Hematopoietic stem cell gene therapy 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

CIRCLE-seq: Circularization In vitro Reporting of CLeavage Effects by Sequencing

CRISPR: Clustered regulatory interspaced short palindromic repeats

CsH: Cyclosporin H

ddPCR: Droplet digital PCR

DSB: Double-strand break

FDA: Food and Drug Administration

GUIDE-seq: Genome-wide, Unbiased Identification of DSBs Enabled by Sequencing

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

HPLC: High-performance liquid chromatopraphy

HSCT: Hematopoietic stem cell transplantation

HSPCs: Hematopoietic stem and progenitor cells

IDLV: Integrase-defective lentivirus

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

KLF1: Kruppel Like Factor 1

MOI: Multiplicity of infection

LVs: Lentiviral vectors

NHEJ: Non-homologous end joining

RFLP: Restriction Fragment Length Polymorphism

SCD: Sickle cell disease

sgRNA: Single guide ribonucleic acid

shmiR: MicroRNA-embedded shRNA

ssDNA: Single-stranded-desoxyribonucleic acid

ssODN: Single-stranded-oligodeoxynucleotide

TALENs: Transcription activator like effector

VCN: Vector copy number

VSV-G: Vesicular-stomatitis-virus glycoprotein

ZFNs: Zinc-finger nucleases

1. Summary

β-hemoglobinopathies, including β-thalassemia and sickle cell disease (SCD), are autosomal recessive inherited disorders caused by various mutations in the *β-globin* gene. The most effective curative therapy involves allogenic hematopoietic stem cell transplantation (HSCT) from an immunologically-matched donor. However, this approach presents some limitations in terms of finding a suitable donor and transplantation related risks, such as a graft-versus-host disease (GvHD). In this thesis, we strongly emphasized that autologous HSCT in combination with gene therapy tools will develop novel treatments for β-hemoglobinopathies.

A promising technique for gene editing has emerged in recent years based on the use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) -associated RNA-guided endonuclease Cas9 (CRISPR/Cas9) technology. This revolutionary tool enables to perform gene disruption, gene correction, and gene addition in a specific locus of interest with high reliability and efficiency. Through this technology, in our first study, we demonstrated that targeting *KLF1* and *BCL11A*, which control the expression of *γ-globin*, as well as the promoter region of *HBG1* and *HBG*2 (*HBG1/2*), resulted in up-regulation *of γ-globin* gene expression and fetal hemoglobin (HbF). Furthermore, after a deep comparison between the three gene editing strategies, we confirmed that BCL11A approach, which is currently in clinical phase, is the safest gene therapy treatment for β-hemoglobinopathies. Nevertheless, HBG1/2 strategy also holds potential for clinical translation.

Alternatively, since *β-globin* gene correction is also a feasible gene therapy approach, in our second study, we attained successfully gene addition in HSPCs by inserting a NheI-tag at the common aberrant splicing mutation point, *HBB* $^{IVS1-110}$ by means of Cas9 mRNA and ssODN electroporation.

In the last project, we thoroughly compared three lentiviral transgenes encoding for *IGF2BP*1, shRNA *BCL11A*, and *γ-globin* to reactivate HbF production in HSPCs. Also, we assessed whether baboon envelope proteins (BaEV and BaEV-RLess) have a beneficial advantage over the regularly used vesicular-stomatitis-virus-G envelope protein (VSV-G). Our results showed

that all treatments using VSV-G envelope proteins resulted in therapeutic levels of HbF. In addition, baboon envelopes, especially BaEV-RLess, achieved decent levels of HbF with less viral particles, which might ameliorate the symptoms of the disease. Finally, even though IGF2BP1 and BCL11A approaches induced higher HbF levels than γ-globin strategy, their role in gene regulation might cause undesired iatrogenic effects. Therefore, we considered γ-globin the best lentiviral gene therapy strategy for the treatment of β-hemoglobinopathies.

During this thesis we evidenced that both gene therapy tools, lentiviral gene transfer and genome editing, provide a successful platform for gene treatment of blood disorders. In addition to the current clinical trial approaches, we strongly believe that resurgence of HbF is the most straight forward strategy together with *β-globin* gene correction. However, due to the low occurrence of gene correction events in HSPCs, further investigation is required.

2. Zusammenfassung

β-Hämoglobinopathien, zu denen die β-Thalassämie und die Sichelzellanämie gehören, sind autosomal rezessiv vererbte Erkrankungen, die durch verschiedene Mutationen im *β-Globin* Gen verursacht werden. Die einzige kurative Behandlung ist eine allogene Stammzelltransplantation (SZT) mit hämatopoetischen Stammzellen (HSZ) eines HLA-identischen Spenders. Diese Behandlung weist jedoch einige Einschränkungen hinsichtlich der Suche nach einem passendem Spender und verschiedenen Transplantationsrisiken auf, wie z. B. einer Transplantat-gegen-Wirt-Reaktion (GvHD). In dieser Arbeit wurde herausgearbeitet, dass sich durch die Kombination der autologen SZT mit gentherapeutischen Methoden neuartige Behandlungsmöglichkeiten für ß-Hämoglobinopathien eröffnen werden.

In den letzten Jahren hat eine innovative Methode, basierend auf der Verwendung der so genannten *„Clustered Regularly Interspaced Short Palindromic Repeats“* CRISPR/Cas9 Technologie, als eine vielversprechende Technik zur Geneditierung, besondere Aufmerksamkeit erlangt. Dieses revolutionäre Werkzeug ermöglicht es, mit hoher Zuverlässigkeit und Effizienz, Genveränderungen, Genkorrekturen und Genadditionen zielgenau durchzuführen. Mit Hilfe dieser Technologie wurde im ersten Teil der Arbeit gezeigt, dass das Ausschalten von *KLF1* und *BCL11A*, welche die Expression von *γ-Globin* regulieren, sowie eine direkte Veränderung der Promotorregionen von *HBG1* und *HBG2* (*HBG1/2*), zu einer Hochregulation der *γ-Globin* Genexpression und fetalem Hämoglobin (HbF) führen. Zudem konnte nach einem umfangreichen Vergleich der drei Geneditierungsstrategien herausgearbeitet werden, dass der *BCL11A* Ansatz, der sich derzeit auch in der klinischen Anwendung in unserer Klinik befindet (Phase I/II Studie), als die sicherste gentherapeutische Behandlung für ß-Hämoglobinopathien darstellt. Darüber hinaus birgt auch die HBG1/2-Strategie das Potential für eine klinische Anwendung.

Da die Korrektur des *β-Globin*-Gens ein weiterer interessanter gentherapeutischer Ansatz ist, wurde in einem zweiten Teil der Doktorarbeit eine

erfolgreiche Genaddition in HSZ an der aberranten Spleißstelle *HBB* $^{IVS1-110}$ mittels Cas9 mRNA und ssODN Elektroporation vorgenommen.

Im dritten Teil-Projekt wurden drei lentivirale Konstrukte, die für *IGF2BP1*, shRNA *BCL11A* und *γ-Globin* kodieren, verglichen, um die HbF-Produktion in HSZ zu erhöhen. Zudem haben wir auch untersucht, ob die innovativen *Baboon* Lentiviren BaEV und BaEV-RLess einen Vorteil gegenüber dem sonst häufig verwendeten *Vesikular-Stomatitis-Virus-G*-Hüllprotein (VSV-G) haben. Unsere Ergebnisse haben gezeigt, dass alle Behandlungen unter Verwendung von VSV-G zu erhöhter HbF-Expression führten. Darüber hinaus erreichte insbesondere der BaEV-RLess Lentivirus gute HbF-Werte, wobei deutlich weniger Viruspartikel benötigt wurden. Obwohl die IGF2BP1- und BCL11A-Ansätze höhere HbF-Expressionen als die γ-Globin-Strategie induzierten, könnte ihre Rolle bei der Genregulation unerwünschte iatrogene Effekte verursachen. Daher betrachteten wir das Einschleusen des *γ-Globin*-Gens als die bevorzugte lentivirale Gentherapiestrategie zur Behandlung von β-Hämoglobinopathien.

In dieser Arbeit wurde gezeigt, dass beide gentherapeutische Werkzeuge, der lentivirale Gentransfer und die Veränderung des Genoms mittels CRISPR/Cas9 System, eine erfolgreiche Möglichkeit für die Genbehandlung von Bluterkrankungen darstellen. Zusätzlich zu den aktuellen Ansätzen der laufenden klinischen Studien konnte gezeigt werden, dass das Reaktivieren von HbF oder die Korrektur des *β-Globin*-Gens zukunftsträchtige Strategien darstellen. Aufgrund der noch niedrigen Genkorrektur-Raten sind fortführende Untersuchungen notwendig.

3. Publications

3.1. Accepted Publications

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

2. 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. Original Article. Doi: 10.1016/j.bcmd.2020.102456

3. **Lamsfus-Calle A***, Daniel-Moreno A*, Ureña G, Raju J, Antony JS, Handgretinger R, Mezger M. Hematopoietic stem cell gene therapy: The optimal use of lentivirus and gene editing approaches. ***Blood Reviews***. 2019. Review. PMID: 31761379

4. Ureña-Bailén G, **Lamsfus-Calle A**, Daniel-Moreno A, Raju J, Schlegel P, Seitz C, Antony JS, Handgretinger R, Mezger M. CRISPR/Cas9 technology: By leaps and bounds towards overcoming the limitations of anticancer CAR-T cells therapies. ***Briefings in Functional Genomics***. 2019. Review. PMID: 31844895

5. 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. Review. PMID: 30903024

6. 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. Original Article. PMID: 30430274

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. Review. Doi: 10.1002/acg2.10

3.2. Manuscripts in preparation

1. **Lamsfus-Calle A***, Daniel-Moreno A*, Ureña-Bailén G, Rottenberger J, Marciano S, Epting T, Heumos L, Baskaran P, Antony JS, Handgretinger R, Mezger M. Universal gene correction approach for β-hemoglobinopathies using CRISPR/Cas9 and AAV6 donor template delivery. **The CRISPR Journal**. 2020. [Submitted]

* Both authors contributed equally.

3.3. Poster and oral presentations

1. Reisensburg Retreat (2019), Günzburg. **Lamsfus-Calle A***, Daniel-Moreno A*, Antony JS, Epting T, Latifi N, Kaftancioglu M, Siegmund D, Kormann MSD, Handgretinger R, Mezger M. Gene editing of hematopoietic stem cells from patients with β-hemoglobinopathies by CRISPR/Cas9. Poster and oral presentation.

2. XXIVth Annual Meeting DG-GT (2018), Freiburg. Antony JS, Kaftancioglu M, Böhringer J, **Lamsfus-Calle A,** Daniel-Moreno A, Klimiankou M, Krogeloh-Mann I, Skokowa J, Handgretinger R, Mezger M. CRISPR/Cas9-mediated hematopoietic stem cell gene therapy for metachromatic leukodystrophy (MLD). Poster presentation.

3. 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 *KLF1*, *BCL11A*, and *HBG1/2* in $CD34^+$ cells by CRISPR/Cas9 as a treatment for β-hemoglobinopathies. Poster presentation.

4. Forschungskolloquium (2018), Tübingen. 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. Poster presentation.

3.4. Contribution to the publications

3.4.1 Hematopoietic stem cell gene therapy: The optimal use of lentivirus and gene editing approaches

In this review article, I designed the outline which gathers pertinent information about gene therapy tools (especially CRISPR/Cas9 and lentivirus), their limitations, and their optimal application in autologous hematopoietic stem cell gene therapy depending on type of disorder and fitness of the edited cells.

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

With the unconditional help from Mr. Daniel-Moreno, I established all gene-editing methods required for this publication, among them, $CD34^+$ HSPC electroporation in Neon Transfection System and CliniMACS Prodigy, gene editing assessment, HSPC differentiation to erythroid precursors, droplet digital PCR (ddPCR), HbF intracellular staining, and library preparation for GUIDE-seq. I further contributed with data analysis and writing the manuscript.

3.4.3. Gene correction of *HBB* mutations in $CD34^+$ hematopoietic stem cells using Cas9 mRNA and ssODN donors

My role in this publication was to perform the *in vitro* gene correction experiments in K-562 by transfection of pX330.sg *HBB*$^{IVS1–110}$ plasmid and multiple ssODN donor templates containing a NheI-tag. I also determined HDR rates by ImageJ software after amplification of *IVS1-110* genomic region by PCR and subsequent restriction fragment length polymorphism (RFLP) detection assay by NheI digestion. Finally, I was responsible of detecting possible off-targets after CRISPR/Cas9 gene editing. To this aim, T7E-assays were conducted for six different genomic regions that were previously detected by *in silico* predicted off-target tools (Zhang Lab).

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

My main contribution to this article was to create a lentiviral production protocol for second and third generation lentiviral systems, which was later improved by Mr. Daniel-Moreno. I also helped in performing the rest of the methodology, data interpretation, and correction of the manuscript.

4. Introduction

4.1. β-hemoglobinopathies

Sickle cell disease (SCD) and β-thalassemia belong to the group of β-hemoglobinopathies, an autosomal recessive inherited blood disorder caused by abnormalities in the adult hemoglobin (HbA) and characterized by hemolytic anemia. In general, β-hemoglobinopathies are the most common genetic diseases in which a single gene is mutated.[1] To date, over 300 mutations have been described in the *β-globin* gene,[2,3] affecting worldwide to approximately 56,000 newborns with β-thalassemia and 270,000 newborns with SCD (Figure 1).[4] Mutations in the *β-globin* gene lead to absent (β^0) or reduced (β^+) expression of *β-globin* in patients with β-thalassemia, whereas a mutation at codon 6 (glutamine > valine) results in a structurally altered protein causing SCD. Since β-thalassemia is an autosomal recessive disorder, only homozygous carriers suffer from clinical symptoms and can exhibit, either a mild form (thalassemia intermedia, $\beta^+\beta^+$ or $\beta^+\beta^0$) or severe form (thalassemia major, $\beta^0\beta^0$).[5]

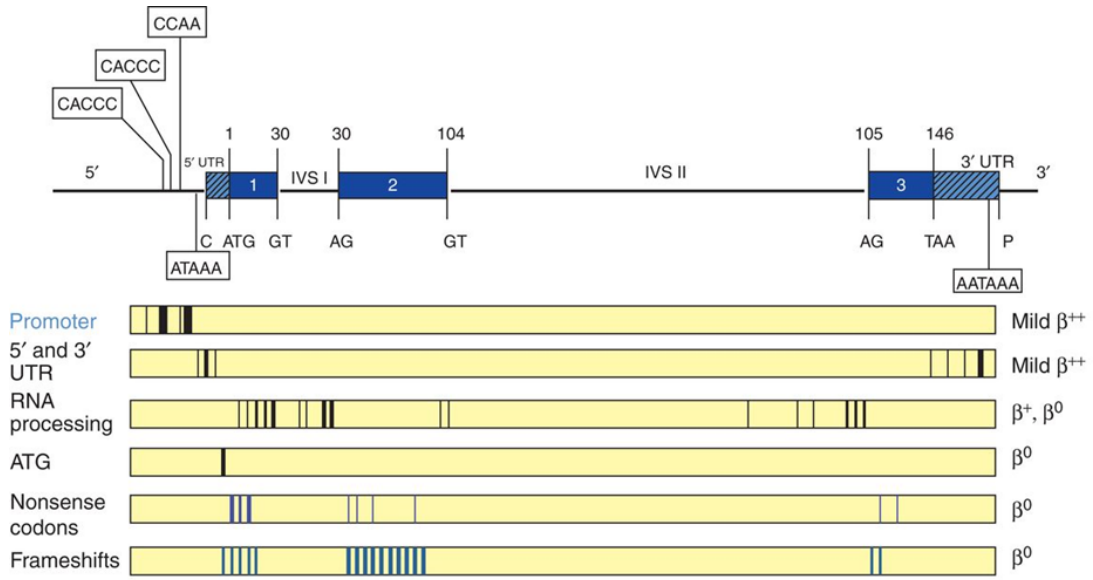

Figure 1. Representation of *HBB* gene and the most prominent point mutations that cause mild or severe form of β-thalassemia. Each long yellow box represents type of mutation (left side) and outcome (right side). The vertical lines inside the boxes refer to common point mutations for each category. Modified from Thein et al 2013.

The impaired expression of *β-globin* causes compensatory up-regulation of *α-globin* resulting in intracellular aggregation of hemoglobin. As a consequence, erythropoiesis is negatively affected, inducing apoptosis of red blood cells and anemia.[6] Symptoms of this disease include fatigue, headaches and dizziness, which later evolve in various skeletal deformities and hepatosplenomegaly, due to disturbed erythropoiesis and extramedullary blood formation.[7]

Because thalassemia major patients present a pronounced anemia, life-long blood transfusions are required, which are associated to immunosuppresion, immunological reactions, and iron overload (hemosiderosis).[8] Therefore, life expectancy of these patients is significantly reduced owing to organ dysfunction as the main cause of death.

In the last decades, hematopoietic stem cell transplantation (HSCT) using allogeneic bone marrow, umbilical cord blood, or mobilized peripheral blood, has shown remarkable success as an effective cure for β-thalassemia. [9] However, allogeneic HSCT is gravely limited by the scarce availability of matched sibling donors and often associated with immunological complications, including graft-rejection and graft-versus-host disease (GvHD).[10] Therefore, autologous HSCT along with gene therapy tools are overtaking allogeneic HSCT for its life-long treatment capacity and fewer side-effects.[11] Gene therapy approaches aim principally to transfer a healthy copy of *β-globin* or re-induce the expression of *γ-globin*, and thus, fetal hemoglobin (HbF).[12] Alternatively to HbA (2α2β), HbF is expressed in newborns and consists of 2 α chains instead of 2 γ chains (2α2γ). Patients with co-inherited β-thalassemia and hereditary persistence of fetal hemoglobin (HPFH) were asymptomatic for the disease, demonstrating the therapeutic value of HbF for the treatment of β-hemoglobinopathies.[13] Therefore, novel gene therapy treatments are focused on blocking the expression of the main regulators involved in γ- to *β-globin* switching (*KLF1*, *BCL11A*)[14-16] and their binding site (*HBG1/2* promoters; Figure 2)[17,18] by CRISPR/Cas9 system, or providing a *γ-globin* gene copy by lentiviral vectors (LVs).[19,20]

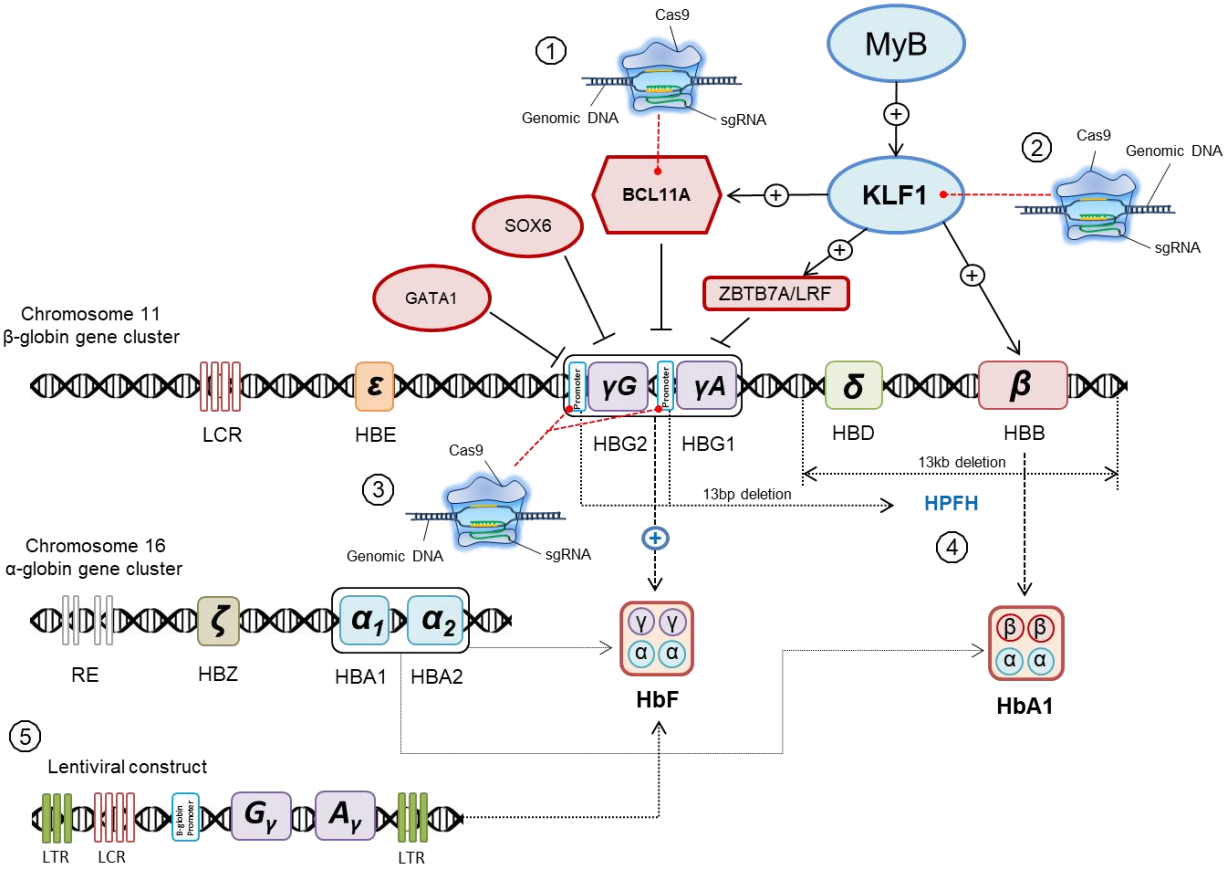

Figure 2. Network of molecular regulators taking part in the fetal (HbF) to adult (HbA) hemoglobin switch, and novel CRISPR/Cas9 genome disruption and lentiviral approaches to increase HbF as a therapy for β-hemoglobinopathies: *β-globin* gene cluster in chromosome 11 is constituted by the locus control region (LCR) and an assembly of ε, γG, γA, δ, and β genes. *α-Globin* gene cluster in chromosome 16 comprises the regulatory elements (RE) and the genes ζ, α1, and α2. *MyB*, *KLF1*, *BCL11A*, *SOX6*, *GATA1*, and *ZBTB7A/LRF* represent the genes regulating *γ-globin* expression. HbF resurgence by CRISPR/Cas9 gene disruption of (1) the *γ-globin* repressor BCL11A, (2) KLF1, and (3) the binding site of *γ-globin* repressors. (4) Recreation of the hereditary persistence of fetal hemoglobin (HPFH) by deleting the *δ*- and *β-globin* genes (13 kb), or deleting the *γ-globin* promoter (13 bp). (5) HbF reactivation by lentiviral gene transfer of *γ-globin* gene under the influence of *β-globin* promoter and LCR. Modified from Antony et al. 2018.

4.2. Gene disruption and gene correction by CRISPR/Cas9 system

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated RNA-guided endonuclease Cas9 (CRISPR/Cas9) system is a very promising and revolutionary technique that has emerged in recent years for creating specific changes into the genome. Nowadays, CRISPR/Cas9 system is considered the best gene-editing technology in comparison to previous gene-engineering tools (ZFNs and TALENs) for its high efficiency, low production costs,

and simple handling.[21] Only two components are required, a protein endonuclease (Cas9) and a single guide RNA (sgRNA). Cas9, considered as a molecular scissors, will be directed by the sgRNA to a specific locus in the genome and induce a double-strand break (DSB).[22] Consequently, two repair mechanisms can repair DSBs: non-homologous end-joining (NHEJ) or homology-directed repair (HDR). The former is often predominant and consists in fast and unreliable correction of the damage by nucleotide insertions or deletions (indels) that can shift the gene reading frame. In contrast, HDR befalls mainly in the late S/G2 phase of the cell cycle, where the DNA damage is meticulously corrected through homologous recombination with the sister chromatid.[23] This way, the activation of NHEJ repair mechanism is used for gene disruption strategies, whereas HDR is utilized for gene correction when delivering a healthy copy of the gene, or gene addition, after providing a transgene with homology sequences to the endogenous gene.[24]

Gene disruption by CRISPR/Cas9 has already reached the clinics, due to its simplicity and efficiency in primary cells. First clinical trials using CRISPR/Cas9 are currently being conducting for the treatment of β-thalassemia (NCT03655678, NCT03745287). The gene disruption approach consist in targeting the enhancer of *BCL11A* by *ex vivo* modification of hematopoietic stem and progenitor cells (HSPCs) and further reinfusion into the same patient (autologous HSCT).

On the other hand, gene correction strategies are still in preclinical phase because the quiescent stage of HSPCs hinders the activation of HDR repair mechanism. However, recent studies have shown elevated gene correction rates by donor template delivery through single-stranded DNA/ODN (ssDNA/ssODN), adeno-associated viruses (AAV), and integrase-defective lentiviruses (IDLV).[14,25-27]

Over the past 10 years, CRISPR/Cas9 has evolved to a safer and more efficient platform. Nowadays, different types of Cas9 protein, CRISPR/Cas9 deliveries, and modified sgRNAs, among others, have increased the efficiency, specificity, and fidelity of the system, reducing the risk of undesired DSBs into the genome (off-targets).[22] Off-targets are the principal concern in the scientific community, and thus, multiple platforms have been developed for their detection

such as GUIDE-seq and CIRCLE-seq.[28,29] However, new off-target detection tools are required to certainly determine the safety profile of the system in preclinical stages.

For further information about CRISPR/Cas9 safety, improvement and recent information about clinical and preclinical trials, please refer to Lamsfus-Calle et al. 2019[22], Daniel-Moreno et al. 2019[11], and Antony JS et al. 2018[14].

4.3. Gene transfer by lentiviral vectors

Lentiviruses are modified HIV retroviruses belonging to *Retroviridae* family, widely utilized to deliver transgenes for the treatment of blood and immune disorders.[30] LVs hold large cargo capacity and can effectively infect a broad range of cell types, inducing low genotoxicity.[31] Due to these characteristics, LVs play an important role for treating diseases caused by loss-of-function mutations.[22] Furthermore, its large-carrying capacity enables not only to deliver the transgene of interest, but also the regulatory elements necessary to induce a similar expression pattern to healthy conditions. However, since proviral information is integrated semi-randomly into the genome, transgene expression can also be affected by surrounding DNA.[1] Therefore, insulators are also included as part of the proviral cargo to avoid uncontrolled expression of the transgene. Another point to be considered is the number of transgene integration events per cell, also known as vector copy number (VCN). U.S. Food and Drug Administration (FDA) reported that in clinically terms, the VCN should not exceed five integration events per cell in order to prevent uncontrolled transgene expression.[31] Thereby, several research groups have generated lentiviruses with baboon retroviral envelope glycoproteins (BaEVs), as an alternative to the common vesicular stomatitis virus G (VSV-G) envelope glycoprotein, since BaEVs tropism permits high transduction efficiencies with lower number of virus particles per cell. Therefore, the safety profile and efficacy of this gene therapy tool is enhanced.[32]

Recent therapeutic approaches for β-hemoglobinopathies are based on the introduction of a healthy copy of the *β-globin* gene by means of lentiviral vectors.[33] A number of different vectors and constructs have been tested in recent years. The first gene therapy trials with β-thalassemia patients were executed in the US and Italy, where clinical success has been reported.[12,34] For more detailed

information about lentiviruses and clinical trials please referred to Lamsfus-Calle et al. 2019.[22]

5. Objectives

The goal of this PhD thesis was to establish a CRISPR/Cas9 and lentiviral platform at the University Children's Hospital Tübingen for the treatment of blood disorders, particularly β-hemoglobinopathies. To this aim, we firstly performed a gene disruption strategy of the principal regulators involved in the fetal-to-adult hemoglobin switching and assessed the induction of HbF at DNA, mRNA, and protein level. Secondly, we conducted gene addition experiments in HSPCs to give clear evidence of *HBB* correction as an alternative treatment. To this purpose, we compared different gene editing tools (TALENs, ZFNs and CRISPR/Cas9) to determine which of them is the most suitable for gene editing. Finally, since the first clinical trials for β-hemoglobinopathies are based on *β-globin* gene transfer by lentiviruses, we compared different lentiviral constructs and envelopes to improve the already existent gene therapy treatments for β-thalassemia and sickle cell disease.

6. Results and Discussion

6.1. Hematopoietic stem cell gene therapy: The optimal use of lentivirus and gene editing approaches

This review article holds important updated information about gene therapy. Along this publication, we focused on autologous hematopoietic stem cell transplantation (HSCT) as an undeniable promising *ex vivo* gene therapy to treat particularly blood disorders caused by monogenetic mutations. Furthermore, we collected basic knowledge about retroviral vectors (retroviruses and lentiviruses) and gene editing tools (meganucleases, TALENs, ZFNs, and CRISPR/Cas9), their limitations, and how they have evolved to a safer platform. Also, we compiled pertinent information that needs to be considered when conducting gene therapy, as for example, which genetic tool is the most suitable depending on gene size and cell fitness. All this information is explained together with current available data about recent clinical trials in the field of gene therapy. Alternatively, we offered through this article, new ideas to treat neurological, lung, and inflammatory disorders based on the concept of “Cell Trafficking”, in which the edited hematopoietic stem cells can be transported to specific inaccessible target tissues. To finalize, in the last section of this publication, we discussed about improvements in the field of gene editing with especial emphasis in lentiviral tropism, enhancement of HDR rates, and novel base editors including the recently discovered prime editing.

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

Recently, gene addition in autologous HSPCs has shown the potential to cure thalassemia major and SCD, resolving the limitations of finding matched donors as well as the immunological complications associated with allogeneic-HSCT.[9] In this approach, lentiviral vectors derived from HIV were used to express a functional copy of *β-globin* gene (*HBB*) in the patient’s own HSPCs.[33] Efficient gene transfer in a high number of HSPCs should be achieved to ensure consistent and therapeutic levels of *β-globin* gene expression. Unfortunately, the efficacy and safety of gene transfer approaches is limited by several factors,

including insufficient HSPC transduction, dysregulated expression of the transgene, and risk of genotoxicity due to random viral vector integration and insertional mutagenesis.[35] Therefore, promising gene therapy alternatives have emerged by using the revolutionary CRISPR/Cas9 system to disrupt the main regulators involved in fetal-to-adult hemoglobin switching. Fetal hemoglobin (HbF) is only expressed during gestation but its reactivation in adults has been observed to be functional, since β-thalassemia patients with hereditary persistence of fetal hemoglobin (HPFH) lack disease symptoms.[13] Multiple gene mutations have been descripted in HPFH patients, especially in *KLF1* gene and promoter regions of *γ-globin* subunit 1 and 2 (*HBG1/2*).[18,36] *KLF1* positively regulates the expression of *HBB* and *BCL11A*, being the latter involved in *γ*-globin repression by binding to the promoter region of *HBG1/2*.[16] However, fully repression of BCL11A is associated with impaired hematopoiesis, and thus, several researches have targeted its enhancer region for HbF reactivation.[37] In fact, two clinical trials are currently in active stage for this strategy to cure β-thalassemia (NCT03655678) and SCD (NCT03745287). In this study, we aimed to compare gene disruption of exonic region of *KLF1,* enhancer of *BCL11A,* and *BCL11A* binding-sites *in HBG1/2* promoter regions to assess which gene editing strategy is superior in terms of safety and HbF resurgence.

6.2.1. *In vitro* gene editing assessment and HbF reactivation in gene edited-HSPCs

DsRed mRNA was utilized to select the most convenient Neon electroporation setting (1650 volts, 10 milliseconds width, and 3 pulses) for $CD34^+$ HSPCs in terms of viability and transfection efficiency. After complex formation of chemically modified sgRNA and Cas9 RNP, $CD34^+$ HSPCs were transfected and subsequently transferred to erythroid-lineage differentiation medium for 21 days. At day 5 post-electroporation, cells were harvested for further DNA isolation and amplification of locus target sites by PCR. DNA amplicons were sent for sequencing to determine the gene editing score by using ICE analysis tool. All sgRNAs (two per target) achieved high insertion and deletion (indels) rates (up to 91%). Gene editing was also reflected at mRNA level on day 21, where all edited samples noticed an increment in *γ-globin* transcripts,

especially for HBG1/2 approach (>6.5 fold). Furthermore, *KLF1* and *BCL11A* mRNA levels dropped for *KLF1*- and *BCL11A*-treated samples. Also, high-performance liquid chromatography (HPLC) and HbF intracellular staining were performed on day 21, showing strong HbF resurgence (HPLC: up to 41.9%; HbF^+: up to 91.7%). *KLF1* T1, *BCL11*A T2, and *HBG1/2* T2 were the sgRNAs that achieved the highest indel rates, *γ-globin* up-regulation, and HbF levels. Finally, flow cytometry staining for erythroid markers (CD71 and CD235a) assured that none of the treatments presented impaired hematopoiesis.

6.2.2. Evaluation of the gene editing safety profile by RNA-seq and GUIDE-seq

Since these strategies hold potential for clinical translation, assessment of the safety profile is demanded. In our study, RNA-seq and GUIDE-seq were conducted with this purpose. RNA-seq results showed similar expression profile for gene-edited samples in comparison to the control (92-99%). Particularly, *BCL11A*-treated samples presented the best gene expression profile. In contrast, *KLF1* and *HBG1/2* gene disruption led to dysregulated genes, being *KLF1* the treatment with the highest number of impaired genes. Noteworthy, impaired genes in *BCL11A*-edited cells were not associated with any oncogene or tumor suppressor gene, whereas in *KLF1*- and *HBG1/2*- treated samples, several genes were involved in cell proliferation, apoptosis, and immune pathways. Most importantly, no off-targets were identified for most sgRNAs after GUIDE-seq analysis, except for *HBG1/2* sgRNA, for which 2 on-targets and 1 off-target were detected. After alignment of the off-target sequence to the human reference genome, this sequence was identified as long non-coding RNA, which could explain the impaired transcription profile due to its role in gene regulation. Interestingly, two on-targets were detected in HBG1/2, which matched with *γ-globin* gene subunit 1 (*HBG*1) and 2 (*HBG2*). However, low numbers of reads were detected for these regions. Since these subunits have homologous sequences in the promoter, *HBG1/2* sgRNA can induce DSBs either in *HBG1, HBG2* or both subunits simultaneously. When the latter occurs, a ~5-kb genome fragment is excised, hindering GUIDE-seq results. To confirm this fact, droplet-

digital PCR (ddPCR) was used to amplify the intergenic region between *HBG1* and *HBG2*, where 43% of treated-HSPCs presented a 5-Kb fragment excision.

6.2.3. Gene editing translation to CliniMACS prodigy

Due to the potential of these sgRNAs to induce HbF up-regulation, we transferred this gene-editing platform to the CliniMACS Prodigy. This GMP-device offers fully automatized cell culture, magnetic sorting, and electroporator in a closed system. As performed in Neon Transfection System, the optimal electroporation setting (square pulse type, 1st pulse: 600V/100μs; 2nd pulse: 300V/2ms) was established using *DsRed* mRNA. After HSPC transfection with *KLF1*, *BCL11A*, and *HBG1/2* sgRNAs and cultivation for 21 days, similar gene editing and HbF results were obtained in comparison to Neon-transfected cells (indels: up to 86%, HbF: up to 45.3%).

In summary, HBG1/2 gene editing strategy was superior over the others in terms of *γ-globin* and HbF reactivation using both types of electroporation devices. However, the doubtful safety profile of this gene editing approach should be further investigated. In contrast, although KLF1 achieved lower gene editing scores and HbF resurgence, this strategy might reduce disease symptoms since >20% of HbF up-regulation has been shown to ameliorate the disease outcome.[38] Nonetheless, its gene expression profile is not suitable for clinical translation. Finally, BCL11A gene editing approach, which is currently in clinical trials, induced significant levels of HbF with unaltered gene expression profile, being the preferred gene-editing strategy up to now for the treatment of β-hemoglobinopathies.

6.3. Gene correction of *HBB* mutations in CD34^{+} hematopoietic stem cells using Cas9 mRNA and ssODN donors

We already described gene disruption as a gene therapy strategy for the treatment of β-hemoglobinopathies. Additionally, we introduced gene correction/addition, which occurs in lower frequencies, but it is considered a valuable approach for gene therapy because it mimics endogenous gene expression after gene modification. In the following study, our first goal was to assess which gene editing platform (CRISPR/Cas9, TALENs, and ZFNs) is the

most adequate for *HBB* gene editing. To this aim, gene editing tools were programmed to target the splicing variant *HBB*$^{IVS1\text{-}110}$, which is commonly detected in β-thalassemia patients, and were transfected in HEK 293T cells at different concentrations (0.5 µg, 1.0 µg, and 1.5 µg). CRISPR/Cas9 tool overperformed TALENs and ZFNs for all concentrations due to its high gene editing scores. Therefore, CRISPR/Cas9 was the gene editing tool of choice for next experiments. Our second aim was to introduce, as a proof of principle, a NheI-tag at IVS1-110 position by electroporation of pX330.sg *HBB*$^{IVS1–110}$ plasmid and single-stranded oligonucleotide (ssODN) donor in K-562 cells. Multiple ssODN NheI-tagged designs differing in their length of homology arms, chemical modification, and target strand were tested, obtaining NheI integration frequencies up to 31% by restriction fragment length polymorphism (RFLP) assay. When transferring this gene editing protocol to CD34^{+} HSPCs, insertion percentages significantly decreased, and clear evidence of cell death was observed. Only by using 5TS-ssODN, 3% of insertion rate was detected by TIDE analysis and 2% by semi-quantitative single-colony sequencing. To improve the effectiveness of gene correction and cell viability, Cas9 pDNA was replaced by chemically modified Cas9 mRNA, which led to a gene editing rate increase to 87% and gene correction to 11%, when using 5TS-,10TS-, 11TS-, and chemically modified-ssODNs. However, gene insertion values for chemically modified-ssODN dropped to 8% after absolute quantification by Next-Generation Sequencing (NGS). Also, no off-target cleavage was identified after screening six *in silico* predicted off-target genes. In brief, among the most efficient gene editing tools, CRISPR/Cas9 system showed superiority in generating genome specific-cleavage and induced 8% of gene insertion in CD34^{+} HSPCs when using Cas9 mRNA and non-viral chemically modified ssODN. Nonetheless, further research to increase gene correction rates in CD34^{+} HSPCs is needed for clinical relevance in *ex vivo* gene therapy.

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

Multiple successful clinical trials have been conducted using lentiviral vectors for the treatment of β-thalassemia. Although there is a risk of insertional mutagenesis, no genotoxicity has been reported, except for a patient who presented a myeloid-biased cell dominant clone expansion due to insertions of the transgene near *HMGA2* oncogene. However, the patient was followed-up during 17 years and did not develop leukemia.[35] Furthermore, to avoid the above-mentioned reasons, safer lentiviral vectors have been designed such as self-inactivating lentiviral vectors and novel insulators.

Alternatively to the already available lentiviral approach consisting in *β-globin* gene delivery, in this study we attempted to induce *γ-globin* up-regulation and subsequent HbF reactivation using three different strategies. The first approach consisted in HSPC transduction of insulin-like growth factor 2 mRNA binding protein-1 (*IGF2BP1*) transgene that is involved in the transcriptional repression of *BCL11A.*[39] Secondly, a lentiviral construct encoding for a microRNA-embedded shRNA (shmiR) against *BCL11A*.[40] The last strategy consists in a lentiviral vector encoding for *γ-globin* under the influence of *β-globin* promoter.[19] Additionally to the three different lentiviral transgene constructs, we also performed a head-to-head comparison between baboon envelope proteins (BaEV, BaEV-RLess) and the often used vesicular-stomatitis-virus-G envelope protein (VSV-G). Baboon envelopes were reported to be superior in terms of HSPCs transduction efficiency over VSV-G,[32,41,42] particularly BaEV-RLess was considered the most efficient due to the deletion of the R-peptide which negatively regulates viral entry.[43] Therefore, the aim of this project was to identify which transgene and envelope combination reflects in strong HbF resurgence for further clinical translation.

6.4.1. GFP-lentiviral transduction efficiencies in HSPCs

GFP-lentiviruses were firstly generated as described by Girard-Gagnepain et al.[32] to ensure proper virus production and transduction efficiencies in Lenti-X

293T, K-562, and HSPC cells. Virus titer was quantified by qPCR, ELISA, and cell-mediated titration. ELISA and qPCR titration resulted in high viral titer for the three types of viruses without pronounce differences. However, the results are overestimated since functional and non-functional viral particles can be measured by these methods. On the contrary, when virus titer was calculated by cell-mediated titration, a clear pattern was observed, where BaEV-lentiviruses production was greater than BaEV-Rless, but lower than VSV-G. VSV-G lentiviruses achieved better transduction efficiencies than baboon envelopes when measuring GFP-positive cells at day 7 post-transduction by flow cytometry. Interestingly, even though the virus titer for BaEV-RLess was lower than BaEV, transduction by BaEV-RLess-lentiviruses was superior over BaEV. The highest number of GFP-transduced cells was detected when infecting K-562 cells (up to 94%). However, for HSPCs transduction further optimization was required, especially for baboon lentiviruses. Thus, to this purpose, RetroNectin and Cyclosporine H (CsH) were tested. No improvements were detected by utilizing RetroNectin, whereas Cyclosporine H significantly increased the number of transduced cells (VSV-G: 18.6%; BaEV-RLess: 25.26%; BaEV: 7.45%). Noteworthy, even though VSV-G lentiviruses attained better transduction efficiencies, fewer BaEV-RLess and BaEV viral particles per cell (MOI) were required to obtain decent efficiencies.

6.4.2. Induction of *γ-globin* and HbF expression by lentiviral constructs

Lentiviruses with the three types of envelope (VSV-G, BaEV-RLess, BaEV) were produced independently in order to transfer *IGF2BP1* (pLVX-IGF2BP1), shRNA *BCL11A* (pCL20-BCL11A), and *γ-globin* (pCL20-γ-globin) transgenes into $1x10^4$ HSPCs. After lentiviral transduction with CsH, cells were differentiated to erythrocyte precursors during 21 days. At day 7, transduction efficiencies were measured for pLVX-IGF2BP1 and pCL20-BCL11A since these constructs also contained a *GFP* reporter gene. As observed previously in GFP-transduced cells, VSV-G was superior over baboons for both constructs, attaining higher efficiencies with pCL20-BCL11A. Importantly, VSV-G MOIs were considerably higher than baboon MOIs. Also, transgene integration events indicated as vector copy number (VCN) were determined for all treated samples

obtaining an average value ranging from 0.1 to 2.2 proviral copies per cell. These values are below the maximum VCN accepted by the U.S. Food and Drug Administration, demonstrating the therapeutic value of these strategies.

Expression analysis was conducted at day 14 post-transduction. For all treatments, *γ-globin* transcripts relative to total (*γ-globin* + *β-globin*) were significantly elevated, especially for VSV-G-treated samples. Furthermore, a strong decay in *BCL11A* transcripts was observed in VSV-G-pCL20-BCL11A samples, while a significant overexpression of *IGF2BP1* was perceived in HSPCs transduced by VSV-G-pLVX-IGF2BP1. Moreover, HbF resurgence was measured by HPLC on day 21, where pLVX-IGF2BP1 and pCL20-BCL11A presented a prominent HbF induction compared to pCL20-γ-globin for all types of envelopes, being extremely significant in VSV-G-treated samples (pLVX-IGF2BP1: up to 51.6%; pCL20-BCL11A: up to 56.5%; pCL20-*γ-globin*: up to 22.6%). Finally, erythroid differentiation was not impaired in all treated samples.

Previous studies reported greater tropism of baboon lentiviruses over VSV-G-lentiviruses in HSPCs, as fewer amounts of viral particles (MOI) were necessary to effectively transduce the cells. Concordantly, our results showed that lower baboon MOIs led to decent transductions efficiencies, whereas higher number of viral particles was needed for VSV-G. The greater performance of GFP-lentiviruses over the other three constructs was probably due to differences in the transgene size and their gene expression under diverse promoters. The transgene size of the GFP construct was similar to BCL11A (~4kb), however, higher percentage of GFP^+-HSPCs were detected for the former by flow cytometer due to GFP expression under the influence of *PGK*, a constitutive promoter. On the contrary, pLVX-IGF2BP1, pCL20-BCL11A, and pCL20-*γ-globin* contain a lineage-specific promoter and their performance was strictly related to its insert size, being pCL20-γ-globin the largest transgene (7.5kb). Furthermore, virus titer data suggested that baboon lentiviruses have lower cargo capacity, affecting the assembly of the viral components. Likewise, baboon virus titer was comparable to VSV-G when measured by qPCR and ELISA, but not through cell-mediated titration.

Finally, VSV-G was superior over baboon counterparts in terms of HbF up-regulation. All constructs delivered by VSV-G envelope achieved therapeutic levels (>20%), being pCL20-*γ-globin* the most straight-forward strategy due to its absent role in signaling pathway. Indeed, a clinical trial (NCT02186418), currently in recruiting stage, has been initiated to implement this approach to the clinic. Nonetheless, further investigation is required for baboon envelopes, whose transduction advantages bring them as potential candidates for the treatment of diseases, where smaller constructs are needed.

7. Concluding remarks

Along this thesis, we have established in our hospital three types of gene modification: gene disruption, gene correction/addition, and gene transfer.

The novelty of our gene disruption project was to include KLF1 and HBG1/2 approach in this study. Gene editing studies have previously been performed for KLF1, but experiments were only conducted in immortalized cell lines.[15] On the other hand, promising outcome about targeting *HBG1/2* promoter was earlier reported in Nature 2016.[17] Nevertheless, CRISPR/Cas9 system was delivered to HSPCs by means of lentiviruses, and thus, the possible drawbacks of both technologies challenged its clinical relevance. Furthermore, above-mentioned strategies were compared under the same conditions than the currently clinical approach, BCL11A, which is being conducted in our University Children's Hospital (NCT03655678).

Regarding gene correction, we were the first group to target the abnormal splicing site $HBB^{IVS1\text{-}110}$ in HSPCs. Further experiments and improvements have been performed, obtaining favorable results, in which, we increased HDR rates up to 30% in comparison to the previous achievement.

Lastly, through the lentiviral study we compared two additional approaches to the already existing clinical trial (NCT02186418). Both of them presented promising results, however, a safety profile assessment for this gene therapy strategy is required. Furthermore, we showed the advantage of baboon envelopes over VSV-G in terms of viral burden needed for successful transduction.

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10. Appendix

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Review

Hematopoietic stem cell gene therapy: The optimal use of lentivirus and gene editing approaches

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Blood disorders

ABSTRACT

Due to pioneering *in vitro* investigations on gene modification, gene engineering platforms have incredibly improved to a safer and more powerful tool for the treatment of multiple blood and immune disorders. Likewise, several clinical trials have been initiated combining autologous hematopoietic stem cell transplantation (auto-HSCT) with gene therapy (GT) tools. As several GT modalities such as lentivirus and gene editing tools have a long developmental path ahead to diminish its negative side effects, it is hard to decide which modality is optimal for treating a specific disease. Gene transfer by lentiviruses is the platform of choice for loss-of-mutation diseases, whereas gene correction/addition or gene disruption by gene editing tools, mainly CRISPR/Cas9, is likely to be more efficient in diseases where tight regulation is needed. Therefore, in this review, we compiled pertinent information about lentiviral gene transfer and CRISPR/Cas9 gene editing, their evolution to a safer platform for HSCT, and their applications on other types of gene disorders based on the etiology of the disease and cell fitness.

1. Introduction

Great advances have emerged in the field of gene therapy (GT) in the last decades, evolving from γ-retroviruses to lentiviruses and from meganucleases to the well-known CRISPR/Cas9 system. Several hematopoietic stem cell transplantation gene therapy (HSCT-GT) trials are currently completed or ongoing with lentivirus or CRISPR/Cas9 (LV-based HSCT and CRISPR-based HSCT) to treat multiple genetic disorders [1–4]. Both types of transplantations consist of the *ex vivo* modification of hematopoietic stem cells (HSCs) from a patient, and the subsequent reinfusion into the same body after gene engineering. This way, the progeny derived from the autologous engraftment of repopulating HSCs will ameliorate the symptoms of the treated disease [5].

Although LV-based HSCT and CRISPR-based HSCT hold enormous potential to treat numerous diseases, both platforms possess different inherent capabilities that can be useful depending on the disease. For example, LV-mediated gene therapy is mostly used to overexpress a transgene, whereas CRISPR/Cas9 system is capable of gene disruption, gene correction, and precise transgene integration (Table 1). These variabilities predetermine the success rate of the gene therapy to treat certain diseases. Despite the fact that it is impossible to precisely categorize which platform is suitable for which disease, in this review article we aim to perform an analysis to determine disease-specific HSCT-GT modality.

2. Gene therapy strategies

To date, several genetic engineering approaches can be applied in gene therapy, from gene integration of a functional gene copy, to gene disruption or gene correction of a target of interest (Fig. 1) [6].

2.1. Gene transfer approaches: highlights and challenges

Thanks to the achievement of controlled production of inactivated retroviruses in the mid-80s, gene transfer emerged as the first gene therapy tool for the treatment of blood and immune disorders [7]. Currently, lentiviruses designed from the human immunodeficiency virus (HIV) are the preferred retroviral vectors to deliver transgenes due to their capability to infect dividing and non-dividing cells, wide

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Table 1
Disease classification based on the fitness of the edited cells, gene therapy strategy and gene therapy tool.

	Fitness			Gene therapy strategy			Preferred strategy
Disease	High	Intermediate	Low	Disruption	Addition/integration	Correction	
B-cell malignancies			X	X	X		Lentivirus (+CRISPR)
T-cell malignancies			X	X	X		Lentivirus (+CRISPR)
X-ALD		X			X	X	CRISPR/Lentivirus
MLD		X			X	X	CRISPR/Lentivirus
WAS	X				X	X	CRISPR/Lentivirus
X-SCID	X				X	X	CRISPR/Lentivirus
X-CGD			X		X	X	Lentivirus
β-thalassemia/SCD	X			X	X	X	CRISPR/Lentivirus
HIV	X			X			CRISPR
Hemophilia		X			X	X	CRISPR/Lentivirus
Fanconi Anemia	X				X	X	CRISPR/Lentivirus
Cystic Fibrosis			X		X	X	CRISPR/Lentivirus
CAPS		X				X	CRISPR

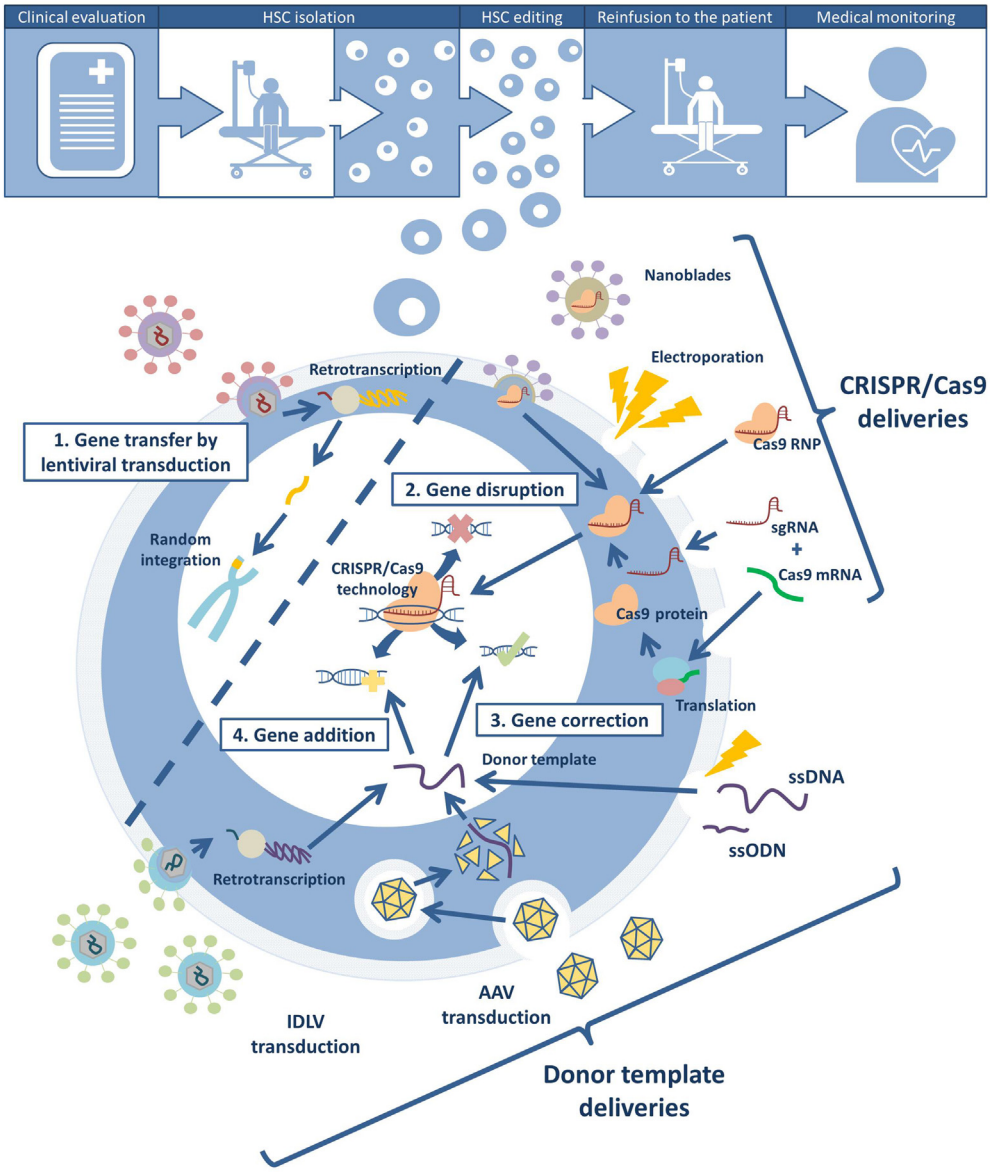

Fig. 1. *Ex vivo* gene therapy approaches for auto-HSCT. The standard procedure for auto-HSCT is illustrated on the top, including: clinical evaluation, blood extraction, HSC isolation, HSC gene editing, and following reinfusion into the patient and medical monitoring. The different gene therapy approaches that can be implemented into the clinic are shown below: [1] gene transfer by lentiviral transduction or [2] gene disruption, [3] gene correction, and [4] gene addition by CRISPR/Cas9. For all gene editing approaches, the preferred delivery of CRISPR/Cas9 components is through electroporation of the RNP complexes or mRNA Cas9 in combination with the sgRNA. Another recently discovered CRISPR/Cas9 transfer approach is through Nanoblades, which can also be used as delivery vehicles. Additionally, a repair template delivery mainly by ssODN/ssDNA electroporation or IDLV/AAV transduction is required for gene addition and gene correction.

tropism, large transgene-carrying capacity (9 Kb), and low genotoxicity [8–10]. Because they have a large transgene-carrying capacity and they integrate semi-randomly into the genome, transgenes can also carry the regulatory elements necessary for the controlled expression in a specific cell type [11]. For this reason, lentiviruses are widely used in diseases that are caused by a loss-of-function mutation. Another important point to be considered is the number of integration events that are taking place per cell (vector copy number, VCN) to achieve a gene expression

level comparable to those exhibited in normal conditions. According to the U.S. Food and Drug Administration (FDA), the VCN should not be more than five in order to avoid uncontrolled transgene expression [12]. Nevertheless, the expression cannot be predicted based on the VCN and it is not always correlated to the therapeutic outcome [8].

Although considered a revolutionary and promising therapy, gene transfer presents several challenging aspects that need to be addressed and further explored. During the first clinical trials by means of γ-retroviruses, most patients exhibited a favorable condition. However, semi-random integration of the provirus close to oncogenes led to the development of leukemia in some of the treated patients [13–15]. Later on, lentiviral gene therapy overtook γ-retrovirus-based gene transfer due to their superior viral transduction, though still presenting a risk of undesired insertions [16,17]. Since then, several safety improvements have been developed in this aspect for further utilization in clinical research. Previously, retroviruses conserved their wild-type LTR regions, and thus, the risk of neighbor gene expression was high since the 5′ LTR contains binding sites for host transcription factors, such as Sp1 and NF-kB [9,18]. Also, recombination can occur between the wild-type LTR regions after integration due to a spontaneous rare event or promoted by natural infection of wild-type retroviruses acting as helper viruses. All these reasons increase the possible risk of gene excision and re-integration in other loci, or the generation of replication-competent lentiviruses [9,19,20]. Therefore, self-inactivating (SIN) vectors were designed to avoid safety concerns by deleting the promoter/enhancer regions of the LTR, allowing the transgene expression to be controlled either by a strong viral promoter such as CMV or RSV, or by an endogenous promoter when a precise transgene expression is required [18,21].

Notably, epigenetics can play an important role in the expression of the transgene after integration. To avoid silencing or overexpression of the transgene, chromatin insulators are utilized as barriers to block nucleosome modifications from surrounding DNA and possible interactions with other enhancers [22], such as the chicken hypersensitive site-4 (cHS4) commonly used in clinical trials [11]. Although new constructs have been developed to increase the safety of this platform, there is still risk of semi-integration near oncogenes, as observed in a β-thalassemia patient after lentiviral gene therapy. In this clinical trial, a patient resulted in a myeloid-biased cell dominant clone expansion after transduction and gene integration of the *β-globin* construct close to the *HMGA2* oncogene; nevertheless, the patient was strictly examined during 17 years and no leukemia progression was detected [17]. To overcome these hurdles, multiple research groups preferred the targeted integration of the transgene in safe genomic harbors, such as adeno-associated virus integration site I (AAVS1), by gene editing tools [23]. However, it is worth noting that targeted integration in primary cells occurs at low frequencies, and thus, lentiviruses are still a leading platform to modify cells on a big scale.

2.2. Gene editing approaches: strengths and limitations

As a promising alternative to gene transfer by lentiviral vectors, gene editing has drawn attention to multiple research groups due to the possibility to perform gene disruption, gene integration, and gene correction approaches. Gene editing is based on the induction of double-strand breaks (DSBs) in the DNA to activate the cell repair mechanisms [24]. The most frequent event that takes place after DSB is the non-homologous end-joining (NHEJ) repair which leads to insertion or deletion of nucleotides due to rapid DNA repair, often introducing frameshift mutations that change the gene reading frame, blocking totally or partially gene transcription and translation [25]. Therefore, NHEJ is mainly used for disrupting target genes. In contrast, the homology-directed repair (HDR) only occurs in the late S/G2 phase of the cell cycle and results in complete gene correction by homologous recombination with the sister chromatid [24,25]. HDR is mainly utilized for gene correction or gene-targeted insertion in a desired locus. The frequency of HDR increases when a healthy gene copy or a cDNA with homologous regions to the endogenous gene is provided either by electroporation of ssDNA/ssODN [26,27] or viral vectors such as integrase-defective lentiviruses (IDLVs) or adeno-associated viruses (AAVs; Fig. 1) [28,29]. However, since HDR is restricted to the S/G2 phase, gene modification in primary cells remains a daunting challenge for the scientific community.

Fast advances were already accomplished in the late 70s when restriction enzymes were discovered and used in the field of gene editing [30]. Nevertheless, even though it was possible to induce gene insertion by homologous recombination after a DSB in yeast and mammalian cells, the frequency of editing was very low (10^{-3} of the transformed cells) [31–34]. On the other hand, higher gene editing efficiencies were attained by means of meganucleases; however, recognition sites are very restricted which hinders gene editing in a desired locus [35]. Alternatively to meganucleases, transcription activator-like effector (TALENs) proteins and zinc finger nucleases (ZFNs) have become a good option for gene editing. TALENs and ZFNs consist of small protein motifs that bind to the DNA by recognition of 1 or 3 base pairs, respectively, and are often delivered in large complexes to increase their specificity to the DNA [36,37]. The fusion of a catalytic domain determined by the nickase Fok-1 to the DNA-binding domain of TALENs and/or ZFNs allows specific target recognition and DSB. Because homodimerization of Fok-1 is a prerequisite to induce DSBs, the specificity and fidelity of these nucleases are really high [37,38]. Nonetheless, though a high number of preclinical and clinical studies have been performed using these nucleases, their production is time-consuming and costly due to complex protein engineering and molecular cloning [30]. Furthermore, the large size of these nucleases hinders their delivery into the cells, often requiring viral vectors especially AAV or IDLV [39,40].

Due to the above-mentioned reasons, the revolutionary CRISPR/Cas9 system formed by an endonuclease (Cas9) and single guide RNA (sgRNA), is currently the gene editing tool of choice. This cost-effective technology is easy to design, produce and deliver, making this technology widely accessible. Although CRISPR/Cas9 can be delivered by adenoviruses (Advs), integrase-defective retroviruses (IDRVs), IDLVs or AAVs [41–44], no vectors are generally needed to deliver the CRISPR/Cas9 system into the cells. A single electroporation of the CRISPR components, either by mRNA or ribonucleoprotein (RNP) complexes, is sufficient to achieve high editing efficiency and low cytotoxicity (Fig. 1) [6]. Furthermore, the RNP delivery reduces the exposure time of the cell to the CRISPR/Cas9 system in order to avoid possible undesired cutting activity in other gene loci (off-targets) [45].

From another point of view, CRISPR/Cas9 is a potential alternative to LV for the treatment of several diseases. Although there have been several safety improvements in the CRISPR field, the screening of undesired DSBs in the genome should always be mandatory, particularly when it holds clinical relevance. Thereby, multiple off-target detection methods have been developed to assess the safety profile of each gene editing strategy, including methods such as LAM-HTGTS, GUIDE-seq, IDLV capture, CIRCLE-seq, BLISS, BLESS, Digenome-seq, and ChIP-seq (Fig. 2) [46–53]. These detection methods may not be completely accurate, and therefore, the development of new off-target screening analyses is one of the main concerns for the scientific community to generate and assess more reliable CRISPR components to increase the specificity and fidelity of this technology. The specificity of the CRISPR system is determined by the Cas9 endonuclease, which recognizes a protospacer adjacent motif (PAM) in the genome. This recognition is paramount to induce a precise DSB in the target gene, and therefore, in the absence of PAM, the accessibility of the Cas9 to the DNA sequence of interest is not feasible [54]. Luckily, PAM sequence recognition varies depending on the type of Cas9 utilized, which broadens accessibility to the gene sequence of interest [30,54]. Cas9 proteins type I and III were mainly found in archaea and classified as class I, whereas type II, IV, V and VI are classified as class II. In the research field, the

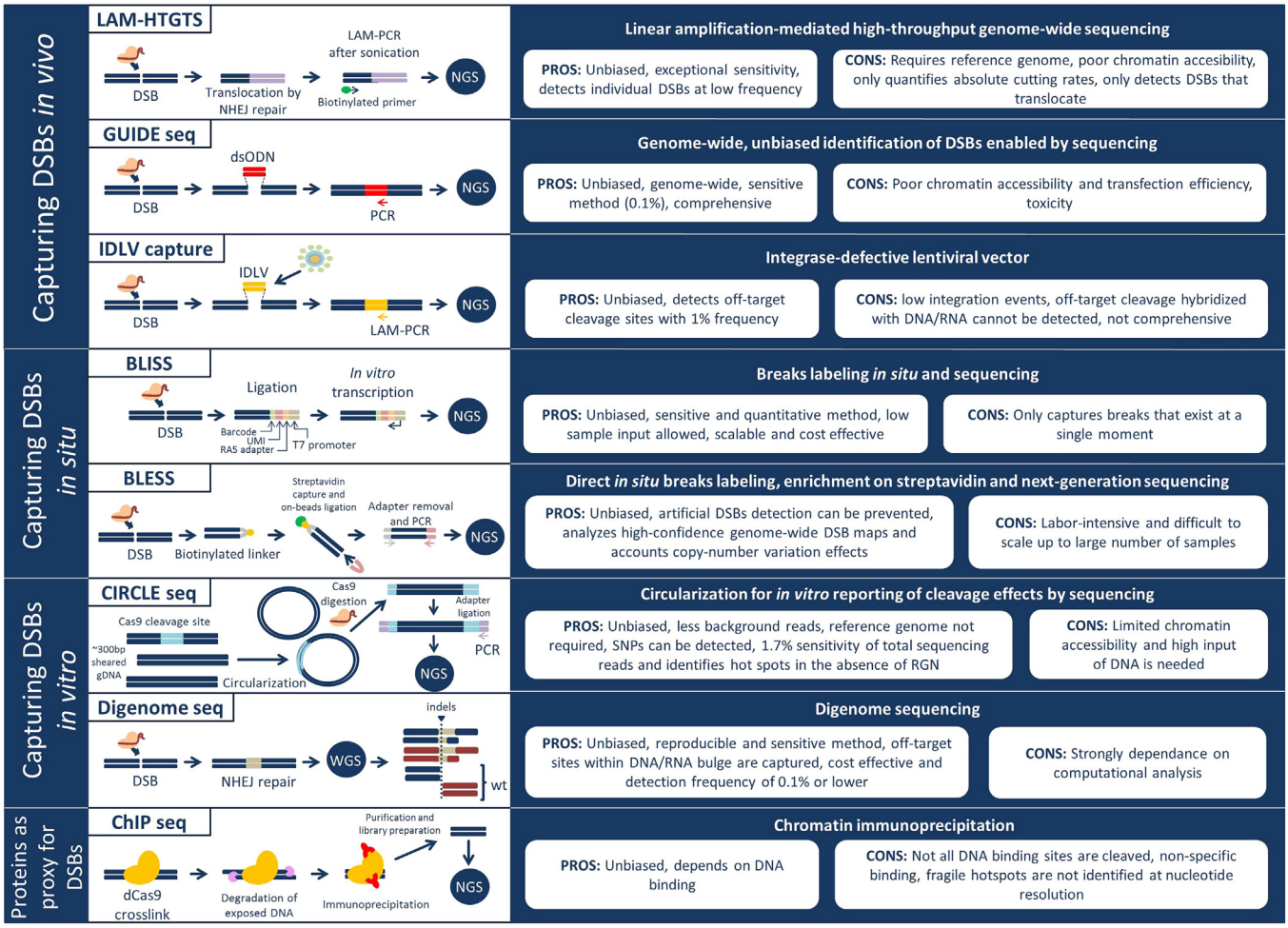

Fig. 2. Off-target screening methods after gene editing by CRISPR/Cas9. Molecular methods are classified depending on the DSB captured *in vitro*, *in vivo,* or using protein-DNA interactions. Brief information about the procedure, advantage, and disadvantages of each method are provided in this figure.

most broadly used is the type II Cas9 system, mainly from *Streptococcus pyogenes*, due to its simple PAM sequence (PAM: NGG) [30]. However, when gene editing is restricted to a specific locus with no accessible PAM, other types of Cas9 have been used, for example, SaCas9 from *Staphylococcus aureus* (PAM: NNGR RT) or NmCas9 from *Neisseria meningitides* (PAM: NNNNG ATT) [55,56]. Another important point to be considered is the fidelity of the system, which is occasionally questionable since the wide flexibility of recognition allows possible mismatches even in the PAM sequence [56]. Nevertheless, truncated sgRNAs (< 18 nt) and new engineered endonucleases based on point mutations in the catalytic domains of Cas9 (RuvC and HNH) have been demonstrated to increase the fidelity of the system [57,58]. This way, two sgRNAs targeting close sequences together with a catalytically inactive Cas9 (dCas9) fused to Fok-1 or a nickase Cas9 (nCas9) protein might increase specificity and fidelity, reducing eventual off-targets [58,59]. Alternatively, Vakulskas et al. performed a screening for different point mutations and discovered a spCas9 variant (R691A) which minimizes off-targets, while maintaining the on-target events compared to the wild-type Cas9 [60]. Also, another nuclease used in recent studies is the type V AsCas12a/AsCpf1 from *Acidaminococcus sp.* (PAM: 5′-TTTV) which presents advantageous properties such as gene editing in organisms with AT-rich genomes, induction of overhang ends with only one single short ~40-nt CRISPR RNA (crRNA), and the possibility to reduce the off-target activity by its high fidelity variant (enAsCas12a-HF1) [61].

3. Gene therapy for blood and immune disorders

HSCT has enormous potential for the treatment of several genetic disorders. Traditional therapies are based on allogeneic HSCT (allo-HSCT), but the difficulty to find a suitable donor together with the strong regimen conditions, risk of graft-*versus*-host disease (GvHD), and strong immune suppression, makes autologous HSCT (auto-HSCT) the current therapy of choice, unless there is a fully matched sibling donor available [5,62]. Despite the fact that auto-HSCT reduces the morbidity and immune suppression after treatment compared to allo-HSCT, the former also presents several limitations, such as the toxicity of the conditioning regimen and the poor repopulation capacity of the modified cells [8,62]. It is pertinent to know that patients undergo chemotherapy prior auto-HSCT in order to eradicate their own cells and facilitate the engraftment of the gene-corrected repopulated cells; however, the toxicity of the chemical compounds also affects negatively the bone marrow environment, and thus, further engraftment [8,63]. Thereby, novel conditioning regimens have emerged to reduce the genotoxicity effect of chemotherapy [64,65]. Another limitation of auto-HSCT is the low engraftment capacity of the repopulated cells due to their poor quality after GT, especially when high percentages of non-edited cells are still present after *ex vivo* modification [8]. In those cases, cell enrichment may improve the engraftment, although additional cell manipulation could lead to cell death. Along these lines, it is necessary to identify which gene therapy tool is the most appropriate for each disease, and whether the modified cells might present a selective advantage to reduce conditioning regimens and increase the cell

Table 2
Clinical gene therapy trials.

Disease	Gene therapy	Goal	NCT	Status	Phase	Delivery	Location
ADA-SCID	γ-retrovirus	Long-term reconstitution of immune functions after retroviral vector-mediated *ADA* gene transfer.	NCT00599781	Complete	I,II	*Ex vivo*	Ospedale San Raffaele - Telethon Institute for Gene Therapy, Milan, Italy (…)
ADA-SCID	γ-retrovirus	Infusion of autologous $CD34^+$ cells transduced with retroviral vector encoding ADA	NCT00598481	Complete	II	*Ex vivo*	Investigational Site, Jerusalem, Israel (…)
ADA-SCID	Lentivirus	Use of the EFS-ADA lentiviral vector to introduce *ADA* cDNA into the hematopoietic progenitors of patients affected with severe combined immunodeficiency due to ADA deficiency.	NCT00018018 NCT01380990 NCT02022696 NCT01852071	Complete Active Complete Complete	I I,II I I,II	*Ex vivo*	National Institutes of Health Clinical Center, Maryland, United States (…)
Fanconi anemia	γ-retrovirus	Transduction of a retroviral vector carrying the *FANCA* gene	NCT00272857	Complete	I	*Ex vivo*	Cincinnati Children's Hospital Medical Center, Ohio, United States
Fanconi anemia	Lentivirus	Transduction of autologous $CD34^+$ cells with the therapeutic *FANCA* lentiviral vector without myeloablation	NCT03157804 NCT03814408 NCT03351868	Active Active Recruiting	I,II I -	*Ex vivo*	Hospital Vall d'Hebron, Barcelona, Spain Stanford University, California, United States Beijing Children's Hospital, China
Hemophilia B	ZFN	Insertion of *FIX* gene into the albumin locus in hepatocytes	NCT02695160 NCT02484092 NCT03307980	Active Active Recruiting	I II II	*In vivo*	UC Davis CTSC Clinical Research Center, California, United States (…)
HIV	ZFNs	Single infusion of autologous $CD4^+$ T cells genetically modified at the *CCR5* gene by ZFNs mRNA	NCT02388594 NCT00842634 NCT02388594NCT00842634 NCT01044654 NCT02500849NCT02225665NCT01543152	Completed	I	*Ex vivo*	University of Pennsylvania, United States (…)
HIV	CRISPR/Cas9	*CCR5* gene disruption in $CD34^+$	NCT03164135	Recruiting	–	*Ex vivo*	Affiliated Hospital to Academy of Military Medical Sciences, Beijing, China
MLD	Lentivirus	Lentivirus-mediated delivery of *ARSA* to the CNS intracerebral injection	NCT03725670	Recruiting	NA	*In vivo*	Lung-Ji Chang Shenzhen, Guangdong, China
MLD	Lentivirus	Autologous $CD34^+$-enriched cell fraction that contains $CD34^+$ cells transduced with a lentiviral vector that encodes for the human *ARSA* cDNA sequence	NCT03392987	Recruiting	II	*In vivo*	Ospedale San Raffaele - Telethon Institute for Gene Therapy, Milan, Italy
MLD	Lentivirus	Autologous $CD34^+$ hematopoietic stem/progenitor cells after introduction of *ARSA* cDNA by means of 3rd generation VSV-G pseudotyped lentiviral vectors	NCT01560182	Active	I,II	*Ex vivo*	Ospedale San Raffaele - Telethon Institute for Gene Therapy, Milan, Italy
MLD/ALD	Lentivirus	Autologous $CD34^+$ hematopoietic stem after transduction of vector-derived *ARSA/ABCD1*	NCT02559830	Recruiting	I,II	*Ex vivo*	Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, Guangdong, China
WAS	γ-retrovirus	Retrovirus-mediated gene transfer of *WAS*	NCT01410825	Active	I,II	*Ex vivo*	Children's Hospital Boston, Massachusetts, United States
WAS	Lentivirus	Autologous $CD34^+$ cells transduced with a lentiviral vector encoding Wiskott-Aldrich syndrome (WAS) protein	NCT01515462	Active	I,II	*Ex vivo*	Ospedale San Raffaele - Telethon Institute for Gene Therapy, Milan, Italy
WAS	Lentivirus	Autologous $CD34^+$ cells transduced with a lentiviral vector harboring the human *WAS* gene.	NCT01347242	Recruiting	I,II	*Ex vivo*	Great Ormond Street Hospital and Royal Free Hospital, London, United Kingdom
WAS	Lentivirus	Autologous $CD34^+$ cells transduced with a lentiviral vector containing human *WAS* gene	NCT01347346 NCT02333760	Complete Recruiting	I,II	*Ex vivo*	Hôpital Necker-Enfants Malades, Paris, France
X-ALD	Lentivirus	Self-inactivating lentiviral vector, TYF-ABCD1, to functionally correct the defective *ABCD1* gene. Intracerebral injection	NCT03727555	Recruiting	NA	*In vivo*	Shenzhen Geno-immune Medical Institute, Guangdong, China
X-CGD	γ-retrovirus	Autologous hematopoietic stem cells transduced with *MT-gp91* retroviral vector	NCT00778882	Active	I,II	*Ex vivo*	Seoul National University Hospital, Korea
X-CGD	γ-retrovirus	Infusion of retroviral gene-corrected autologous $CD34^+$ cells of the peripheral blood	NCT00927134	Complete	I,II	*Ex vivo*	University Children's Hospital, Zürich, Switzerland
X-CGD	γ-retrovirus	Gene-corrected autologous $CD34^+$ cells, using a SIN γ-retroviral vector	NCT01906541	Recruiting	I,II	*Ex vivo*	University Hospital Frankfurt, Germany
X-CGD	γ-retrovirus	Retrovirus-mediated gene transfer of *MFGS-p47phox* or *MFGS-gp91phox*	NCT00001476	Complete	I	*Ex vivo*	National Institutes of Health Clinical Center, Maryland, United States
X-CGD	Lentivirus	Autologous $CD34^+$ cells transduced with a lentiviral vector containing *GP91PHOX* gene	NCT01855685	Recruiting	I,II	*Ex vivo*	University Hospital Frankfurt and Institute for Biomedical Research, Germany (…)

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Table 2 (*continued*)

Disease	Gene therapy	Goal	NCT	Status	Phase	Delivery	Location
X-CGD	Lentivirus	Autologous $CD34^+$ stem cells corrected with *G1XCGD* lentiviral vector	NCT02234934	Recruiting	I,II	*Ex vivo*	University of California, United States (…)
X-SCID	γ-retrovirus	Gene-transduced autologous $CD34^+$ stem cells	NCT00028236	Complete	I	*Ex vivo*	National Institutes of Health Clinical Center, Maryland, United States
X-SCID	γ-retrovirus	Gene transfer using a self-inactivating (SIN) γ-retroviral vector	NCT01129544	Active	I,II	*Ex vivo*	Mattel Children's Hospital – UCLA, California, United States (…)
X-SCID	Lentivirus	Autologous $CD34^+$ hematopoietic stem/progenitor cells after introduction of a normal copy of the *IL2RG* gene	NCT03601286	Recruiting	I	*Ex vivo*	Great Ormond Street Hospital for Children NHS Foundation Trust, London, United Kingdom
X-SCID	Lentivirus	Infusion of transduced autologous $CD34^+$ hematopoietic stem cells	NCT01306019	Recruiting	I,II	*Ex vivo*	National Institutes of Health Clinical Center, Maryland, United States
X-SCID	Lentivirus	Autologous $CD34^+$ HSCs transduced with the lentivirus vector VSV-G pseudotyped CL20-4i-EF1α-hγc-OPT	NCT03315078	Recruiting	I,II	*Ex vivo*	National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Maryland, United States
β-thalassemia/SCD	Lentivirus	Autologous $CD34^+$ hematopoietic stem cells (HSCs) transduced with the LentiGlobin BB305 lentiviral vector encoding the human *beta-A-T87Q globin* gene	NCT02140554 NCT02151526 NCT01745120 NCT02906202 NCT03207009	Active Complete Complete Recruiting Recruiting	I,II I,II I,II III III	*Ex vivo*	Paris, France California, United States (…)
β-thalassemia/SCD	Lentivirus	Autologous $CD34^+$ cells transduced with TNS9.3.55. An open-label study using a non-myeloablative conditioning regimen of busulfan and one or several infusions of autologous hematopoietic stem cells transduced with a lentiviral vector encoding the human *β-globin* gene.	NCT01639690	Active	I	*Ex vivo*	Memorial Sloan Kettering Cancer Center, New York, United States
β-thalassemia/SCD	Lentivirus	Autologous hematopoietic stem cells genetically modified with GLOBE lentiviral vector encoding for the human *β-globin* gene	NCT02453477	Active	I,II	*Ex vivo*	Ospedale San Raffaele, Milan, Italy
β-thalassemia/SCD	CRISPR/Cas9	Autologous $CD34^+$ after *BCL11A* enhancer disruption for HbF resurgence	NCT03655678 NCT03745287	Recruiting	I,II	*Ex vivo*	University Hospital Regensburg, Germany Children's Hospital Tübingen, Germany (…)
β-thalassemia/SCD	ZFN	Autologous $CD34^+$ after *BCL11A* enhancer disruption for HbF resurgence	NCT03432364 NCT03653247	Recruiting	I,II	*Ex vivo*	University of California, United States UCSF Benioff Children's Hospital, California, United States (…)

engraftment capacity. In this part of the review, we describe the most suitable gene therapy method for different blood and immune disorders depending on the fitness of the edited cells (Table 1) while reporting the current clinical trials in complete, active or recruiting stage (Table 2). We refer to fitness in transplantation as cell survival success and how effectively cells adapt to its niche. Taking this definition into consideration, we classify the different diseases into three fitness categories.

3.1. High fitness of edited cells

In this first category, we allude to high fitness when edited cells present a strong repopulating potential due to a selective advantage to the non-edited cells, and thus, lower numbers of engineered cells are needed to ameliorate the symptoms of the disease [66]. In this class are grouped all blood disorders in which the hematopoiesis is impaired, including Fanconi anemia (FA), adenosine deaminase severe combined immunodeficiency (ADA-SCID), X-linked severe combined immunodeficiency (X-SCID), Wiskott-Aldrich syndrome (WAS), and β-hemoglobinopathies. Because the cause of these diseases is due to a loss-of-function mutation, the most suitable gene therapy is gene transfer by retroviruses or gene correction/addition by gene editing tools.

The fitness of the edited cells is particularly high for FA patients since no conditioning is required due to the acquired resistance advantage of edited HSCs over the fragile stem cells in the bone marrow. Recently, the first clinical results of the active FA clinical trial (NCT03157804) have revealed engraftment and proliferation advantages, acquired resistance to DNA cross-linking agents, and multipotent nature of edited HSCs [67]. On the other hand, there are no gene addition clinical studies for FA; however, one preclinical study achieved 14% of gene addition after delivering a wild-type copy of the *FANCA* gene into *AAVS1* safe harbor locus of HSCs by ZFNs and IDLV-repair template, which might be sufficient to alleviate the disease symptoms [68].

Interestingly, the first *ex vivo* gene therapy drug (Strimvelis) was approved by the European Medicines Agency (EMA) at the beginning of 2016, consisting of a γ-retroviral-GT for the treatment of ADA-SCID patients. Notably, 75% of patients presented functional ADA activity, immune reconstitution, and long-term engraftment after reinfusion of transduced HSCs by γ-retroviruses carrying an ADA cDNA copy (NCT00598481). However, insertion of the transgene adjacent to or within oncogenes was detected, though no events of leukemia have been reported in any of the patients [69]. Therefore, to further improve the safety of ADA-SCID therapy, other multiple clinical trials are on the way using lentiviral vectors (NCT00018018, NCT01380990, NCT02022696, and NCT01852071). Unlike the previous outcome for ADA-SCID γ-retroviral-GT, T cell lymphoblastic leukemia was detected in 25% of X-SCID patients treated with γ-retroviruses (NCT00028236). However, in the next X-SCID clinical trial utilizing SIN-γ-retroviruses, 90% of the patients exhibited corrected IL-2RG expression levels without insertional mutagenesis (NCT01129544) [70,71]. A similar scenario was reported for WAS-GT, where the first attempts with γ-retroviral vectors improved immunological parameters of the patients, but 7 out of the 9 patients developed acute leukemia [72]. Thereafter, a viable strategy through SIN-lentiviral vectors was used in WAS patients with no detected integrations near oncogenes and sustained clinical benefit in all treated patients [73,74].

The marked incidence rate of β-hemoglobinopathies has drawn attention from the gene engineering community, incrementing the number of clinical studies. The first human clinical trial (LG001) was sponsored by BlueBird Bio in 2006. Although the β^E/β^0 patient became transfusion-independent a year after treatment with the HPV569 lentiviral vector (*β-globin*^{T87Q}), a transient clonal dominance was observed due to abnormal splicing of the *HMG2* oncogene [17]. After optimization of the vector, multiple clinical trials were conducted in France, USA, Thailand, and Australia (NCT02151526, NCT01745120, NCT02140554, NCT02906202, NCT03207009). Based on the available information, approximately 73% of patients with different genotypes were transfusion-independent after GT-HSCT with no apparent insertional mutagenesis [75]. Furthermore, other two clinical trials are currently in active stage (NCT01639690, NCT02453477), with special emphasis on the clinical trial conducted in Italy, where the edited-HSCs were for the first time reintroduced through intrabone infusion [75]. Alternatively, gene disruption can be an optimal strategy for those diseases in which there is tight gene regulation. For instance, other recent clinical trials for β-hemoglobinopathies are focused on the reactivation of fetal hemoglobin (HbF) by disruption of a transcription factor (BCL11A) involved in fetal-to-adult hemoglobin switching (NCT03655678, NCT03745287). Likewise, a similar gene disruption approach can be applied for other diseases such as acquired immune deficiency syndrome (AIDS), since the disruption of the entry co-receptor (CCR5) for HIV in HSCs leads to a T-cell progeny with a survival advantage over the unedited cells (NCT02388594, NCT03164135).

3.2. Intermediate fitness of edited cells

We gather in this category diseases in which the modified cells do not present any repopulating advantage, but a low number of corrected cells are enough to positively improve the patient outcome [66]. For instance, hemophilia is a hereditary X-linked hemorrhagic disorder caused by mutations in the coagulation factors FVIII (hemophilia A) and FIX (hemophilia B) that are involved in the normal blood clot. Around 60% of the patients suffer from the severe form of the disease, where the expression of normal blood clot factor is < 1% [76,77]. Thereby, low rates of correction are sufficient to switch from the severe form to a moderate form, as observed in hemophilia B mice after > 1% of FIX *in vivo* correction by CRISPR/Cas9 [77]. Currently, there are three *in vivo* clinical trials for hemophilia B consisting in the insertion of the *FIX* gene into the albumin locus in hepatocytes by intravenous injection of AAV ZFNs (NCT02695160, NCT03307980, NCT02484092) [78].

3.3. Low fitness of edited cells

For some diseases, the edited cells do not show a significant change in fitness or may even present a fitness disadvantage. For this reason, a high number of cells have to be modified to attain therapeutic benefits, unlike the two previous fitness categories aforementioned, as edited cells will not preferentially expand or might even be outcompeted by the disease cells [66]. In this fitness category, we find blood sicknesses such as X-linked chronic granulomatous disease (X-CGD), a disorder in which neutrophils do not produce reactive oxygen species adequately to eliminate pathogens [79]. After gene therapy, the corrected cells in X-CGD do not exhibit an advantage, and therefore, given the current situation of technology, lentiviral gene transfer would be the most appropriate approach to treat this type of diseases due to its capacity to genetically engineer a vast percentage of the cell population with ease. Nowadays, there are two ongoing clinical trials in recruiting stage in the EU (NCT01855685) and in the US (NCT02234934) using the G1XCGD lentiviral vector to transduce human CD34$^+$ HSCs from X-CGD patients. There is no current outcome for these clinical trials; however, there is a bright future ahead since preclinical studies showed no evidence of toxicity in human CD34$^+$, whilst NADPH oxidase activity was restored to normal levels after engraftment in NSG mice [80].

In this category, B-cell and T-cell malignancies can also be included; but these disorders are tackled with a completely different approach. The use of chimeric antigen receptor (CAR)-T cells is an innovative treatment utilized as therapy for this type of malignancies, which consists in the expression of CARs on the cell surface of T-cells after lentiviral gene transfer with the aim to target cancerous cells [81]. Next-generation CAR-T cells have been also developed to generate

allogeneic universal CARs by disruption of the TCR receptor, MHCI or inhibitory receptors such as CTL4 and PD-1 using CRISPR/Cas9 [82]. In spite of this, malignant cells may accumulate in reservoirs and might ultimately resurge to outcompete healthy cells hindering the efficacy of this therapy, unless the number of corrected T-cells is sufficient to overcome the tumor reservoirs. Another solution is a persistent pool of anti-cancer CAR-T cells continuously produced by transduced HSCs with the CAR transgene under the influence of a lymphoid-specific promoter. Concretely, the latter option would improve the life quality of the patients, as observed in an NSG humanized mouse model, with only one conditioning regimen necessary to ensure long-term engraftment [83,84].

4. Extended applications for gene-modified autologous stem cells

At first glance, auto-HSCT GT gives the impression to be limited only to the treatment of blood and immune disorders; however, other genetic diseases such as lysosomal storage disorders (LSDs), cystic fibrosis (*CF*) and cryopyrin-associated periodic syndromes (CAPS) can also be tackled using this type of therapy. The treatment for these diseases is based on the concept of "edited-cell trafficking", which consists in the migration of blood cells derived from gene-edited HSCs located in the bone marrow to other target organs.

As aforementioned, LSDs are one example of illnesses that can be treated through trafficking of gene-edited cells. These inherited disorders are caused by loss-of-function mutations in genes involved in the metabolic arrangement of the lysosomes, resulting in aberrant or absent production of catalytic lysosomal enzymes. Their symptoms range from cognitive disability to skeletal deformity, and often premature death [85]. The treatment of LSDs is based on the uptake of functional enzymes by deficient cells, the so-called 'cross-correction', in which 5 to 10% uptake of the recombinant enzyme is sufficient to induce a therapeutic clinical outcome [86]. Thus, LSDs can be included in the intermediate fitness category. During the first clinical trials, the enzyme was intravenously introduced to ameliorate the symptoms of the disease; however, the blood-brain barrier hindered its administration, minimizing the potential benefit for the disease (NCT01510028) [87,88]. Consequently, allo-HSCT became a more effective therapy to replace defective macrophages/microglia with healthy donor cells, but as we previously mentioned, the limitations of allo-HSCT can be overcome using auto-HSCT in combination with gene therapy [5]. The feasibility and benefit of LV-based HSCT in monogenetic neurological disorders were successfully demonstrated for Metachromatic Leukodystrophy (MLD) and X-linked Adrenoleukodystrophy (X-ALD) by the Italian Telethon Foundation and BlueBird Bio, respectively [89,90]. Especially, the MLD trial showed 100% survival, clinical benefit in eight out of nine children (89%), improved motor skills, good engraftment of gene-corrected cells, and reconstitution of ARSA enzyme activity in peripheral leukocytes. Though clinical data supported the safety and efficacy of LV-based HSCT in MLD, the random and non-targeted integration of ARSA-expressing lentivirus resulted in > 10.000 integration sites in gene-corrected HSCs, which holds the risk of insertional mutagenesis, though no malignancy has been observed to date [89,91]. Therefore, the ideal HSCT-GT ensures targeted integration of therapeutic transgenes, preferentially in the endogenous locus by CRISPR/Cas9 [92]. Alternatively to *ex vivo* modification, *in vivo* delivery of lentivirus with a cDNA ARSA copy by intracerebral injection might be an alternative treatment for this disease (NCT03727555, NCT03725670, NCT03392987). Nevertheless, the risk of adaptive immune response activation against lentiviruses may lead to strong inflammation in the brain, although recent advances enabled the evasion of the immune response for other diseases such as Pompe disease by means of AAV [93].

Likewise, HSCT-GT can be implemented for the treatment of cystic fibrosis (*CF*). *CF* is an autosomal recessive disease caused by loss-of-function mutations in the *CF* transmembrane conductance regulator (*CFTR*) gene, which encodes for a cAMP-regulated anion channel [94]. Normal expression of *CFTR* in the epithelial cells of the lungs helps to maintain the homeostasis of a thin liquid layer known as the periciliary liquid (PCL), whose thickness is important to facilitate proper movement of the cilia, and subsequent, mucus clearance [95]. On the contrary, when the channels are impaired, osmotic forces reduce the PCL volume, hindering mucus clearance and fomenting a niche for bacteria, which is the second cause of death for this disease [95,96]. Interestingly, *CFTR* mutations also affect the ion homeostasis in neutrophils and degranulation of antimicrobial peptides as a protective mechanism against bacteria [97,98]. Therefore, alternatively to other types of therapies, we propose a combined treatment that encompasses auto-HSCT-GT to correct *CFTR* mutations in neutrophils, along with the administration of hypertonic solutions to increase the antimicrobial activity and the PCL thickness, in order to enhance the quality and life expectancy of the patients. Since corrected cells do not have any advantage compared to unedited cells, we classify this disease as low fitness, hence a massive quantity of cells must be corrected. Based on these lines, lentivirus gene therapy would be a preferable platform for this treatment rather than CRISPR/Cas9, also because the transgene size is considerably large and its delivery would not be successful by AAV, unless CRISPR/Cas9 is used for the correction of specific mutations.

Other disorders that could be treated through gene-edited cell trafficking are cryopyrin-associated periodic syndromes (CAPS). CAPS are rare hereditary inflammatory disorders caused by 100 different gain-of-function pathogenic mutations in the *NLRP3* gene (NM_001243133.1, Infevers; NG_007509.2, NCBI), which encodes for cryopyrin, a protein expressed in monocytes and neutrophils that is involved in the regulation of the innate immune response [99,100]. Cryopyrin forms inflammasomes that help to cleave pro–interleukin (IL)-1β into its mature form, while its defectiveness leads to over-activation of the inflammasome, increased IL-1β secretion, and systemic inflammation [101]. Current treatments target IL-1β or its receptor by monoclonal antibodies or inhibitory compounds, respectively [99]. However, these treatments are life-long administrated and present some side effects. Since *NLRP3* plays a critical role in the innate immune response, full knockout of this gene would result in immunosuppression [100]. Therefore, a personalized HSC gene correction approach by CRISPR/Cas9 for each pathogenic mutation would be a potential GT treatment, also considering that a low number of gene-edited cells could sufficiently reduce inflammatory symptoms (intermediate fitness).

5. Future perspective of gene therapy tools

In this part of the review, we emphasize current investigations on lentivirus and CRISPR/Cas9 variants to improve existing gene editing tools and develop new approaches for gene therapy. As an instance, new envelopes have been designed to widely increase the application of lentivirus in the field of gene engineering. The most frequently used is the vesicular stomatitis virus-G (VSV-G) envelope derived from murine leukemia virus (MLV) that utilizes phosphatidylserine residues present in all mammalian cells as way of entry [9]. However, other research groups opted for other types of envelopes, such as baboon envelopes (BaEVs), due to their high tropism through two viral entry receptors, ASCT-1 and ASCT-2, and their capacity to transduce quiescent HSCs without activating the complement system [102–104]. Thereby, since ASCT-1 and ASCT-2 are highly expressed in HSCs, fewer viral particles would be enough to transduce the same number of cells, leading to a reduction in cytotoxicity and genotoxicity, and probably to an increment in the engraftment capacity. More recently, novel glycoprotein-displaying LVs (hemagglutinin and fusion protein LVs [H/F-LVs]) were able to transduce quiescence HSCs with higher efficiencies than Baboon envelopes [105].

Regarding CRISPR/Cas9, there is no current clinical trial by gene correction, because HDR frequencies in primary cells have to be further

improved. Therefore, multiple research groups investigate the mechanisms behind gene repair to increase gene correction and gene addition frequencies by inhibiting factors involved in NHEJ, such as DNA Ligase-1, or overexpressing factors involved in HDR, such as RAD51 [106–109]. Furthermore, the so-called second-generation of CRISPR gene editing tools is slowly gaining territory since this technique is able to induce changes in the gene expression patron without DSBs. To this aim, nickase Cas9 (nCas9) or catalytically inactivated Cas9 (dCas9) are fused to enzymes that are able to induce a single nucleotide conversion (base-editing), inhibit or activate the transcription of a target gene, or modulate the epigenetic regulation of a gene in a precise locus [59,110–112]. Especially nCas9 fused to cytosine base editor (G-C to T-A) or adenine base editor (T-A to G-C) might be valuable to treat diseases caused by a single point mutation [113]. For instance, non-functional β-globin chains are produced in β-thalassemia patients when a single point mutation in the first intron of *HBB* (IVS1–110 G > A) induces aberrant splicing after transcription. On the other hand, this technology is especially advantageous for diseases caused by a gain-of-function mutation, because it can convert a nucleotide to induce a STOP codon in a gene, hence repressing its expression [114].

The latest revolution in gene editing has come to light very recently, with the potential to correct the vast majority of pathogenic human genetic variants, overcoming base editing limitations such as the absence of PAM close to the target site and unwanted edits due to the broad activity window of the base editor enzyme [115]. This technology is known as prime editing (PE), and consists of a nCas9 and a prime editing guide RNA (pegRNA) fused to a reverse transcriptase (RT). The pegRNA includes an extension in the 3′ end with the RT template which is incorporated into the genome sequence with low indel formation. Furthermore, this system has similar HDR frequencies with lower indel rates in comparison to spCas9 and ssDNA donor template, resulting in a safer off-target profile [116]. The unique limitation that the second-generation of CRISPR/Cas9 technology might present is the inefficient RNP complex delivery due to the increased size of the Cas9 protein fused to other enzymes. For this reason, Cas9 proteins from different species may facilitate the delivery due to their smaller size in comparison to the conventional Cas9 protein from *Streptococcus pyogenes* [30]. Moreover, engineered murine leukemia virus (MLV)-like particles (LPs) or lentivirus-like particles (LVLPs) loaded with Cas9-sgRNA ribonucleoproteins (Nanoblades) could help to palliate size concerns, as Nanoblades are delivery vehicles lacking viral genome that facilitate the dispatch of CRISPR/Cas9 components to induce efficient genome editing in a wider range of cell lines and primary cells (Fig. 1) [117,118].

Thanks to the galloping development of gene editing techniques, a new alternative form of end-joining that could revolutionize CRISPR/Cas9 gene disruption and correction approaches has been recently reported. This DSB repair mechanism, known as microhomology-mediated end-joining (MMEJ), consists in annealing short homologies (5-25 bp) flanking a DSB, which results in deletions between these homologous sequences [119,120]. The application of this mechanism, active only during M and early S phases unlike HDR, could enhance gene disruption strategies by causing more robust deletions and frameshifts, as well as presenting the possibility to be used as a novel knock-in system [119,121]. For instance, the disruption of the binding sites of the HbF repressor BCL11A has been previously proposed as an alternative therapy for β-hemoglobinopathies [122]. In this regard, when implementing the MMEJ repair mechanism, a 13 bp natural-occurring deletion can be mimicked in the BCL11A binding site, impeding the activity of the aforementioned repressor and enabling subsequent therapeutic HbF production [75]. Also, through the newly developed MMEJ-mediated knock-in system named Precise Integration into Target Chromosomes (PITCh), accurate gene insertions can be accomplished using shorter homology regions, facilitating repair template cloning and reducing its *in vitro* production cost [119], even in primary cells where HDR occurs at a reduced rate [121]. For all these reasons, exciting future approaches can be expected for this novel repair mechanism which could arise promising therapies to be applied into the clinics.

6. Conclusion

Certainly, the effectiveness of auto-HSCT-GT for the treatments of non-malignant diseases cannot be denied. We only have to take a glance at the multiple GT studies and clinical trials currently ongoing. Despite the fact that investigations on genome editing techniques have skyrocketed in the last decade, lentiviruses still represent an effective alternative for many loss-of-function diseases where high transgene expression is needed for therapeutic effects. Nonetheless, gene editing approaches, primarily through the cost-effective CRISPR/Cas9 system, are becoming a great GT alternative for disorders where tightly-controlled gene expression is essential. In this context, this review aimed to provide a general overview of which GT modality would be more effective and safer according to existing preclinical studies and clinical trials. Indeed, the current tendency seems to favor CRISPR/Cas9 as the most promising gene editing technology; however, there is still a long developmental pathway ahead in which the aforementioned drawbacks and difficulties of this method have to be addressed to ensure high safety profile and effective curative outcome.

As a final point, as can be perceived throughout this review, GT is a powerful platform that could be, to a certain extent, utilized for more than curative purposes. Thus, it is important to ensure exclusively somatic GT treatments to guarantee the ethical usage of this technology for the good of humankind.

Practice points

- Lentivirus is utilized for semi-random gene insertion, whereas CRISPR/Cas9 technology is widely used for gene disruption, gene correction, and targeted gene insertion.
- To overcome possible side-effects after gene transfer treatment, new lentiviral constructs have been designed during the last decades based on new insulators and self-inactivating (SIN) vectors. Equally occurred for CRISPR/Cas9 with the emergence of different Cas9 variants and truncated sgRNAs.
- Auto-HSCT-GT is steadily overtaking allo-HSCT.
- Cautious assessment of the disease based on fitness and loss- or gain-of-function mutations is necessary in order to select the most appropriate gene therapy treatment, either by lentivirus or gene-editing.
- The concept of “edited-cell trafficking” will lead to novel GT treatments for inflammatory, lung, and neurological diseases, among others.
- New lentiviral envelopes and second-generation of CRISPR/Cas9 will arise promising therapies in the near future.

Research agenda

- Safer vectors for reducing insertional mutagenesis and better control of transgene expression.
- New techniques to increase HDR rates in primary cells.
- Sensitive high throughput analysis to identify off-targets.

Author contributions

A.L.C and A.D.M wrote the main part of the manuscript, followed by contributions of G.U·B and J.R, who created figures and tables and gave input to the manuscript. R.H performed proofreading. M.M and J.S.A drafted the final version of the manuscript. All authors read and approved the final manuscript.

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Declaration of Competing Interest

None of the authors state any conflicts of interest.

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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

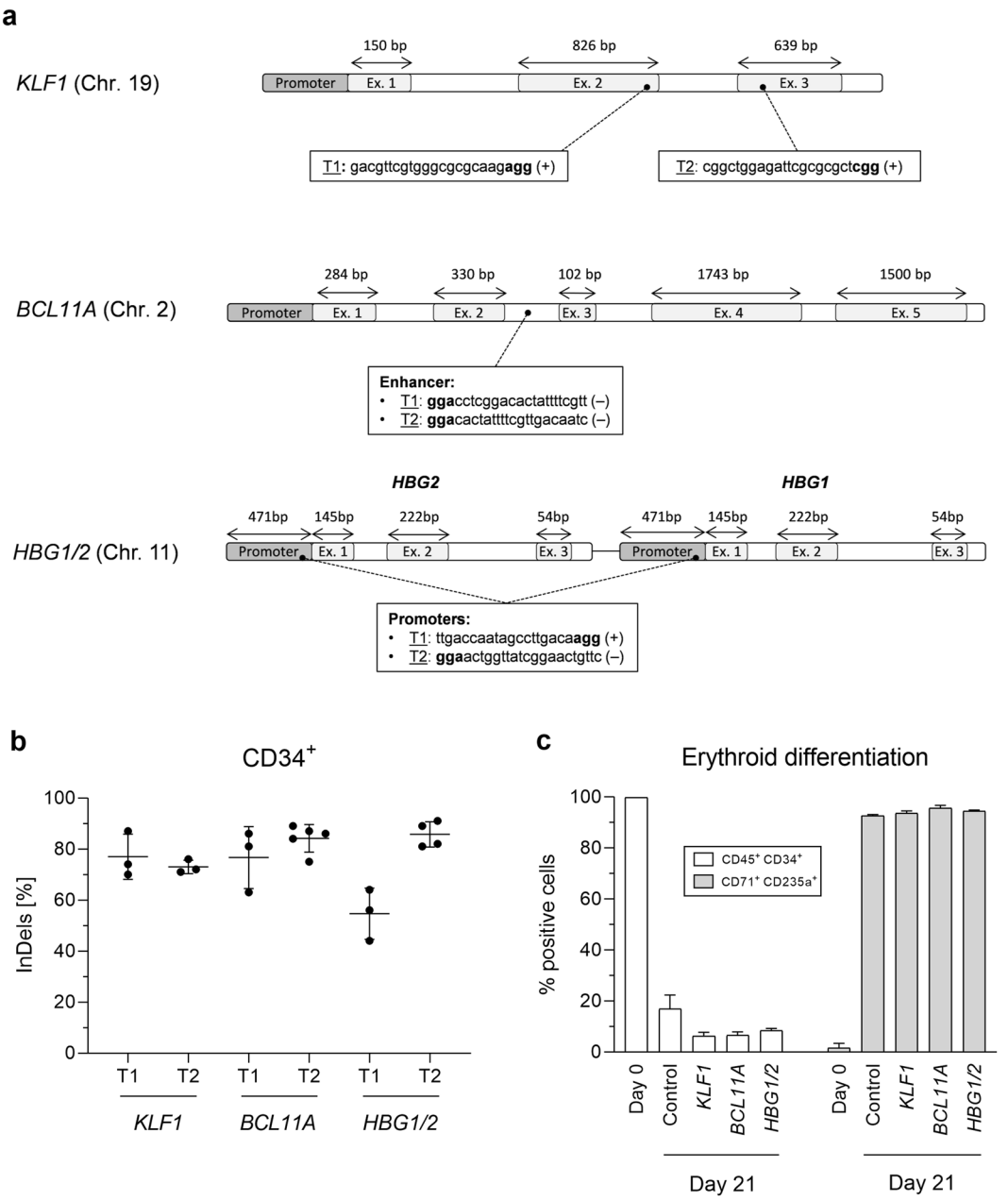

Figure 1. Gene editing of human CD34$^+$ HSPCs. (**a**) Schematic representation of the genome-editing strategies and target sequences for each sgRNA. *KLF1*: targets 1 (T1) and 2 (T2) are localized in the second and third exon, respectively; *BCL11A*: both targets are situated in the enhancer region 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).

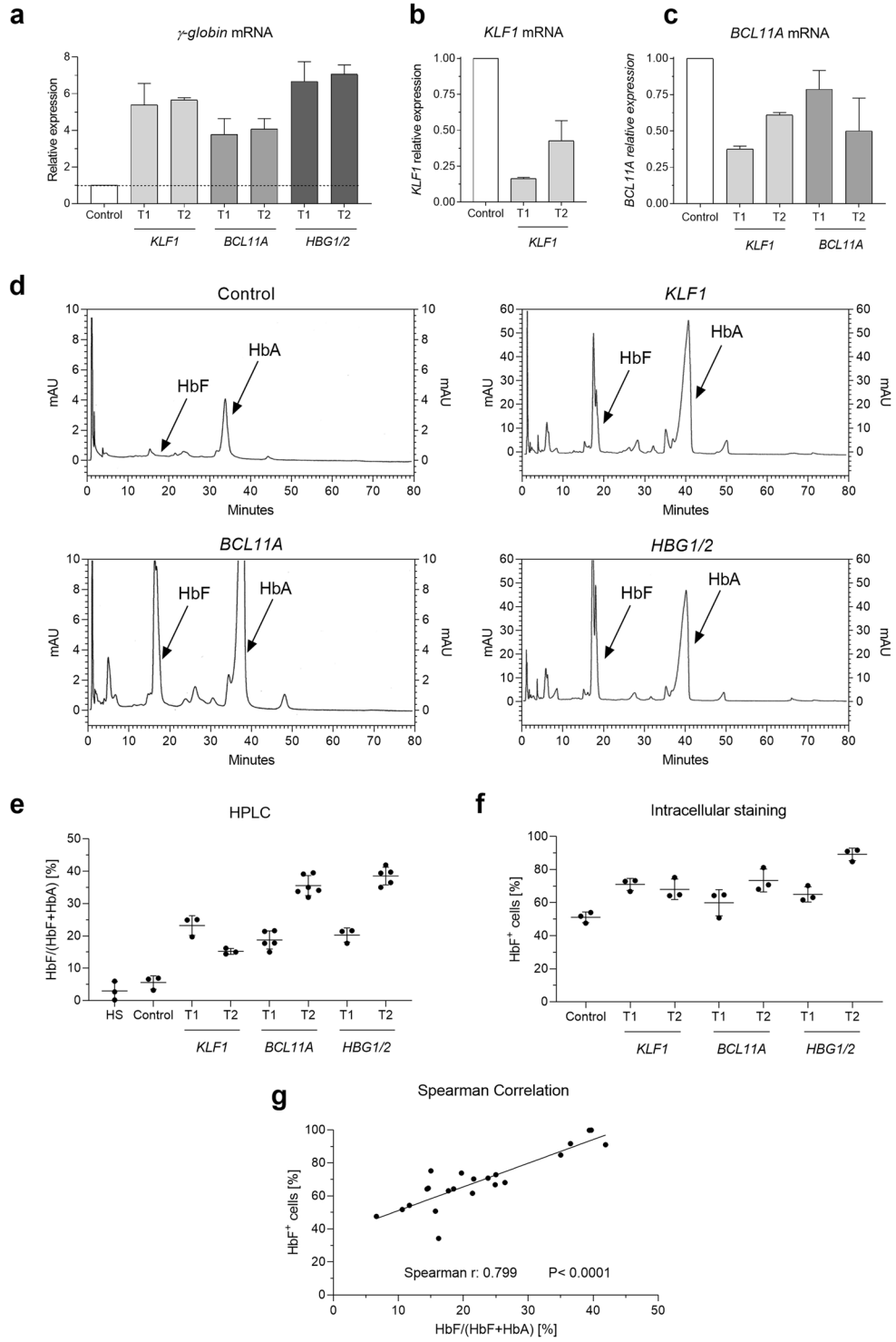

Figure 2. mRNA and protein analysis of gene-edited CD34$^+$ HSPCs. (**a**) *γ-globin* expression analysis by qRT-PCR on day 21. (**b**) Decline of *KLF1* transcripts after treatment with *KLF1* T1 and T2. (**c**) Down-regulation of *BCL11A* transcripts in *KLF1* and *BCL11A* samples. (**d**) HPLC histograms 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

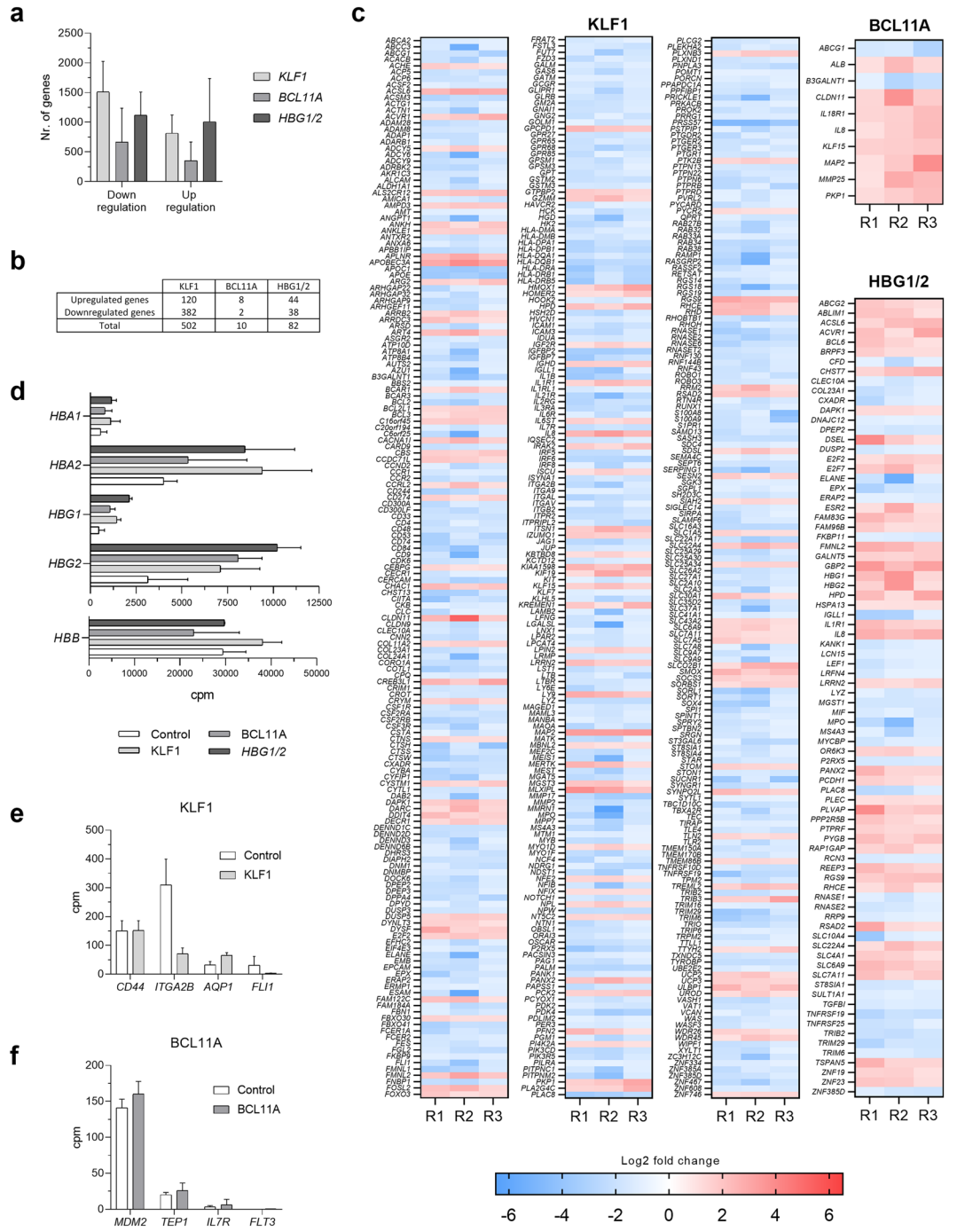

Figure 3. RNA-seq analysis. (**a**) Number of genes down- and up-regulated after targeting *KLF1*, *BCL11A*, and *HBG1/2*. (**b**) Number of common dysregulated genes for the three individual experiments. (**c**) Heatmap showing the Log2 fold change of the common differentially expressed genes after 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).

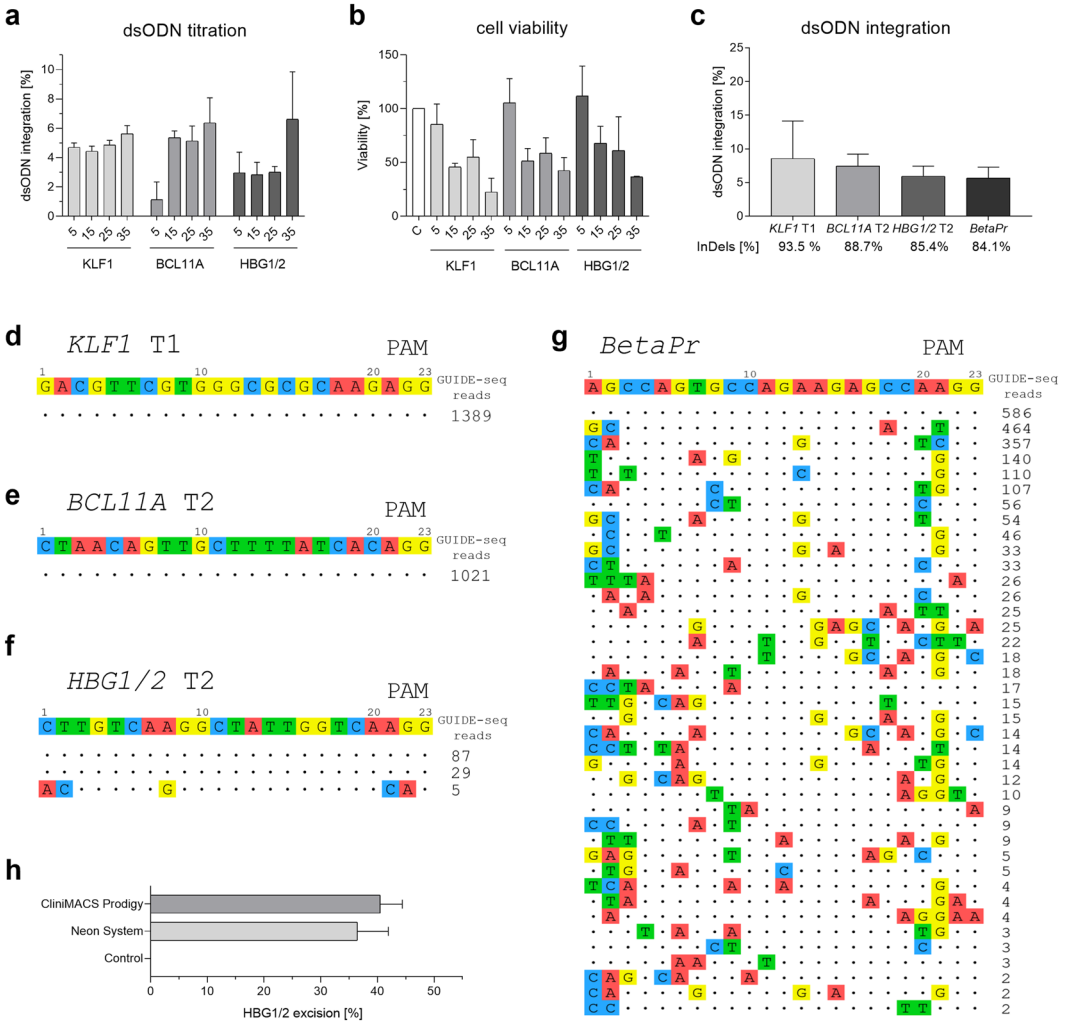

Figure 4. GUIDE-seq analysis. (**a**) dsODN titration in K-562 for GUIDE-seq optimization. The percentage of dsODN integration for each specific dsODN count of particle (5, 15, 25, and 35 pmol) is depicted. (**b**) Cell viability determined by cell counting on day 5 post-electroporation. (**c**) Percentage 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

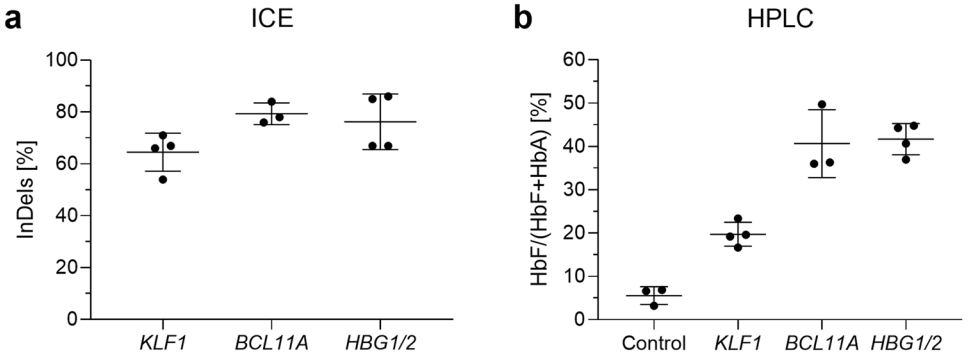

Figure 5. Translation of the CRISPR/Cas9 gene editing platform to the CliniMACS Prodigy GMP-grade device. (**a**) Percentage of insertions and deletions (InDels) detected by ICE analysis. (**b**) HbF levels (%) in edited human CD34$^+$ HSPCs measured by HPLC.

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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SUPPLEMENTARY FIGURES AND TABLES

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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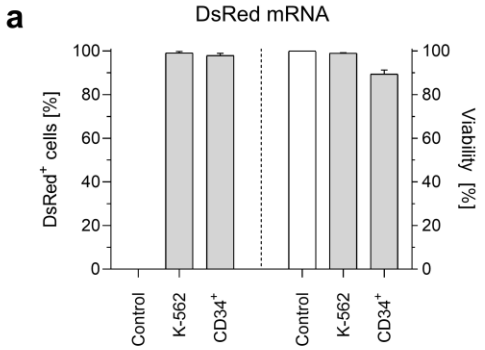
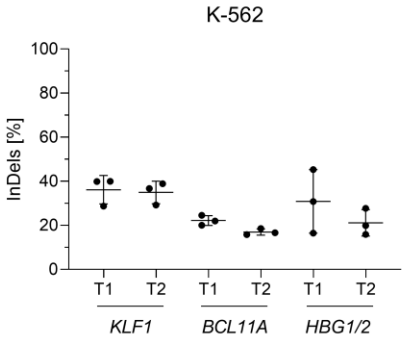
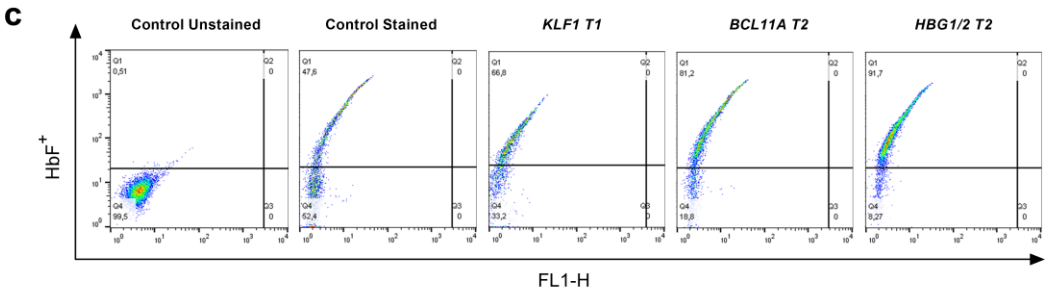
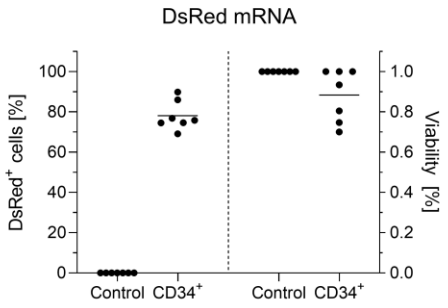
SUPPLEMENTARY FIGURES









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Sample	InDel [nt]	Wt sequence: CCTTGCCTTGA|CCAATAGCCTTG	InDel[%]
1	-13	CCTTGCCT---|----------TG	50.6
	-2	CCTTGCCTTGA|--AATAGCCTTG	8.2
	-1	CCTTGCCTTGA|-CAATAGCCTTG	6.4
			...
2	-13	CCTTGCCT---|----------TG	42.2
	-2	CCTTGCCTTGA|--AATAGCCTTG	9.5
	-3	CCTTGCCTTGA|---ATAGCCTTG	7.3
			...
3	-13	CCTTGCC----|---------TTG	38.3
	-1	CCTTGCCTTGA|-CAATAGCCTTG	3.4
	-3	CCTTGCCTTG-|--AATAGCCTTG	3.1
			...

Figure S1. Gene editing in K-562 and human CD34$^+$ HSPCs using Neon Transfection System and CliniMACS Prodigy. (**a**) Transfection efficiency and viability for K-562 and CD34$^+$ utilizing the Neon Transfection System and DsRed reporter mRNA. (**b**) Indel rates measured by T7E1-assay in K-562 cells after electroporation of recombinant pX-330 for each sgRNA. (**c**) Representative intracellular staining dot-plots showing HbF$^+$ CD34$^+$ HSPCs on day 21 for the most efficient treatments. (**d**) DsRed mRNA transfection into human CD34$^+$ HSPCs using the CliniMACS Prodigy with respective cell viability. (**e**) Representation of the 13-nt deletion detected by ICE analysis after *HBG1/2* T2 gene editing. The rectangle indicates the binding site for BCL11A which is disrupted in all gene-edited samples compared to the wild-type (wt) control sequence. Underlined are represented short homology sequences involved in MMEJ-based repair.

SUPPLEMENTARY TABLES

Table S1. Target sequences, oligonucleotides utilized for cloning into pX-330 vector, sgRNAs, and respective references for all studied genes.

Gene	Target	Target strand (5'-3')	Oligonucleotides (5'-3')	sgRNA (5'-3')	Reference
KLF1	1	**CCT** CTTGCGCGCCCACGAACGTC	For: AAAC-CTTGCGCGCCCACGAACGTC Rev: CACC-GACGTTCGTGGGCGCGCAAG	GACGTTCGTGGGCGCGCAAG	17
	2	**CCG** AGCGCGCGAATCTCCAGCCG	For: AAAC-AGCGCGCGAATCTCCAGCCG Rev: CACC-CGGCTGGAGATTCGCGCGCT	CGGCTGGAGATTCGCGCGCT	17
BCL11A	1	**CCT** GGAGCCTGTGATAAAAGCAA	For: AAAC-GGAGCCTGTGATAAAAGCAA Rev: CACC-TTGCTTTTATCACAGGCTCC	TTGCTTTTATCACAGGCTCC	43
	2	**CCT** GTGATAAAAGCAACTGTTAG	For: AAAC-GTGATAAAAGCAACTGTTAG Rev: CACC-CTAACAGTTGCTTTTATCAC	CTAACAGTTGCTTTTATCAC	43
HBG1/2	1	**CCT** TGTCAAGGCTATTGGTCAAG	For: AAAC-TGTCAAGGCTATTGGTCAAG Rev: CACC-CTTGACCAATAGCCTTGACA	CTTGACCAATAGCCTTGACA	14
	2	**CCT** TGACCAATAGCCTTGACAAG	For: AAAC-TGACCAATAGCCTTGACAAG Rev: CACC-CTTGTCAAGGCTATTGGTCA	CTTGTCAAGGCTATTGGTCA	14
BetaPr	1	**CCT** TGGCTCTTCTGGCACTGGCT	-	AGCCAGTGCCAGAAGAGCCA	55

Table S2. Oligonucleotide sequences utilized for PCR, qRT-PCR, and ddPCR.

Gene	qRT-PCR / PCR / ddPCR	Target	For/Rev	Primer Sequence	Reference
α-globin	qRT-PCR	-	For	CTGGCGAGTATGGTGCG	56
			Rev	GAAGTGCGGGAAGTAGGTC	56
β-globin	qRT-PCR	-	For	TGCACGTGGATCCTGAGAACT	56
			Rev	AATTCTTTGCCAAAGTGATGGG	56
γ-globin	qRT-PCR	-	For	TGGCAAGAAGGTGCTGACTTC	57
			Rev	TCACTCAGCTGGGCAAAGG	57
B2M	qRT-PCR	-	For	GATGAGTATGCCTGCCGTGT	58
			Rev	AATTCATCCAATCCAAATGAG	58
	ddPCR	Intron 1	For	TGGCTGTGATACAAAGCGGT	Own design
			Rev	GGAAACAACCAGGCAAAGAG	Own design
			HEX probe	GATGAAGAAACTAAGGCACCG	Own design
KLF1	PCR	1	For	AAGGGCACTTCCAGCTCTTC	Own design
			Rev	GTGGTCAGAGCGCGAAAAAG	Own design
		2	For	TCCTTCCTGAGTTGTTTGG	17
			Rev	GATGTCCAAACTGTCGTGCAA	Own design
	qRT-PCR	-	For	CACACAGGGGAGAAGCCATA	17
			Rev	GTCAGAGCGCGAAAAAGC	
BCL11A	PCR qRT-PCR	1	For	GTGTATGTGCTGATTGAGGGC	Own design
			Rev	GGACAGCCCGACAGATGAAA	Own design
		2	For	GTGTATGTGCTGATTGAGGGC	Own design
			Rev	GGACAGCCCGACAGATGAAA	Own design
HBG1/2	PCR	*HBG1*	For	AACCACTGCTAACTGAAAGAGACT	14
			Rev	GGCGTCTGGACTAGGAGCTTATTG	
		HBG2	For	GCACTGAAACTGTTGCTTTATAGGAT	
			Rev	GGCGTCTGGACTAGGAGCTTATTG	
	ddPCR	Intergenic region	For	TTCAGTGAAGGGGGCTGAAC	Own design
			Rev	ATGACACGCTGATGCTGACT	Own design
			FAM probe	ACAGGGAGGTTGAGGTGTTA	Own design

Table S3. *In silico* off-target predictions utilizing online tool CHOPCHOP.[59]

Gene	Target	Target sequence	off-targets *in silico*
KLF1	1	GACGTTCGTGGGCGCGCAAG AGG	0
KLF1	2	CGGCTGGAGATTCGCGCGCT CGG	0
BCL11A	1	TTGCTTTTATCACAGGCTCC AGG	n/d
BCL11A	2	CTAACAGTTGCTTTTATCAC AGG	n/d
HBG1/2	1	CTTGACCAATAGCCTTGACA AGG	5
HBG1/2	2	CTTGTCAAGGCTATTGGTCA AGG	2
BetaPr	-	AGCCAGTGCCAGAAGAGCCA AGG	16

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Molecular and Cellular Pediatrics

SHORT COMMUNICATION Open Access

Gene correction of *HBB* mutations in CD34$^+$ hematopoietic stem cells using *Cas9* mRNA and ssODN donors

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Abstract

Background: β-Thalassemia is an inherited hematological disorder caused by mutations in the human hemoglobin beta (*HBB*) gene that reduce or abrogate β-globin expression. Although lentiviral-mediated expression of β-globin and autologous transplantation is a promising therapeutic approach, the risk of insertional mutagenesis or low transgene expression is apparent. However, targeted gene correction of *HBB* mutations with programmable nucleases such as CRISPR/Cas9, TALENs, and ZFNs with non-viral repair templates ensures a higher safety profile and endogenous expression control.

Methods: We have compared three different gene-editing tools (CRISPR/Cas9, TALENs, and ZFNs) for their targeting efficiency of the *HBB* gene locus. As a proof of concept, we studied the personalized gene-correction therapy for a common β-thalassemia splicing variant $HBB^{IVS1-110}$ using *Cas*9 mRNA and several optimally designed single-stranded oligonucleotide (ssODN) donors in K562 and CD34$^+$ hematopoietic stem cells (HSCs).

Results: Our results exhibited that indel frequency of CRISPR/Cas9 was superior to TALENs and ZFNs ($P < 0.0001$). Our designed sgRNA targeting the site of $HBB^{IVS1-110}$ mutation showed indels in both K562 cells (up to 77%) and CD34$^+$ hematopoietic stem cells—HSCs (up to 87%). The absolute quantification by next-generation sequencing showed that up to 8% site-specific insertion of the *Nhe*I tag was achieved using *Cas9* mRNA and a chemically modified ssODN in CD34$^+$ HSCs.

Conclusion: Our approach provides guidance on non-viral gene correction in CD34$^+$ HSCs using *Cas*9 mRNA and chemically modified ssODN. However, further optimization is needed to increase the homology directed repair (HDR) to attain a real clinical benefit for β-thalassemia.

Keywords: *HBB*, Beta-thalassemia, Gene correction, CRISPR/Cas9, IVS1–110, *Cas9* mRNA

Background

β-Thalassemia (OMIM: 613985) is an inherited hematological disorder caused by mutations of the human hemoglobin beta (*HBB*) gene, leading to deficient β-globin expression and severe anemia [1]. The current treatment options include allogeneic bone marrow transplantation and hematopoietic stem cell (HSC) transplantation but are limited to histo-compatible donors. However, gene therapy based on autologous transplantation of a lentiviral-transferred *HBB* gene to HSCs resulted in remarkable clinical benefit [2, 3]. Though the safety and efficacy of lentiviral-based gene therapy is positive in a treated patient, the transactivation of the proto-oncogene *HMGA2* and more than 3500 unique integration sites in tested mouse model forewarns the possibility of insertional mutagenesis [2, 4]. Earlier retroviral gene therapy studies on other inherited diseases reported the treatment-related leukemogenesis [5–7], and lentiviral

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therapy resulted in T cell lymphoma in a mouse model of X-SCID due to random integration into oncogenes [8]. Therefore, the ideal gene therapy with viral vectors must ensure targeted integration of a therapeutic *HBB* transgene in the endogenous locus. Otherwise, personalized gene-correction therapy with programmable nucleases and non-viral repair templates such as single-stranded oligonucleotides (ssODNs) must be employed as it is less likely to randomly integrate into the genome and result in a safe and precise gene editing [9]. Though several studies targeted *HBB* gene with ZFNs, TALENs, and CRISPR/Cas9, no study has ever compared all the three gene-editing platforms simultaneously. Therefore, in the present study, we compared these three approaches for their target efficiency in parallel. Here, we tested the gene correction efficiency of strategically designed ssODNs as repair templates to target *HBB* gene. This is an important measure while editing the highly proliferating stem cell population to avoid clonal selection and thereby triggering oncogenesis.

Correspondence/findings

First, we designed ZFNs, TALENs, and CRISPR/sgRNA for targeting the promoter region of the *HBB* gene (Additional file 1: Figure S1). The *HBB* gene-targeting efficacy of designed ZFNs, TALENs, or CRISPR/Cas9 was determined by T7 endonuclease-I (T7EI) assay in HEK293 cells. Interestingly, CRISPR/Cas9 exhibited higher indels for all three different concentrations (0.5 μg, 1.0 μg, and 1.5 μg) compared to ZFNs and TALENs (Fig. 1a; Additional file 1: Figure S1). Similar results were observed earlier for the $HBB^{IVS2-654}$ mutation where gene-targeting efficiency of CRISPR/Cas9 was superior to TALENs [10].

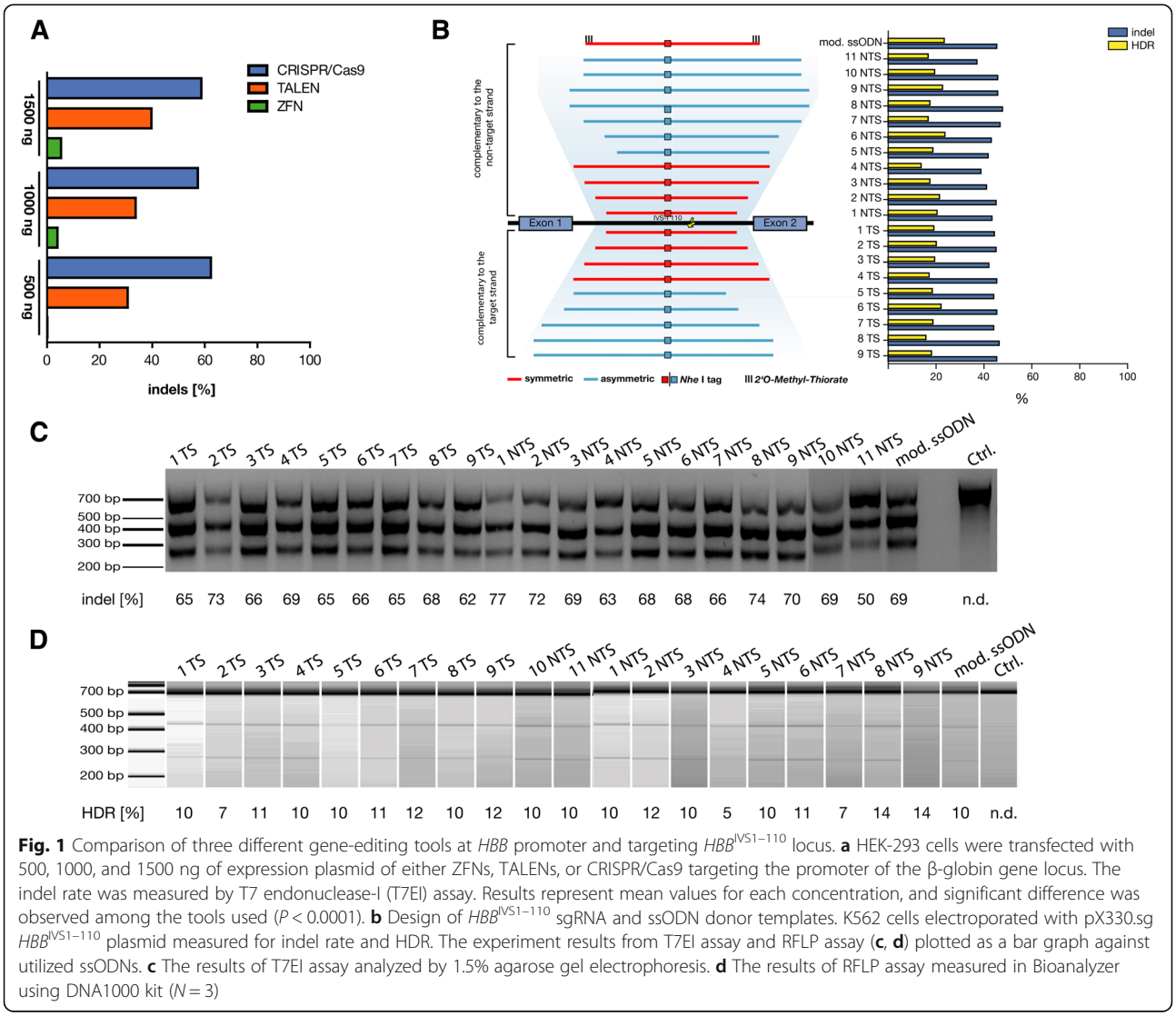

Fig. 1 Comparison of three different gene-editing tools at *HBB* promoter and targeting $HBB^{IVS1-110}$ locus. **a** HEK-293 cells were transfected with 500, 1000, and 1500 ng of expression plasmid of either ZFNs, TALENs, or CRISPR/Cas9 targeting the promoter of the β-globin gene locus. The indel rate was measured by T7 endonuclease-I (T7EI) assay. Results represent mean values for each concentration, and significant difference was observed among the tools used ($P < 0.0001$). **b** Design of $HBB^{IVS1-110}$ sgRNA and ssODN donor templates. K562 cells electroporated with pX330.sg $HBB^{IVS1-110}$ plasmid measured for indel rate and HDR. The experiment results from T7EI assay and RFLP assay (**c**, **d**) plotted as a bar graph against utilized ssODNs. **c** The results of T7EI assay analyzed by 1.5% agarose gel electrophoresis. **d** The results of RFLP assay measured in Bioanalyzer using DNA1000 kit ($N = 3$)

Therefore, we focused on CRISPR/Cas9 to examine the gene correction efficiency of non-viral repair templates. Several studies have targeted the *HBB* gene using CRISPR/Cas9 system in HSCs, induced pluripotent stem cells, and human embryos [11–16]. Most of these studies were either focused on *HBB* gene addition or targeting sickle cell disease mutation. To the best of our knowledge, this is the first study that attempted to target a common β-thalassemia splicing variant $HBB^{IVS1-110}$ (rs35004220), which leads to an alternative splice site and reduced β-globin expression with a non-viral strategy [17].

To target the $HBB^{IVS1-110}$ locus, we designed a sgRNA and several ssODNs (Fig. 1b; Additional file 1: Figure S1) with varying lengths of homology arms, symmetrical difference, and chemical modifications and complimentary to the strand targeted or not targeted by the guide-RNA [18]. To evaluate the ability of CRISPR/Cas9 to correct the $HBB^{IVS1-110}$ mutation by an exogenous DNA sequence, we introduced an *Nhe*I-tag (restriction site) into the ssODNs that can be stably integrated upon successful homology-directed repair (HDR) (Fig. 1b). We electroporated pX330.sg $HBB^{IVS1-110}$ plasmid and ssODN donors harboring *Nhe*I-tag using a Neon system into K562 cells and evaluated the frequency of indels by T7EI assay and HDR-driven *Nhe*I integration by restriction fragment length polymorphism (RFLP) assay (Figs. 1c/d). Mean indel frequencies for the targeted loci were 44 ± 18%. We next determined whether any of the rationally designed ssODNs could stimulate gene targeting by HDR and found that most of the tested ssODNs resulted in similar HDR frequencies with the median of 20% (range 5–31%).

To assess our non-viral correction strategy with bone marrow-derived $CD34^+$ HSCs, we co-delivered pX330.sg $HBB^{IVS1-110}$ plasmid and several ssODNs. Unlike our results with K562 cells, the indel frequencies in HSCs were relatively low with a median of 30% (range 0–56%) and only one ssODN (5TS) exhibited 3% HDR rate in TIDE analysis (Fig. 2a; Additional file 1: Figure S3.A). We next sought to determine HDR rate for 5TS-ssODN by a semi-quantitative single-colony sequencing and found that 2% (3/172) of clones showed *Nhe*I insertions (Fig. 2b). We observed that pDNA resulted in lower transfection rate and higher cell death in HSCs (data not shown). Due to the low indel rate achieved by pDNA-encoded Cas9, we aimed to increase the efficiency and viability by using mRNA-encoded *Cas*9 as others and ourselves earlier reported the superiority of mRNA over pDNA [19–21]. In addition, transiently expressed *Cas*9 mRNA resulted in reduced off-targets compared to long-term Cas9 expression systems [22]. Therefore, we delivered *Cas*9 mRNA and chemically modified sgRNA with four different ssODNs (chemically modified, 2 PAM-depleted, 5TS) using a 4D-Nucleofector. Genomic analysis with the T7EI assay resulted in high indels ranging from 65% (mod. ssODN) to 87% (11 NTS), and the RFLP assay showed up to 11% integration of *Nhe*I tag at the $HBB^{IVS1-110}$ locus (Fig. 2c). Our results clearly imply the superiority of *Cas*9 mRNA over pDNA (Additional file 1: Figure S3.B). We noticed significant enrichment of 6-bp insertion (range 2–9%) by *Nhe*I integration at the target locus for tested ssODNs (Fig. 2d; Additional file 1: Figure S4).

However, the absolute quantification of site-specific insertion events of the *Nhe*I tag at the $HBB^{IVS1-110}$ locus by next-Generation sequencing showed 8% correction for modified ssODN (Fig. 2e; Additional file 1: Figure S5). All the analyses spotted improved gene correction for chemically modified ssODN in HSCs as reported earlier [23]. No detectable off-target cleavage was found in six of the in silico predicted off-targets (Additional file 2: Table S4; Additional file 1: Figure S6) for the sgRNA-targeted $HBB^{IVS1-110}$ locus**.** We found that CRISPR/Cas9 greatly facilitates targeted genome modification compared to TALENs and ZFNs, and introduction of new DNA sequences in HSCs using *Cas*9 mRNA and ssODN is feasible without viral vectors. However, correction of 8% in HSCs at ex vivo setting is sub-optimal. Therefore, further improvement on HDR efficacy and selection of corrected cells is needed to attain a meaningful gene correction of *HBB* mutations to treat β-thalassemia and other related genetic diseases.

Online methods

Design of gene-editing tools

The targeting efficacy at the promoter of the *HBB* gene locus (200 bp upstream of the transcription start site) between three different gene-editing tools (CRSIPR/Cas9, TALENs, and ZFN) was evaluated (Additional file 1: Figure S1). The ZFN constructs targeting the promoter were ordered from Sigma-Aldrich (http://www.sigmaaldrich.com). The full amino acid sequences of the ZFN pair are shown in Additional file 2: Data S1. The targeting TALEN pair was designed with the help of the online tool ZiFiT Targeter Version 4.2 (http://zifit.partners.org) and assembled by fast ligation-based automatable solid-phase high-throughput (FLASH). Plasmids encoding TALE repeats harboring different repeat variable di-residues (RVDs) with their FLASH IDs are summarized in Additional file 2: Table S1. The sgRNAs for promoter (5′-AGCCAGTGCCAGAAGAGCC A-3′) and $HBB^{IVS1-110}$ (5-GGGTGGGAAAATAGACCAA T-3′) were designed using CRISPR design tool. The respective oligo pairs encoding the 20-nt guide sequences are annealed and ligated into a pX330 vector consisting of a *Sp*Cas9 expression cassette and a U6 promoter driving the expression of chimeric sgRNA. The chemically modified sgRNA for $HBB^{IVS1-110}$ mutation was synthesized by incorporating 2-*O*-methyl 3′phosphorothioate

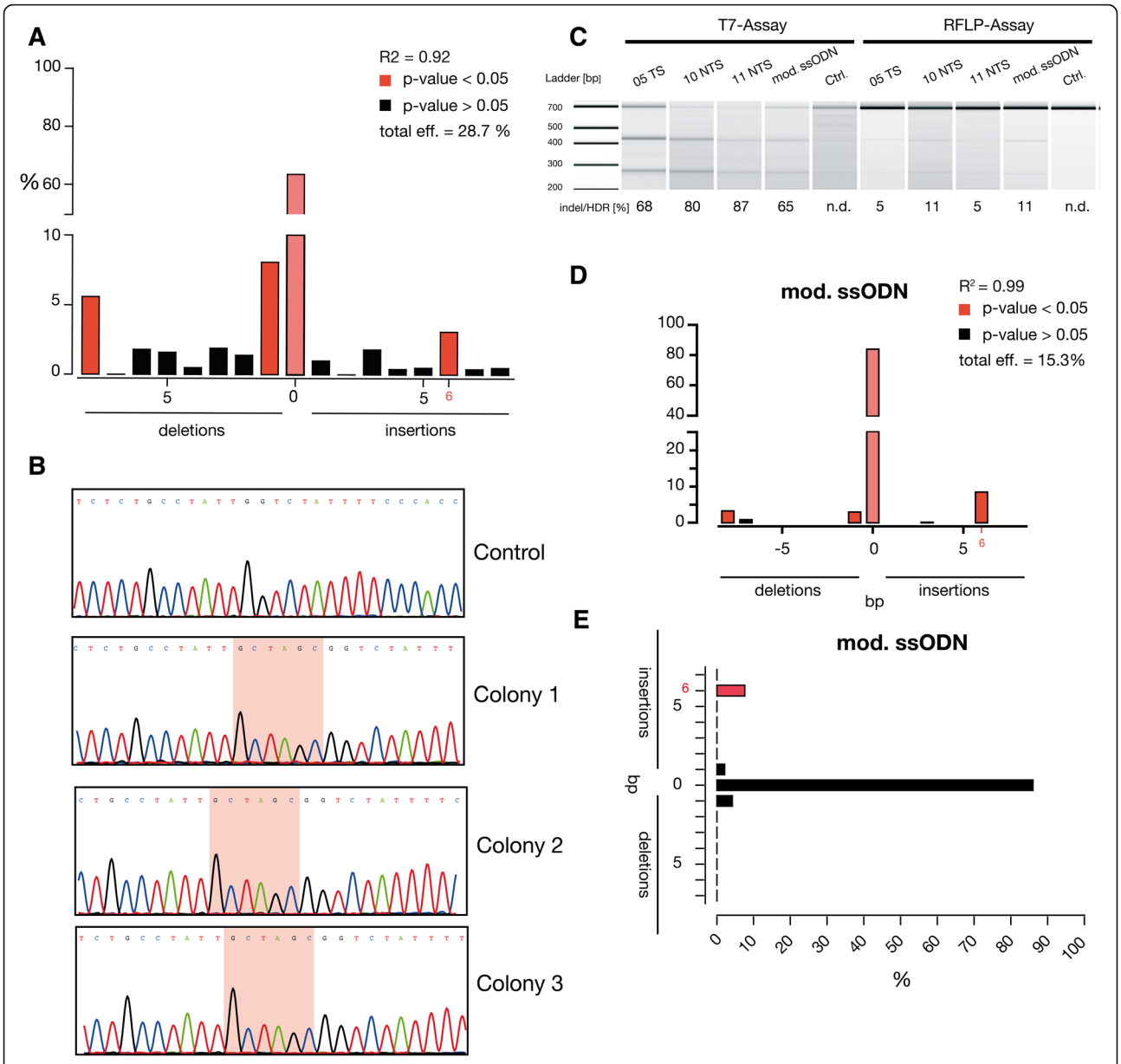

Fig. 2 Gene correction of *HBB*$^{IVS1-110}$ in CD34^{+} HSCs using CRISPR/Cas9 and ssODNs. **a** CD34^{+} HSCs co-nucleofected with pX330.sg *HBB*$^{IVS1-110}$ plasmid and 5TS ssODN. Indel frequency was measured by TIDE analysis and 3% of 6 bp insertions were spotted. **b** Single-colony sequencing analysis samples showed successful gene insertion of *Nhe*I restriction-site tag to the *HBB* gene by HDR in pX330.sg *HBB*$^{IVS1-110}$/5TS ssODN-treated sample. **c** Indel and HDR rate of CD34^{+} HSCs co-nucleofected with specified ssODN and Cas9 mRNA measured by T7 assay and RFLP assay in Bioanalyzer using DNA1000 kit. **d** TIDE analysis exhibited the significant enrichment of 6 bp insertions up to 8%. **e** The absolute quantification by next-generation analysis displayed the significant enrichment of 6 bp insertions up to 8% ($N = 1$)

(MS) modification at three terminal nucleotides at both the 5′ and 3′ ends.

Cell culture and transfection

HEK-293 cells were cultured in DMEM (https://www.thermofisher.com) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. In HEK-293 cells, pDNA-encoded gene-editing tools (CRSIPR/Cas9, TALENs, and ZFN) were transfected using Lipofectamin 3000 at three different concentrations of 0.5, 1, and 1.5 μg. K562 cells were cultured in RPMI 1640 (https://www.thermofisher.com) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. In K562 cells, 200 ng of pX330-Chimeric vector targeting *HBB*$^{IVS1-110}$ loci was co-electroporated with 10 pmol of different ssODN

using Neon Transfection System (https://www.thermofisher.com). Bone marrow-derived $CD34^+$ HSCs from healthy donors were cultured in StemSpan™ serum-free medium II (SFEMII) containing StemSpan™ Cytokine Cocktail 110 (https://www.stemcell.com). 1×10^5 $CD34^+$ HSCs were electroporated with 1.2 μg pX330 vector targeting $HBB^{IVS1-110}$ mutation and 100 pmol of specified ssODN repair template using Lonza© 4D-Nucleofector™ (Program: EO 100). The similar amount of p.Max eGFP pDNA (1.2 μg) was electroporated as a transfection control. For Cas9 mRNA experiments, 1×10^5 $CD34^+$ HSCs were co-electroporated with 5 μg chemically modified sgRNA, 10 μg Ca9 mRNA vector, and 100 pmol of specified ssODN repair template using Lonza© 4D-Nucleofector™ (Program: EO 100).

Repair templates

The ssODNs were synthesized by Metabion, Germany (www.metabion.com). The ssODNs were designed with an insertion site harboring an *Nhe*I recognition site (GCTAGC). The ssODNs were complimentary to either the strand targeted or not targeted by the gRNA, asymmetric or symmetric to the *Nhe*I tag. The chemically modified ssODN for $HBB^{IVS1-110}$ mutation was synthesized by incorporating 2-*O*-methyl 3′phosphorothioate (MS) modification at three terminal nucleotides at both the 5′ and 3′ ends with 72 bp homology arms. The complete details can be found in Additional file 2: Table S2.

T7 endonuclease assay and RFLP assay

Five days post transfection, genomic DNA was isolated using Merchery-Nagel NucleoSpin Tissue Kit following the manufacturer's instructions. The promoter region was amplified using the primer pair Prom-For_5′-GTAGACCACCAGCAGCCTAA-3′ and Prom-Rev_5′ TGGAGACGCAGGAAGAGATC-3′, and the region covering $HBB^{IVS1-110}$ mutation was amplified using the primer pair IVS1–110-For_5′-GGGTTTGAAGTCCAACTCCTAA-3′ and 3′UTR-For_5′-AGAAAACATCAAGCGTCCCATA-3′. The target regions were amplified using the AmpliTaq® Gold 360 MasterMix (https://www.thermofisher.com). The cycling parameters for both amplicons were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min. PCR products were purified by ethanol precipitation, and 1 μg of PCR product was used for T7 endonuclease assay. Likewise, 1 μg of PCR product was used for the RFLP assay where amplicons bearing the *Nhe*I tag upon successful gene correction will result in a specific cleavage after the treatment with the *Nhe*I restriction enzyme. The readout of the T7 endonuclease assay and the RFLP assay were determined on a 1.5% agarose gel and on a Bioanalyzer chip using DNA1000 kit. The band intensities were quantified using ImageJ embedded in Fiji software (www.fiji.sc).

TIDE analysis and single-colony and next-generation sequencing

For analyzing allele modification frequencies with non-enzymatic assays, we used TIDE (Tracking of Indels by Decomposition) analysis by Sanger-sequencing the purified PCR products used for T7 assay and examined with the online TIDE software (http://tide.nki.nl.) with the respective control sample. To precisely measure the events of site-specific insertion of *Nhe*I tag at $HBB^{IVS1-110}$ loci in $CD34^+$ HSPCs, we performed two different approaches: (i) Sanger-based single-colony sequencing and (ii) next-generation sequencing. For single-colony sequencing, the $HBB^{IVS1-110}$ region was PCR amplified from 5TS ssODN gene-corrected $CD34^+$ HSCs and cloned into pJET1.2 vector using CloneJET PCR Cloning kit (https://www.thermofisher.com) and transformed into Top10 competent cells using standard cloning techniques. Closely, 192 (two 96-well plates) single colonies were processed for Sanger sequencing with pJET1.2 forward sequencing primer (5′-CGACTCACTATAGGGAGAGCGGC-3′) and analyzed for the presence for *Nhe*I recognition site (GCTAGC) using Geneious-R6 software. Three of 192 clones resulted in *Nhe*I insertion (1.5%). In case of next-generation sequencing (NGS), new primers were designed with the amplicon length of 150 bp to be sequenced with the Illumina platform (Forward 5′-AGAAACTGGGCATGTGGAGA-3′; Reverse 5′-CCATAACAGCATCAGGAGTGG-3′). Further, barcode-tagged PCR primers were used to multiplex samples (Additional file 2: Table S3), and are adapter ligated, size selected, and bridge amplified and proceeded with amplicon sequencing in HiSeq 4000 system (http://www.illumina.com). The standard R-Package was used for NGS analysis where the sequencing reads were pre-filtered for low-quality reads and mapped to the reference sequence using a BWA tool. Further, the number of indel-carrying sequence reads was calculated using SAM tools. The distribution plot was generated by calculating the size of the indels in sequence and calculating the median percentage for each indel class.

In vitro transcription (IVT) of Cas9 mRNA

The open reading frame of SpCas9 was PCR amplified from the pX330 vector and sub-cloned into the pVAX.120 vector consisting of a T7 promoter and 120 bp length of a poly-A tail using standard molecular biology techniques. The IVT reaction was performed in linearized plasmid using T7 RNA polymerase in MEGAscript T7 kit (https://www.thermofisher.com). All mRNAs were produced with an anti-reverse CAP analog (ARCA; [m7G(5′)G]) at the 5′ end (https://www.trilinkbiotech.com/). The IVT-mRNAs were made with following chemical modifications in the indicated ratios: 100% Pseudo-UTP and 25% s2-thio-UTP/5-methyl-CTP

(https://www.trilinkbiotech.com/). All IVT mRNAs were purified using the MEGAclear kit (https://www.thermofisher.com) and quantified with nano-photometer and bioanalyzed for quality using the RNA6000 kit in Agilent 2100 Bioanalyzer (https://www.agilent.com).

Statistics

Kruskal-Wallis or Wilcoxon-Mann-Whitney rank-sum tests were applied wherever appropriate to analyze the differences in indel induction among the gene-editing technologies and comparison between encoded pDNA Cas9 and *Cas*9 mRNA using Graphpad Prism v.6.0d (https://www.graphpad.com).

Additional files

Additional file 1: Figure S1. Strategy for targeting the promoter and IVS1–110 mutation of the *HBB* gene. A) The promoter region of *HBB* gene targeted with three different gene-editing tools, $HBB^{IVS1-110}$ targeted with CRISPR/Cas9. B) The design of three different gene-editing tools at sequence level. **Figure S2** Comparison of three different gene-editing tools at *HBB* promoter. The complete raw data of Fig. 1a. **Figure S3** Gene correction of $HBB^{IVS1-110}$ in $CD34^+$ HSCs using pX330.sg $HBB^{IVS1-110}$ and ssODNs. A) $CD34^+$ HSCs nucleofected with pX330.sg $HBB^{IVS1-110}$ plasmid and ssODNs and measured for indel rate by T7 assay and HDR by TIDE analysis. Only 5TS resulted 3% HDR rate in TIDE analysis (as in Fig. 2a). B) Gene-editing capacity of pDNA-encoded Cas9 and mRNA-encoded *Cas*9 were compared, and superiority of Cas9 mRNA was observed ($P < 0.0001$). **Figure S4** TIDE analysis-gene correction of $HBB^{IVS1-110}$ in $CD34^+$ HSCs using Cas9 mRNA and ssODNs. TIDE analyses of four different ssODNs resulted in varying levels of 6 bp insertions that rely with the ssODN design. Modified ssODN resulted up to 8% HDR rate. **Figure S5** NGS analysis-gene correction of $HBB^{IVS1-110}$ in $CD34^+$ HSCs using Cas9 mRNA and ssODNs. The absolute quantification of *Nhe*I insertion by NGS analyses for four different ssODNs showed distinct rate of 6 bp insertions and correlate with ssODN design. Modified ssODN resulted up to 8% HDR rate. **Figure S6** Off-target analysis for the in silico predicted sites. The indel rate was measured by T7 endonuclease-I (T7EI) assay for six different off-target sites predicted through in silico (Additional file 2: **Table S4**) in K562 cells. We preselected top three hits in intronic and three hits in an exonic region. (PDF 2219 kb)

Additional file 2 Data S1 The complete amino acid sequences of the *HBB* targeting ZFNs. **Table S1** TALE FLASH IDs and RVDs targeting the promoter of *HBB* gene. **Table S2** Details of ssODNs (sequence, symmetry, and length of homology arms). Table S3 Barcode and sample details of next-generation sequencing. **Table S4** Details of off-target position, primer details, and indel frequency. (PDF 601 kb)

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Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its Additional files).

Authors' contributions

NL, JSA, RH, and MSDK conceived and designed the experiments. NL, JSA, AH, ALC, ADM, SG, and PW performed the experiments. JSA, NL, and PB analyzed the data. MSDK, MM, and RH contributed reagents/materials/analysis tools. JSA, NL, and MSDK wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval for using $CD34^+$ hematopoietic stem cells from healthy donors was obtained from Ethics commission, University Clinic of Tuebingen, Germany (829/2016BO2).

Consent for publication

Not applicable

Competing interests

M.S.D.K. is listed as main inventor on a patent application related to Nuclease encoding modified mRNA. M.S.D.K. is a main inventor on a patent licensed to the biopharmaceutical company, Ethris GmbH. M.S.D.K., A.D., A.H., and J.S.A. are inventors (with M.S.D.K. as main inventor) on a patent application related to modified mRNA encoding hCFTR.

Publisher's Note

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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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BaEV

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).

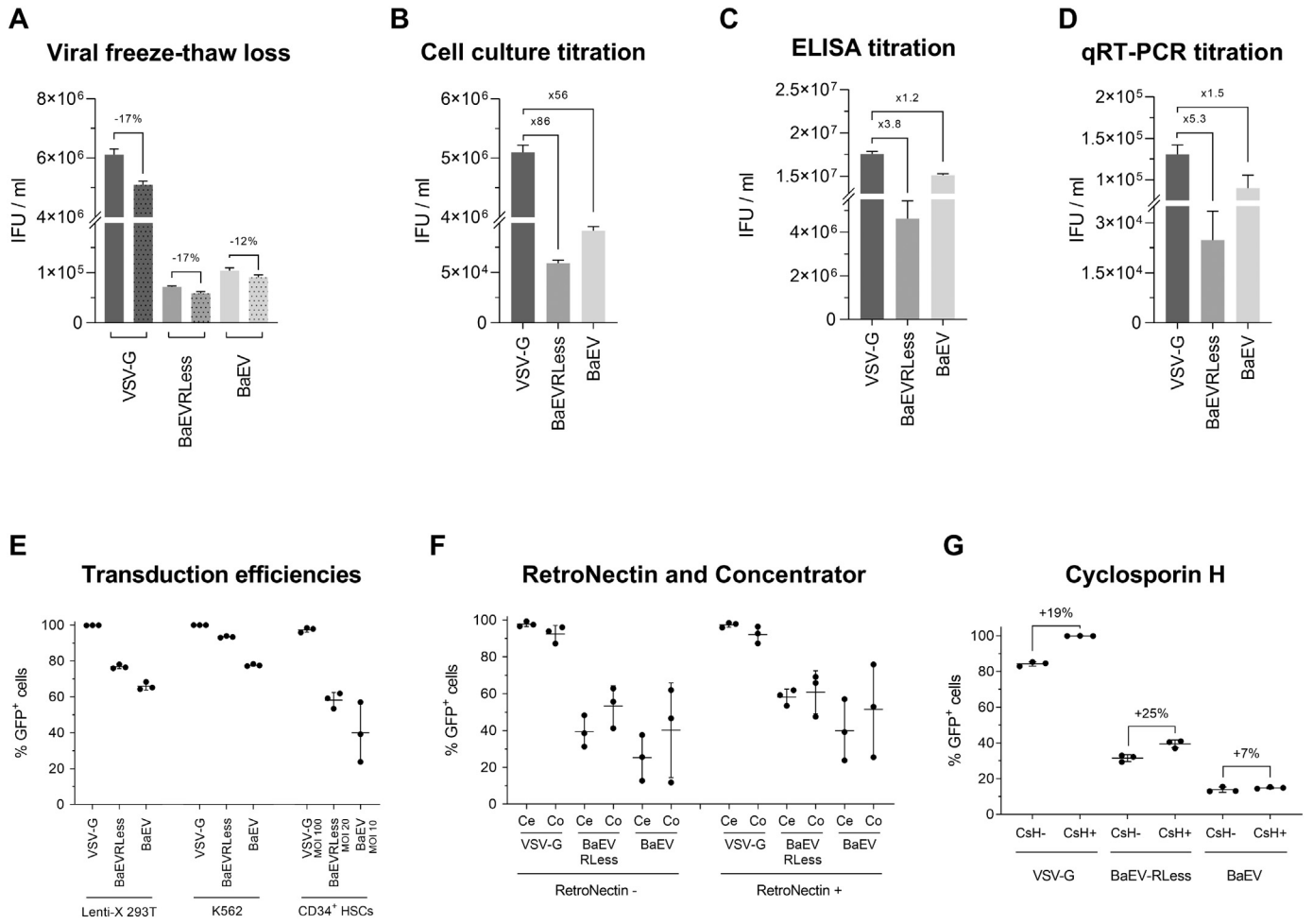

Fig. 1. Lentivirus production and transduction efficiency for alternative envelope proteins. Lentiviral vector particles encoding for a PGK-regulated GFP cassette and pseudotyped with VSV-G, BaEV-RLess, or full-length BaEV were produced by transient transfection as described in the materials and methods 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

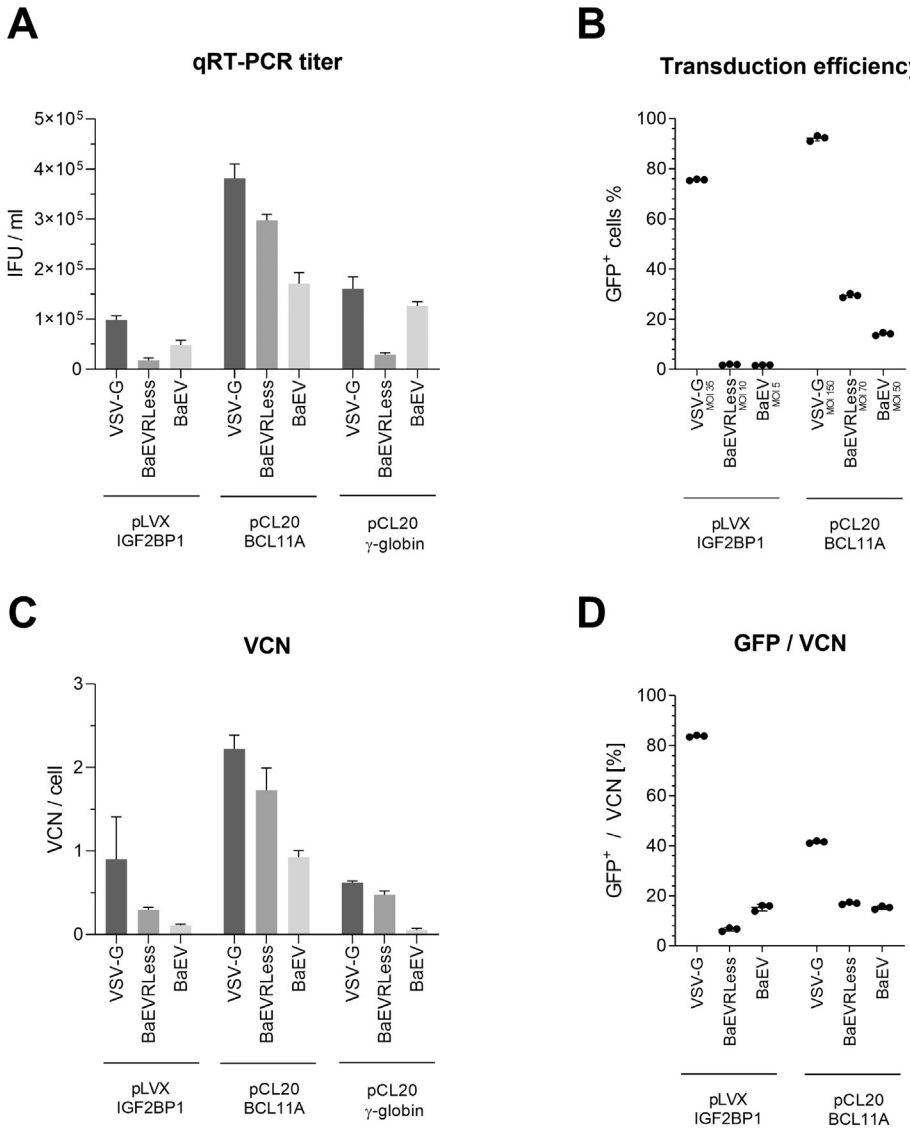

Fig. 2. Impact of alternative envelope proteins on lentivirus-mediated gene transfer of HbF inducing factors in human CD34$^+$ cells. Lentiviral vector particles encoding for expression of IGF2BP1 or BCL11A shmiR under control of an erythroid-specific promoter and coupled to expression of GFP and pseudotyped 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.

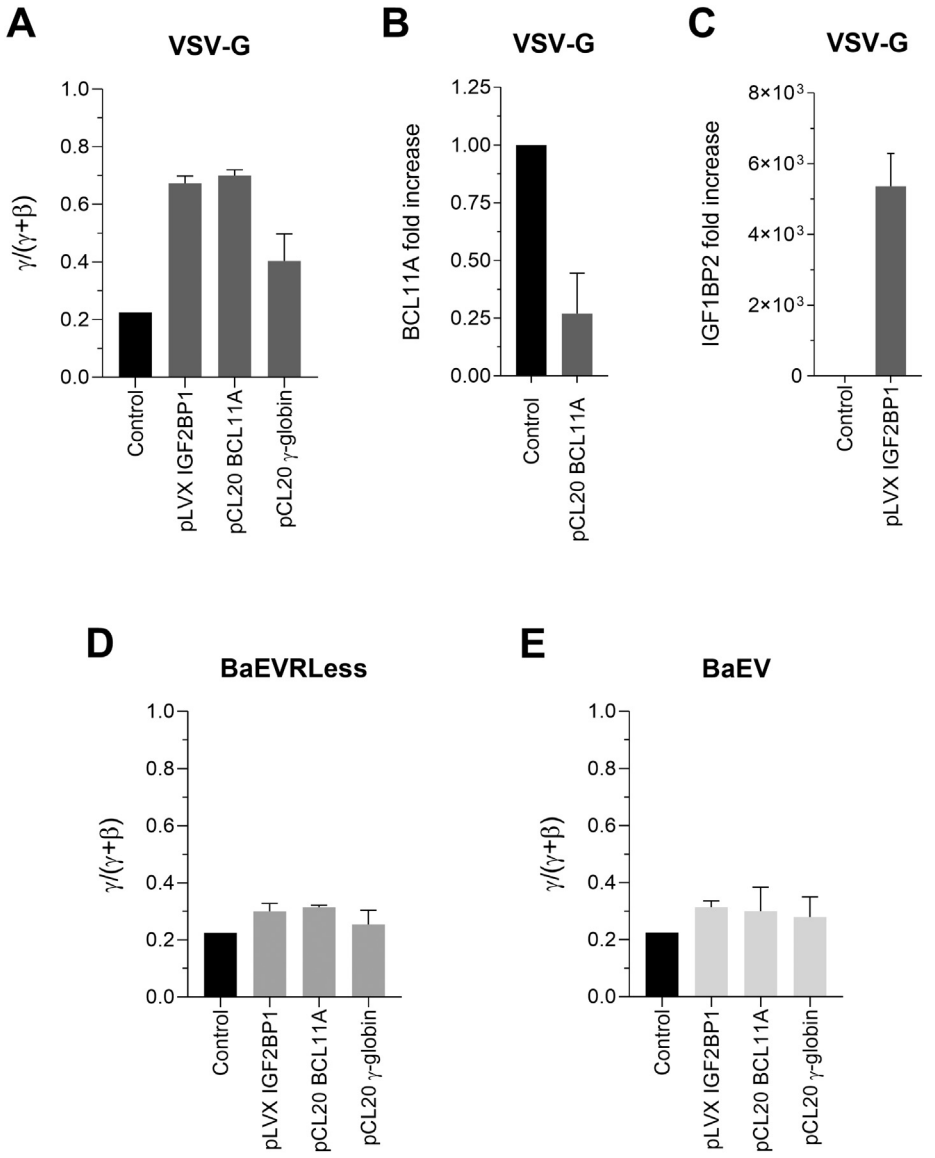

Fig. 3. Functional gene expression analysis of HbF inducing factors in culture-differentiated erythroblasts. Human $CD34^+$ cells left untreated (control) or transduced with lentiviral vectors encoding for erythroid-specific expression of γ-*globin* genomic sequences, shmiR sequences targeting *BCL11A*, 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

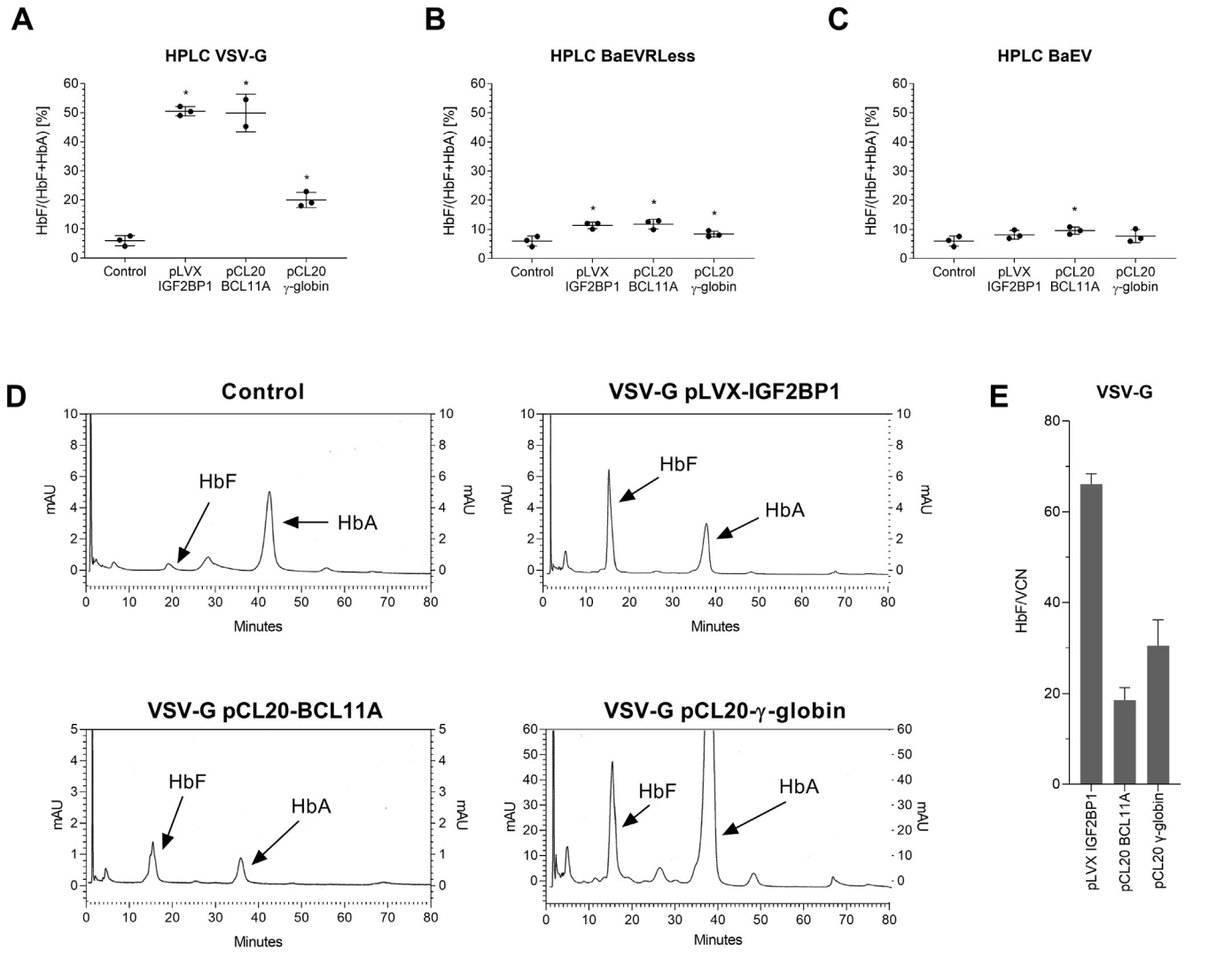

Fig. 4. Fetal hemoglobin levels for terminally differentiated erythroblasts. Untreated human CD34$^+$ cells (control) or transduced with lentiviral vectors encoding for erythroid-specific expression of γ-*globin*, *BCL11A* shmiR, or *IGF2BP1* and pseudotyped with either VSV-G, BaEV-RLess, or BaEV envelope 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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