001$; Fig. 2g).

Expression pattern analysis by RNA-seq. Since KLF1 and BCL11A are transcription factors involved in several signaling pathways, RNA-seq analysis was performed to determine the safety of each gene editing profile. We accounted for relatively similar ICE scores (KLF1 T1: 77 ± 8.9%; BLC11A T2: 86 ± 2.5%; HBG1/2 T2:84.7 ± 9.3%) and HbF levels (KLF1 T1: 23.2 ± 3%; BLC11A T2: 34.3 ± 0.7%; HBG1/2 T2: 39.6 ± 0.2%) to choose the samples for RNA-seq. We noticed that the expression patterns of all three gene-disrupted $CD34^+$ HSPC treatments showed high similarity rates to the control sample (from 92% to 99%). Importantly, a mean value of 1017 genes showed dissimilarity in the BCL11A sample, while KLF1 and HBG1/2 gene disruption led to 2327 and 2129 impaired genes, respectively (Fig. 3a). However, when considering the common differentially expressed genes across all replicates, a clear pattern was observed, where KLF1 resulted in 502 impaired genes, whereas BCL11A and HBG1/2 accounted for 10 and 82 dysregulated genes, respectively (Fig. 3b,c). From those genes, a deeper screening for dysregulated oncogenes or tumor suppressor genes was performed to assess the safety profile of each gene editing approach. These results showed the presence of several disturbed genes involved in cell cycle (*E2F2*, *E2F7*), ERK/MAPK and p53 signaling (*DUSP2*, *PPP2R5B*, *TRIM29*), apoptosis (*DAPK1*), and

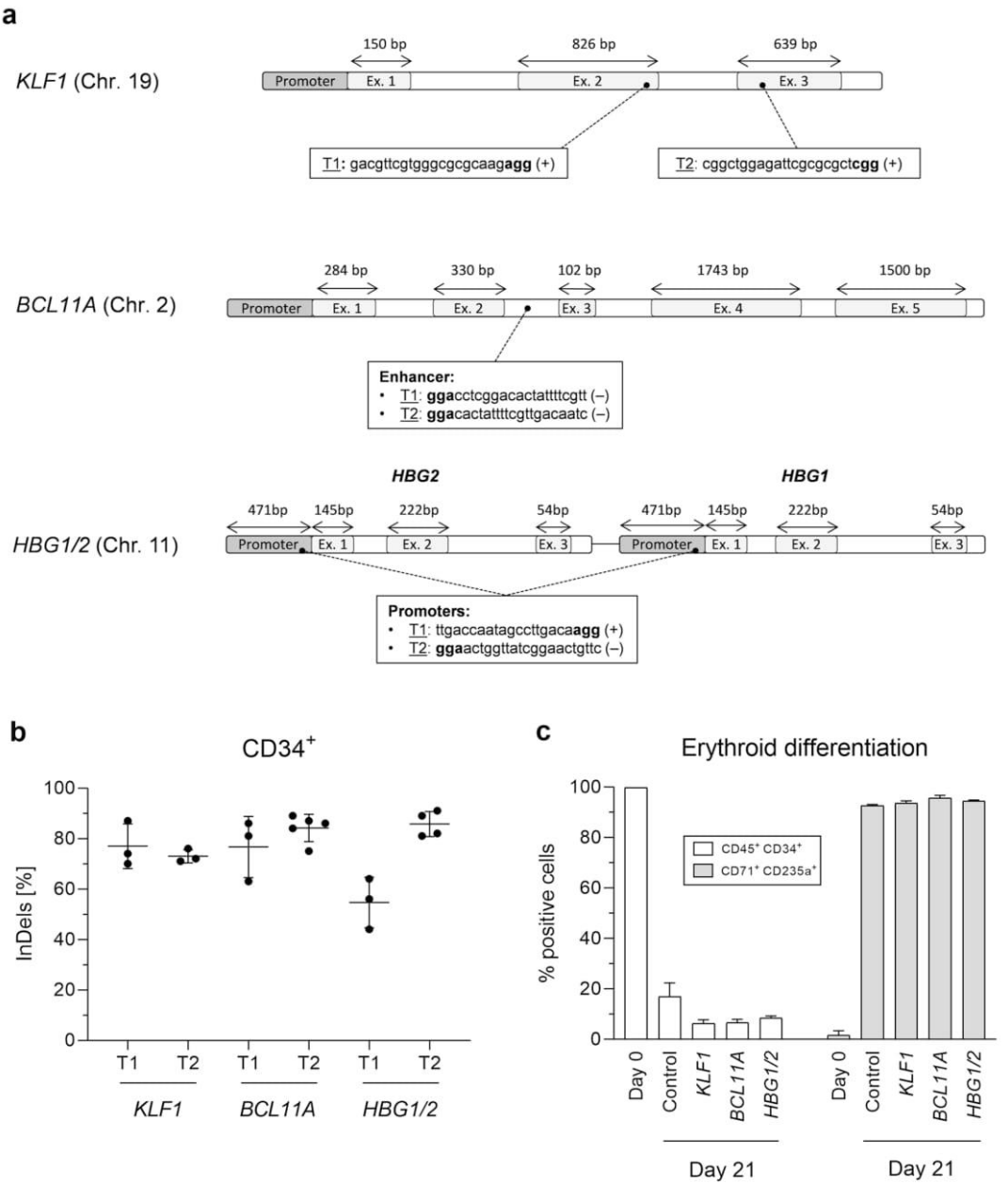

Figure 1. Gene editing of human CD34$^+$ HSPCs. (**a**) Schematic representation of the genome-editing strategies and target sequences for each sgRNA. *KLF1*: targets 1 (T1) and 2 (T2) are localized in the second and third exon, respectively; *BCL11A*: both targets are situated in the enhancer region of the second intron; *HBG1/2*: both sgRNAs target *HBG1 and HBG2* promoters. (**b**) Indel percentage in CD34$^+$ HSPCs measured by ICE analysis after electroporation of Cas9 RNP and chemically-modified sgRNAs for T1 and T2 in *KLF1*, *BCL11A*, and *HBG1/2*. (**c**) Flow cytometry analysis after immunostaining of CD34$^+$ HSPCs to follow differentiation into erythrocytes precursors: percentage of double positive cells for CD34$^+$ (hematopoietic stem cells) and CD45$^+$ (leukocytes), and for CD71 (erythroid precursors) and CD235a (erythrocyte) on day 0 and 21, respectively.

immune pathways (*BCL6*) for *KLF1*- and *HBG1/2*-treated samples. In contrast, neither oncogenes nor tumor suppressor genes were found for BCL11A samples. Noteworthy, all dysregulated genes for *BCL11A*-treated samples were found in KLF1 expression panel, except for *ALB*, *IL18R*, and *MMP25*.

We also assessed the expression values indicated as clusters per million (cpm) for genes of interest involved in hematopoiesis (Fig. 3d). Predictably, adult-to-fetal hemoglobin switching was noticed in our RNA-seq results, where *HBG1/HBG2* up-regulation was noted in all treated samples. Furthermore, up-regulation of *HBA1/HBA2* was observed in those treatments where high levels of *γ-globin* transcripts were perceived. Alternatively, no major changes were detected in *HBB* expression (Fig. 3d). We also screened for important genes regulated by KLF1 and BCL11A with no dissimilarities found when compared to the control, except for *ITGA2B* (Fig. 3e,f).

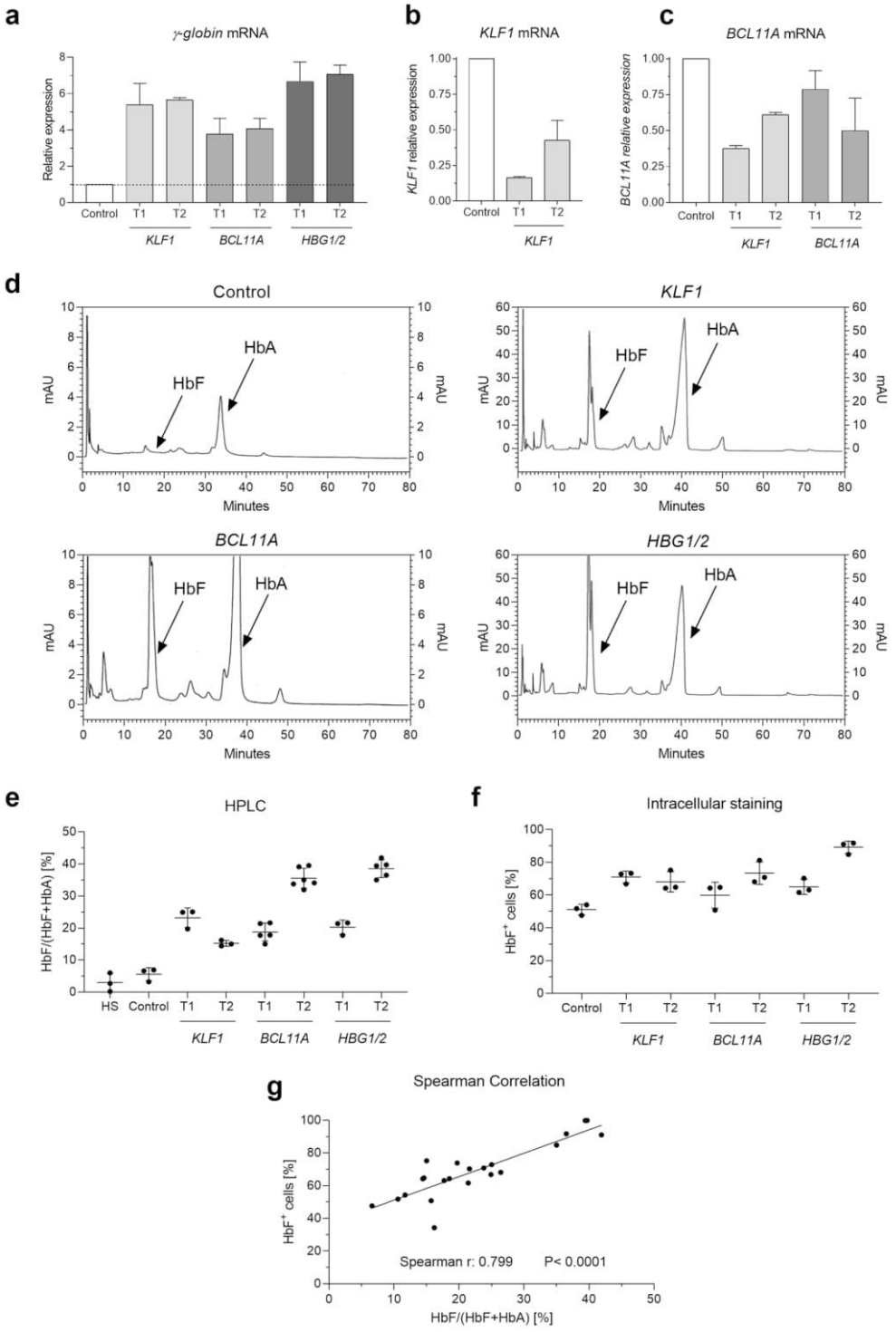

Figure 2. mRNA and protein analysis of gene-edited CD34$^+$ HSPCs. (**a**) *γ-globin* expression analysis by qRT-PCR on day 21. (**b**) Decline of *KLF1* transcripts after treatment with *KLF1* T1 and T2. (**c**) Down-regulation of *BCL11A* transcripts in *KLF1* and *BCL11A* samples. (**d**) HPLC histograms of control, *KLF1*, *BCL11A*, and *HBG1/2* samples. (**e**) Percentage of HbF for human standard (HS), control, and the different gene editing treatments by HPLC on day 21. (**f**) HbF Intracellular staining in differentiated CD34$^+$ HSPCs on day 21. (**g**) Spearman correlation of HPLC and HbF intracellular staining.

Off-target analysis by GUIDE-seq. Most importantly, GUIDE-seq analysis was performed after assessing the optimal dsODN concentration to achieve the highest integration rate with reduced cytotoxicity (Fig. 4a,b). Using 25 pmol of dsODN, we obtained adequate integration index for the most efficient targets (*KLF1* T1: 8.55 ± 5.6%, *BCL11A* T2: 7.45 ± 1.8%; *HBG1/2* T2: 5.95 ± 1.5%; Fig. 4c). We also included a sgRNA that targeted

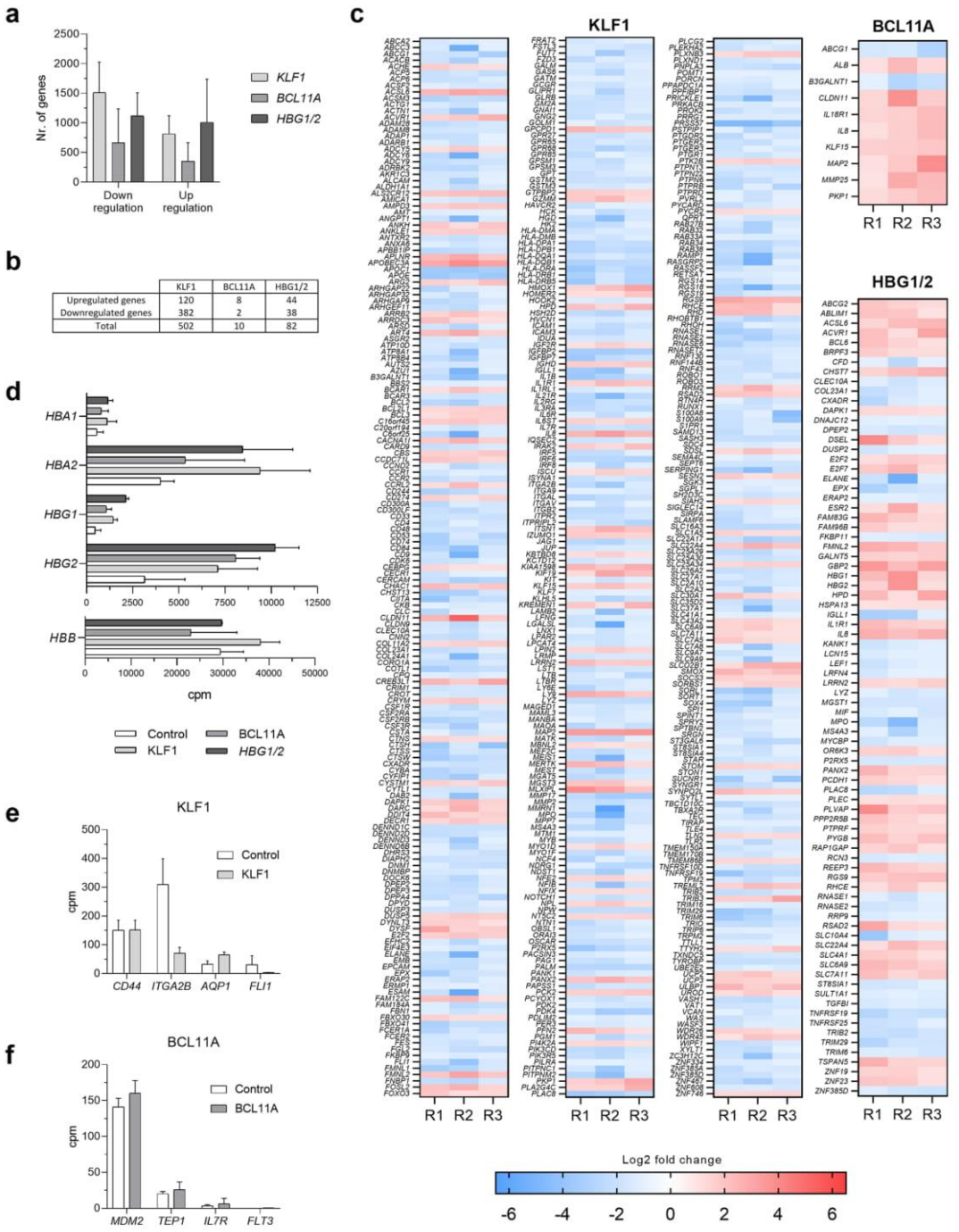

Figure 3. RNA-seq analysis. (**a**) Number of genes down- and up-regulated after targeting *KLF1*, *BCL11A*, and *HBG1/2*. (**b**) Number of common dysregulated genes for the three individual experiments. (**c**) Heatmap showing the Log2 fold change of the common differentially expressed genes after gene editing in *KLF1*, *BCL11A*, and *HBG1/2*. R1, R2, and R3 represent the three performed replicates. Dysregulated genes are depicted in a blue-to-red color gradient, where down-regulated genes are shown in blue and up-regulated genes in red. (**d**) Number of reads indicated as clusters per million (cpm) for hemoglobin genes in edited samples relative to the control sample. (**e**,**f**) Cpm values for the most relevant genes regulated by KLF1 (**e**) and BCL11A (**f**).

the promoter region of *HBB* (*BetaPr*) for which numerous *in silico* off-targets were predicted (Table S3, Fig. 4c). This way, our GUIDE-seq analysis resulted in no detectable off-targets, except for HBG1/2 and BetaPr, where 1 and 39 off-targets were determined (Fig. 4d–g). Interestingly, two on-targets were identified with low number of reads for *HBG1/2* T2 after GUIDE-seq (Fig. 4f). We hypothesized that, after gene editing, a 5-kb deletion between *HBG1* and *HBG2* could restrain GUIDE-seq results. With this aim in mind, we designed ddPCR oligonucleotides within the intergenic region of *HBG2* and *HBG1* (Table S2) and observed up to 43% of 5-kb excision in gene-edited samples after *HBG1/2* T2 transfection utilizing our electroporation devices (Fig. 4h).

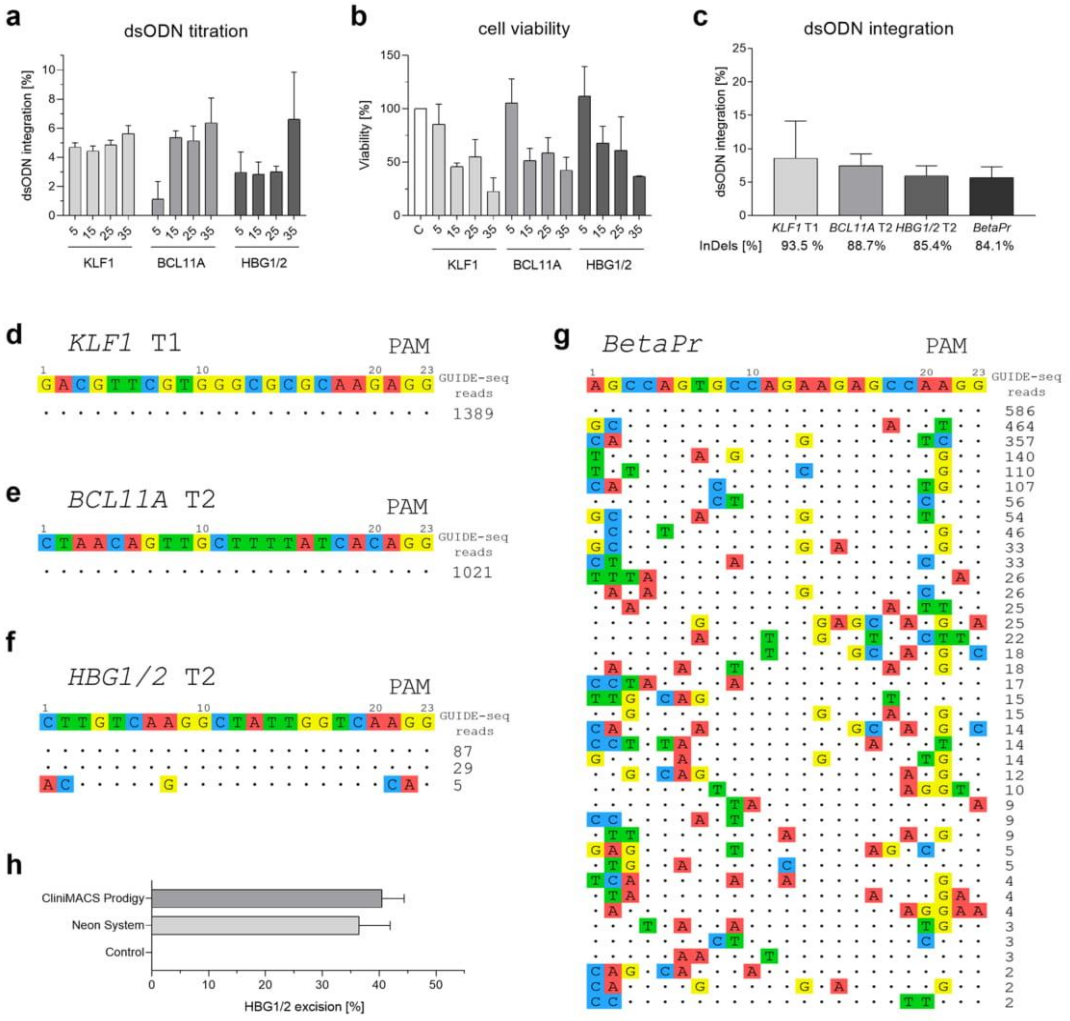

Figure 4. GUIDE-seq analysis. (**a**) dsODN titration in K-562 for GUIDE-seq optimization. The percentage of dsODN integration for each specific dsODN count of particle (5, 15, 25, and 35 pmol) is depicted. (**b**) Cell viability determined by cell counting on day 5 post-electroporation. (**c**) Percentage of dsODN integration at the target sites of interest utilizing 25 pmol of dsODN. Mean indel rates determined by ICE analysis are shown for each sgRNA. (**d**–**g**) Off-target illustration for *KLF1* T1 (**d**), *BCL11A* T2 (**e**), *HBG1/2* T2 (**f**), and *BetaPr* (**g**). Target sequences are shown on the top; matches are represented with dots, while mismatches are highlighted underneath. GUIDE-seq reads are shown on the right of each on-target/off-target site. (**h**) Percentage of $CD34^+$ HSPCs with excised intergenic region of *HBG1/2* (5-Kb) after gene editing by Neon System and CliniMACS Prodigy.

Translation of the gene editing platform to CliniMACS Prodigy. Due to the promising outcome of all gene editing strategies, especially HBG1/2 and BCL11A, the next step was to translate this platform to the GMP-grade CliniMACS Prodigy. As previously performed in the Neon System, the best electroporation setting was selected based on DsRed mRNA transfection efficiency in $CD34^+$ HSPCs, where the pulse mode 'Square', 600 V/100 µs first pulse, and 300 V/2 ms second pulse, was the setup combination that attained the highest percentage of $DsRed^+$ cells (78.1 ± 7.2%) and cell viability (88.4 ± 13%; Supplementary Fig. S1d). Subsequently, *KLF1* T1, *BCL11A* T2 and *HBG1/2* T2 sgRNAs were transfected with the above-explained CliniMACS Prodigy settings, noticing excellent gene editing performance comparable to Neon Transfection System (range of 54–86%; Fig. 5a). Likewise, after erythroid differentiation for 21 days, HSPCs showed similar HbF resurgence by HPLC compared to Neon-transfected cells (*KLF1* T1: 19.7 ± 2.8%, *BCL11A* T2: 40.67 ± 7.8%; *HBG1/2* T2: 41.7 ± 3.6%; Fig. 5b), indicating once again the potential of these approaches for clinical translation.

Discussion

Earlier studies relied on the fact that β-thalassemia patients with natural mutations in genetic regulators involved in the fetal-to-adult hemoglobin switching, reactivate the expression of HbF, ameliorating the disease symptoms[24]. Furthermore, mutations or epigenetic modifications in the distal promoter of *HBG1/2* can induce a similar outcome, as observed in the rare benign HPFH[14,25–28]. Above-mentioned genetic variants trigger a dynamic process, in which the HbF levels increase whereas HbA decreases. When HbF levels are above 20%, it

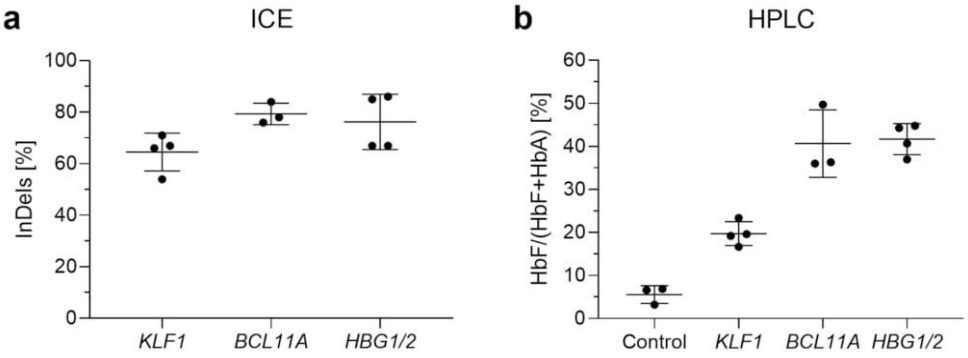

Figure 5. Translation of the CRISPR/Cas9 gene editing platform to the CliniMACS Prodigy GMP-grade device. (**a**) Percentage of insertions and deletions (InDels) detected by ICE analysis. (**b**) HbF levels (%) in edited human $CD34^+$ HSPCs measured by HPLC.

is demonstrated to be therapeutically beneficial, especially in SCD patients due to the reduction of hemoglobin polymerization[24,26,29,30]. Therefore, owing to its great clinical impact, several investigations were attempted to induce HbF, either by gene disruption or by gene transfer[10,14,17,31]. Though gene disruption is a promising option, no comparative analyses were performed to date for these genetic loci to choose the best approach for possible clinical applications. Hence, we chose *KLF1*, *BCL11A*, and *HBG1/2* promoters as the three prominent gene regulators of 'Globin Switching' and performed one-to-one comparison for HbF resurgence efficacy and safety using CRISPR/Cas9-mediated gene disruption.

Several mutations have been described for *KLF1* in HPFH patients which generates variable levels of HbF (3–30.9%)[32]. In particular, our study is the first lentiviral-free attempt to use CRISPR/Cas9 to target exon 2 and 3 of *KLF1* in $CD34^+$ HSPCs. We successfully induced high indel rates in both exons, down-regulation of *KLF1* and *BCL11A* transcripts, elevated *γ-globin* mRNA expression, and significant HbF levels comparable to HPFH mutations such as K288X and S270X[32,33]. Although *KLF1* gene disruption resulted in increased HbF (up to 25%) and no off-targets were detected by GUIDE-seq, the negative effect of *KLF1* knock-down was observed in our RNA-seq analysis, where several genes with different biological functions were dysregulated compared to the non-edited sample, which could raise major safety concerns. Previous studies have documented that impaired expression of *KLF1* might affect the expression of genes involved in cell-cell interaction (*CD44* and *ITGA2B*), microcytosis (*AQP1*) and cancer (*FLI-1*)[32,34]. Nevertheless, in our RNA-seq analysis no differences were perceived for those genes, except for *ITGA2B*, which its down-regulation is associated to Glanzmann thrombasthenia, a bleeding disorder characterized by a lack of platelets aggregation[35].

Another important transcription factor implicated in γ- to *β-globin* switching is BCL11A[24,36–39], which has become a promising target for HbF resurgence. Likewise, long-term engraftment and normal hematopoiesis could be attained down-regulating *BCL11A* by targeting its enhancer (GATAA box)[16,23,40], whilst impaired erythropoiesis and limited engraftment have been detected in complete knock-down of *BCL11A* gene[23,30,36]. Therefore, in our study, we selected two sgRNAs matching the GATAA box and achieved high level of genetic disruption with excellent HbF increment up to ~40% for sgRNA T2. Due to the essential role of *BCL11A* in globin switch, regulation of proto-oncogenes (*MDM2* and *TEP1*), and genes involved in immune cell development (*IL7-R* and *FLT3*)[37,41,42], we performed RNA-seq analyses for *BCL11A* T2 gene-edited samples. BCL11A gene disruption approach resulted in normal expression of the above-mentioned genes and led to the lowest gene expression variation of all different strategies. This low variation was expected since we are targeting the enhancer box located in the second intron of the *BCL11A* gene, and therefore, its expression declines but there is no total suppression which could affect hematopoiesis. Indeed, dysregulated genes for the BCL11A approach could also be found in KLF1 treated samples, where their expression was highly impaired since KLF1 knock-out induces stronger down-regulation of BCL11A than targeting directly its enhancer. Also, no off-targets were detected for *BCL11A* T2, hence this strategy showed high level of safety profile. In a complementary manner, a recent study performed by the group of Wu *et al.* confirmed the safety and efficacy of this sgRNA, which together with our findings could contribute in the current clinical trial for the treatment of β-thalassemia (NCT03655678) and SCD (NCT03745287)[43].

The gamma chain of HbF is encoded by the *HBG1/2* genes and transcriptionally controlled by several elements in the *β-globin* gene cluster. Interestingly, the elevation of HbF by natural-occurring deletions in the *β-globin* cluster, varying from 13 bp, 7.2 kb (Corfu), 12.9 kb (Sicilian) and 13.6 kb, have been identified in HPFH individuals[7,14,26–28]. Furthermore, previous studies using ChIP-seq and CUT&RUN analyses elucidated the consensus binding site of BCL11A (TGACCA) repressor, situated upstream of the transcription start site of the *γ-globin* gene (−115 bp)[28,44]. Therefore, similarly to what was previously performed by Traxler *et al.*, we utilized two sgRNAs to target the binding site of BCL11A, mimicking the 13-bp natural-occurring deletion described previously in HPFH individuals. This 13-bp deletion has been described before after gene editing, and might occur via microhomology-mediated end-joining (MMEJ) due to short homology sequences flanking the target site that can be observed in our ICE analysis results (Supplementary Fig. S1e)[14,28,44,45] Notably, *HBG1/2* T2 induced higher *γ-globin* and HbF levels compared to KLF1 and BCL11A approaches. Nonetheless, RNA-seq analysis showed that HBG1/2 approach has a better safety profile than KLF1 but lower than BCL11A. After screening for possible

oncogenes or tumor suppressor genes, we detected potential genes involved in oncogenesis as also observed in RNA-seq for *KLF1*-treated samples. However, to definitely confirm that those genes will not promote cancer development, gene dysregulation should also be assessed by other methods such as qRT-PCR, clonal expansion assays, and *in vivo* models. In fact, a recent publication has reported long-term engraftment of *HBG1/2*-edited HSPCs in rhesus primates and no toxic effects were found in mature blood lineages after a follow-up of 1.5 years[46].

Since *HBG1/2* consists of two homologous subunits (*HBG1* and *HBG2*) with just some differences in the upstream region of the distal promoter, this sgRNA cleaves twice in the genome. In the present study, we confirmed that, when the sgRNA cuts simultaneously in both subunits, a 5-kb fragment is excised in high frequencies (up to 43%). Thus, the detection of the on-target reads during GUIDE-seq analysis is hindered and can only be identified when the DSB occurs either in *HBG1* or *HBG2*. These two on-targets were found in our GUIDE-seq results at low number of reads due to the above-mentioned reasons. Also, one off-target at low frequency (5 reads) was detected by GUIDE-seq for HBG1/2 sgRNA. BLAST analysis matched the off-target sequence with an intergenic region (NC_000002.12) located in chromosome 2 downstream of the ATP synthase F(0) complex subunit C3 gene and upstream of the endoplasmic reticulum junction formation factor gene. This region is associated with a long-non coding RNA (lncRNA), which might have several biological roles including epigenetic regulation[47]. This could elucidate the variation found in the transcript expression profile after gene editing, but the low number of detected reads and the uncertain function of this lncRNA lead to the inability to draw final conclusions.

To evaluate whether these approaches can be transferred to a GMP-grade electroporation device, CliniMACS Prodigy was utilized for the best sgRNAs of our study. The system is noteworthy due to its GMP-compatibility and offers automated electroporation of CRISPR components, cell culture, and direct application into humans with a clinical grade quality. Most importantly, similar results to the Neon Transfection System were attained, demonstrating the clinical potential of these gene therapy approaches. Nonetheless, *in vivo* experiments must be performed to evaluate the engraftment capacity of gene-edited cells.

Based on this thorough comparative analysis of different HbF-inducing gene editing strategies, we concluded that *KLF1* is not a suitable approach for clinical translation due to impaired gene expression after gene editing. On the contrary, *BCL11A* is a great candidate for the treatment of β-hemoglobinopathies, with high HbF resurgence, no off-targets, and unaltered gene expression. In addition, the above-explained *HBG1/2* approach also yielded clinically relevant levels of HbF with mediocre safety profile, and thus, after further investigations, this strategy could be considered a promising alternative gene therapy for β-hemoglobinopathies.

Materials and Methods

Ethics approval. Human mobilized peripheral blood $CD34^+$ HSPCs from individual donors were adquired using protocols approved by the local ethics committee/institutional review board (IRB; ethic number: 829/2016BO2), University Children's Hospital. Written informed consents were obtained from all the participants in the study. All methods were carried out in accordance with relevant guidelines and regulations.

Cell culture. K-562 cells were acquired from Sigma-Aldrich and cultured at 37 °C with 5% CO_2 in RPMI (Biochrom) supplemented with 10% FBS (Gibco), 1% L-glutamine (Biochrom), and 1% Penicillin/Streptomycin (Biochrom).

Immunomagnetic enrichment of HSPCs was performed using magnetic-activated cell sorting system (CliniMACS System, Miltenyi Biotec), according to the manufacturer's instructions. $CD34^+$ HSPCs were then cultured at 37 °C with 5% CO_2 in StemMACS HSC Expansion Media (Miltenyi Biotec) supplemented with human cytokines (Miltenyi Biotec): SCF (100 ng/ml), TPO (20 ng/ml), and Flt3-L (100 ng/ml).

DsRed mRNA *in vitro* synthesis. Before mRNA transcription, 20 µg $pCS2^+$ DsRed was digested by XbaI (New England Biolabs) for 1 hour at 37 °C. Linearized plasmid was then purified using QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's instructions. Finally, DsRed mRNA *in vitro* transcription, poly(A) tailing, and mRNA clean up were performed in accordance with the manufacturer's protocols of mMESSAGE mMACHINE SP6 Transcription kit (Life Technologies), Poly (A) tailing kit (Ambion), and MEGAclear kit (Ambion), respectively.

Cloning of oligonucleotides in pX330. sgRNAs for each targeted gene were designed (Fig. 1a) and oligonucleotides cloned into the chimeric pX-330 vector (Addgene #42230; Table S1). All constructs were confirmed by Sanger sequencing. The amplification of the vectors was performed in DH5α competent cells (Sigma-Aldrich) and the purification of the plasmids by means of standard plasmid isolation kits (Peqlab Biotechnologie).

***In vitro* differentiation of $CD34^+$ HSPCs into erythrocyte precursors.** $CD34^+$ HSPCs were cultured according to the three-phase differentiation protocol from Dever *et al.*[48]. Subsequent erythroid differentiation and maturation were monitored by flow cytometry (BD FACSCalibur) using FITC-conjugated anti-CD34 (Miltenyi Biotec), PE-conjugated anti-CD235a (Miltenyi Biotec), PerCP-conjugated anti-CD45 (Miltenyi Biotec) and APC-conjugated anti-CD71 (Miltenyi Biotec) at two different time points, day 0 and day 21.

K-562 and $CD34^+$ HSPCs cell transfection. To transfect 1×10^6 K-562 cells using the 100 µl Neon transfection kit (Thermo Fisher Scientific), 200 ng of recombinant pX-330 were utilized. Electroporation settings for this cell line were 1,450 V, 10 ms, and 3 pulses. T7 endonuclease-I (T7E1) assay was performed on day 5 after electroporation.

Chemically modified sgRNAs (Synthego; Table S1) and Cas9 ribonucleoprotein (RNP; IDT) were incubated at a molar ratio of 1:2 (45 pmol to 90 pmol) at room temperature for 15 minutes. After complex formation, 1×10^5 $CD34^+$ HSPCs were transfected using the 10 µl Neon transfection kit (Thermo Fisher Scientific) or the Test Cuvette Adaptor (TCA; Miltenyi Biotec) with the following electroporation settings: 1,650 V, 10 ms, 3 pulses (Neon System) or Square mode, 600 V/100 µs, 300 V/2 ms (CliniMACS Prodigy). Subsequently, cells were transferred to stem cell differentiation culture media. On day 5 post-electroporation, cells were harvested for further DNA isolation, T7E assay, and ICE analysis. On day 21, erythrocyte precursors were collected for RNA isolation, qRT-PCR, and HbF quantification. Primer sequences are listed in Table S2.

T7E1 assay and ICE analysis. Genomic DNA was isolated 5 days post-transfection using NucleoSpin Tissue Kit following the manufacturer's instructions (MACHERY-NAGEL). The target regions were amplified using the GoTaq Colorless Master Mix (Promega). Primers for each target region are listed in Table S2. PCR products were purified by utilizing QIAquick PCR Purification Kit (QIAGEN) and 1 µg of PCR product was used for T7E1 assay in accordance with the manufacturer's protocol (New England Biolabs). Readouts of the assay were determined on a 2% agarose gel and analyzed by ImageJ (Fiji software). Indel rates for each target were evaluated by the web tool 'ICE' (Inference of CRISPR Edits; https://ice.synthego.com/) after Sanger-sequencing of the purified PCR products.

RNA isolation, cDNA synthesis, and qRT-PCR assays. $CD34^+$ HSPCs were harvested on day 21. Total RNA was isolated by using the RNeasy Mini Kit and QiaShredder spin columns (QIAGEN), in accordance with the manufacturer's protocol. RNA at a concentration of 500 ng was used for cDNA synthesis with the QuantiTect Reverse Transcription Kit (QIAGEN). Amplification and quantification of cDNA were performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). PCR was run utilizing the KAPA SYBR FAST 2x MasterMix (KAPA Biosystems). Primer sequences are listed in Table S2. Results were normalized against the expression of the housekeeping gene *β2-microglobulin* (*β2M*). The crossing point (CP) values for the unknown samples were evaluated with the formula 2 (CP *β2M* - CP target gene)[49].

HbF quantification. For high-performance liquid chromatography (HPLC), frozen cell pellets were lysed in 200 µl deionized sterile water and ultrasonicated for 5 minutes. Cell debris was removed by centrifugation at 13,000 g. The supernatant was then concentrated to a final volume of 30 µl using a Nanosep molecular filter (PALL Corporation) with a 10 kDa membrane by centrifugation at 13,000 g. Hemoglobin species from cell lysates were separated using a PolyCAT A cation exchanger column (PolyLC Inc). The analysis was performed on an elite-LaChrom HPLC-system (Merck-Hitachi) using a gradient elution mode with a bis-tris buffer system (buffer A: bis-tris 20 mM, NH4-acetate 13 mM, KCN 1 mM and buffer B: bis-tris 20 mM, Na-acetate 38 mM, KCN 1 mM, NaCl 200 mM). Hemoglobin proteins were detected by absorbance measurements at 415 nm. Intracellular HbF was determined 21 days after erythroid differentiation utilizing the kit 'Monoclonal antibodies directed to HbF' (Life Technologies).

RNA-seq. Total RNA from edited HSPCs was isolated after 21 days of erythrocyte differentiation by RNeasy Mini Kit (QIAGEN). RNA quality was determined by measuring 260/280 and 230/260 absorbance ratios on a spectrophotometer (Nanodrop ND-1000, Peqlab Biotechnologie) and the RNA concentration using the Qubit Fluorometric Quantitation and RNA Broad-Range Assay (Thermo Fisher Scientific). The RNA Integrity Number (RIN) was determined using the Lab-on-a-Chip-System Bio-analyzer 2100 and the RNA 6000 Nano assay (Agilent).

For library preparation, mRNA fraction was enriched using polyA capture from 100 ng of total RNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). Next, mRNA libraries were prepared using the NEB Next Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs) according to the manufacturer's instructions. Then, the library molarity was determined by measuring the library size (approximately 400 bp) using the Bioanalyzer 2100 with the High Sensitivity DNA assay, and the library concentration (approximately 10 ng/µl) using Qubit Fluorometric Quantitation and dsDNA High sensitivity assay (Thermo Fisher Scientific). For the first experiment, the libraries were denaturized according to the manufacturer's instructions, diluted to 1.2 pM and sequenced as paired-end 75 bp reads on an Illumina NextSeq500 (Illumina) with a sequencing depth of >22 million clusters per sample. For the second experiment, the libraries were denatured, diluted to 270 pM and sequenced as paired-end 50 bp reads on an Illumina NovaSeq6000 (Illumina) with a sequencing depth of approximately 20 million clusters per sample.

Read quality of RNA-seq data in FASTQ files was assessed using ngs-bits (v.2019_03) to identify sequencing cycles with low average quality, adaptor contamination, or repetitive sequences from PCR amplification. Reads were aligned using STAR v2.7.3a[50] to the grch37 and the alignment quality was analyzed using ngs-bits (v.2019_11). Normalized read counts for all genes were obtained using Subread (v2.0.0) and edgeR (v3.26.8). The distribution of logarithmized cpm-normalized expression values showed similar characteristics over all samples. Based on the filtered data set, samples were investigated with respect to their pairwise similarity. Spearman's rank correlation coefficient was calculated for each pair of samples. A hierarchical clustering was performed on the resulting similarity values. Differential gene expression analysis was conducted based on the filtered gene expression data set and a statistical model incorporating the group property of samples was tested by fitting a negative binomial distribution using a generalized linear model (GLM) approach. For the analysis, genes were classified when their gene expression fold change (log2 fold change) were equal or greater than +1, and equal or minor than −1. Only genes that were impaired in the three independent experiments were considered. Finally, a screening for oncogenes or suppressor genes was performed using Ingenuity Pathway Analysis (IPA; QIAGEN) to determine

the safety of each gene therapy strategy. FASTQ files for all replicates were uploaded to Sequence Read Archive (SRA) at NCBI website (http://www.ncbi.nlm.nih.gov/bioproject/606664).

DNA library preparation. DNA library preparation for GUIDE-seq analysis was performed as described earlier in K-562 cells[51,52]. The optimal dsODN concentration based on integration efficiency by ICE analysis and cell viability by cell counting was determined after electroporation of 100,000 cells with different dsODN concentrations (5, 15, 25, and 35 pmol). 25 pmol of dsODN was used for further transfections together with sgRNA and Cas9 RNP at molar ratio of 2:1. After 5 days in culture, DNA was isolated with DNeasy Blood & Tissue Kit using standard protocols (QIAGEN). DNA fragments of 200–450 bp were generated and subsequently ligated to adaptors by utilizing NEBNext Ultra II kit (New England Biolabs). NEBNext Ultra II Q5 Master Mix (New England Biolabs) was used for the first DNA amplification, whereas KAPA SYBR FAST 2x MasterMix (KAPA Biosystems) was utilized for the second amplification. The libraries were pooled and loaded into 3 lanes of an Illumina GAIIx single-read flow cell and two MiSeq flow cells. Bound molecules were clonally amplified on a cBot instrument. Subsequently, the first 50 nucleotides from each fragment were sequenced followed by a seven nucleotide sequencing run to decipher the barcode sequence in the adapter (Illumina).

GUIDE-seq. Demultiplexing, PCR duplicate consolidation, cleavage site recognition, off-target activity identification, and visualization was performed with the GUIDE-Seq Analysis Package v1.0.1[53] using the GRCh37.75 human genome as reference. The read alignment step of the pipeline was conducted using BWA-MEM v0.7.17[52] and bedtools v2.28[54] was used for downstream analysis.

Droplet digital PCR (ddPCR). PCR mastermix was prepared by adding ddPCR Multiplex Supermix (Bio-Rad Laboratories), primers (950 nM), probes (250 nM), and DNA (350 ng) at a final volume of 20 µl. Next, QX200 ddPCR droplet generator (Bio-Rad Laboratories) was utilized to separate the DNA into 20,000 droplets, which were transferred to a 96-well plate and sealed to avoid evaporation using the PX1 PCR Plate Sealer (Bio-Rad Laboratories). Finally, the PCR was run on the C1000 Touch Thermal Cycler (Bio-Rad Laboratories) with the following thermal parameters: 10 min at 95 °C, 40 cycles comprising 30 s at 95 °C, 1 min at 61 °C, and 2 min at 72 °C, followed by enzyme inactivation at 98 °C during 10 min. Finally, PCR products were examined using the QX2000 droplet reader (Bio-Rad Laboratories) and analyzed with the QuantaSoft 1.6.6 software (Bio-Rad Laboratories).

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Author contributions

Performed experiments and analyzed data: A.L.C. and A.D.M. Performed hemoglobin electrophoresis: T.E. Analyzed GUIDE-seq data: L.H. and P.B. RNA-seq data: J.A. and N.C. Contributed reagents/materials/analysis tools: J.S.A., N.L., D.M.S., M.S.D.K., R.H. and M.M. Wrote the paper: A.L.C., A.D.M., J.S.A. and M.M. Conceived, designed and supervised the experiments: M.M., A.L.C., A.D.M. and J.S.A. All authors read and approved the final manuscript. A.L.C. and A.D.M. contributed equally to this work.

Competing interests

The authors declare no competing interests.

Additional information

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PAPER 3

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REVIEW ARTICLE

CRISPR/Cas9-modified hematopoietic stem cells—present and future perspectives for stem cell transplantation

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Abstract
Allogeneic hematopoietic stem cell transplantation (HSCT) is a standard therapeutic intervention for hematological malignancies and several monogenic diseases. However, this approach has limitations related to lack of a suitable donor, graft-versus-host disease and infectious complications due to immune suppression. On the contrary, autologous HSCT diminishes the negative effects of allogeneic HSCT. Despite the good efficacy, earlier gene therapy trials with autologous HSCs and viral vectors have raised serious safety concerns. However, the CRISPR/Cas9-edited autologous HSCs have been proposed to be an alternative option with a high safety profile. In this review, we summarized the possibility of CRISPR/Cas9-mediated autologous HSCT as a potential treatment option for various diseases supported by preclinical gene-editing studies. Furthermore, we discussed future clinical perspectives and possible clinical grade improvements of CRISPR/cas9-mediated autologous HSCT.

Allogeneic hematopoietic stem cell transplantation

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative approach for many malignant and non-malignant diseases, such as leukemia, lymphoma, aplastic anemia, thalassemia, and other genetic diseases [1]. In the case of several malignant diseases, allogeneic HSCT is strongly recommended due to the broad anti-tumor effect of donor immune cells over the recipient's cancer cells. There are different aims for HSCT: (1) to replace the hematopoietic system of the patient with the one from the donor, e.g., in patients with severe blood disorders; (2) to treat a cancer patient from the toxic side effects of chemotherapy and/or radiation; (3) to enhance the immune-attack towards malignant cells by the donor's immune cells; and (4) to abolish autoimmunity [2]. HSCT is usually performed with allogeneic stem cells from a foreign donor [2]. Recent advances in transplantation technology and supportive care practices had substantially improved the long-term survival after allogeneic transplantation and observed to become safer [3].

Limitations of allogeneic hematopoietic stem cell transplantation

Despite the fact that allogeneic HSCT is becoming safer, the utilization of allogeneic stem cells is limited by a number of important difficulties including the lack of a suitable donor, graft-versus-host disease, and infectious complications due to immune suppression [4, 5]. The primary limitation is associated with the lack of finding a suitable HLA-matched donor for the transplantation. Worldwide, only 30% of patients who need allogeneic HSCT will have a matched sibling donor [6]. Although the alternative graft sources are available, it has been reported that the likelihood of finding an appropriate unrelated donor with HLA-match and willingness to donate is confounded by racial and ethnic background [7].

Hence, the proposed option is limited to the patient's ethnicity that certain groups, including African and Asian descendants, are expected to have less than 40% of adult donors with complete HLA-match [7]. It is worth to note

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that African and Asian ethnic groups are majorly affected by several blood disorders, therefore more allogeneic transplantation might be needed for these ethnic groups than others [8]. The next severe and potentially lethal complication of allogeneic HSCT is the development of graft-versus-host disease (GvHD). The etiology of GvHD is multifactorial and its exact incidence is unknown, but it is reported to be in the range between 9 and 50% in optimal HLA-matched transplant recipients [9, 10].

Autologous hematopoietic stem cell transplantation

In autologous HSCT, a wild-type copy of a mutated gene is introduced, either using viral vectors or gene-editing into a recipient's own HSCs in ex vivo condition, to re-establish lost of gene function during disease state [11]. The autologous HSCT is becoming an alternate approach to the burdensome allogeneic HSCT in order to treat patients with diverse inherited monogenic diseases and certain neoplastic tumors. For example, autologous HSCT is a preferred option for multiple myeloma, as this therapeutic intervention permits the injection of high-dose chemotherapy without prolonged bone marrow aplasia [12]. Moreover, autologous HSCT avoids immune suppression and development of GvHD, and provides survival advantage over allogeneic HSCT [11, 13, 14].

Earlier gene therapy clinical trials on autologous HSCT, which utilized viral vectors to deliver therapeutic transgenes, showed clinical efficacy for patients with hemoglobinopathies, metabolic storage diseases, and immunodeficiency disorders [14–17]. However, the few studies reported that gamma-retroviral vectors lead to the activation of proto-oncogenes and treatment-related leukemogenesis [18–20]. Unlike the gamma-retroviral vectors, no insertional mutagenesis has been reported for lentiviral vectors up to date in human, but the development of T-cell lymphoma was observed in a mouse model of X-SCID due to random integration into oncogenes [21]. Moreover, the lentiviral treatment exhibited a transcriptionally activated monoclonal expansion, and a huge volume of random integration loads (5–20 million/kg body weight), which forewarns for random integration-mediated mutagenesis [22, 23]. Though it is feasible to regulate the viral vector expression specifically to blood cells using transcriptional control elements, it is observed that a variety of immature precursors were expressing the transgene despite a specific stimulus [22]. Besides, the random integration events of viral vectors lead to uncontrolled transgene expression [22].

To overcome the limitation of random integration inherent to viral-mediated autologous HSCT, the use of gene-editing tools ensure Targeted-Transgene Integration (TTI) at either safe-harbor or endogenous locus that provides several advantages [24]. Hence, combining autologous HSCT with gene-editing tools (ZFNs, TALENs CRISPR/Cas9) warrants a superior safety profile, and offers a dynamic treatment option for various genetic and oncological diseases.

CRISPR/Cas9 technology: a breakthrough in gene therapy

CRISPR/Cas9 system was the latest technology to emerge and the most powerful; owning to the best gene-editing efficiency, ease of use, specificity, and lower costs in comparison to former technologies [25, 26]. The Cas9 endonuclease is guided by a single-guide RNA (sgRNA) to a specific site in the genome, resulting in a double-stranded break (DSB), which ensures targeted gene-editing or integration of therapeutic transgenes controlled by natural regulatory elements of the promoter [24]. Cells try to repair the DSB by non-homologous end-joining (NHEJ) or homology-directed repair (HDR). Error-prone NHEJ often results in mutations (insertions/deletions) near the cutting site, while HDR repairs the mutations by homologous recombination [27]. Thus, autologous CRISPR/Cas9 gene-edited HSCs could be relocated into the patient, diminishing the negative effects of allogeneic transplantation and retroviral gene therapy. In this review, we provide a detailed up-to-date overview of the different CRISPR/Cas9-mediated gene-editing studies in preclinical phase targeting HSCs and other related cell types. We also discuss the future perspectives that might be applied into the clinic for the treatment of several genetic diseases (Table 1).

Nonmalignant blood disorders

Blood disorders comprise inherited illnesses affecting society worldwide. Currently, the public health sector is striving to minimize the incidence of these diseases by developing new therapies in order to prolong and improve the life quality and expectancy of the patients. Blood disorders can affect different stages during hematopoiesis, ranging from bone marrow failure (Fanconi anemia and amegakaryocytic thrombocytopenia) to abnormal functioning of blood cells and coagulation factors (β-hemoglobinopathies and hemophilia). In this part of the review, we describe the preclinical studies that utilized CRISPR/Cas9 technology to tackle some of the blood disorders.

β-Hemoglobinopathies

β-Hemoglobinopathies are congenital hematologic diseases, of which approximately 56,000 and 270,000 newborns are

Table 1 Most relevant gene-editing studies utilizing CRISPR/Cas9 in human cells for HSC-related diseases

	Disease	Type of gene-editing	Gene-editing tool	Cell line	Gene	Nuclease delivery	Donor template delivery	Gene-editing efficiency	Reference
Nonmalignant blood disorders	Amegakaryocytic thrombocytopenia	Gene correction	CRISPR/Cas9	HSCs	*MPL* (W272R)	RNP	ssODN	NA	[81]
	β-hemoglobinopathies	Gene addition	CRISPR/Cas9	iPSCs	*HBB*	pDNA	pDNA (HBB cDNA)	NA	[41]
		Gene correction	CRISPR/Cas9	HSCs	*HBB* (Ex.1)	RNP	ssAAV6	90% HDR[a]	[34]
				HSCs	*HBB* (-69 A > T)	mRNA	IDLV	20% HDR	[35]
				HSCs	*HBB* (-69 A > T)	RNP	ssODN	33% HDR	[36]
				HSCs	*HBB* (IVS-110)	mRNA	ssODN	8% HDR	[37]
				HSCs	*HBB* (-69 A > T)	RNP	AAV6	70% HDR[a]	[38]
				HSCs	*HBB* (-69 A > T)	RNP	ssDNA	20% HDR	[40]
		Gene disruption	CRISPR/Cas9	HSCs	*BCL11A* (GATA 1)	LV	–	NA	[46]
				HSCs	*HBG1/2*	LV	–	77% NHEJ	[44]
				HSCs	*HBG-HBD, HBD-HBB*	pDNA	–	24% NHEJ[a]	[47]
	Fanconi anemia	Gene addition	ZFN	HSCs	*AAVS1*	mRNA	IDLV (FANCA)	14% HDR	[82]
	Hemophilia	Gene addition	ZFN	hMSCs	*AAVS1*	pDNA	pDNA (F9)	7% HDR	[83]
			CRISPR/Cas9	mZygote	*F9*	AAV8	AAV8 (F9)	NA	[84]
		Gene correction	CRISPR/Cas9	Germline cells	*F9* (Exon 8, 8-bp del.)	RNP	ssDNA	53% HDR	[85]
Immunodeficiencies	Chronic granulomatous disease	Gene addition	TALENs	iPSCs	*AAVS1*	pDNA	pDNA (CYBB cDNA)	50% HDR[a]	[86]
			ZFN	HSCs	*AAVS1*	mRNA	AAV6 (CYBB cDNA)	57% HDR	[15]
				iPSCs	*AAVS1*	mRNA	pDNA (CYBB cDNA)	80% HDR[a]	[87]
			CRISPR/Cas9	iPSCs	*CYBB*	LV	AAV6 (CYBB cDNA)	50%–67% HDR[a]	[88]
		Gene correction	CRISPR/Cas9	iPSCs	*CYBB* (Int.1 T > G)	pDNA	pDNA	17% HDR	[89]
				HSCs	*CYBB* (C676T)	mRNA	ssODN	21% HDR	[90]
	Human immunodeficiency virus	Gene disruption	ZFN	HSCs	*CCR5*	Ad5/F35	–	31% NHEJ	[91]
				T-cell	*CCR5*	pDNA	–	28% NHEJ	[68]
			CRISPR/Cas9	T-cell	*CCR5*	Ad5/F35	–	33% NHEJ	[92]
				HSCs	*CCR5*	pDNA	–	27% NHEJ	[93]
	Wiskott-Aldrich Syndrome	Gene addition	ZFN/CRISPR/Cas9	K-562	*WAS*	IDLV	IDLV	88/83% HDR[a]	[25]
	X-linked severe combined immunodeficiency	Gene addition	TALENs	mESC	*IL2RG*	pDNA	pDNA (IL2RG cDNA)	4% HDR[a]	[51]
			ZFN	HSCs	*IL2RG*	mRNA	IDLV (*IL2RG* cDNA)	6% HDR	[53]
				HSCs	*CCR5*	IDLV	IDLV (*GFP*/*Puro*)	85% HDR[a]	[54]
		Gene correction	ZFN	HSCs	*IL2RG* (R226H)	mRNA	AAV6	27% HDR	[56]
			CRISPR/Cas9	T-cell	*IL2RG* (c.800delA/A530G)	RNP	ssDNA/dsDNA	25/22% HDR	[57]
				iPSCs	*JAK3* (C1837T)	pDNA	pDNA	73% HDR[a]	[55]

Table 1 (continued)

	Disease	Type of gene-editing	Gene-editing tool	Cell line	Gene	Nuclease delivery	Donor template delivery	Gene-editing efficiency	Reference
Cancer and CAR-T cell immunotherapy	Leukemia (ALL, AML, MML)	Gene addition	CRISPR/Cas9	T-cell	*TRAC*	mRNA	AAV (CAR19)	40% HDR	[64]
		Gene disruption	TALENs	T-cell	*TRAC/CD52*	mRNA	LV (CAR19)	70% NHEJ	[61]
			CRISPR/Cas9	T-cell	*TRAC/B2M/PD-1*	RNP	LV (CAR19)	NA	[60]
				RM-HSCs	*CD33*	RNP	LV (CAR33)	40% NHEJ	[62]
				T-cell	*TRAC/B2M/PD-1/CTLA-4*	mRNA	LV (CAR19)	90% NHEJ	[63]
	Human immunodeficiency virus	Gene addition	megaTAL	T-cell	*CCR5*	mRNA	AAV (CAR19)	51% HDR[a]	[65]

HSCs hematopoietic stem cells, *iPSCs* induced pluripotent stem cells, *pDNA* plasmid DNA, *ssODNs* single-stranded oligodeoxynucleotides, *NHEJ* non-homologous end joining, *IDLV* integrase-deficient lentivirus, *RNP* ribonucleoprotein; *AAV* adeno-associated virus

[a]Gene-editing efficiency attained by means of selection markers

affected worldwide by β-thalassemia and sickle cell disease (SCD), respectively [28]. β-Hemoglobinopathies are caused by over 300 mutations in the human *β-globin* gene (HBB) [29], which results in disturbed erythropoiesis, increased apoptosis of red blood cells, and anemia [30], leaving patients to life-long blood transfusions with the associated risks of infection, immunological reactions, and iron overload [31]. Previous approaches relied on lentiviral vectors as gene therapy for β-hemoglobinopathies by introducing a healthy copy of the *β-globin* or *γ-globin* gene into HSCs [17, 32, 33]. However, novel studies for β-hemoglobinopathies are based on three different approaches using the CRISPR/Cas9 system: (1) gene correction in which specific mutations are corrected with a repair template, (2) gene addition of an *HBB* transgene to the endogenous locus, and (3) gene disruption in which the expression of fetal hemoglobin (HbF) is induced by suppressing genes that are involved in HbF regulation.

Most CRISPR studies performing gene correction strategies target sites near SCD and β-thalassemia mutations [34–39]. The correction of point mutations has been accomplished utilizing ssODNs [36, 37, 39, 40], adeno-associated virus 6 (AAV6) [34, 38], and integrase-defective lentivirus (IDLV) [35]. In addition, one study has developed a universal correction by gene addition of *HBB* cDNA in iPSCs after cutting the endogenous gene with two sgRNAs, restoring *β-globin* expression [41]. Nonetheless, gene correction and gene addition have always been more challenging in HSCs since HDR only occurs in the G2 phase of the cell cycle and these cells are generally in a quiescent stage (G0) [24]. Considering that non-homologous end joining (NHEJ) occurs more frequently than homologous-direct repair (HDR), many investigations are focused on gene deletion or suppression by CRISPR/Cas9 [42]. Therefore, researchers have centered on reactivating HbF (2α2γ) by gene disruption of silencing factors and regulators such as *BCL11A*, *KLF1*, and *ZBTB7A*, since more than 30% of HbF presence reduces disease severity for β-hemoglobinopathies [43]. Alternatively, other studies were focused on the establishment of naturally occurring hereditary persistence of fetal hemoglobin (HPFH) by reactivation of fetal *γ-globin* by mutating *BCL11A* and *ZBTB7A* binding sites in the *HBG1*/*HBG2* promoter sequence [44-46] or by deleting the *δ*-globin and *β-globin* genes (13 kb) via CRISPR/Cas9 editing [47, 48]. All the studies are summarized in Table 1.

Immunodeficiencies

The immune system is compromised or partially absent in patients with immunodeficiencies. These diseases could be successfully treated by genome-editing-mediated therapy since a small number of corrected HSCs could ameliorate the symptoms of the patients.

Wiskott–Aldrich syndrome Wiskott-Aldrich Syndrome (WAS) is an X-linked severe primary immunodeficiency caused by mutations in the *WAS* gene encoding for WAS protein (WASP) [25, 49]. The WASP is a regulator of the actin cytoskeleton in all hematopoietic cells, thus, its deficiency causes thrombocytopenia, lymphoid/myeloid cell dysfunction, recurrent infections, and tumor development [49, 50]. There is only one gene addition approach in K-562 cells using IDLV for the delivery of ZFNs or CRISPR/Cas9 together with the *WAS* donor template attaining up to 88% HDR after selection (Table 1) [25]. This proof of principle could be potentially applied to HSCs. Nevertheless, further studies are required to demonstrate *WAS* gene correction in a pooled HSCs without any selection markers.

X-linked severe combined immunodeficiency (X-SCID) The X-SCID consists of mutations in the *interleukin-2 receptor γ chain* (*IL2RG*) gene, which is common for several interleukin receptors (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) and crucial to develop functional lymphocytes [20, 51, 52]. As a consequence, X-SCID patients are characterized by the absence of T and NK cells, and showed impaired function of B-cells [51, 52]. Furthermore, individuals with mutations in the *JAK3* gene develop similar SCID phenotype. Gene addition and correction strategies were performed in HSCs, iPSCs, and T-cells by different research groups [53–57]. Interestingly, Schiroli et al. achieved functional gene correction (27%) for *IL2RG* gene in HSCs using ZFNs and AAV6 repair template (Table 1) [56]. As CRISPR/Cas9 is more efficient than ZFNs it is feasible to attain even higher therapeutic level of gene correction (>27%) for *IL2RG* locus to treat X-SCID.

Blood cancers and chimeric antigen receptor (CAR)-T cell therapy

Immunotherapy with CAR in T cells and HSCs has created big hope for treating blood cancers [58]. CARs include an extracellular single-chain variable fragment (ScFv) to recognize tumor antigen and an intracellular chimeric signaling domain for T-cell activation [59]. Several studies in T-cells aimed to knock-out the endogenous MHC-related genes (*TRAC* and *B2M*, among others) using endonucleases including CRISPR/Cas9, while LV-mediated integration of CD19-CAR or CD33-CAR were used to treat different lymphoid and myeloid malignancies [60–63]. As discussed earlier, viral free strategies are preferred for gene therapy, several research lines focused on CRISPR-based CAR-T cell immunotherapy. Eyquem et al. and Sather et al. utilized CRISPR/Cas9 technology to target *CCR5* and *TRAC* in order to incorporate CAR19 by AAV-donor for the treatment of HIV and AML, respectively (Table 1) [64, 65]. The similar strategy could be applied into HSCs with the T-cell specific promoter in cancer patients, so that they acquire long-lived immunological memory against specific cancer types [66].

The clinical use of CRISPR/Cas9 in autologous HSCT: perspectives

Since the year 1968, allogeneic HSCT has been used in the clinic to cure several genetic disorders and some types of cancer [67]. It is evident that gene-engineered autologous HSCT might be superior with several advantages than allogeneic HSCT as discussed earlier. For example, we have compared the survival outcome of both allogeneic and lentiviral modified autologous HSCT for the treatment of metachromatic leukodystrophy (MLD), a lysosomal storage disorder. We observed that allogeneic HSCT resulted in 5-year survival of 79%, whereas lentiviral-mediated autologous HSCT displayed 100% survival and signifies the survival benefit of the autologous system [13, 14].

The real excitement of gene-editing in autologous cells in humans started when the ZFNs lead to the attainment of genetic resistance to HIV infection in infected patients by gene disruption of *CCR5* gene in autologous $CD4^+$-T cells [68]. The study provided a great prospect of operating gene-editing tools in autologous HSCT setting. Excitingly, a similar approach is being tested in clinical trials using ZFNs in autologous HSCs to induce resistance against HIV infection by *CCR5* disruption (NCT02500849), and HbF upregulation through interference of the enhancer of *BCL11A* (NCT03432364). Compared to the 20 years of experience in research and development of ZFNs, the CRISPR/Cas9 system is still in its beginning stage with less than 5 years. However, it is very imperative to note that 13 different clinical trials have been registered to utilize the CRISPR/Cas9 technology to attain therapeutic benefit (http://clinicaltrials.gov/). In particular, three clinical trials are registered and in recruiting stage which utilize CRISPR/Cas9 technology to modify autologous HSCs to fight against β-thalassemia, sickle-cell disease, and HIV infection (Table 2).

In theory, CRISPR/Cas9-mediated autologous transplantation is appropriate on many occasions wherever allogeneic HSCT is feasible, to treat various monogenic diseases. In Fig. 1, we summarized the diseases that can be targeted through CRISPR/Cas9-mediated gene therapy. The rationale behind using HSCT as a treatment is that differentiated gene-corrected stem cells can migrate to several organs. For instance, human monocytes, an important population of circulating mononuclear cells, travel to targeted organs as different macrophage subtypes, including brain (microglial cells), liver (Kupffer cells), skin (Langerhan's cells), lungs (alveolar macrophages), and spleen

Table 2 Overview of clinical trials that utilize CRISPR/Cas9 in autologous HSCs

S. No	NCT number	Disease	Target gene	Strategy	Country	Industry sponsership	Status	Location
1	NCT03655678	Beta Thalassemia	BCL11A	Gene Disruption	Germany	CRISPR Therapeutics	Recruiting	University of Tuebingen/ Regensburg
2	NCT03745287	Sickle Cell Disease	BCL11A	Gene Disruption	USA	CRISPR Therapeutics	Recruiting	Nashville, Tennessee
3	NCT03164135	HIV infection	CCR5	Gene Disruption	China	307 PLA General Hospital	Recruiting	Chinese PLA General Hospital

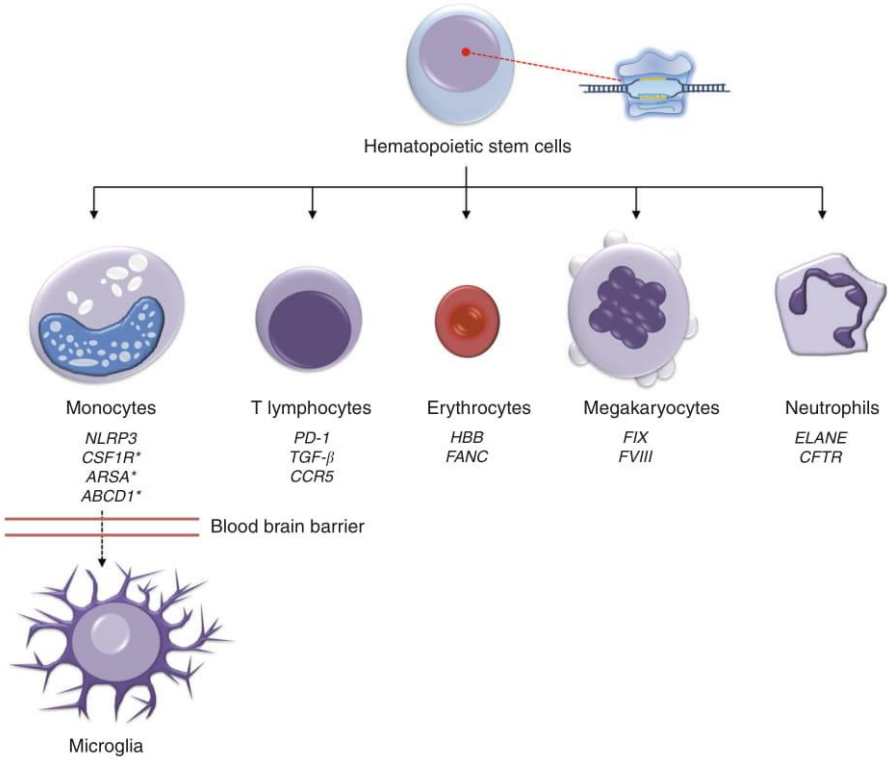

Fig. 1 Functional myeloid, lymphoid, and erythroid lineages after autologous transplantation of edited HSCs. Genes associated with diverse diseases are listed below each cell type. Gene correction or gene addition of those genes in HSCs results in normal and functional differentiation into the different lineages. Gene-edited HSCs might also cure some neurogenetic diseases, such as ALD or MLD, due to monocyte migration through the blood—brain barrier (BBB) to the brain (respective genes denoted with an asterisk symbol)

(macrophages) [69]. This process is commonly known as 'Monocyte trafficking' and can deliver the deficient protein/enzyme [69]. For example, the brain can be targeted through HSCT as gene-corrected monocytes migrate from bone marrow into the brain as microglial cells and correct the deficient neuronal cells through a phenomenon referred as 'Cross correction' [69]. The mentioned mechanism offers an ideal option to treat lysosomal storage disorders and is noted to be helpful in treating patients with metachromatic leukodystrophy and adrenoleukodystrophy using autologous HSCT after lentiviral-transfer of corrected genes [14, 16]. Therefore, it is evident that similar to targeting brain with monocytes, the diseases in other organs including skin, lung, and liver can be targeted through CRISPR/Cas9-mediated autologous HSCT.

In this part of the review, we provide some exciting models that utilize CRISPR/Cas9-mediated autologous HSCT to treat some rare diseases (Fig. 1). Since the gene-corrected monocytes migrate to skin and differentiate into Langerhan's cells, it is possible to target congenital auto-inflammatory skin disorders through CRISPR/Cas9 corrected HSCs [70]. The monogenetic nature of some skin disorders including cryopyrin-associated periodic syndrome (CAPS) and familial Mediterranean fever (FMF), make the CRISPR/Cas9-mediated gene therapy as an attractive option to replace pathogenic inflammasomes by edited macrophages [71, 72]. Likewise, the monocytes migrate to lungs and differentiate into alveolar macrophages. It has been reported that in cystic fibrosis (CF) patients the function of alveolar macrophages is impaired with sub-optimal phagocytosis in clearing infections, exhibiting a hyper-inflammatory immune response in the lungs [73]. Moreover, an interesting study reported that the neutrophils of CF patients have impaired degranulation and defective ion

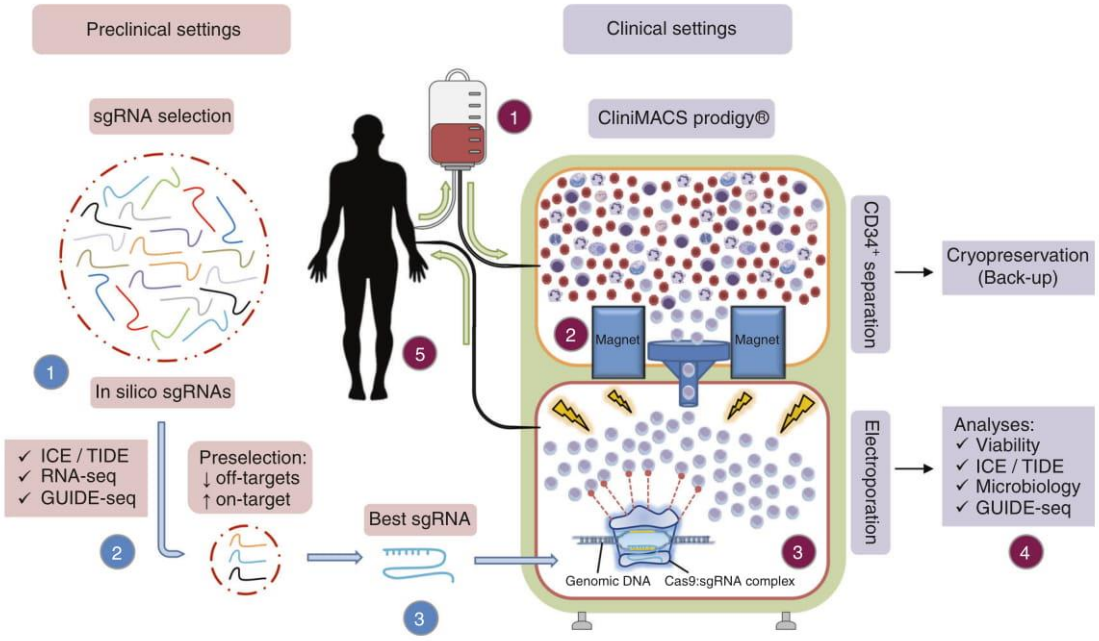

Fig. 2 CRISPR/Cas9 gene therapy procedure. Preclinical settings are shown in blue, including: (1) sgRNA in silico predictions; (2) sgRNA preselection based on the on-target efficiency and number of off-targets after ICE/TIDE analyses, RNA-seq, and GUIDE-seq; (3) final sgRNA exhibiting the highest on-target efficiency and the lowest number of off-targets. Clinical settings are represented in green, including: (1) mobilization of $CD34^+$ HSCs to the bloodstream and blood sampling into the CliniMACS Prodigy (Miltenyi Biotec); (2) $CD34^+$ HSC separation and cell cryopreservation for possible future back-ups; (3) Electroporation of $CD34^+$ HSCs together with the Cas9:sgRNA complex for gene-editing; (4) Post-electroporation analyses comprising a second off-target study, cell viability, and microbiology assays; (5) Autologous HSC transplantation in the patient

homeostasis including chloride, sodium, and magnesium [74]. This observation led to a new paradigm that in addition to bronchial epithelial cells, the expression of CFTR channel is essential in neutrophils too [75]. Therefore, CRISPR/Cas9-mediated autologous HSCT could be beneficial in CF patients to provide healthy immune cells with improved function.

However, the proposed strategy might not provide a beneficial effect in the pulmonary system, but might halt the immune-mediated lung damage. Together, the prospect seems very bright for CRISPR/Cas9-mediated autologous HSCT, and the following years will definitely bring several treatments to the clinic. In addition, the improvement in automation technology provided new devices, including CliniMACS Prodigy® (www.miltenyibiotec.com), which are GMP compatible to automatic separation of stem cells, electroporation with CRISPR components, cultivation, and application into humans with a clinical grade quality (Fig. 2).

Challenges and optimization of CRISPR/Cas9 to edit human HSCs

High proliferating capacity of HSCs demands the need for safety assurance while gene-editing this stem cell population. Although CRISPR/Cas9 is in clinical trial for gene-editing in HSCs, the experts still fear the possibility of off-target mediated toxicity and poor gene-repair efficacy. We critically reviewed these challenges in the context of HSCs gene-editing earlier [24]. Since the CRISPR/Cas9 mediated genetic alterations are permanent, the adverse effect from off-target mutations might decrease the fitness of the gene-corrected HSCs and might influence engraftment potential.

Due to the ex vivo nature of autologous HSCT, the gene-engineered HSCs can be efficiently monitored for off-target effects and their engraftment potential. Recent improvements in sgRNA design (truncated version/GC-content/DNA-RNA chimera), Cas9 variants (high-fidelity Cas9/nickase/dCas9 with *Fok*I/enhanced specificity Cas9/hyper-accurate Cas9), and transient Cas9 delivery system (RNP/mRNA) were observed to increase the specificity while reducing the off-targets [24, 76]. Moreover, a recent study reported that the labeling of the sgRNA/RNP complex was observed to aid the enrichment of gene-modified HSCs [77]. Interestingly, the utilization of Zebrafish model to assess the viability and efficacy of human HSC grafts after gene modification would be beneficial prior to transplantation into patient as the model possess several advantages over humanized NSG mouse model [78]. Importantly, the $CD34^+$ HSCs cells encompass a mixed population of primitive and more differentiated cells, where targeting the most primitive cells ($CD34^+CD133^+CD90^+$) would be advantageous [79].

The autologous HSCT typically need 1–20 million/kg of gene-edited HSCs, and to attain therapeutic level it is mandatory to achieve correct gene modification in at least 10–30% of $CD34^+$ HSCs [80]. It is worth to note that, the initial proof-of-concept studies carried out in $CD34^+$ HSCs at the level of small scale using laboratory equipped electroporators (Neon/4D-Nucleofector) might not provide sufficient detailed results which could be translatable to clinic at large scale gene-editing with high number of HSCs. Since the CRISPR/Cas9 system is an efficient endonuclease system, even a tiny miscalculation in the quantity of theses nucleases during the clinical translation of laboratory finding could lead to under performance (poor gene-editing) or over performance (more off-targets). This limitation can be overcome with the new instrument of CliniMACS Prodigy-Electroporator system as it offers such an option with two different selections such as Cuvette system (small scale) and Cyclic system (large scale) with similar efficacy (Fig. 2).

Conclusions

CRISPR/Cas9 technology is a very promising gene therapy invention that possibly provides curative treatments for numerous diseases in the field of hematology, neurology, gastroenterology, pulmonology, and oncology. However, the discussed limitations related to safety and efficacy must be duly considered and evaluated by high-throughput techniques. We strongly deem that, CRISPR/Cas9 will facilitate future advancements in autologous HSCT and will be a better alternative to viral vectors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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