Preclinical Evaluation of a Novel Lentiviral Vector Driving Lineage-Specific BCL11A Knockdown for Sickle Cell Gene Therapy

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In this work we provide preclinical data to support initiation of a first-in-human trial for sickle cell disease (SCD) using an approach that relies on reversal of the developmental fetal-to-adult hemoglobin switch. Erythroid-specific knockdown of BCL11A via a lentiviral-encoded microRNA-adapted short hairpin RNA (shRNAmiR) leads to reactivation of the gamma-BCL11A via a lentiviral-encoded microRNA-adapted short adult hemoglobin switch. Erythroid-speciﬁc approach that relies on reversal of the developmental fetal-to-adult switch in sickle cell disease (SCD) is the most common monogenic disease in the world.1 Around 2.1% of adults globally are carriers,2 and an estimated 100,000 individuals are affected in the United States alone.3

SCD is an inherited disorder caused by the E6V missense mutation [rs334] in the β-globin gene, leading to the production of mutant sickle hemoglobin S (HbS). Homozygosity of this mutation, coinheritance with β-globin thalassemic variants (β0 or β- mutations), and the hemoglobin C (HbC) mutation (E6K) in the second β-globin allele all lead to SCD.4-7 HbS has an increased propensity to polymerize under low oxygen conditions, which causes formation of sickled, inflexible red blood cells (RBCs). Sickled and sickle-polymer-containing RBCs are associated with hemolytic anemia, vasculopathy, and vaso-occlusive events (VOEs) that are characteristic for SCD and subsequently result in serious acute and chronic complications.

Treatments to reduce SCD complications include fluid administration, acute analgesia for VOEs, and daily administration of hydroxyurea, which has been used for more than 20 years to increase fetal hemoglobin (HbF) expression.8 In some cases, erythrocyte transfusions are administered to improve oxygen delivery in the presence of sickle RBCs. These treatments are largely symptomatic and transient, and the only available curative therapy for SCD is hematopoietic stem cell transplantation (HSCT).9 Around 2,000 SCD patients in the world have received HSCT from an allogeneic donor, and survival rates can exceed 90% in Europe and the United States, with the best outcomes in SCD attained using histocompatibility leukocyte antigen (HLA)-matched related donors.10,11 Only a minority of SCD patients can benefit from this therapy because of the unavailability of HLA-matched unaffected related donors.12-14 Genetically engineering autologous cells offers two major benefits over allogeneic HSCT: it eliminates the need to find a suitable hematopoietic stem cell (HSC) donor, and it eliminates the risks of graft-versus-host-disease (GVHD) and immune-mediated graft rejection, both serious complications of allogeneic HSCT. Gene therapy has been used to successfully treat multiple rare genetic conditions, including adenosine

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deaminase deficiency,15 X-linked severe combined immunodeficiency (SCID),16–18 X-linked chronic granulomatous disease,19 Wiskott-Aldrich syndrome,20,21 childhood cerebral adrenoleukodystrophy,22,23 and metachromatic leukodystrophy.24 More recently, ex vivo lentiviral vector (LVV)-based gene therapy has shown promise as a treatment for severe SCD and β-thalassemia.19,25 In previous and ongoing trials, this approach relies on the regulated expression of β-globin, a modified anti-sickling β-globin (HbA187Q), or transgenic expression of γ-globin in erythroid cells. Several clinical trials that rely on a similar strategy but use different beta-like globin variants are ongoing.

An alternative approach to gene therapy for SCD aims at reversing the fetal-to-adult hemoglobin switch by interfering with the transcriptional repressor BCL11A. BCL11A was first identified as a potent regulator of the hemoglobin switch in genome-wide association studies (GWASs) in healthy individuals with higher levels of Hbf.26–29 In erythroid cells, BCL11A functions as a developmental stage-specific repressor of Hbf expression,30 but it is also essential for B lymphocyte development, and more recently was identified by us and others as critical for HSC function.31–33 A proof-of-concept study showing phenotypic correction of SCD has been reported in a transgenic mouse model of SCD with genetic deletion of Bcl11a, which displayed pancellular induction of Hbf.37 Interfering with BCL11A takes advantage of this developmentally regulated physiological switch and has the potential added benefit over gene addition strategies of not only providing therapeutically relevant levels of functional γ-globin, but simultaneously reducing expression of the adult β-globin gene, thereby concurrently and coordinately reducing the concentration of HbS in erythrocytes.38 The increase in Hbf, which itself has potent anti-sickling properties,39,40 and concomitant reduction in HbS provide an extremely powerful approach to attenuating the sickling tendency of RBCs.

We have previously reported on an LVV that mediates potent erythroid-specific knockdown of BCL11A via RNA interference (RNAi) using a microRNA (miRNA)-adapted short hairpin RNA (shRNAmir).35,41 In order to circumvent poor titers seen during large-scale production of the original research construct, we transferred the hairpin cassette into an LVV backbone that has consistently demonstrated enhanced titer characteristics.35,29,37 We present data on the safety and efficacy of this LVV and show large-scale validation experiments using clinical-grade LVV to genetically modify SCD patient cells. The data presented here form the basis for an ongoing NIH-funded pilot and feasibility clinical trial (ClinicalTrials.org: NCT03282656).

RESULTS

Design and Production of the BCH-BB694 Anti-BCL11A LVV for Hbf Induction

Inducing Hbf is a promising approach to correcting the phenotype of β-thalassemic or SCD erythrocytes.43 We have previously designed an LVV (LCR-shRNAmir) for erythroid-specific BCL11A knockdown, which resulted in robust induction of γ-globin and increased production of Hbf.35 With the aim of conducting Investigational New Drug (IND)-enabling studies and to initiate a clinical trial, we performed pilot large-scale production of purified LVV. HEK293T cells were expanded and transfected with helper constructs and the plasmid pLCR-shRNAmir. After clarification, nuclease digestion, and filtration, vector particles were purified by ion exchange chromatography and concentrated by tangential flow filtration before final filtration and vialing. The titer of this vector in pilot production runs was lower than desired, which prompted us to test the therapeutic shRNAmir cassette in an alternative LVV backbone, which was previously used in clinical trials.26,29 Similar to the research vector, this optimized self-inactivating (SIN) third generation LVV BCH-BB694 expresses the therapeutic shRNAmir targeting BCL11A under transcriptional control of regulatory elements derived from the β-globin locus (Figure 1). The regulatory elements are comprised of portions of DNase hypersensitive sites 2 and 3 (HS2 and HS3) of the β-globin locus control region (LCR) fused to the minimal proximal promoter element from the β-globin gene. The Venus fluorescent reporter present in LCR-shRNAmir is not present in this clinical BCH-BB694 LVV. The optimized backbone is substantially smaller in size (6 versus 10 kb) and was associated with a nearly 5-fold increase in titers as assessed in three independent production runs (Figure S1), with the titer of the final Good Manufacturing Practices (GMP) grade vector batch being 6.47E+8 transducing units/ml (TU/ml). In a side-by-side comparison between the original LCR-shRNAmir and the refined BCH-BB694 LVVs, we confirmed comparable levels of Hbf induction per vector insertion. CD34+ cells from two different healthy donors (HDs) were left unmodified (mock) or transduced with an LVV.
containing a non-targeting control hairpin (NT), the original LCR-shRNA
research LVV, or BCH-BB694 LVV (Figure S2). After erythroid differentiation in liquid
culture in vitro, the levels of HbF in-
duction were determined by high-performance liquid chromatog-
raphy (HPLC) on the total cell population, which owing to the lack
of a selectable marker included untransduced cells. At similar vector
copy numbers (VCNs; /C24
1 copy/diploid genome [c/dg]), the fractions
of HbF were comparable for LCR-shRNAmiR and BCH-BB694 LVVs,
ranging between 23% and 33% in these mixed cell populations. For
comparison with previous experiments, cells transduced with LCR-
shRNAmiR were additionally sorted by flow cytometry for Venus

expression (data not shown), and induction
levels of HbF were between 44% and 69% of total
hemoglobin, consistent with previous findings.35

Assessment of Transduction Efficacy and
HbF Induction Using HD and SCD CD34+
Cells In Vitro
To further characterize the performance of the
BCH-BB694 LVV, we transduced human mobi-
lized peripheral blood (mPB) CD34+ cells from
HD and SCD donors at a multiplicity of infec-
tion (MOI) of 25. After transduction the cells
were kept in culture for 2 weeks under condi-
tions supporting erythroid differentiation.44,45
Ion exchange-HPLC (IE-HPLC) analysis was
performed to monitor expression of adult type
(HbA or HbS) hemoglobin or HbF (Figures
2A and 2B), and the VCN was assessed by
qPCR. The levels of HbF in mock-treated con-
trol groups were 22% and 32%, on average, for
HD and SCD groups, respectively. BCH-
BB694 LVV-transduced samples showed significant induction of
HbF averaging 74% and 70% for HD and SCD groups at VCNs of
2.78 ± 0.08 and 1.16 ± 0.03 c/dg, respectively. To further assess the
rate of transduction, we utilized progenitor colony-forming assays
as a surrogate readout for HSC transduction and to assess HbF induc-
tion at the clonal level. Transduced CD34+ cells were plated in semi-
solid media under conditions supporting myeloid and erythroid col-
ony formation. The total number of colonies was similar between
mock and transduced groups (Figure S3). Individual erythroid col-
ones were picked and analyzed by HPLC (Figures 2C and 2D).
Similar to the results from erythroid differentiation cultures, the

Figure 2. In Vitro Assays with Human CD34+ Cells
Human CD34+ cells from a healthy donor (HD) and a
sickle cell disease (SCD) donor were transduced with
BCH-BB694. (A) The proportion of HbF (A) of total he-
moglobin was assessed by ion exchange HPLC in bulk
erythroid liquid cultures. The percentage of HbF was
calculated based on peak areas. The average VCN was
measured by qPCR: VCN HD, 2.78 ± 0.08 copies per
diploid genome [c/dg]; SCD, 1.16 ± 0.03 c/dg. (B) Colony
assays were performed, and the fraction of transduced
colonies containing the vector was assessed in individual
replicates. (C and D) HbF induction was analyzed in in-
dividual erythroid colonies by ion exchange HPLC for HD
(C) or SCD (D) samples. (E and F) The percentage of HbF
(%HbF) in each colony plotted as a function of the vector
copy number (VCN) per diploid genome in individual
erythroid colonies for HD (E) or SCD (F) samples. Open
circles indicate mock groups; closed circles indicate
BCH-BB694-transduced groups. R2 = 0.83 and 0.79,
respectively. The average VCN on pooled colonies was
HD: 3.54 ± 0.82 and SCD: 1.95 ± 0.11 c/dg. Two-sided
unpaired t test, *p < 0.05, **p < 0.01, ***p < 0.005
immortalization of primary lineage-negative mouse bone marrow (BM) cells caused by insertional mutagenesis. The assay includes a positive control vector RSF91 previously demonstrated to induce immortalization. At MOIs of up to 500 and VCNs of ~4–11 (mean 7.5 c/dg), there was no difference in the frequency of immortalization of BCH-BB694 LVV-transduced cells in comparison with mock-transduced cells, whereas the positive control showed the expected high rate of immortalization in this assay (Figure 3). In addition, BCH-BB694 LVV-transduced cells showed no differences in proliferation or viability compared with mock-transduced cells, indicating the absence of detectable signs of cellular toxicity (Figure S4). These results indicate low genotoxicity of the BCH-BB694 LVV.

**Competitive Transplantation Assay for the Detection of Phenotoxicity Due to BCL11A Knockdown**

We and others have previously shown that the transcription factor BCL11A is essential for the engraftment of HSCs. Knockdown of BCL11A in all hematopoietic cells is associated with a rapid and near-complete loss of transduced cells after transplantation in vivo. Thus, a tightly regulated erythroid-specific knockdown of BCL11A mediated by the transcriptional control elements present in BCH-BB694 and LCR-shRNA**miR** LVVs is essential to allow for stem cell engraftment, long-term reconstitution, and therapeutic efficacy. To confirm the lack of any negative effect of LCR-shRNA**miR** transduction on engraftment, we performed competitive transplantation experiments. Lineage-negative BM cells from CD45.1** B6 mice were transduced with the LCR-shRNA**miR** LVV (which contains the Venus fluorescent reporter) and mixed with an equal number of cells transduced with an LVV encoding only the dTomato fluorochrome under transcriptional control of the LCR as a neutral competitor (Figure S5). Transduction rates of both competitor populations were similar as assessed by colony assays (Figure S6), with an average VCN of ~1 c/dg across both groups. The relative frequency of different colony types and the absolute number of colonies were also comparable between groups. The cell mixture was transplanted into congeneric CD45.2 B6 recipients, and the relative ratio of the two populations in peripheral blood (PB) was monitored in 4-week intervals over a 16-week reconstitution period (Figure 4). Two independent experiments were performed that differ in their irradiation dose used for conditioning. For the first experiment, recipient animals received 10 Gy total body irradiation, which led to incomplete and heterogeneous engraftment of both groups of donor cells (Figure 4A, open circles). Consequently, in a second independent experiment, the dose of irradiation was increased to 11.5 Gy, which led to near-complete donor chimeraism (Figure 4A, closed circles). We were able to assess the frequency of Venus- and dTomato-positive cells in all animals from both experiments and calculate log2-transformed relative ratios to obtain Gaussian distribution for statistical analysis. As shown in Figure 4B, the competitor populations showed equal engraftment, suggesting that LVV-mediated erythroid lineage-specific knockdown of BCL11A is compatible with normal HSC engraftment and function in this assay. Furthermore, there were no signs of skewing in hematopoietic lineages in the BM, PB, or spleen of transplanted animals (Figures 4C–4E).
Xenotransplantation of Gene-Modified CD34+ Cells into NSG Mice

With the aim of confirming these findings in a model system with primary human cells, a xenograft transplantation experiment was performed using HD mPB CD34+ cells transduced with BCH-BB694 LVV or a neutral control vector expressing only GFP. Similar engraftment of human CD45+ cells was observed in PB 4 and 8 weeks post-transplantation and in spleen and BM at 8 weeks post-transplantation for both groups (Figure 5A). The lineage distribution in PB, BM, spleen, and thymus (Figure 5B) and the frequency of BM CD34+ and CD34+/CD19- cells (Figure 5C) were comparable between groups. The frequency of B cells was similar between groups, indicating normal BCL11A function in lymphoid cells. To determine any toxicity in the engrafting HSPC population, we divided the VCN determined in BM cells 8 weeks post-transplantation by the pre-transplant VCN, determined from a cell product that was kept in culture for 14 days. The VCNs from harvested total BM (post-transplant) (Figure 5D) were similar to the VCN of the cells transplanted (pre-transplant) in both groups. Similar results for VCNs were obtained with samples from PB, human CD45+ (hCD45+) BM, hCD34+ BM, spleen, and thymus (Table S1). These data demonstrate the absence of engraftment defects or toxicity mediated by the LVV. Because human erythropoiesis is poorly supported in NSG mice, we isolated hCD34+ cells from BM of transplanted animals and induced erythroid differentiation in vitro. HbF levels in erythroid cells derived from the BCH-BB694 LVV treatment group demonstrated 39% ± 8% HbF compared with 10% ± 3% in the control GFP LVV group (Figure 5E) as determined by HPLC, with the amount of HbF closely correlating with the VCN in differentiated samples (Figure S7).

Large-Scale Validations Using SCD Patient CD34+ Cells and GMP-Grade LVV

Lastly, we performed large-scale transduction experiments using the manufacturing protocol to be utilized for the generation of the cell products for the planned clinical trial (ClinicalTrials.gov: NCT03282656). The goal of these large-scale transductions was to establish the appropriate MOI to achieve a VCN in the desired range (0.7–5 c/dg). Four independent engineering runs were performed on plerixafor mPB CD34+ cells from SCD subjects. The VCNs were assessed after 7 days in culture (Table 1). The first engineering run was performed on previously cryopreserved cells with research-grade BCH-BB694 LVV at an MOI of 6 or 25, leading to VCNs of 3.7 and 11.1 c/dg, respectively. After this initial pilot experiment, three other engineering runs were performed under GMP conditions with clinical-grade BCH-BB694 LVV using MOIs ranging from 15 to 37, which resulted in VCNs between 3.5 and 7.5 c/dg. The last two engineering runs used an MOI of 15, resulting in a VCN within the desired range. The cell products generated at the end of each run successfully passed sterility, endotoxin, and mycoplasma tests.

Transduced SCD CD34+ cells from one donor were erythroid differentiated in vitro to quantify HbF and physiological analyses using a
sickling assay. After 18 days of differentiation, the average VCN was 2.8 c/dg, resulting in 39% ± 1% HbF of total hemoglobin (Figure 6). Enucleated RBCs derived from this sample were exposed to the potent sickling inducer metabisulfite. Although >90% of mock-treated cells demonstrated sickling or dysmorphic appearance caused by hemoglobin polymerization, this number was significantly reduced to 48% in cells treated with BCH-BBB694 LVV (Figure 6) coincident with the presence of increased levels of HbF.

DISCUSSION

We previously developed an LVV that mediates downregulation of BCL11A via RNAi selectively in erythroid cells using a microRNA-adapted short hairpin RNA (shRNA<sup>mir</sup>). Significant downregulation of BCL11A in erythroid cells leads to sustained reactivation of γ-globin, the production of HbF, reduced polymerization of sickle-containing hemoglobin, and significant mitigation of the hematologic effects of SCD. We<sup>35</sup> Our strategy and vector configuration underwent several stages of development to address a variety of problems limiting clinical applicability. First, a polymerase II-driven shRNA<sup>mir</sup> replaced the polymerase III-driven shRNAs to reduce non-specific cytotoxicity related to excessive shRNA expression and allowing expression via more physiological promoters.<sup>31</sup> Second, the shRNA<sup>mir</sup> was placed under control of erythroid-specific promoter/enhancer elements to avoid downregulation of BCL11A in HSCs and B cell progenitors, cells in which BCL11A expression is essential.<sup>31–36</sup> As described here, we also changed the vector backbone in order to improve viral titers to a level that is required for the efficient genetic modification of patient primary HSCs at large scale, an essential step to bring this therapy to the clinic. Low viral titers are a practical bottleneck particularly for LVVs containing the globin LCR regulatory elements and β-globin sequences due to their large size,<sup>38–40</sup> and LVV-expressing miRNA hairpins have reduced titers because of the partial degradation of vector genomes through the cellular miRNA-processing complex DROSHA during virus production.<sup>31–33</sup> Low titers are problematic because they limit the achievable transduction rate and VCN, particularly within the rare long-term HSC compartment, and ultimately the therapeutic efficacy for the patient.

We performed preclinical experiments showing that clinical-grade vector can be produced for the BCH-BBB694 LVV vector at high titers in GMP production at large scale. In colony assays, we provide evidence that we consistently obtained transduction of >80% of hematopoietic progenitor-derived colony-forming units, and that almost all transduced erythroid colonies show high levels of HbF expression in vitro. These parameters are particularly important in SCD gene therapy because a too-large fraction of unmodified cells may lead to a less than complete reversal of the clinical phenotype. RBCs without sufficient HbF will remain prone to intracellular HbS polymerization and sickling.<sup>54</sup> Although the therapeutic efficacy of gene therapy approaches for SCD is augmented by a substantial selective advantage of high HbF-containing mature RBCs due to an increased lifespan,<sup>55–66</sup> and potentially also due to reduced ineffective erythropoiesis,<sup>61</sup> it is difficult to extrapolate the fraction of gene-modified HSCs that is required for the prevention of specific clinical manifestations. Two somewhat related parameters are the main determinants of therapeutic success. First, PB in gene therapy patients treated with BCH-BBB694 LVV and other gene therapy approaches, including methods of gene editing, will consist of a mixture of gene-modified and unmodified cells, the latter of which contain mainly HbS and are still prone to sickling. The number of RBCs
containing mostly HbS depends in large part on the efficiency of genetic modification of long-term reconstituting stem cells and the effectiveness of the conditioning regimen to prevent endogenous HSC recovery. In our and others’ previous work, it has been estimated that 20% gene-corrected HSCs would lead to 80% non-sickling erythrocytes in the periphery. Second, the gene-modified cells will contain varying levels of HbF depending on the efficacy of the vector in expressing the heterologous gene, in this case the shRNA\textsuperscript{miR}, while in other cases a hemoglobin gene. Although the level of intracellular HbF required to prevent HbS polymerization \textit{in vivo} is not currently known, the use of a physiological switch to both increase HbF and concurrently reduce HbS has clear theoretical advantages. HbF has potent anti-sickling characteristics, as shown in previous work that has suggested a level of HbF of approximately one-third of the total cellular content of hemoglobin would prevent HbS polymerization, while the concurrent reduction in intracellular HbS further attenuates the tendency for polymer formation.

An additional concern of lineage-targeted BCL11A knockdown is that any residual expression of the shRNA\textsuperscript{miR} in HSC or B cells may negatively affect engraftment or B cell reconstitution because BCL11A is associated with high VCNs due to the potential risk of insertional oncogenesis, and also against economic considerations of using high volumes of costly vector. We demonstrate at VCNs of 4–11 c/dg the safety of the BCH-BB694 LVV vector using the IVIM assay. This confirms findings by others who used very similar vector configurations, although the predictive value of available genotoxicity assays is limited.

An additional concern of lineage-targeted BCL11A knockdown is that any residual expression of the shRNA\textsuperscript{miR} in HSC or B cells may negatively affect engraftment or B cell reconstitution because BCL11A is essential in those cells. We addressed this concern by stringent competitive transplantation experiments, where cells transduced with the BCL11A knockdown vector competed with cells transduced with a neutral LVV encoding only a fluorescent reporter. Equivalent frequencies of both competitor populations were detected in transplanted animals, suggesting the absence of biologically relevant levels of leaky expression in HSCs. To account for potential species-specific differences, we performed xenotransplantation experiments using human CD34\textsuperscript+ cells that were transduced to high VCNs (up to 9.35 c/dg \textit{in vivo}) with BCH-BB694 LVV or a neutral GFP LVV. Even minimal leakiness of shRNA\textsuperscript{miR} expression by the vector at this high VCN would be predicted to lead to loss of gene-modified cells as previously demonstrated. However, cells transduced with BCH-BB694 LVV showed similar engraftment characteristics as cells transduced with the neutral control vector. Overall, in the studies reported here, human cell engraftment, lineage distribution, and specifically B cell reconstitution were similar, as was the recovery rate of VCN.
pre-versus post-transplantation in both groups. No evidence of transduced B or stem cell impairment has been observed. These data are consistent with a previous report that showed therapeutic efficacy and the complete absence of detectable expression in CD34+ BM cells isolated from transplanted NSG animals.35 In summary, all experimental evidence indicates that the BCH-BB694 LVV is safe for use in HSC gene therapy.

One hurdle in the clinical translation of gene therapies is the scale-up of cell manufacturing protocols, which may lead to lower than desired gene modification rates. We performed a series of large-scale validation runs that identified suitable conditions for transducing SCD CD34+ cells to generate cell products within a desired target VCN range and confirmed the compatibility of the procedures with therapeutic efficacy in an in vitro sickling assay, underscoring the potency of our vector.

In conclusion, the data presented here form the basis for an ongoing pilot and feasibility clinical trial for the treatment of SCD (ClinicalTrials.org: NCT03282656) using autologous HSCs transduced with our vector.

Figure 6. In Vitro HbF Induction and Sickling Assay with BCH-BB694-Transduced SCD Cells Manufactured under GMP Conditions
CD34 cells transduced with BCH-BB694 under GMP conditions were differentiated into erythrocytes. The VCN and HbF induction were determined, and enucleated erythrocytes were embedded and were analyzed by IF-EHPLC to assess metabisulfite-induced sickling. The relative proportion of dysmorphic versus normally shaped cells was determined by light microscopy. n = 3 technical replicates. Average VCN of total cells prior to FACS: 2.8. Error bars: SD. Statistical test: two-sided t test, **p ≤ 0.01, ***p ≤ 0.001.

Erythroid Differentiation in Liquid Culture
Following transduction, a subset of cells was cultured in erythroid differentiation media in a standard humidified tissue culture incubator for 2 weeks at 37°C and 5% CO2. The erythroid differentiation media consisted of Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 1% penicillin/streptomycin (Pen/Strep), 20 ng/mL hSCF, 1 ng/mL hIL-3 (all PeproTech, Rocky Hill, NJ, USA), 2 IU/mL erythropoietin (R&D Systems, Minneapolis, MN, USA), and 20% fetal bovine serum. After 14 days, cells were centrifuged (~300 × g, 10 min), washed in PBS, and lysed in HPLC-grade water. After high-speed centrifugation (20,000 × g, 30 min at 4°C), supernatants were analyzed by IE-HPLC. Alternatively, the three-stage erythroid differentiation protocol developed by Giarratana et al.35 was followed.

Hemoglobin Analysis by IE-HPLC
After erythroid differentiation, cells were washed in PBS (Sigma-Aldrich, St. Louis, MO, USA) and analyzed by IE-HPLC on a PolyCATA 200 × 2.1 mm 5 μm 1,000 Å (PolyC#202CT0510; PolyLC, Columbia, MD, USA) using the mobile phases: phase A, Tris 40 mM, KCN 3 mM, in HPLC-grade water adjusted to a pH 6.5 with acetic acid (reagents from Sigma-Aldrich, St. Louis, MO, USA); and phase B, Tris 40 mM, KCN 3 mM in HPLC-grade water, NaCl 0.2M adjusted to a pH 6.5 with acetic acid (reagents from Sigma-Aldrich, St. Louis, MO, USA). A timed 24-min program was used to create a 2%–100% B gradient with a flow rate of 0.3 mL/min using a Shimadzu Prominence Chromatograph (Shimadzu Scientific Instruments, Columbia, MD).
USA). The column oven temperature was 30°C, and the sample tray was kept at 4°C. The peaks were detected at 418 nm. Retention times were determined using the AFSC hemoglobin standard (Helena Laboratories, Beaumont, TX, USA).

Clonogenic Cultures
Approximately 500 CD34+ cells in total were seeded into MethoCult Classic H4434 (STEMCELL Technologies, Vancouver, BC, Canada) for triplicates in 3.5-cm plates following the manufacturer’s protocol. After 2 weeks of culture, colonies were scored by morphology, enumerated, and either plucked as individual colonies or pooled and subjected to qPCR for assessment of VCNs (c/dg).

Mouse Transplantations
B6 (C57BL/6), BoyJ (B6.SJL-Ptprc<sup>+</sup> Pecp<sup>+</sup>/BoyJ), and NSG mice (NOD.Cg-Pkdc<sup>+</sup>Il2rg<sup>+</sup>/SzJ) aged 4–8 weeks were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). B6 animals were conditioned with 10 or 11.5 Gy and NSG mice with 2.7 Gy, and transplanted retro-orbitally with 1 × 10⁶ cells per animal. Blood samples were obtained via retro-orbital bleeds. For final analysis, mice were sacrificed using CO₂. Spleen and thymus were removed and ground over a 100-µm mesh (Merck Millipore, Danvers, MA, USA) to obtain cell suspensions; femur and tibia were flushed out and filtered through a 100-µm mesh to obtain BM cells. The procedures were approved under Boston Children’s Hospital Institutional Animal Care and Use Committee (BCH IACUC) #17-01-3364R.

Flow Cytometry and Fluorescence-Assisted Cell Sorting
Cells suspensions from cultured cells or from transplanted mice were stained with combinations of surface antibodies for mCD45-VioBlue, hCD3-allophycocyanin (APC) (both Miltenyi, Germany), hCD45-PerCP-Cy5.5, hCD235a-phycocerythrin (PE) (both BioLegend, San Diego, CA, USA), hCD4-PE-Cy7, hCD71-APC, hCD19-PE-Cy7, hCD33-PE, hCD71-APC, hCD19-PE-Cy7, hCD235a-phycoerythrin (PE) (both BioLegend, San Diego, CA, USA), hCD3-allophycocyanin (APC) (both Miltenyi, Germany), and hCD34-APC (BD Biosciences, San Jose, CA, USA). Mouse and human Fc-Block were added when staining cells isolated from mice. Analyses were performed on BD Fortessa SOP or BD LSRII SOP equipped with UV, 405-, 488-, 561-, and 633-nm lasers, and cell sorting was performed on a BD AriaII machine. Analysis of flow cytometry data was performed using the BD Diva and Tree Star FlowJo software.

PCR and VCN Assay
Genomic DNA was extracted using the QIAGEN DNeasy protocol (Qiagen, Hilden, Germany). VCN was assessed by real-time polymerase chain reaction, performed using TaqMan Fast Master Mix (Invitrogen, Carlsbad, CA, USA) and 0.9 mM GAG forward (5'-GGAGCTAGAACGATTCGCAGTTA-3') and reverse (5'-GTTTGTAGCTGTCCCAGTATTTGTC-3') primers, GAG FAM probe (5'-[FAM]-ACAGCCCTGCTGTTCTCTAAGGGCCAGG-TAMRA]-3'), and RNASE-P-VIC control TaqMan assay (Invitrogen). PCR was run using Fast program on Applied Biosystems StepOnePlus real-time thermocycler (Invitrogen, Carlsbad, CA, USA). VCN was assessed relative to a reference known to contain one copy of integrated viral DNA per haploid genome.

Assessment of VCN and HbF on Individual BFU-E Colonies
Erythroid colonies were plucked individually under a microscope. Each colony was washed in PBS (~300 × g for 10 min) and resuspended in 100 µL of HPLC-grade water. A total of 20 µL was used for VCN assessment by qPCR, and 80 µL was used for hemoglobin analysis by IE-HPLC. Genomic DNA was column purified using the DNA Extract All Reagents Kit (Thermo Fisher, Waltham, MA, USA).

IVIM
The IVIM assay was conducted as previously described. Lineage-negative cells from B6 mice were isolated using the murine lineage depletion kit (Miltenyi, Germany) according to the manufacturer’s instructions and transduced in StemSpan-medium (STEMCELL Technologies, Cologne, Germany) supplemented with 1% Pen/Strep, 50 ng/mL mSCF, 100 ng/ml hFLT-3-L, 100 ng/mL hIL-11, and 20 ng/mL m-IL-3 (all PeproTech, Hamburg, Germany). Cells were further expanded in IMDM supplemented with the above-mentioned antibiotics and cytokines, as well as 10% fetal calf serum and 2 mmol/L glutamine for 15 days. A total of 100 cells/well was seeded on 96-well plates and incubated for 14 days in IMDM conditions. Mock cells usually do not grow under these conditions. Wells containing proliferated insertional mutants were detected by microscopic evaluation and an enzymatic assay (MTT). The positive wells were used to calculate the replating frequencies (RFs) using the R package limdil. According to metadata, plates with insertional mutants in three or more wells (Q1 level) were counted as positive assays. The incidence of positive assays was compared by Fisher’s exact test with Benjamini-Hochberg correction for multiple comparisons.

Large-Scale Engineering Runs of CD34+ Cell Transduction under Good Manufacturing Practices
For engineering runs, large-scale vector preparations were produced by transient transfection of HEK293T cells with LVV construct and packaging plasmids, followed by microfiltration, ion exchange chromatography, concentration, and diafiltration by tangential flow filtration and fill-finish. All vector preparations were titrated on HOS cells. Four large-scale validations were performed at Connell and O’Reilly Families Cell Manipulation Core Facility at Dana-Farber Cancer Institute (Boston, USA). Plerixafor mobilized CD34+ cells enriched by CliniMACS device (Miltenyi Biotec, Auburn, CA, USA) were pre-stimulated in CellGenix SCGM media supplemented with recombinant human (rh)TPO, rhSCF, and rhFLT3-L (all 100 ng/mL from CellGenix, Portsmouth, NH, USA) at a density of 1 × 10⁶ cells/mL for 44 ± 4 h at 37°C and 5% CO₂. Cells were then transduced at MOIs indicated in Table 1 at 5 × 10⁶ cells/mL with BCH-BB694 LVV for 22 ± 4 h as previously described. The final product was cryopreserved in Cryostor5 (BioLife Solutions, Bothell, WA, USA) prior to downstream analyses.

In Vitro Sickling Assay
At the end of in vitro erythroid differentiation, enucleated RBCs were sorted using Hoechst 33342 (5 µg/mL; Invitrogen, Carlsbad, CA, USA). Sorted cells were resuspended in 50 µL PBS, mixed with...
an equal volume of 2% metaphosphite in PBS, and incubated at 37°C for 20 min in a chamber slide (Nunc/Life Technologies, Grand Island, NY, USA) prior to recording and analysis. Cells with irregular structure, protruding spikes, or sickle shape were counted as sickling cells.

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism 8.3 software (GraphPad Software, San Diego, CA, USA). The standard statistical test used in most cases is a two-sided unpaired t test unless indicated otherwise. Statistical significance is indicated with a p value; N.S. denotes p > 0.05. Statistical significance differences are indicated with asterisks: *p < 0.05, **p < 0.01, ***p < 0.005. For Figures 2E and 2F we applied a Gompertz model, and the three parameters characterizing the Gompertz model have been estimated by means of the easynls package available in R.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.03.015.

**AUTHOR CONTRIBUTIONS**

C.B., O.N., and D.A.W. wrote the manuscript; C.B., O.N., M.R., M. Bonner, A.S., and D.A.W. designed experiments; C.B., O.N., M.R., S.G., G.P., C.H., M.M., M.A., D.A., D.K., M. Bentler, and M. Bonner performed experiments; A.S., J.M., G.V., E.E., S.G., G.P., C.H., M.M., M.A., D.A., A.T., D.K., M. Bentler, H.N., O.N., G.P., L.C., M. Bonner, and G.V are employees and shareholders of bluebird bio, Inc. DAW licensed IP relevant to BCL11A vector to O.N., G.P., L.C., and M. Bonner performed experiments; A.S., J.M., G.V., E.E., and M.A. provided technical help and advice; D.P. performed statistical analyses.

**CONFLICTS OF INTEREST**

O.N., G.P., L.C., M. Bonner, and G.V are employees and shareholders of bluebird bio, Inc. DAW licensed IP relevant to BCL11A vector to bluebird bio and has the potential to receive future royalty/milestone payments. DAW has received payment in the past through BCH institutional licensing agreement with bluebird bio.

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