Molecular Therapy
Original Article

Coordinated β-globin expression and α2-globin reduction in a multiplex lentiviral gene therapy vector for β-thalassemia

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A primary challenge in lentiviral gene therapy of β-hemoglobinopathies is to maintain low vector copy numbers to avoid genotoxicity while being reliably therapeutic for all genotypes. We designed a high-titer lentiviral vector, LVβ-shα2, that allows coordinated expression of the therapeutic $\beta^{A\text{-}T87Q}$-globin gene and of an intron-embedded miR-30-based short hairpin RNA (shRNA) selectively targeting the α2-globin mRNA. Our approach was guided by the knowledge that moderate reduction of α-globin chain synthesis ameliorates disease severity in β-thalassemia. We demonstrate that LVβ-shα2 reduces α2-globin mRNA expression in erythroid cells while keeping α1-globin mRNA levels unchanged and $\beta^{A\text{-}T87Q}$-globin gene expression identical to the parent vector. Compared with the first $\beta^{A\text{-}T87Q}$-globin lentiviral vector that has received conditional marketing authorization, BB305, LVβ-shα2 shows 1.7-fold greater potency to improve α/β ratios. It may thus result in greater therapeutic efficacy and reliability for the most severe types of β-thalassemia and provide an improved benefit/risk ratio regardless of the β-thalassemia genotype.

INTRODUCTION

β-Thalassemia is the result of reduced or absent β-globin chain synthesis.[1,2] During normal erythropoiesis, β-globin is synthesized in equimolar quantities with α-globin. Two chains of each protein associate to form the tetrameric adult hemoglobin (HbA; $\alpha_2\beta_2$). In β-thalassemia, a quantitative reduction or absence of β-globin chain synthesis disrupts the α:β-globin chain balance, leading to accumulation of unpaired α-globin in developing erythrocytes. Excess α-globin forms toxic aggregates, causing apoptosis of immature red blood cells (RBCs).[3] The most severe forms of β-thalassemia manifest as dyserythropoiesis and hemolytic anemia, requiring monthly blood transfusions and lifelong iron chelation therapy.

Several lentiviral β-globin gene therapy vectors (LVβs) encompassing elements of the locus control region (LCR)[4] are in clinical trials for β-hemoglobinopathies.[5,6] Notably, the LentiGlobin BB305 gene therapy vector, encoding $\beta^{A\text{-}T87Q}$-globin, originally designed by Leboulch et al.[7] and Pawliuk et al.[8] to inhibit hemoglobin S (HbS) polymerization, has achieved clinical efficacy and safety in a large number of transfusion-dependent individuals with β-thalassemia[9–12] and sickle cell disease in phase II and III trials.[13,14] With regard to gene therapy for β-thalassemia, virtually all individuals with β-thalassemia with residual β-globin synthesis (non-β^0/β^0 genotype) (e.g., HbE/β^0-thalassemia), have discontinued transfusions following BB305 gene therapy, with near-normal blood Hb levels.[9,11,12] Importantly, molecular and cellular hallmarks of dyserythropoiesis were corrected in individuals who achieved near-normal blood Hb values. As a result, autologous hematopoietic $CD34^+$ cells transduced with BB305 are the first gene therapy product (Zynteglo) to be granted conditional approval in Europe for transfusion-dependent non-β^0/β^0 genotype individuals.[15]

Received 17 August 2020; accepted 27 April 2021;
https://doi.org/10.1016/j.ymthe.2021.04.037.

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However, β^0/β^0-genotype individuals have been proven to be more challenging to treat with BB305 gene therapy.[9–12] Only a subset of β^0/β^0-genotype individuals achieved transfusion independence, although most displayed a decrease in transfusion requirements.[9,10,12,16] The clinical efficacy of lentiviral β-globin gene therapy vectors is directly correlated with the number of proviral integration events in hematopoietic cells and overall output of therapeutic β-globin chain synthesis.[11,17] Achieving an optimal therapeutic effect in all β^0/β^0-genotype individuals, who have the greatest requirement for therapeutic β-globin output, would benefit from a lentiviral vector capable of minimizing the detrimental effects of free α-globin chains while continuing to express high levels of $\beta^{A\text{-}T87Q}$-globin in an erythroid-specific manner. Such a combination vector would theoretically result in greater therapeutic efficacy and reliability at lower vector copy numbers (VCNs) than BB305. Reaching the therapeutic threshold at lower VCNs would reduce the risk of untoward insertional mutagenesis for all individuals with β-thalassemia regardless of genotype.

To enhance the therapeutic efficacy of the BB305 gene therapy vector for the most severe β^0/β^0-genotype individuals, we developed a novel multiplexed lentiviral β-globin gene vector that allows coordinated expression of the therapeutic $\beta^{A\text{-}T87Q}$-globin gene with concomitant reduction in α-globin chain synthesis. Our approach was guided by the knowledge that co-inheritance of an α-thalassemia trait along with a variety of β-thalassemia mutations reduces the clinical severity of β-thalassemia. A single α-globin gene deletion ($-\alpha/\alpha\alpha$) has a minimal effect, but double ($-\alpha/-\alpha$ or $--/\alpha\alpha$) or triple ($--/-\alpha$) α-globin gene deletions have a major beneficial effect on disease severity by normalizing α:β-globin chain balance and minimizing the harmful effects of free α-globin chains.[18–21]

Here we modified LentiGlobin BB305 by inserting a miR-30 short hairpin RNA (shRNA) expression cassette into intron 2 of the $\beta^{A\text{-}T87Q}$-globin gene to derive the LVβ-shRNA vector, allowing stable erythroid-specific expression of functional shRNA from a common primary transcript. We further configured the LVβ-shRNA vector to specifically target the human α2-globin (*HBA2*) mRNA to generate the LVβ-shα2 gene therapy vector. We demonstrate that the LVβ-shα2 vector yields a high viral titer, transduction efficiency, and erythroid $\beta^{A\text{-}T87Q}$-globin gene expression equivalent to the parent BB305 vector. Notably, LVβ-shα2 shows the additional property of decreasing human α2-globin mRNA levels without affecting α1-globin (*HBA1*) mRNA expression levels in transduced human erythroid cell lines and primary human $CD34^+$ hematopoietic cells derived from normal individuals and those with β-thalassemia. Importantly, the LVβ-shα2 vector is able to achieve a greater degree of correction of α/β-globin mRNA ratios in cells from individuals with β-thalassemia at identical VCNs compared with the parent BB305. We define a novel therapeutic strategy that could improve gene therapy outcomes in individuals with β-thalassemia irrespective of genotype.

RESULTS

Generation of the LVβ gene therapy vector carrying a functional intron-encoded miR30-shRNA expression cassette

To examine whether the LentiGlobin BB305 vector could be used to deliver miR30-shRNA expression cassettes embedded in an intron of the vector-bearing $\beta^{A\text{-}T87Q}$-globin gene (Figure 1A; Figure S1), LVβ-shRNA vectors carrying the enhanced green fluorescent protein (EGFP)-specific shRNA (LVβ-shGFP) and scrambled negative control (LVβ-shSCR) vectors were generated and validated in the murine erythro-leukemia (MEL)-βEGFP reporter cell line, in which EGFP expression is driven by the human β-globin promoter in the context of a chromosomally integrated, large segment of the human β-globin locus.[22,23] Following viral transduction with BB305, LVβ-shSCR, and LVβ-shGFP vectors, bulk-transduced MEL-βEGFP cells were induced to undergo erythroid differentiation after exposure to dimethyl sulfoxide (DMSO). We observed that LVβ-shGFP significantly reduced EGFP fluorescence and mRNA expression, whereas BB305 and LVβ-SCR vectors had no significant effect (Figures 1B and 1C).

Next, $\beta^{A\text{-}T87Q}$-globin mRNA expression was measured following erythroid differentiation of the transduced MEL-βEGFP cells. We observed that $\beta^{A\text{-}T87Q}$-globin transgene expression by the LVβ-shGFP and LVβ-shSCR vectors was equivalent to the parent BB305 vector (Figure 1D). To assess whether the miR30-shRNA expression cassette may yield alternative or aberrant mRNA splicing events, $\beta^{A\text{-}T87Q}$-globin expression was analyzed by RT-PCR using primers located on either side of the human $\beta^{A\text{-}T87Q}$-globin exon 2-3 boundary (Figure S2A). A single PCR product of the expected size (168 bp), representing the correctly spliced $\beta^{A\text{-}T87Q}$-globin mRNA, was identified in cells transduced with the BB305, LVβ-shSCR, and LVβ-shGFP vectors (Figure S2B). These results indicate that inserting the miR30-shRNA expression cassette at the intervning sequence 2 (IVS2) breakpoint site allows normal mRNA splicing and does not result in aberrant splice products detected by the assay.

To investigate the relationship between $\beta^{A\text{-}T87Q}$-globin transgene expression and functional EGFP shRNA, transduced MEL-βEGFP clones (n = 10) were analyzed following erythroid differentiation. Consistent with the bulk-transduced cells, the LVβ-shGFP vector significantly diminished EGFP fluorescence in individual clones (Figure 1E). In LVβ-shGFP-transduced clones, EGFP shRNA and $\beta^{A\text{-}T87Q}$-globin transgene expression levels showed a significant positive correlation between them ($p < 0.0001$) (Figure 1F), whereas $\beta^{A\text{-}T87Q}$-globin transgene expression levels and EGFP fluorescence displayed a significant negative correlation ($p = 0.0005$) (Figure 1G). As expected, there was no correlation between $\beta^{A\text{-}T87Q}$-globin expression and EGFP fluorescence in BB305- or LVβ-shSCR-transduced clones ($p = 0.7811$) (Figure 1H). These results demonstrate that the LVβ-shGFP vector produces a functional intron-encoded shRNA whose amount depends on the expression level of the $\beta^{A\text{-}T87Q}$-globin transgene.

Validation in K562 cells of LVβ-shRNA lentiviral vectors tailored to reduce human α-globin expression

We then explored the possibility of using small interfering RNAs (siRNAs) to reduce human α-globin gene expression. Comparative analysis of 5 siRNAs targeting different α-globin locations was performed following electroporation into K562 cells. Four siRNAs

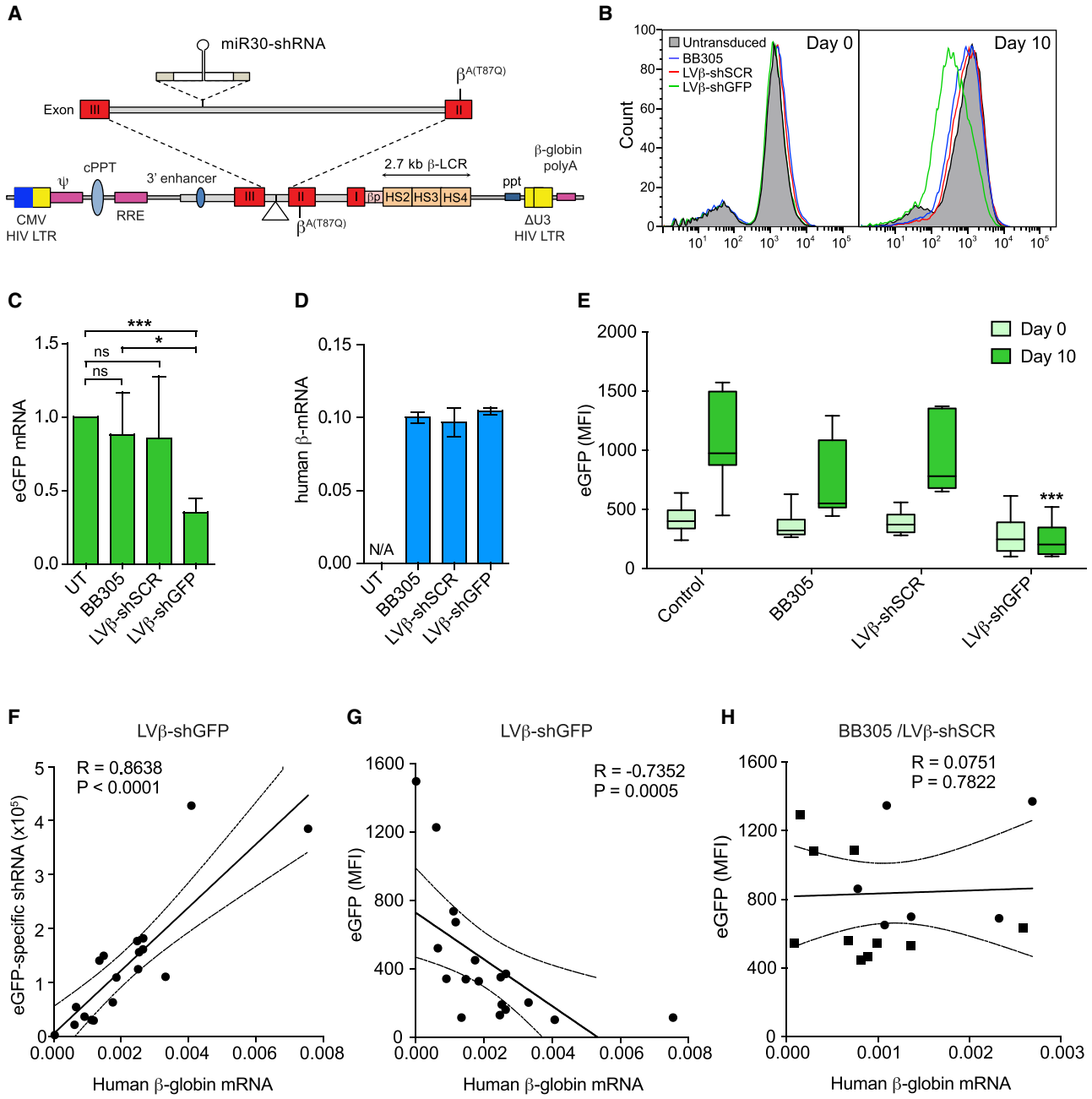

Figure 1. The LVβ gene therapy vector containing intronic miR30-shGFP reduces target gene expression in erythroid cells

(A) Schematic of the LVβ gene therapy vector, LentiGlobin BB305, modified to express the miR30-shRNA cassette. (B) Representative histograms of EGFP expression by MEL-βEGFP cells transduced with the BB305, LVβ-shSCR, and LVβ-shGFP vectors at equivalent MOIs. (C and D) Relative EGFP mRNA expression (C, n = 3) and $\beta^{A\text{-}T87Q}$-globin mRNA expression (D) in transduced MEL-βEGFP cells normalized to β-actin. Data are presented as mean ± SD of at least three separate experiments relative to untransduced (UT) cells following 10 days of DMSO induction. Significance was calculated by unpaired Student's t test; *p ≤ 0.05, ***p ≤ 0.001. (E) Flow cytometry analysis of individual MEL-βEGFP clones (n = 10) transduced with the BB305, LVβ-shSCR, and LVβ-shGFP vectors. Data represent median (lines), the 25th and 75th percentiles (boxes), and the 90th and 10th percentiles (error bars) (***p ≤ 0.0001). (F–H) Correlation analysis of (F) shGFP expression to β-globin mRNA, (G) EGFP fluorescence to β-globin mRNA in LVβ-shGFP-transduced clones, and (H) EGFP MFI to β-globin mRNA in BB305-transduced (squares) and LVβ-shSCR-transduced (circles) clones following 10 days of DMSO induction. Correlation analysis was performed using the Spearman rank method. Scatterplot linear regression lines with 95% confidence intervals (dotted lines) are displayed for (F)–(H). A positive correlation between shGFP expression and β-globin mRNA expression in LVβ-shGFP clones was detected (n = 18, R = 0.864, p < 0.0001). A negative correlation between EGFP MFI and LV β-shGFP β-globin mRNA expression was detected (n = 18, R = −0.735, p < 0.0005). EGFP MFI and β-globin mRNA expression in BB305-transduced (squares) and LVβ-shSCR-transduced (circles) clones did not correlate (n = 16, R = 0.0751, p = 0.7811).

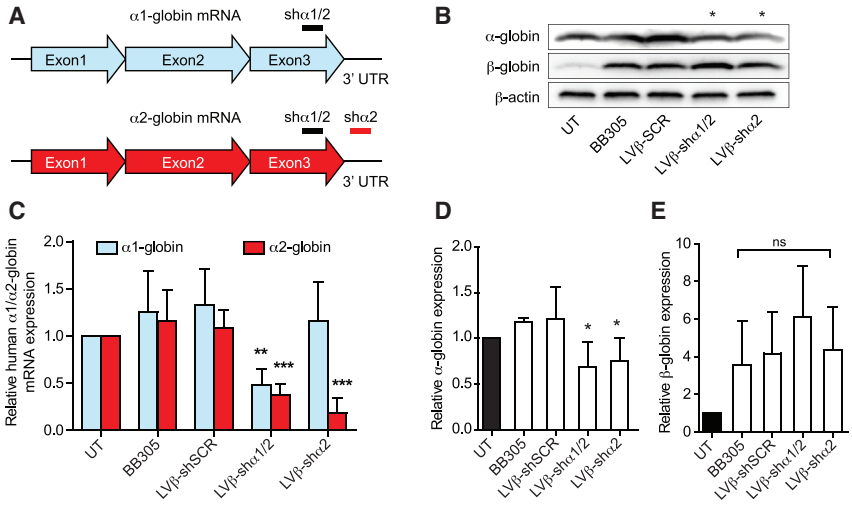

Figure 2. The LVβ gene therapy vector containing the miR30-shRNA expression cassette can be used to reduce α-globin expression in K562 cells

(A) Schematic of α-globin mRNA showing the location of the shα1/2 and shα2 target sites. (B) Relative α1 and α2-globin mRNA expression levels in K562 cells transduced with the BB305, LVβ-shSCR, LVβ-shα1/2, and LVβ-shα2 vectors at equivalent MOIs following 5 days of hemin induction. α1/α2-globin mRNA expression levels were calculated by normalizing to β-actin in UT controls. (C) Representative western blot analysis of K562 cells transduced with the BB305, LVβ-shSCR, LVβ-shα1/2, and LVβ-shα2 vectors following hemin induction. (D and E) Relative quantification of (D) α-globin and (E) β-globin chains. The level of β-actin expression in UT cells was used to normalize signal intensity values between samples. Data represent mean ± SD of three independent experiments and vector preparations. Statistical analysis was performed using unpaired Student's t test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

were identified to significantly reduce human α-globin mRNA (Figure S3). Because si-α3 and si-α4 showed a suitable reduction in human α-globin expression, they were configured into the LVβ-shRNA vector system to generate the LVβ-shα1/2 and LVβ-shα2 vectors, respectively. Because of a number of nucleotide differences between human α1 and α2 globin mRNAs in their 3′ untranslated regions (UTRs), the LVβ-shα2 was designed to specifically target human α2-globin mRNA without affecting human α1-globin mRNA, whereas LVβ-shα1/2 targets the third exon of the α1-globin and α2-globin genes (Figure 2A). LVβ-shRNA vectors yielded titers equivalent to that of the parental BB305 vector (Figure S4).

The human α-globin-specific mRNA knockdown activity of the LVβ-shα1/2 and LVβ-shα2 vectors was evaluated in K562 cells by qRT-PCR analysis (Figure 2B). The LVβ-shα1/2 vector reduced α1- and α2-globin mRNA by approximately 50%. Notably, the LVβ-shα2 vector reduced α2-globin mRNA expression by 78% ± 13% ($p < 0.001$), whereas α1-globin mRNA remained unaffected (Figure 2B). Using primers designed to amplify across the exon 2-to-exon 3 junction, the correctly spliced human $\beta^{A\text{-}T87Q}$-globin mRNA was detected as a single RT-PCR product (168 bp) (Figure S2C). Importantly, western blot analysis of proteins extracted from transduced K562 cells confirmed a substantial reduction of α-globin chain expression by the LVβ-shα1/2 and LVβ-shα2 vectors (35% ± 15% and 30% ± 18%, respectively; $p < 0.05$) compared with negative control groups (Figures 2C and 2D), whereas β-globin expression remained unchanged at the mRNA and protein levels (Figures 2C and 2E). To ensure that the α-globin knockdown would not be complete, we decided to focus on the LVβ-shα2 vector, which specifically targets the α2-globin mRNA, leaving α1-globin expression untouched.

The LVβ-shα2 lentiviral vector reduces α2-globin expression in β^0-HUDEP-2 cells

The HUDEP-2 cell line recapitulates the human adult erythroid differentiation program.[24] To evaluate LVβ-shRNA vectors in a sustainable β-thalassemia environment, a HUDEP-2 cell line containing biallelic β^0-globin mutations, β^0-HUDEP-2, was created using CRISPR-Cas9 genome editing (Supplemental materials and methods). The absence of β-globin chain synthesis was confirmed by high performance liquid chromatography (HPLC) analysis (Figure 3C). β^0-HUDEP-2 cells failed to progress beyond the Poly-E stage of differentiation and morphologically exhibited an increased level of cell death by day 8, recapitulating the β-thalassemia environment (Figures 3A and 3B).

We next evaluated erythroid maturation of β^0-HUDEP-2 cells following transduction with the BB305, LVβ-shSCR, and LVβ-shα2 vectors at equivalent multiplicities of infection (MOIs). Initial transduction efficiency, as measured by intracellular β-globin staining 48 h after transduction, ranged from 27.0%–40.7%, and after one cryopreservation cycle, the majority of transduced β^0-HUDEP-2 cells (88.3% ± 6.0%) expressed the $\beta^{A\text{-}T87Q}$-globin transgene (data not shown). Therefore, $\beta^{A\text{-}T87Q}$-globin transgene expression appears to provide a survival advantage in bulk-transduced β^0-HUDEP-2 cells. This is in agreement with the observations made by Miccio et al.,[25] who have reported a selective survival advantage of genetically corrected β-thalassemic erythroid progenitor cells in mice. A similar survival advantage because of $\beta^{A\text{-}T87Q}$-globin transgene expression was also observed during a 10-day time course of erythroid differentiation with β^0-HUDEP-2 cells transduced with the BB305, LVβ-shSCR, or LVβ-shα2 vector (Figure 3D). Morphological analysis of β^0-HUDEP-2 cells transduced with the BB305, LVβ-shSCR, or LVβ-shα2 vector revealed a significant reduction in the proportion of Baso-E and a concomitant increase in Ortho-E, which was equivalent to normal HUDEP-2 cells (Figures 3A and 3B). Importantly, these results demonstrate that $\beta^{A\text{-}T87Q}$-globin transgene expression by either of the three vectors is sufficient to restore β^0-HUDEP-2 erythroid maturation to the normal range.

Next, β^0-HUDEP-2 cells were transduced at two different MOIs to result in low and high VCNs (1–2 and 12–15), and α- and β-globin

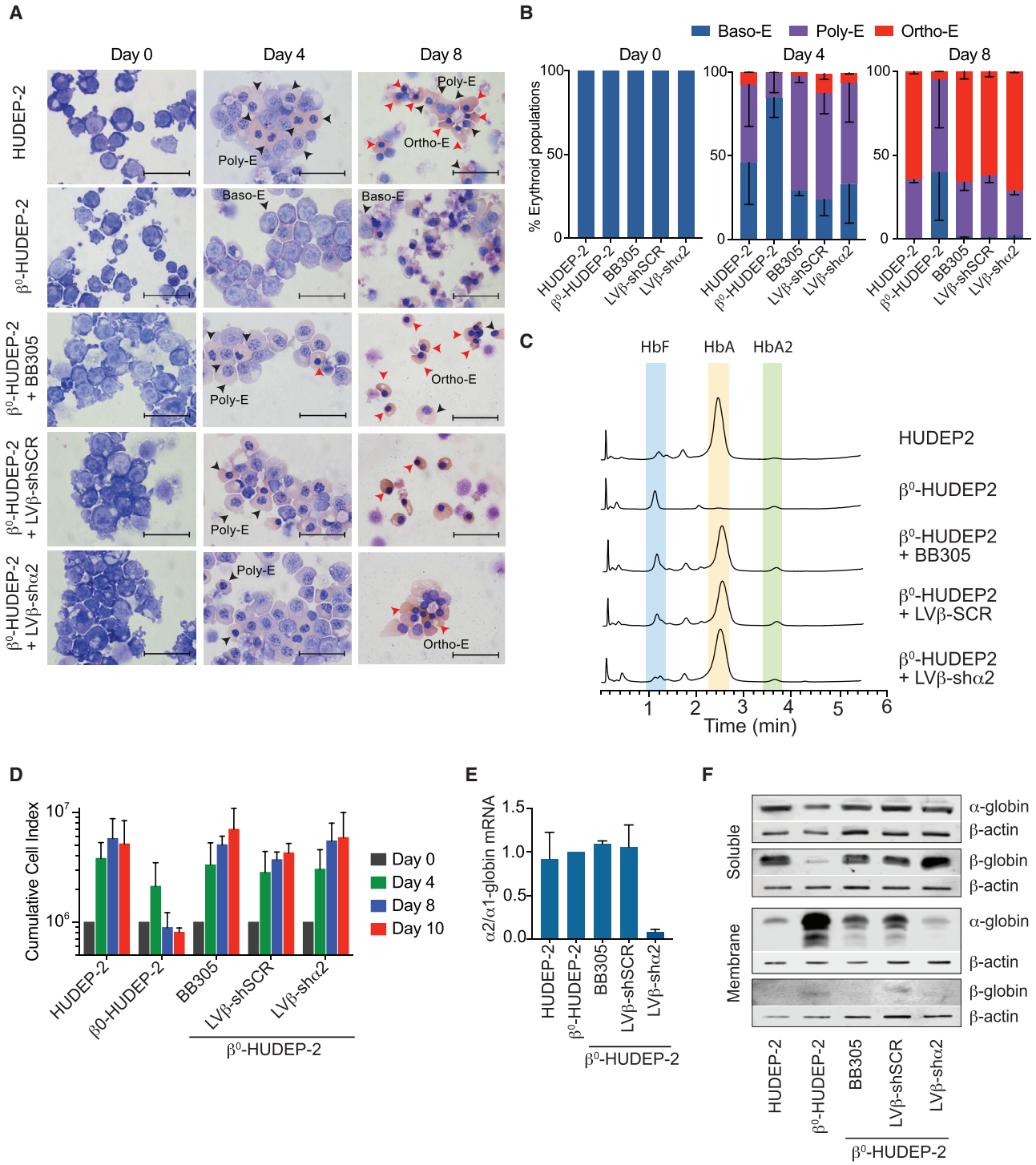

Figure 3. Analysis of β^0-HUDEP-2 differentiation following transduction with the LVβ-shα2 lentiviral vector

(A) Morphological changes of HUDEP-2 and β^0-HUDEP-2 cells following differentiation. Day 0, day 4, and day 8 samples were stained with May-Grünwald Giemsa. Arrows indicate advanced stages of erythroid differentiation (Ortho-E, Poly-E, and Baso-E are indicated on day 4 and day 8). Magnification: 1,000×; scale bars, 25 μm. (B) Erythroid progenitor populations on day 0, day 4, and day 8 of differentiation. Cells at different stages of differentiation were counted from images taken at 400× magnification from cytospin slides. Percentages of different erythroid populations (Ortho-E, Poly-E, and Baso-E) for day 4 and day 8 of differentiation are shown (n = 2, >200 cells counted per cytospin). (C) Hemoglobin variant analysis by HPLC of the soluble cellular fraction obtained from HUDEP-2 and β^0-HUDEP-2 cells transduced with BB305, LVβ-shSCR, and

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mRNA expression levels were assessed by qRT-PCR analysis during the expansion phase. Remarkably, the LVβ-shα2 vector reduced α2-globin transcription levels by more than 3- and more than 20-fold, respectively, compared with that observed in untreated and BB305- and LVβ-shSCR-transduced β^0-HUDEP-2 cells (Figure S5). Accordingly, the reduction in α2-globin mRNA was reflected in the reduction of the α2/α1-globin mRNA ratio (by up to ~95%) by the LVβ-shα2 vector, whereas α1-globin mRNA levels were equivalent in BB305- and LVβ-shSCR-transduced β^0-HUDEP-2 cells (Figure 3E; Figure S5). Similarly, α- to β-globin mRNA ratios in LVβ-shα2-transduced cells decreased by approximately 25% (1–2 VCNs) and 50% (12–15 VCNs) (Figure S5). Because unpaired α-globin chains precipitate and are thus excluded from the water-soluble protein fraction, we also prepared the water-insoluble fraction for globin chain examination. Western blot analysis demonstrated reduced levels of α-globin chains in the water-insoluble cell fraction of β^0-HUDEP-2 cells transduced with BB305, LVβ-shSCR, or LVβ-shα2. This was the result of a greater degree of α-globin chain assembly into hemoglobin complexes resulting from $\beta^{A\text{-}T87Q}$-globin transgene expression from BB305 and LVβ-shSCR. In the case of β^0-HUDEP-2 cells transduced with LVβ-shα2, the amount of α-globin chain in the water-insoluble fraction was further reduced to normal levels through the combined effects of $\beta^{A\text{-}T87Q}$-globin transgene expression and decreased α2-globin chain synthesis (Figure 3F).

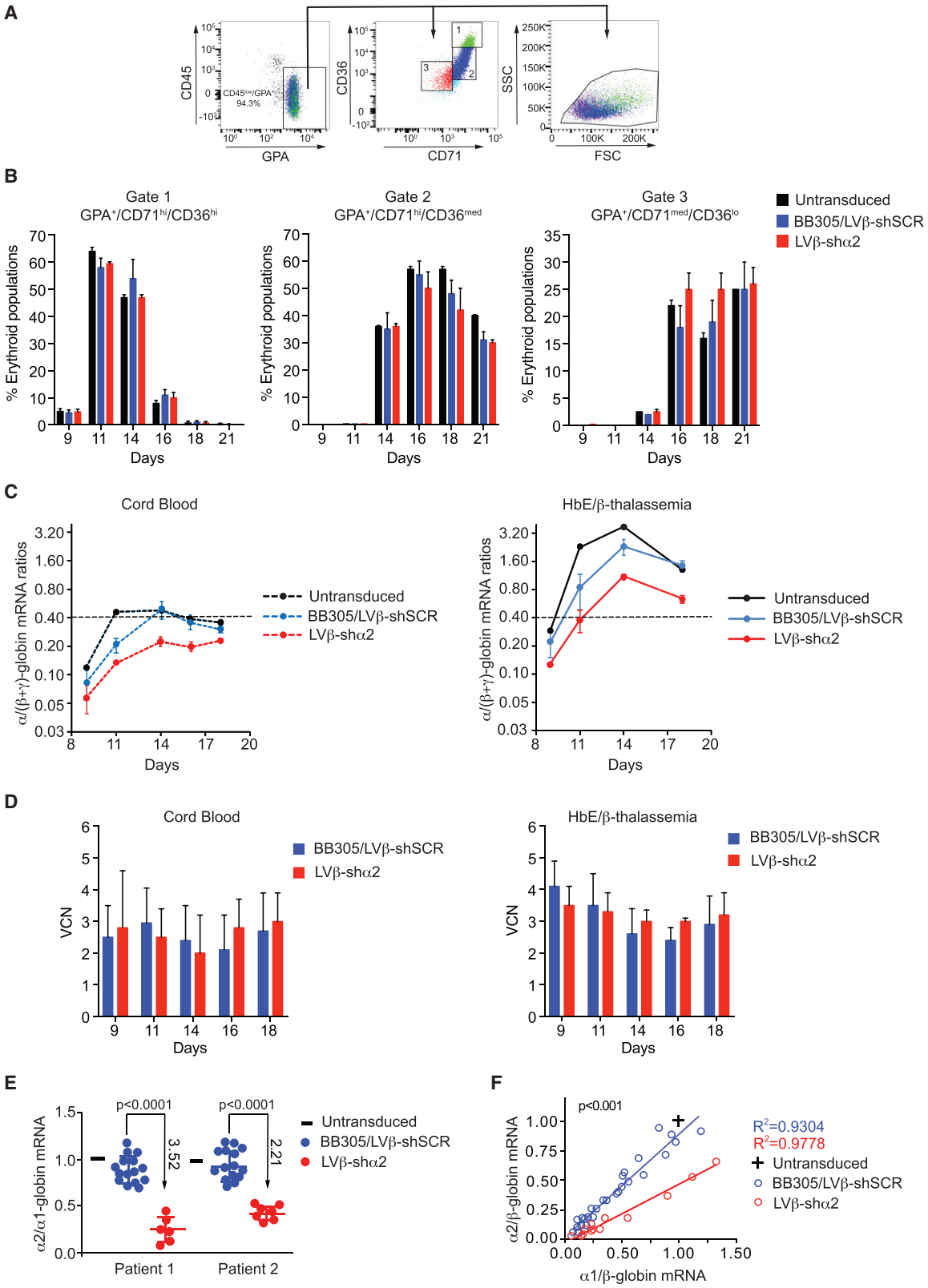
LVβ-shα2 normalizes α/β-globin mRNA ratios in primary human erythroid cells from individuals with β-thalassemia by decreasing α2-globin mRNA levels

$CD34^+$ cells from human cord blood (CB) and individuals with β-thalassemia (n = 2) were transduced at clinically relevant VCNs with the BB305, LVβ-shSCR, or LVβ-shα2 vector (Figure S6). We chose to perform experiments in cells from individuals with HbE/β-thalassemia because use of non-transduced β^0/β^0 cells would not have provided a negative control for α/β mRNA ratios following vector transduction because this cell population does not properly differentiate and survive during *in vitro* erythroid differentiation.[26] Furthermore, transduction of cells from individuals with β^0/β^0-thalassemia with β-globin gene expressing vectors is sufficient to rescue erythroid cell proliferation and differentiation *in vitro*, indistinguishably from normal cells.[26] Thus, a reduction in the α/β mRNA ratio by co-expression of α-globin-specific shRNAs would not produce further observable phenotypic correction *in vitro*.

Erythroid differentiation profiles of transduced $CD34^+$ cells were determined by measuring the percentages of erythroid precursor cells (Figure 4A). Following erythroid differentiation, no gross differences were found between transduced erythroblasts based on the cell surface expression profiles of erythroid markers (Figure 4B). The cell surface expression profile of glycophorin A $(GPA)^+$ cells followed the generally accepted differentiation dynamics with CD36 and CD71 cell surface expression already present on erythroblasts, increasing during differentiation but decreasing at the end of differentiation. The final stages of differentiation were associated with a reduction in cell volume, as seen by a decrease in forward scatter (FSC) (Figure 4A). These data indicates that the LVβ-shSCR or LVβ-shα2 vectors were not associated with any deleterious effects on erythroid differentiation, as assessed over 21 days of culture.

We next measured the mRNA ratios of α/(β-like)-globins (i.e., $\alpha/(\beta^E+\beta^{A\text{-}T87Q}+\gamma^A+\gamma^G)$ in erythroid cells obtained by culture of $CD34^+$ cells from CB of a normal individual and from bone marrow of an individual with HbE/β-thalassemia following transduction with the BB305, LVβ-shSCR, or LVβ-shα2 vector (Figure 4C). The γ-globin mRNAs were quantified only when CB cells were included in the comparison. As expected, the α/(β like)-globin mRNA ratios in the absence of vector transduction were higher in HbE/β-thalassemia cells than in normal CB cells (Figure S7A) because of the β-thalassemia phenotype. Either of the control vectors (BB305 or LVβ-shSCR) led to partial normalization of the α/(β-like)-globin mRNA ratios because of expression of $\beta^{A\text{-}T87Q}$-globin gene (Figures S7B and S7C). Furthermore, when α/(β-like)-globin mRNA ratios were gathered at different time points during erythroid differentiation culture, normalized to ratios in non-transduced cells, and plotted for comparison between BB305 and LVβ-shSCR, no difference was observed (Figures S7B and S7C), indicating that the miR30-shRNA expression cassette does not interfere with vector-derived or endogenous β-like-globin expression levels. This allowed us to pool data collected for BB305 and LVβ-shSCR-transduced cells to form a single control group for statistical comparison with data obtained with LVβ-shα2-transduced cells (Figure 4C). Most significantly, LVβ-shα2 reduced α/(β-like)-globin mRNA ratios in adult HbE/β-thalassemia erythroid cells to levels seen with normal CB cells (Figure 4C), whereas BB305- and LVβ-shSCR-transduced cells had less of an effect on α/(β like)-globin mRNA ratios, particularly during the final stages of erythroid differentiation (day 18). Importantly, these results were obtained at similar VCNs (Figure 4D). We then set out to quantify differences between vector groups in the erythroid cell population of transduced and cultured $CD34^+$ marrow cells from 2 adults with HbE/β-thalassemia (P1 and P2). The (α/β like)-globin mRNA ratios decreased by 2.34- and 1.25- fold with LVβ-shα2 versus the control BB305/LVβ-shSCR group at mean VCN levels of approximatively 3 and 0.75 for P1 and P2, respectively (Figure S8). Importantly the vector encoded $\beta^{A\text{-}T87Q}$-globin chain was detected at similar levels with all three vectors (Figure S9), supporting the conclusion that variation of the α/ββ ratio is solely due to α-globin mRNA reduction in cells transduced with the LVβ-shα2 vector.

LVβ-shα2 on day 10 of erythroid differentiation. (D) Cumulative cell index of HUDEP-2, β^0-HUDEP-2, and β^0-HUDEP-2 cells transduced with the BB305, LVβ-shSCR, and LVβ-shα2 vectors over a 10 day time-course of erythroid differentiation. Data are presented as mean ± SD of two independent experiments. (E) Relative α2/α1-globin mRNA expression levels in HUDEP-2, β^0-HUDEP-2, and β^0-HUDEP-2 cells transduced with the BB305, LVβ-shSCR, and LVβ-shα2 vectors. (F) Western blot analysis of soluble and membrane fractions of HUDEP-2, β^0-HUDEP-2, and transduced β^0-HUDEP-2 cells after 10 days of erythroid differentiation. The antibody used for β-globin detection cross-reacts with δ-globin and therefore contributes to the faint band observed with β^0-HUDEP-2 cells.



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With regard to differences between the 2 α-globin species, the mean α2/α1-globin mRNA ratio after BB305/LVβ-shSCR transduction was equivalent to that measured in non-transduced cells. In contrast, α2/α1-globin mRNA ratios were 3.52- and 2.21-fold lower with LVβ-shα2 than with the control vectors in P1 and P2, respectively (Figure 4E). We then plotted α2/β-globin mRNA ratios relative to α1/β-globin mRNA ratios in the various groups (Figure 4F; Figure S8). As expected, the regression line obtained after transduction with the control vectors BB305/LVβ-shSCR fit the data points for untransduced cells. The slope of the regression line for LVβ-shα2-transduced cells was significantly lower than the slope for the control vectors ($p < 0.001$), indicating that the decreased α2/α1-globin mRNA ratio is caused by a decrease in α2-globin mRNA levels and not by an increase in α1-globin mRNA levels.

LVβ-shα2 improves (α/β)-globin mRNA ratios at lower VCNs compared with BB305

We next measured (α/β-like)-globin mRNA ratios relative to VCN in P1 and P2 after transduction with the different vectors (Figure 5A). $CD34^+$ cells were transduced at different MOIs (Figure S6). The α/(β-like)-globin mRNA ratios in BB305- and LVβ-shSCR-transduced cells were inversely correlated to the VCNs (Figures S7B and S7C), and these mRNA ratios (y axis) as a function of VCN values (x axis) best fit an "exponential decay model" (Figure S10). No significant difference was observed between the decay rate constants K for BB305 and LVβ-shSCR, indicating that β-globin transgene expression levels were equivalent within this control vector group. In contrast, transduction of HbE/β-thalassemia cells with the LVβ-shα2 vector decreased α/β-globin mRNA ratios to a greater degree than with the vector control group for a given VCN value (Figure 5B), yielding differential values for K. The mean VCNs were 2.8 and 3.1 for BB305/LVβ-shSCR- and LVβ-shα2-transduced P1 cells, respectively, and 0.81 and 0.66 for BB305/LVβ-shSCR- and LVβ-shα2-transduced P2 cells, respectively (Figure S8). According to the model, the relative fold decreases in α/(β-like) ratios with the LVβ-shα2 vector compared with the control vector group, calculated from the best fit curves, were 2.16 ($e^{(3.1 \times 0.5211)-(2.8 \times 0.3013)}$) and 1.25 ($e^{(0.66 \times 1.282)-(0.81 \times 0.7705)}$) in samples from P1 and P2, respectively (Figure 5B). These calculated values were comparable with the actual measures of 2.34 and 1.25 for P1 and P2, respectively (Figure S8), suggesting that the model fits the data well. The model takes into account all relevant parameters, which include α-globin mRNA fold reduction by the shRNAs, increased β-like-globin mRNA production because of vector-derived $\beta^{A\text{-}T87Q}$-globin expression, and VCN levels.

An important corollary to the model is that one can derive the "gain" in VCN values between the LVβ-shα2 vector and the control vector group to achieve the same degree of normalization (decrease) of the (α/β-like)-globin mRNA ratios, establishing the differential potency of the various vectors. This is obtained by simply calculating the ratio of the K decay rate constants for each of the two groups, and the factor so derived is 1.7. Importantly, this determination is independent of VCN levels. In other words, 1.7-fold fewer integrated vector copies of the LVβ-shα2 vector than the parent vector BB305 are sufficient to achieve the same degree of (α/β-like)-globin mRNA ratio improvement.

To further validate the LVβ-shα2 vector, we investigated a third individual with β°/β^+-thalassemia (P3) with a particularly severe compound heterozygous genotype (IVS2-654C > T and −28A > G). We also made use of pooled erythroid colonies grown in methylcellulose instead of liquid cultures, used previously for P1 and P2, to diversify the experimental approach. $CD34^+$ cells were transduced at a low MOI to maximize the number of cells with a single integration event. After transduction (n = 2), cells were grown in methylcellulose for 2 weeks and analyzed by qPCR for VCN determination and concurrent globin mRNA assays by qRT-PCR. The mean population VCNs were 0.23, 0.77, and 0.80 for LVβ-shSCR-, BB305-, and LVβ-shα2-transduced cells, respectively (Figure 6A). Again, the LVβ-shα2 vector showed a greater potency to decrease α/β-globin mRNA ratios than BB305 at low VCNs (Figure 6B).

DISCUSSION

In this study, we report an innovative gene therapy strategy and vector to improve the clinical efficacy and safety of current lentiviral gene therapy for individuals with β-thalassemia. The gene therapy vector derived from BB305 comprises the therapeutic anti-sickling $\beta^{A\text{-}T87Q}$-globin gene configured with an intronic miR30-shRNA tailored to quantitatively reduce α-globin chain synthesis and correct the α:β-globin chain imbalance. LVβ-shα2 was designed to specifically target human α2-globin mRNA without affecting human α1-globin mRNA. This ensures that there will be no risk of inducing clinically meaningful α-thalassemia.

Our results demonstrate that LVβ-shα2 vector titers and transduction efficiency in primary human hematopoietic $CD34^+$ cells are equivalent to the parent BB305 gene therapy vector. LVβ-shα2 retains vector-encoded $\beta^{A\text{-}T87Q}$-globin expression profiles similar to BB305 in erythroid cell lines and erythroid progeny of transduced normal and β-thalassemia $CD34^+$ cells. The reduction in α2-globin mRNA levels, in concert with vector-encoded $\beta^{A\text{-}T87Q}$-globin expression,

Figure 4. Analysis of LVβ-shα2-mediated knockdown of α2-globin in primary erythroid cells

(A) Erythroid differentiation profile of cells from P1 grown in medium 1 after transduction with BB305, LVβ-shSCR, or LVβ-shα2 at equivalent MOIs. (B) Mean percentages of populations 1 ($CD71^{hi}/CD36^{hi}$), 2 ($CD71^{hi}/CD36^{med}$), and 3 ($CD71^{med}/CD36^{lo}$) in GPA^+ cells. (C) Analysis of α/(β+γ)-globin mRNA ratios in CB and P1 cells transduced with the BB305, LVβ-shSCR, and LVβ-shα2 vectors. Results from BB305 and LVβ-shSCR were pooled. (D) VCNs in the samples analyzed in (C). Data are presented as mean +/- SD of three replicates (E) Analysis of α2/α1-globin mRNA ratios in cells from 2 individuals with HbE/β-thalassemia (P1 and P2). For each analyzed sample, ratios were normalized to those measured at the same time point in non-transduced cells. (F) Correlation between α2/β-globin and α1/β-globin mRNA ratios in erythroid cells of P1 (days 11, 14, and 16) and P2 (days 11, 15, 18, and 21).

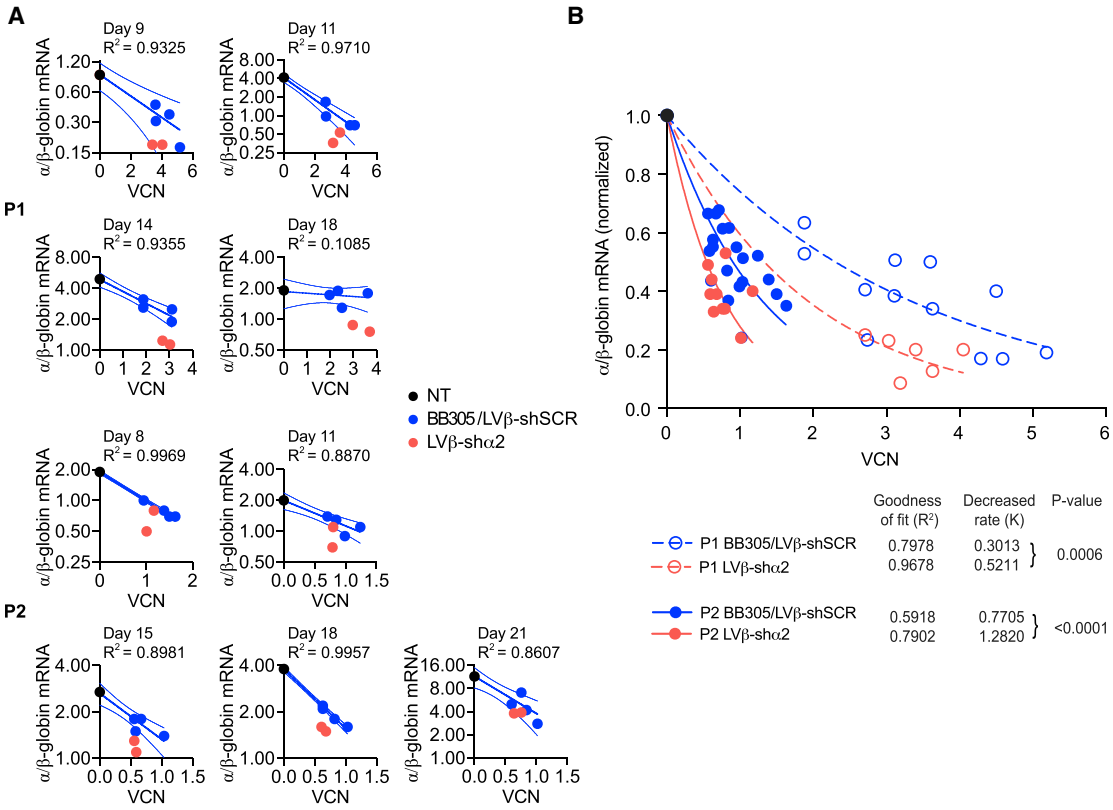

Figure 5. Analysis of α/β-globin ratio to VCN in transduced primary HbE/β-thalassemia erythroid cells

(A) α/β-globin mRNA ratio in transduced primary cells from two individuals (P1 and P2) with HbE/β-thalassemia. Cells were transduced at MOI 5 and 10 (P1) or 1 and 3 (P2) and grown for 3 weeks in medium 1 (P1) or medium 2 (P2). (B) Curve fitting to normalized data, 95% confidence intervals, and significance of differences between decay rates (K) calculated from data obtained in BB305/LVβ-shSCR- and LVβ-shα2-transduced cells. Results from day 18 in (A) were not included to construct the best fit curve because the data did not follow the shared model. Determination of p values for comparison of decreased rates in samples transduced with the BB305/LVβ-shSCR and LVβ-shα2 vectors was performed, assuming ratios of 1 in unmodified cells and 0 at infinite VCN.

improved α:β-globin ratios in β-thalassemia erythroid cells to the same degree at VCN levels 1.7-fold lower than those of the parent vector BB305. Importantly, α1-globin mRNA levels are unaffected.

Although BB305 has proven highly effective and reliable for successful treatment of β^+-thalassemia (e.g., HbE/β^0-thalassemia), it has faced more challenges for individuals with a β^0/β^0 genotype because of the very large amount of therapeutic protein that needs to be produced *in vivo* in this population.[10,11] Preliminary data from a recent clinical trial of BB305 that focuses on individuals with β^0/β^0-thalassemia (HGB-212) indicate that higher levels of hematopoietic stem cell (HSC) transduction were able to increase Hb levels to 13.2 g/dL in one individual and to 10.4 g/dL, respectively, in two individuals with the β^0/β^0 genotype after 12 months of follow-up.[10,27] Although these clinical data are encouraging, there is room for further improvement in this challenging population. Although continuous optimization in transduction conditions may play a major role in increasing clinical efficacy, further augmenting VCN values in hematopoietic cells raises the concern of an increased risk of oncogenicity by insertional mutagenesis.[27,28]

Previous efforts to increase β-globin gene synthesis explored the use of larger DNase hypersensitive site (HS) fragments or adding the LCR HS 1 (HS1) element in LVβ gene therapy vectors, and this provided only modest improvements.[29,30] The use of chromatin insulators, such as the chicken β-globin locus 5′ HS4 element, has also been explored to prevent the repressive influences of surrounding chromatin on the integrated vector and reduce possible *cis*-acting effects of the LCR enhancers on neighboring genes.[31,32] However, these elements were associated with reduced transduction efficiency and genetic instability.[33,34]

Clinical evidence indicates that moderate reduction of α-globin chains via co-inheritance of the α-thalassemia trait decreases the severity of β-thalassemia.[19,35] Decreasing the levels of unpaired α-globin has been proposed on a theoretical basis as a complementary approach for treatment of β-thalassemia.[18,36–38] Our previous investigations demonstrated phenotypic improvement of primary erythroid cells derived from β-thalassemic mice ($Hbb^{th3/+}$) when treated with α-globin-specific siRNA.[21] However, lifelong delivery of siRNAs are a major limitation for the utility of therapeutic

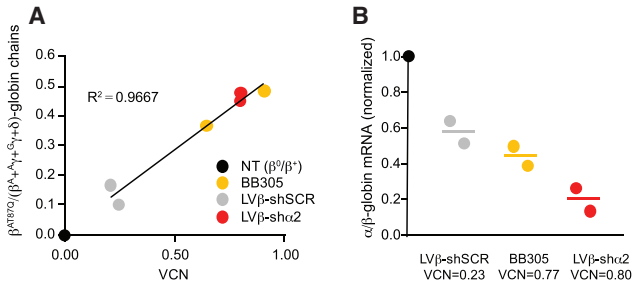

Figure 6. Analysis of α/β-globin ratios in transduced primary β°/β⁺-thalassemia erythroid cells

$CD34^+$ cells from a transfusion-dependent individual with β°/β⁺-thalassemia were transduced (n = 2) with the BB305, LVβ-shSCR, and LVβ-shα2 vectors at low MOI, grown in methylcellulose for 2 weeks, and analyzed by qPCR, qRT-PCR, and HPLC for VCN determination and globin gene expression. (A) Correlation of the β^{T87Q}-globin chain fraction relative to VCN. (B) α/β-mRNA ratios relative to UT cells.

siRNAs for β-hemoglobinopathies. Approaches based on transduction of HSCs with LV vectors harboring shRNAs are an effective delivery strategy for clinical translation of RNAi-based therapies. Such a strategy was used to deliver a sickle (β^S)-globin-specific shRNA embedded in intron 2 of the γ-globin gene because a moderate decrease in β^S-globin expression may substantially improve sickle cell disease and abrogate the need for high-level expression of the vector-encoded globin gene.[39]

More recently, by extending the design principles of microRNAs (miRNAs), an optimized BCL11A-shRNA embedded in a miRNA was used to achieve ubiquitous knockdown of BCL11A and promote production of HbF in erythroid cells.[40,41] Utilizing the erythroid-specific β-globin LCR to drive expression of a miRNA-containing BCL11A-specific shRNA and minimize potential off-target side effects averted negative effects on HSC engraftment.[42–44] Similarly, LV vectors encoding erythroid-specific shRNA were designed to target the aberrant mRNA in $\beta^{IVS1\text{-}110}$-thalassemia because it may potentially interfere with *HBB* expression.[45] These findings signify the importance of lineage-specific gene silencing because it can be used as a flexible tool for analysis of gene function and development of gene-specific therapeutic agents.

The strategy to combine β-globin gene addition with reduction of α-globin chains has been evidenced previously using foamy virus vector-expressing β-globin regulated by the HS40 element derived from the α-globin locus and polymerase III (Pol III)-directed, α-globin-specific shRNA.[46] This vector has been reported to restore globin chain balance in β^0-thalassemic erythroid cells. However, lack of proper control and the deleterious effect of Pol III-directed shRNA limits its use.[46] An alternative approach to reduce α-globin expression, focused on disrupting the core α-globin gene enhancer MCS-R2 (HS-40) in primary human HSCs using genome editing technology.[47] This strategy was used to emulate a natural α-thalassemia mutation and reduce α-globin expression to levels beneficial for individuals with β-thalassemia. In cells with HbE/β-thalassemia mutations, targeted deletion of MCS-R2 ameliorated the α/β-globin chain ratio to beneficial levels without perturbing erythroid differentiation or having detectable off-target events. If genome editing of MCS-R2 is to become a realistic therapeutic approach for β-thalassemia, then such an approach will be generally applicable to non-β^0/β^0-genotype individuals in whom the endogenous output of β-globin is relatively high.

Even in individuals with β-thalassemia with non-β^0/β^0 genotypes, where BB305 has proven so effective, further increase in safety could be obtained by using a vector that can achieve a similar degree of therapeutic efficacy while minimizing the number of chromosomal integration events. This would be especially valuable for individuals with a possible increased predisposition to hematologic malignancies, as in those with sickle cell disease.[48,49] Because optimal therapeutic benefits are likely to require a vector capable of completely restoring equimolar expression of α- and β-globin, the LVβ-shα2 gene therapy vector may afford an improved benefit/risk ratio for all individuals with β-thalassemia regardless of genotype. Clinical trials of LVβ-shα2, anticipated to be initiated shortly, should provide answers regarding the safety and efficacy of this approach for gene therapy of β-thalassemia.

MATERIALS AND METHODS

Lentiviral vector design, production, titration, and characterization in human cell lines

The LentiGlobin BB305 gene therapy vector has been described previously.[50] It is a self-inactivating (SIN), Tat-independent vector that contains a cytomegalovirus (CMV) promoter and enhancer instead of the HIV U3 region at the 5′ long terminal repeat (LTR) and a deleted 3′ U3 region and encodes the altered adult $\beta^{A\text{-}T87Q}$-globin gene (Figure 1).[51] The $\beta^{A\text{-}T87Q}$-globin gene in the BB305 gene therapy vector features a 374-bp purine-rich deletion in IVS2, located between +580 and +953 of *HBB* ("GenBank: MG657341"), identified previously to increase viral titers.[52] The IVS2 breakpoint site was used to insert the miR30-shRNA expression cassette flanked by approximately 125 bases 5′ and 3′ of the miR30 sequence to ensure β-globin-coupled shRNA expression in erythroid cells. This design mimics natural intronic miRNAs in protein-coding genes, which provide expression of the miRNAs and the protein-coding gene from a single transcript.[53] The miR30-shRNA expression cassette was selected for its well-characterized loop/bulge structure and accurate processing of shRNA/pre-miRNA ends.[54,55]

A description of the cloning procedures is provided in Figure S1. Briefly, the miR30-shRNA cassette was amplified from the MSCV/LTRmiR30-PIGΔRI (LMP) vector.[56] Two partially complementary oligonucleotides (5′BP-miR30 and 3′BP-miR30; Table S1) with a 40-bp homologous sequence immediately flanking the deletion breakpoint of the β-globin intron 2 region was used to amplify the miR30-shRNA expression cassette. The resultant fragment was then used as the downstream megaprimer for the overlap extension PCR cloning strategy to insert the miR30-shRNA expression cassette into the β-globin IVS2 breakpoint site, which contained part of

β-globin exon 2, intron 2, exon 3, and 3′ enhancer region cloned into the BamHI site of pBluescript II KS (+).[57] β-Globin intron 2 containing the miR30-shRNA cassette was then cloned into pBB305, replacing the existing BamHI fragment, and verified by sequencing. Using this approach, LVβ-shRNA viral vector derivatives were created by incorporating shRNA sequences targeting EGFP (LVβ-shGFP) and the negative control vector containing a scrambled shRNA sequence (LVβ-shSCR) (Table S2). Oligonucleotides and primers used for cloning are listed in Table S1.

Lentiviral particles were produced by co-transfecting the lentiviral transfer vector with a packaging plasmid system as described previously.[58] The lentiviral titer was determined by transducing NIH/3T3 cells.[59] Lentiviral particles were evaluated in the MEL-βEGFP, K562, and HUDEP-2 cell lines as described previously.[22] The down-regulation efficiency of EGFP expression was determined by measuring the median fluorescence intensity (MFI) by flow cytometry. Transduced K562 cells were incubated with hemin to induce erythroid differentiation. Human $\beta^{A\text{-}T87Q}$-globin expression was determined by intracellular antibody staining. β^0-HUDEP-2 cells, containing biallelic β^0-globin mutations, were created using CRISPR-Cas9 technology. Details can be found in the Supplemental materials and methods.

Human CD34$^+$ transduction and culture

Human CD34$^+$ cells were cultured in StemPro34 SFM (serum free medium, Thermo Fisher Scientific) supplemented with the human cytokines fms-related tyrosine kinase 3 ligand (hFlt3L), Stem Cell Factor (hSCF), and interleukin-3 (hIL-3) (at 50, 50, and 10 ng/mL, respectively; all from Miltenyi Biotech) in the presence of 20% serum substitute (BIT 9500, STEMCELL Technologies). After 7 days, erythropoietin (EPO) at 3 IU/mL was used to replace FLt3L, SCF, and IL-3 (medium 1). Alternatively, CD34$^+$ cells were cultured in Iscove's modified Dubelcco's medium (IMDM) supplemented with SCF (100 ng/mL), IL-3 (5 ng/mL), and EPO (3 U/mL) from day 0, supplemented with SCF and EPO from day 8, and with EPO only from day 11 (medium 2). When indicated, CD34$^+$ cells were transduced 24 h after the beginning of culture in medium containing protamine sulfate (8 μg/mL) at MOIs between 2 and 10.

VCN determination

Genomic DNA from CD34$^+$-derived cells was prepared from liquid culture or pooled colonies using the NucleoSpin Blood Kit (Macherey Nagel). VCNs per cell were determined by the delta-delta-Ct method (ΔΔCt) method as described previously.[60] Primers and probes used for quantitative polymerase chain reaction (qPCR) are listed in Table S3.

RNA preparation and qRT-PCR

Total RNA was isolated using TRI Reagent solution or PureLink RNA Mini Kit (Thermo Fisher Scientific), and first-strand cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen) or EuroScript Reverse Transcriptase Core Kit (Eurogentec, Seraing, Belgium) according to the manufacturers' protocols. Gene expression was quantified by quantitative reverse-transcriptase PCR (qRT-PCR), performed with gene-specific primers and probes (Table S4) and 2X qPCR Master Mix (Eurogentec) using the 7300 ABI Prism detection system. Relative expression of target genes was quantified based on the efficiency-corrected calculation method.[61] For analysis of shRNA expression, first-strand cDNA synthesis was performed using specific stem-loop RT primers and subsequently analyzed by qPCR.[62,63] For relative quantification of α-globin versus β-globin and γ-globin gene expression, the recombinant DNA plasmid pT7-αβγ containing *HBA*, *HBB*, and *HBG* cDNAs in tandem was used to generate standard curves.[64]

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad). A p value of less than 0.05, generated by a Student's t test, was considered statistically significant. The decreased rates of α/β-globin mRNA ratios and differences between groups using the exponential decay equation model (Supplemental materials and methods) were determined using GraphPad Prism 6.0.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2021.04.037.

ACKNOWLEDGMENTS

We thank Drs. Ryo Kurita and Yukio Nakamura for providing the HUDEP-2 cell line. We also acknowledge Monash Pathology for assistance with HPLC and hemoglobin variant analysis of HUDEP-2 cells and the Fondation Générale de Santé (FGDS) and the Assistance Publique-Hôpitaux de Paris (AP-HP) for collecting and testing cord blood cells. This work was supported by the European Research Projects on Rare Diseases (GETHERTHAL); the European Commission; the National Health and Medical Research Council (NHMRC), Australia (Project Grant APP1147867); the Victorian Government's Operational Infrastructure Support Program; the Thalassaemia and Sickle Cell Society of Australia, the Thalassaemia Society of New South Wales; The Greek Conference; the National Research Council of Thailand (NRCT); the Thai Program Management Unit for Human Resources & Institutional Development, Research and Innovation (B05F630062); and Mahidol University (MRC-MGR 01/2563).

AUTHOR CONTRIBUTIONS

T.N., K.S.-F., M.G., B.M., E.P., P.L., and J.V. designed the experiments. T.N., K.S.-F., M.G., B.M., J.G., A.G., H.P.J.V., and H.Y.T. performed experiments. T.N., K.S.-F., M.G., B.M., J.G., A.G., H.P.J.V., H.Y.T., and E.P. analyzed data. G.G., S.S., S.F., and S.H. provided samples and resources. T.N., P.L., E.P., and J.V. wrote the manuscript. P.L., E.P., and J.V. conceptualized the idea and supervised the project.

DECLARATION OF INTERESTS

P.L. is a scientific founder and shareholder of bluebird bio, Inc. He is an inventor of awarded patents that cover the $\beta^{A\text{-}T87Q}$-globin gene and the BB305 LentiGlobin vector.

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YMTHE, Volume *29*

Supplemental Information

Coordinated β-globin expression and α2-globin reduction in a multiplex lentiviral gene therapy vector for β-thalassemia

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Supplementary Information

SUPPLEMENTAL MATERIAL AND METHODS

Validation of the α-globin-specific siRNA

In order to identify and evaluate effective α-globin-specific siRNA target sequences, 5 siRNAs targeting human α-globin were electroporated into K562 cells (Supplemental Table S2). Four sequences (siα-1, siα-2 siα-3 and siα-4) generated significant reductions in human α-globin mRNA compared to negative controls (mock and siLuc electroporated K562 cells) 24 hrs and 48 hrs post electroporation as detected by real-time PCR. In brief, 5×10^6 K562 cells mixed with siRNA in 0.5ml of Opti-Mem, were transferred to 0.4 cm cuvettes and electroporated with the Gene Pulser (Bio-Rad, Hercules, CA, USA) using the following conditions: 226 Volts, 950 μF, ∞ resistance. Cells were then cultured in 10 ml DMEM containing 10% FCS and 30 μM hemin for erythroid differentiation and incubated at 37°C. Relative α-globin RNA expression levels were detected by real-time PCR 24 and 48 hours post electroporation. The most effective siRNA, siα-1 and siα-2, reduced α-globin mRNA by 95%±5% and 83%±6% relative to negative controls, while siα-3 and siα-4 generated modest, though significant, reductions of 35%±13% and 47%±14% respectively ($p<0.05$), and siα-5 had no effect. Analysis of efficacy over time demonstrated that 1 μg of siα-3 and siα-4 generated modest reductions in α-globin mRNA expression which remained significant at 48 hours relative to negative controls (Figure S3). In humans, the α-globin is encoded by two highly homologous *HBA1*(α1) and *HBA2*(α2)

genes. These genes differ significantly over the 3' UTRs. Whereas siα-1, siα-2 and siα-3 target both genes, siα-4 specifically targets *HBA2*.

Lentiviral vector comparison

Vesicular stomatitis virus glycoprotein (VSVG) pseudotyped lentiviral supernatants were produced by transient transfection of HEK293T cells with the plasmid vector and the 3-plasmid packaging system made of HPV275, ψN15 and p633.[1] Lentiviral particles were produced and purified with a Mustang Q anion exchange membrane cartridge (Pall, Saint Germain-en-Laye, France) and a 40K ZebaSpin desalting column (Thermo Fisher Scientific, Villebon, France) before concentration by overnight precipitation with Lentivirus precipitation solution (Alstem, Richmond, CA). Infectious titers were determined in NIH3T3 cells. Notably, no observable difference was identified in the titres of LVβ-shRNA vectors from culture supernatants harvested 24 hours post-transfection of HEK 293T, before and after purification and ultracentrifugation (Figure S4). Based on these results, the insertion of the miR30-shRNA expression cassette into LVβ gene therapy vector did not interfere with LV vector production or transduction.

Flow cytometry

To determine knockdown efficiency of LVβ-shGFP, eGFP expression of transduced-MEL-βeGFP cells were determined by flow cytometry at day 3-10 of erythroid differentiation. Cells were washed and resuspended in 1X PBS. Cells were analysed using BD LSR II flow cytometer (BD Biosciences). $\beta^{A\text{-}T87Q}$-globin expression of transduced cells was assessed by intracellular staining. Briefly, 1×10^6 cells were fixed with 100 µl of ice-cold

4% paraformaldehyde at room temperature for 10 minutes. Cells were washed with 1X PBS and permeabilized with 200 µl of ice-cold 0.1% Triton X-100 (v/v) in 2% FCS-PBS at room temperature for 5 minutes. Cells were washed with 2% FCS-PBS. 1×10^5 cells were stained with 2 µl of antibody against human β-globin and incubated on ice for 30 minutes. Cells were washed 3 times with 2% FCS-PBS and subjected to analysis using BD LSR II flow cytometer (BD Biosciences). Data were acquired on a LSR II cytometer (BD, San Jose, CA) using FACS Diva software. For determination of erythroid differentiation profiles, cells were stained with antibodies directed against CD235A, CD36, and CD71 and subjected to analysis using MACSQuant cytometer (Miltenyi Biotech) and analysed using FlowJo software (Tree Star, OR, USA). Antibodies used are listed in Supplemental Table S5.

High performance liquid chromatography

Percentage of the globin chains in human samples was determined by high-performance liquid chromatography (HPLC) with a Prominence chromatograph (Shimadzu). Cells lysates were injected onto an Aeris Widepore 3.6 µm C4 column 250×4.6 mm (Phenomenex). Elution was achieved with a gradient of buffer A (30% acetonitrile (Carlo Erba), 0.07% trifluoracetic acid (Sigma Aldrich) in Milli-Q water and buffer B (50% acetonitrile, 0.05% trifluoroacetic acid), with 48% as an initial percentage of buffer B, in 75 minutes at a total flow rate of 0.8 mL/mn at 30°C.The detection wavelength was 220 nm. Data acquisition was performed with the LC Solution software (Shimadzu).

Analysis of globin precipitates by western blot

Cells were lysed in Milli-Q water then centrifuged to obtain water-soluble cell fractions. Membrane fractions was extensively washed in 0.05% PBS and resuspended in 56 mM sodium borate (pH 8.0) with 0.5% Triton X-100. After 20 minutes incubation on ice, membrane fractions was centrifuged at 16,000xg, 4°C for 10 minutes. Precipitated globins were dissolved in 1X sample loading buffer for Western blot analysis. [2, 3]

Protein analysis by western blot

Total cell lysate was prepared using RIPA lysis buffer system (SantaCruz Biotechnology, Dallas, TX). Briefly, 5×10^6 washed-cells were gently resuspended in 200 µl of ice cold RIPA lysis buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide), 2 mM PMSF solution, 1 mM sodium orthovanadate solution and 10% (v/v) protease inhibitor cocktail solution (Sigma Aldrich). Lysis reaction was incubated on ice for 30 minutes and centrifuged at 10,000xg, 4°C for 10 minutes. The supernatant which is total cell lysate was collected to new tube and stored at -20°C.

Quantification of proteins

Proteins in total cell lysates were quantified using BCA protein assay kit (Thermo Scientific) according to the manufacturer's protocol. Briefly, BSA standard curve was generated in the range of 0-1,000 µg/ml. A working reagent (WR) solution was prepared by mixing BCA Reagent A with BCA Reagent B at ratio 50:1. 1 ml of WR solution was combined to 50 µl of protein sample or standard BSA and incubated at 37°C for 30

minutes. After incubation, the reaction was transferred to cuvette and measured using a spectrophotometer at OD562. Protein quantity was calculated using linear regression.

Western blot analysis of globin chains

K562: A total 10 µg of protein cell lysate was denatured by heating at 95°C for 5 minutes in 1X sample loading buffer (50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.02% bromophenol blue) and separated on 4-15% SDS-polyacrylamide Mini-PROTEAN® TGX™ precast gel (Bio-Rad, Hercules, CA) in Tris-glycine running buffer (25 mM Tris pH 8.3, 192 mM Glycine, 0.1% SDS) at 120 volts for 40 minutes. After electrophoresis, protein was transferred to 0.22 µm PVDF membrane (GE healthcare, Chicago, IL) in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) at 100 V for 90 minutes in cold room. After blocking the membranes in 1X Tris buffer saline (TBS) with Tween-20 (20 mM Tris-Hcl pH 7.5, 154 mM NaCl, 0.1% Tween-20 supplemented with 3% bovine serum albumin (BSA)) at room temperature for 2 hours, membranes were incubated with primary antibody either against human α-globin (at dilution 1:10,000 in 3% BSA-TBS-T or human β-globin (at dilution 1:10,000 in 3% BSA-TBS-T or human β-actin antibody conjugated with horseradish peroxidase (HRP) (at dilution 1: 20,000 in 3% BSA-TBS-T at 4°C overnight. Membranes were washed 3 times with 1X TBS-T for 10 minutes each wash before incubated with secondary antibody conjugated with HRP at dilution 1:5,000 in 3% BSA-TBS-T at room temperature for 2 hours when required. Membranes were washed 3 times with 1X TBS-T for 10 minutes each wash before protein was detected by ImageQuant LAS4000 (GE Healthcare) using ECL solution (1.25 mM Luminol, 200 µM p-Coumaric acid, 0.01% H_2O_2, 100 mM Tris-HCl pH 8.5) according to the manufacturer's protocol.

Cell lines

i) The MEL-βeGFP cell is derived from the MEL (murine erythroleukemia) cell line modified to carrying the 180kb BAC human genomic fragment containing the β-globin locus, where the coding regions of the β-globin has been replaced by the eGFP reporter.[4] Cells were grown and maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 g/ml streptomycin at 37°C 5% CO_2. Cells were induced to undergo erythroid differentiation by culturing in medium containing 2% DMSO at initial concentration 2×10^5 cells/ml for 7 to 10 days.

ii) Human erythroleukemia (K562) cell line, derived from chronic myeloid leukemia patient, predominantly expresses embryonic hemoglobin, Hb Gower I ($\zeta_2\varepsilon_2$), Hb Portland ($\zeta_2\gamma_2$) and fetal hemoglobin, HbF ($\alpha_2\gamma_2$).[5-7] Cells were grown and maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 g/ml streptomycin at 37°C 5% CO2. K562 cells were induced to differentiate by culturing in media containing 30 μM Hemin for 3-5 days.

iii) HUDEP-2 cell line was previously created by lentiviral transduction of human cord blood haematopoietic stem cells with doxycycline (DOX)-inducible human papilloma virus 16 E6/E7 expression system.[8] The cells express mostly adult hemoglobin (HbA). HUDEP-2 cells containing biallelic β^0-globin mutations, termed β^0-HUDEP-2 cells, were created using CRISPR-Cas9 genome editing to recapitulate a sustainable in vitro model of β^0-thalassemia. Following clonal analysis, a β^0-HUDEP-2 *cell line with biallelic* β-globin *gene*

disruptions was identified by sanger sequencing (manuscript in preparation). Cells were grown at a density of 0.1-2 × 10^6 cells/ml in StemSpan SFEM (Stem Cell technologies) supplemented with human stem cell factor (hSCF, 50 ng/ml), erythropoietin (EPO, 3 IU/ml), dexamethasone (Dex, 1 µM), and doxycycline (DOX, 1 µg/ml), 1X pen/strep/glutamine (PSG). Differentiation of HUDEP-2 cells was induced by culture in IMDM supplemented with EPO (10 IU/ml), mifepristone (1 µM), human AB serum (5%), holotransferrin (500 µg/ml), heparin (2 IU/ml) and insulin (10 µg/ml), 1X PSG. Differentiation media was replaced every two days and cell concentration was kept at 0.5-1 × 10^6 cells/ml.

Human hematopoietic cells

Human cells of de-identified donors were obtained from normal cord blood (CB) from Saint-Louis Hospital (Paris, France) and from non-mobilized peripheral blood of thalassemia patients from Ramathibodi Hospital (Bangkok, Thailand), following patient informed consent. They were enriched using the CD34 progenitor cell isolation kit (Miltenyi Biotec and the autoMACSPro instrument) according to manufacturer instructions. The study was approved by the ethics evaluation committees of the French Institute of Medical Research and of the Faculty of Medicine Ramathibodi Hospital, under protocol numbers 16-315 and 06-57-07 respectively.

Mathematical model of α to β-globin mRNA ratio decay according to vector copy number

In order to compare the level of globin transcripts between groups of cells transduced with BB305 or LVβ-shRNA, α-globin to β-globin mRNA ratios were determined at several time

points after erythroid differentiation and eventually normalized to those measured in non-transduced cells at the same time point. The resulting values were plotted as a function of VCNs, and the exponential decay equation model ($Y=e^{-kx}$, were Y is the mRNA ratio, K is the decay rate and X is the vector copy number) was used to fit the data. For comparison of groups using this model, the transcript ratios were fixed to 0 at infinite VCNs (bottom plateau) and to 1 in the absence of transduction when normalized. The influence of shRNA on α/β globin mRNA ratio and the differential vector efficiencies were calculated according to this model. When $Y1=e^{-k1x1}$ and $Y2=e^{-k2x2}$, the fold difference between α/β mRNA ratios is equal to e^{-k1x1}/e^{-k2x2}. Therefore, the mean fold variation of α/β mRNA ratios per vector copy is equal to $e^{(k2-k1)}$. When the α-globin to β-globin mRNA ratio are equal (Y1=Y2), $e^{-k1x1}= e^{-k2x2}$. Therefore, at equal α:β mRNA ratio, the VCN ratio (x1/x2) is equal to k2/k1.

SUPPLEMENTAL FIGURES

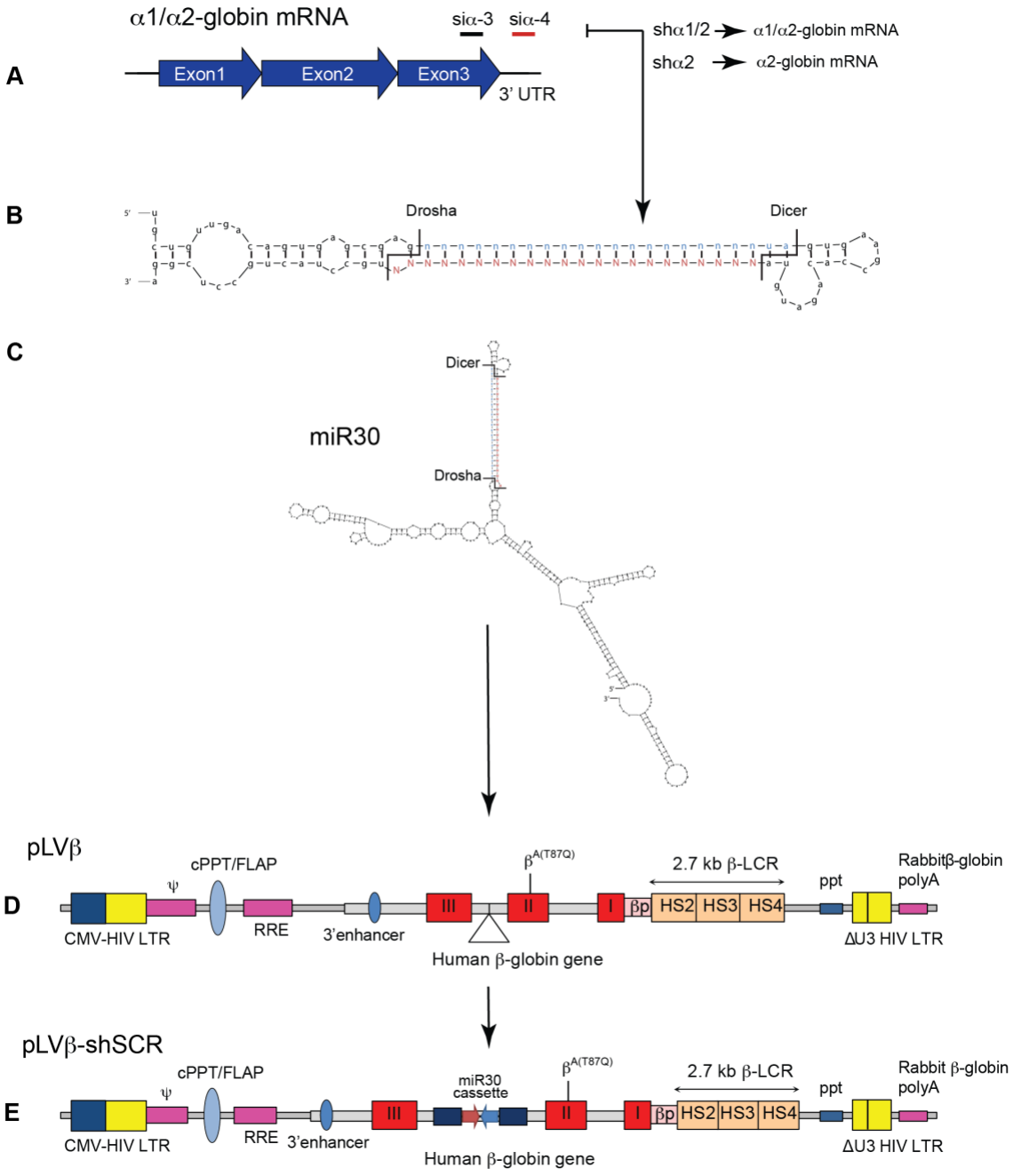


Figure S1. Schematic diagram illustrating the construction of the LVβ-shRNA vector containing miR30-shRNA expression cassette. A) Schematic diagram of human α-globin mRNA showing the target sites for siα-3 and siα-4 which are adapted into **B**) miR30-based shRNA; shα1/2 and shα2, respectively. **C**) Predicted secondary structure of miR30-shRNA expression cassette generated by mfold. miR30-based shRNA is flanked by 125-nt of miR30 flanking region on either side of the hairpin. Sequence for target site (sense orientation) is shown in blue lowercase letter, while the guide strand sequence is shown in red uppercase letters. Putative Drosha and Dicer cleavage sites are indicated by horizontal lines.[9] miR30-shRNA expression cassette is inserted into **D**) the LentiGlobin BB305 (pLVβ) gene therapy vector encoding the $\beta^{A\text{-}T87Q}$-globin gene to generate **E**) pLVβ-shRNA vector containing the intronic miR30-shRNA expression cassette. The $\beta^{A\text{-}T87Q}$-globin gene is placed under control of the human β-globin promoter (βp) and elements from the human β-globin locus control region (LCR), 5'DNaseI hypersensitive sites (HS2-4), truncated β-globin Intron 2 containing a 374bp *Rsa*I site deletion (Δ), long terminal repeat (LTR), extended packaging signal (ψ), miR30-shRNA expression cassette flanked by the 5'miR30 and 3'miR30 scaffold, hybrid human immunodeficiency type-1 virus long terminal repeat (HIV-LTR) whereby the U3 region of 5' HIV LTR is replaced with cytomegalovirus (CMV) promoter (CMV-R-U5), deletion in the U3 (ΔU3) region of 3' HIV-LTR, Rev-responsive element (RRE) and polypurine tract (ppt).

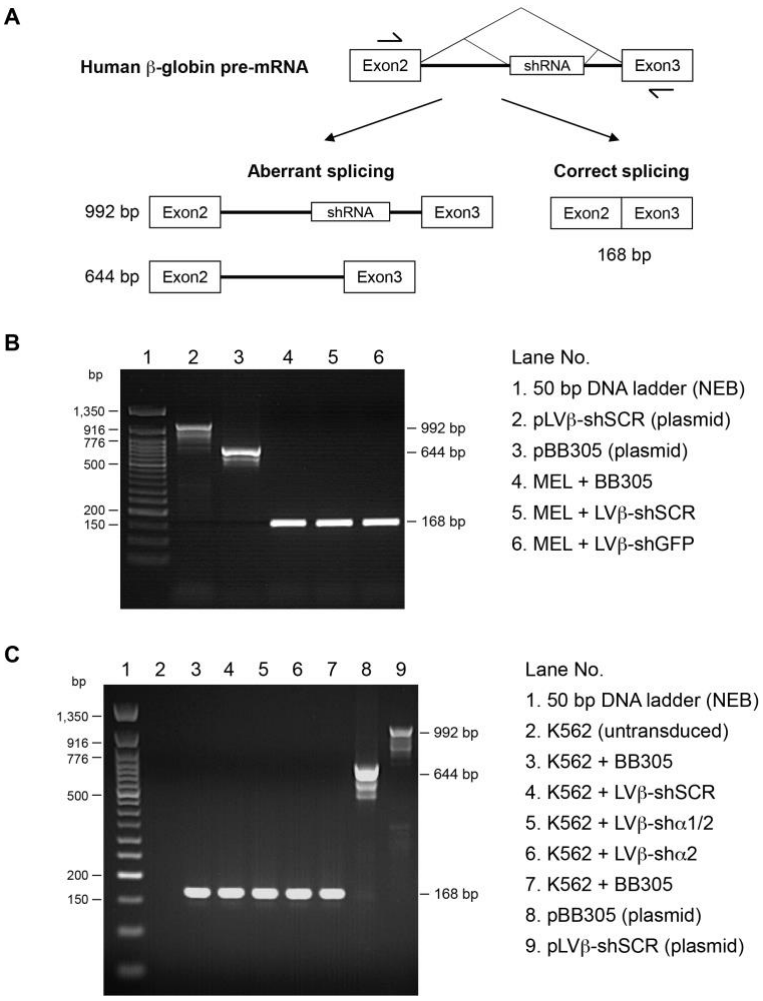


Figure S2. Analysis of human β-globin mRNA splicing following transduction of BB305 and LVβ-shRNA vectors. A) Diagram of human β-globin splicing and location of PCR spanning β-globin exon 2 to exon 3. **B**) Analysis of human β-globin mRNA splicing in MEL-βeGFP cells transduced with BB305 and LVβ-shGFP vectors **C**) Human β-globin mRNA splicing in K562 cells transduced with BB305, LVβ-shα1/2 and LVβ-shα2 vectors. PCR products were visualized on 1% agarose gel electrophoresis. A single PCR product of the expected size (168 bp) representing the correctly spliced $\beta^{A\text{-}T87Q}$-globin mRNA was identified in cells transduced with BB305 and LVβ-shRNA vectors (**b**: Lanes 4-6; **c**: Lanes 3-7).

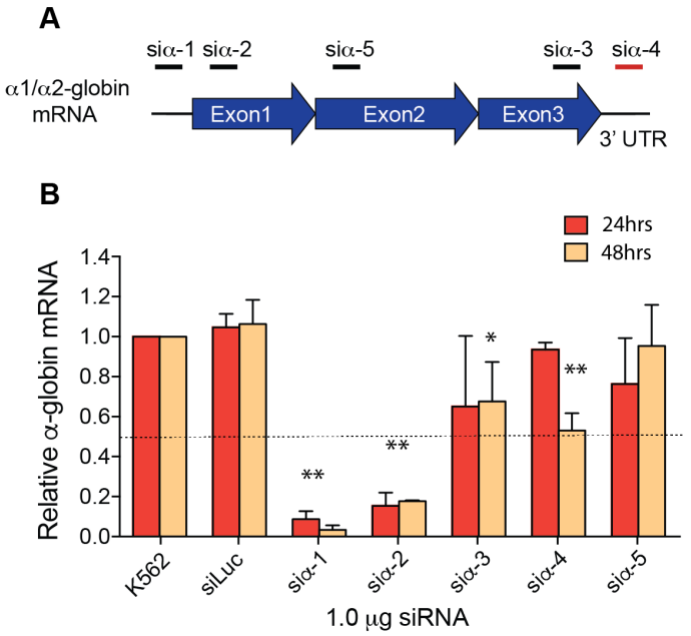
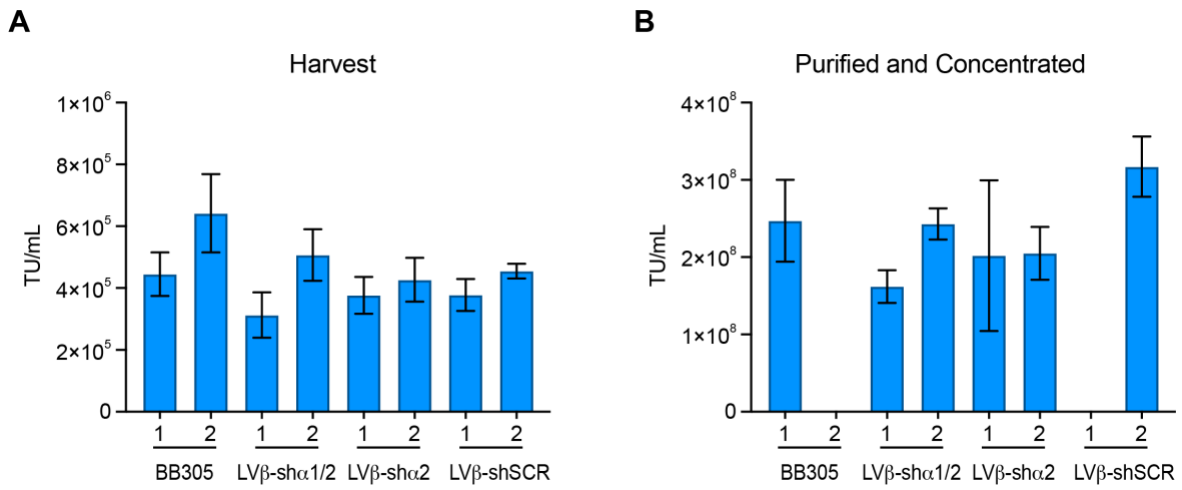


Figure S3. Relative α-globin mRNA expression in K562 cells following electroporation of human α-globin-specific siRNAs. **A**) Schematic diagram of human α-globin mRNA showing the target sites for siα-1, siα-2, siα-3, siα-4 and siα-5 siRNAs. **B**) Relative α-globin RNA expression post electroporation of 1 μg of siRNA. Relative expression of α-globin was normalized to relative expression levels in mock electroporated K562 cells using β-actin expression as an RNA loading control. An siRNA sequence targeting luciferase (siLuc) was included in all experiments as an irrelevant control. Values represent the mean average of at least three independent experiments (mean±SD). Statistical analysis was performed using one-way ANOVA (*p≤0.05, **p≤0.01).



225

226 **Figure S4. Comparison of lentiviral vector titres. A**) Lentiviral titre from culture
227 supernatants harvested 24 hours post-transfection of HEK 293T cells (2 independent
228 productions per vector) **B**) Lentiviral titre of harvested lentiviral vector following purification
229 by ion exchange chromatography and 1000-fold concentration.

230

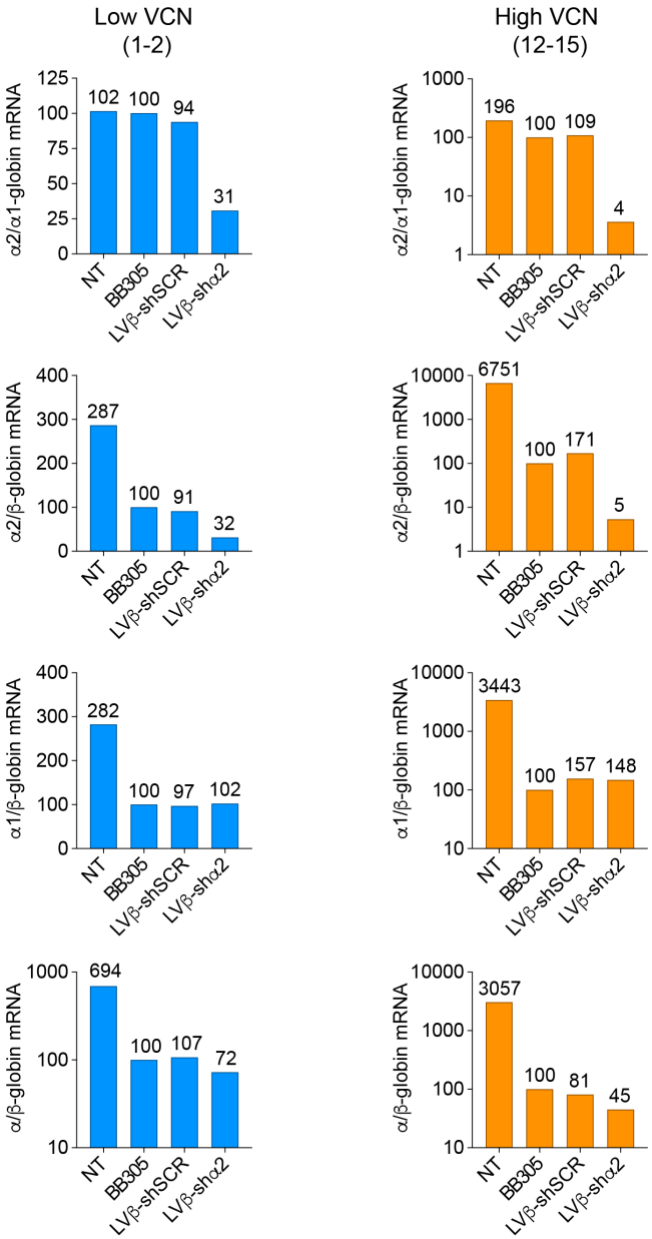


Figure S5. Analysis of globin mRNA ratio in transduced β^0-HUDEP-2 cells. RT-qPCR were made from RNA extracted in proliferating β^0-HUDEP-2 cells transduced at low VCN (1-2) and high VCN (12-15).

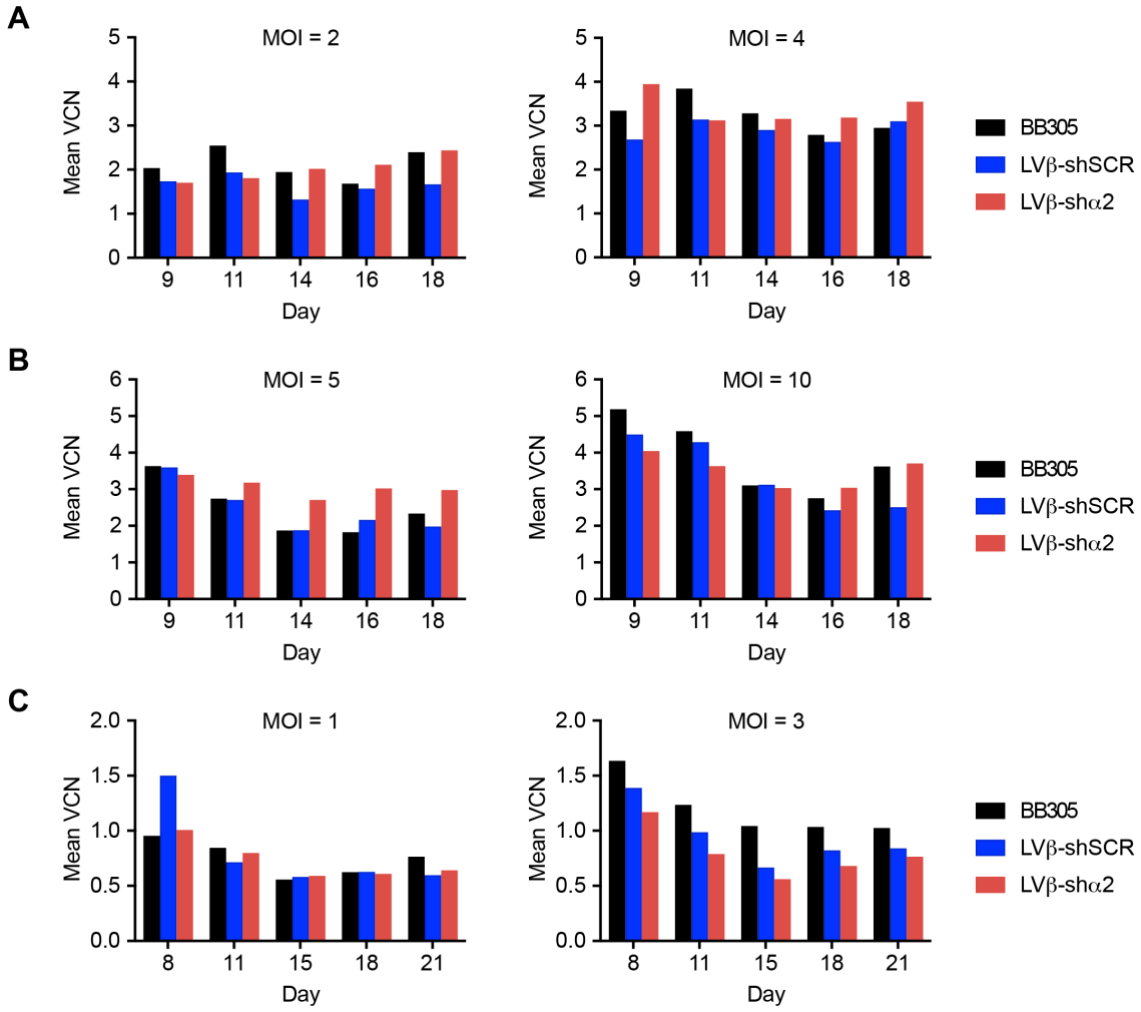


Figure S6. Vector copy number in transduced primary cells. Cord blood (**A**) and HbE/β-thalassemia erythroid cells from patient 1 (**B**) and patient 2 (**C**) were transduced with BB305, LVβ-shSCR and LVβ-shα2 vectors, and grown in differentiation medium 1 (**A**, **B**) or differentiation medium 2 (**C**).

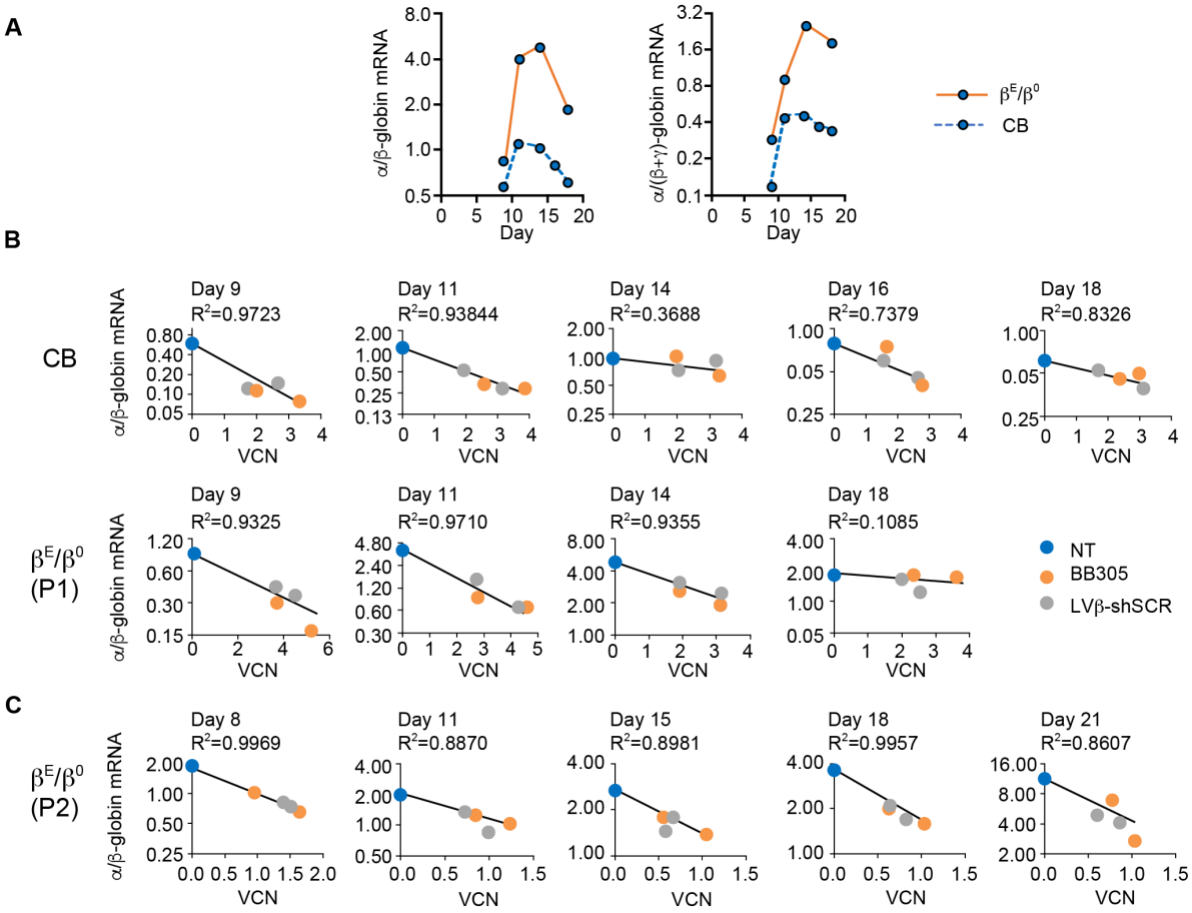


Figure S7. Analysis of α/(β like)-globin mRNA ratio relative to VCN A) Analysis of α/(β like)-globin mRNA ratio in unmodified primary cells from cord blood (CB) and HbE/β-thalassemia patient collected at several time points after erythroid differentiation. **B**) Globin mRNA ratios in transduced cells from CB and HbE/β-thalassemia patient (P1) relative to VCN. **C**) Globin mRNA ratios in unmodified and transduced cells from second HbE/β-thalassemia patient (P2), grown three weeks in medium 2. The values are distributed in a similar way around the regression line, whether they come from samples transduced by BB305 or LVβ-shSCR.

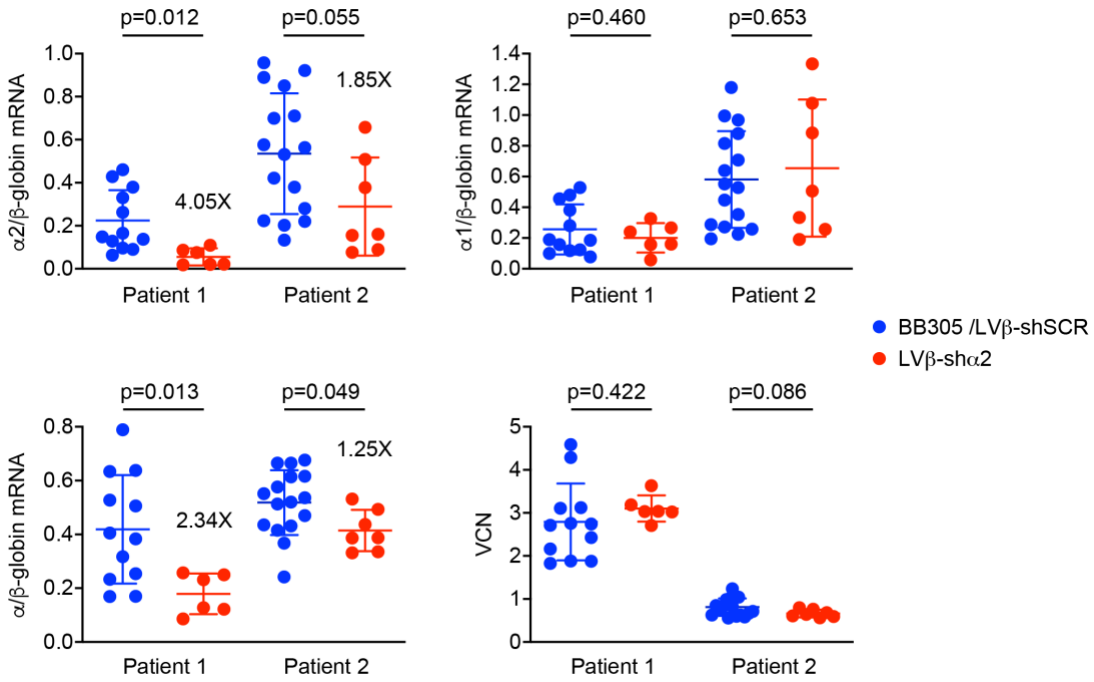


Figure S8. Analysis of α/β-globin ratios and VCNs in transduced primary HbE/β-thalassemia erythroid cells. α2/β -, α1/β-, α/β-globin mRNA ratios and VCNs in transduced primary cells from two patients with HbE/β-thalassemia. Cells were grown for three weeks in medium 1 (P1) or 2 (P2) and transduced at MOI 5 and 10 (P1) or 1 and 3 (P2). mRNA ratios were normalized to non-transduced cells (set to 1).

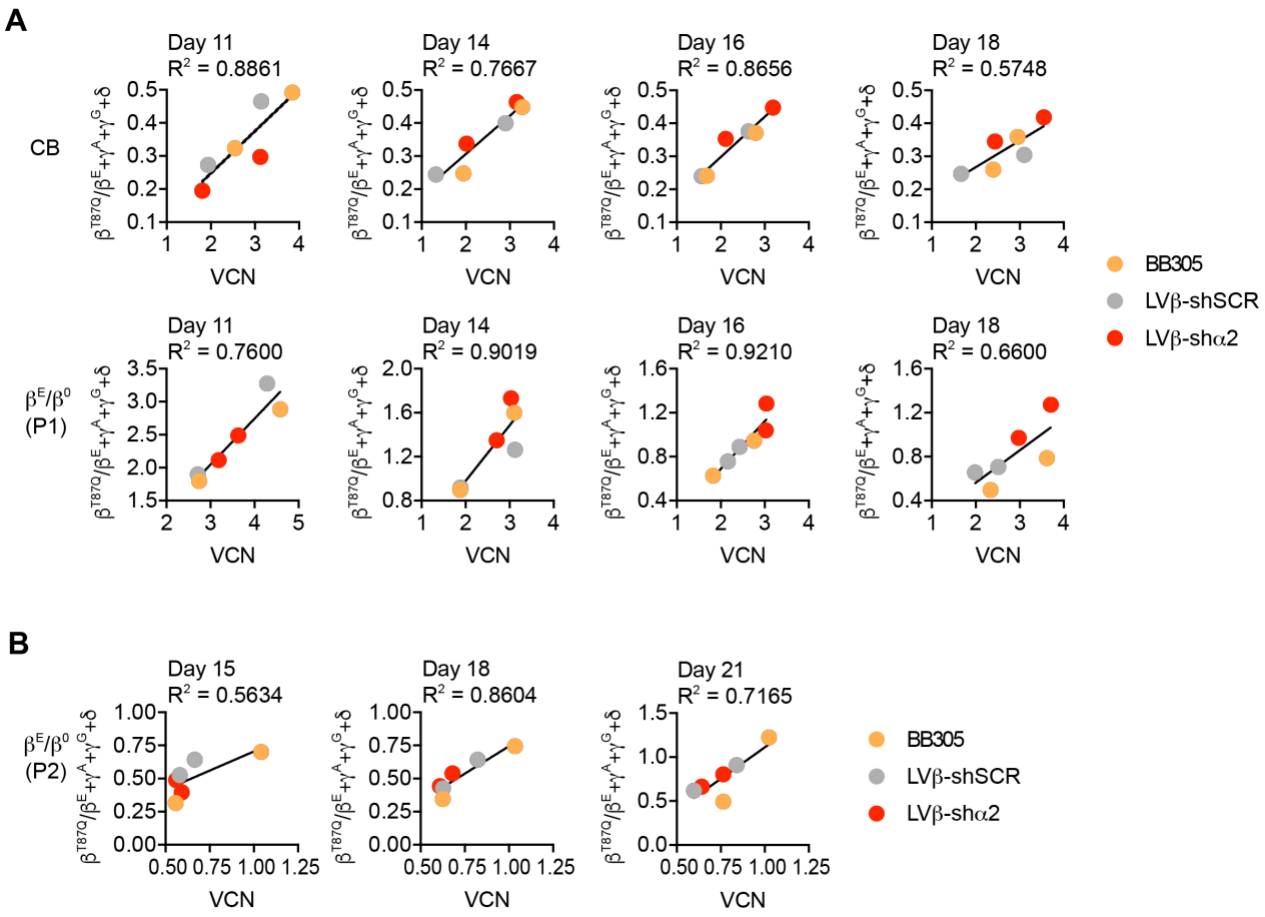


Figure S9. Expression of $\beta^{A\text{-}T87Q}$-globin is not affected by shRNA. $\beta^{A\text{-}T87Q}$-globin expression relative to other β-like globin chains, determined by RP-HPLC in erythroid cell derived from cord blood (CB) and HbE/β-thalassemia samples (patient 1; P1 and patient 2; P2) grown in medium 1 (**A**) or 2 (**B**), after transduction with BB305, LVβ-shSCR, or LVβ-shα2.

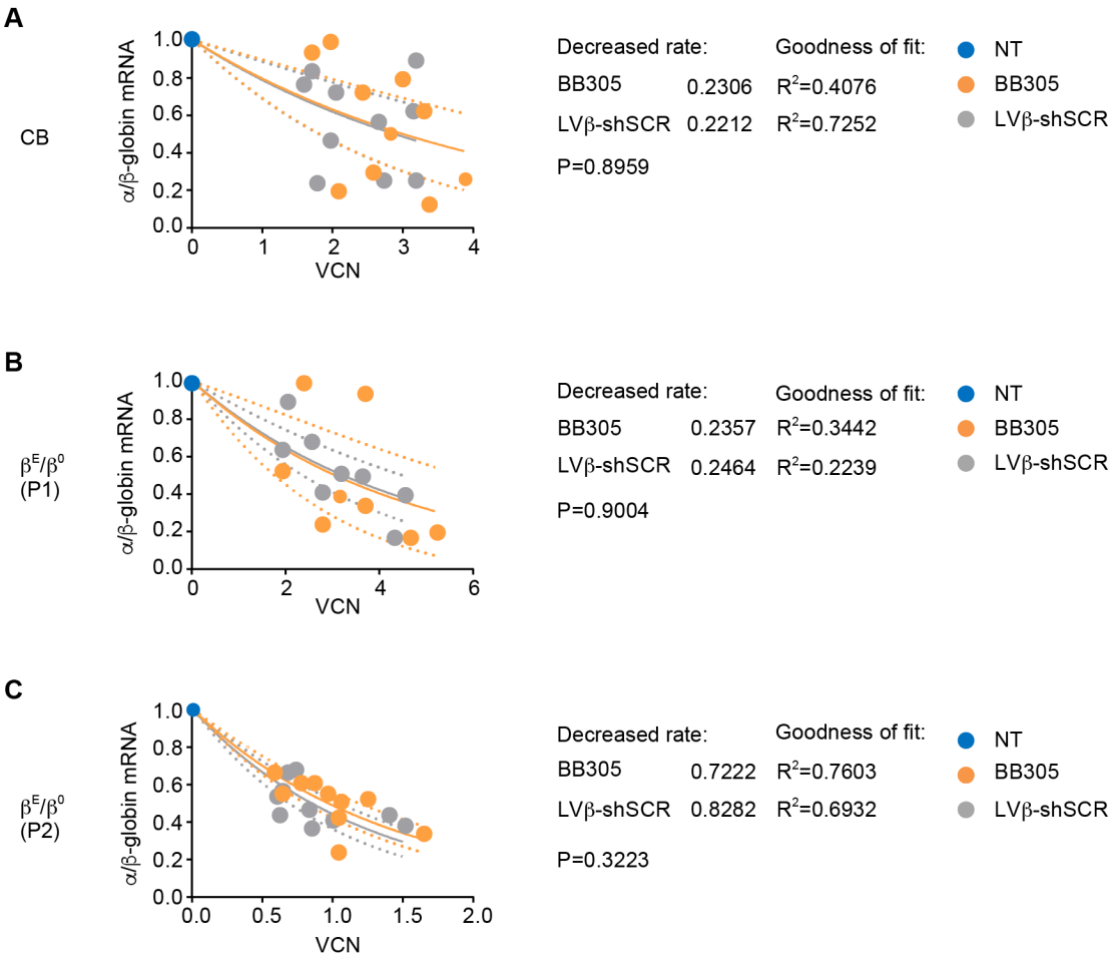


Figure S10. Analysis of α/β-globin mRNA ratio relative to VCN. **A**) Analysis of α/β-globin mRNA ratio in transduced cord blood (CB), **B**) HbE/β-thalassemia (P1) and **C**) HbE/β-thalassemia (P2). Globin mRNA ratios are calculated from transduced samples after normalization to values measured in non-transduced cells. Best fit curves and 95% confidence intervals are shown. Determination of P values for comparison of decreased rates in samples transduced with BB305 and LVβ-shSCR vectors were performed assuming ratios of 1 in unmodified cells and 0 at infinite VCN.

SUPPLEMENTAL TABLES

Table S1: Oligonucleotides and primers use for construction of the LVβ-shRNA vectors

Assay	Primers	Sequence (5'-3')
Generation of shGFP	shGFP Fwd	ACTCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGA**GCAC AAGCTGGAGTACAACTA***TAGTGAAGCCACAG* *
	shGFP Rev	ATTGAATTCCGAGGCAGTAGGCA**CACAAGCTGGAGTACAAC TA***TACATCTGTGGCTTC* *
Generation of shα1/2	shα1/2 Fwd	ACTCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGA**TGAG CACCGTGCTGACCTCCA***TAGTGAAGCCACAG* *
	shα1/2 Rev	ATTGAATTCCGAGGCAGTAGGCA**GTGAGCACCGTGCTGACC TCCA***TACATCTGTGGCTTC* *
Generation of shα2	shα2 Fwd	ACTCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGA**CGGC CCTTCCTGGTCTTTGAA***TAGTGAAGCCACAG* *
	shα2 Rev	ATTGAATTCCGAGGCAGTAGGCAC**CGGCCCTTCCTGGTCTT TGAA***TACATCTGTGGCTTC* *
Screening of pLVβ-shRNA	BG-Ex1	CTGACACAACTGTGTTCACT
	shRNA Rev	GTGCAGGGTCCGAGGT
Screening of pIntron2 shuttle vector	T7	GATATACGACTCACTATAGGGC
	BG-Int2	CTGTGGCAGCAAGATAAGAG

* Underline: restriction sites for shRNA cloning, bold: anti-sense and sense sequences, italic: loop sequences

Table S2: siRNA and shRNA sequences

shRNAs	Sequence (5'-3')	Target
siα-1 sense	r(CAGACUCAGAGAGAACCCA)dTdT	5'UTR
siα-1 anti-sense	r(UGGGUUCUCUCUGAGUCUG)dTdG	5'UTR
siα-2 sense	r(CCGACAAGACCAACGUCAA)dTdT	Exon 1
siα-2 antisense	r(UUGACGUUGGUCUUGUCGG)dCdA	Exon 1
siα-3 sense	r(CCGUGCUGACCUCCAAAUA)dTdT	Exon 3
siα-3 antisense	r(UAUUUGGAGGUCAGCACGG)dTdG	Exon 3
siα-4 sense	r(GGCCCUUCCUGGUCUUUGA)dTdT	3'UTR of α2-globin mRNA
siα-4 antisense	r(UCAAAGACCAGGAAGGGCC)dGdG	3'UTR of α2-globin mRNA
siα-5 sense	r(GACCUACUUCCCGCACUUC)dTdT	Exon 2
siα-5 antisense	r(GAAGUGCGGGAAGUAGGUC)dTdT	Exon2
shα1/2	tgctgttgacagtgagcgaGAGCACCGTGCTGACCTCCAA TA*gtgaagccacagatgta***TTGGAGGTCAGCACGGTGCT CG**tgcctactgcctcgga	Exon 3 of α1 and α2-globin mRNAs
shα2	tgctgttgacagtgagcgaCGGCCCTTCCTGGTCTTTGAA TA*gtgaagccacagatgta***TTCAAAGACCAGGAAGGGC CGG**tgcctactgcctcgga	3' UTR of α2-globin mRNA
shGFP	tgctgttgacagtgagcgaGCACAAGCTGGAGTACAACTA TA*gtgaagccacagatgta***TAGTTGTACTCCAGCTTGTG CC**tgcctactgcctcgga	eGFP mRNA
shSCR	tgctgttgacagtgagcgaTCTCGCTTGGGCGAGAGTAAG TA*gtgaagccacagatgta***CTTACTCTCGCCCAAGCGAG CG**tgcctactgcctcgga	Non-targeting control

Bold uppercase: antisense sequences, regular uppercase: sense sequences, italic: loop sequence.

Table S3: Primers and probes used for vector copy number determination [10]

Assay	Amplicon	Primer and probe	Sequence (5'-3') or TaqMan gene expression assay number*	Modification	Conc.
Vector copy number	Lentiviral vector	GAG F	GGAGCTAGAACGATTCGCAGTTA	-	720nM
		GAG R	GGTTGTAGCTGTCCCAGTATTTGTC	-	720nM
		GAG P	ACAGCCTTCTGATGTCTCTAAAAGGCCAGG	5'FAM 3'TAMRA	140nM
	Human β-actin	hbAct F	TCCGTGTGGATCGGCGGCTCCA	-	900nM
		hbAct R	CTGCTTGCTGATCCACATCTG	-	900nM
		hbAct P	CCTGGCCTCGCTGTCCACCTTCCA	5'FAM 3'TAMRA	250nM
NIH3T3 titer	Mouse β-actin	mbAct assay	Mm00607939_S1*	5'FAM 3'NFQ-MGB	1x

FAM, 6-carboxyfluorescein ester; TAMRA, tetramethyl-6-carboxyrhodamine; NFQ, nonfluorescent quencher; MGB, minor groove binder; Conc., concentration

* Applied Biosystems.

Table S4: Primers and Probes used for RT-qPCR

Assay	Amplicon	Primer and probe	Sequence (5'-3') or TaqMan gene expression assay number*	Modification	Conc.
eGFP expression (SYBR Green)	EGFP	eGFP F	AACGTCTATATCATGGCCGA	-	250nM
		eGFP R	TGCACGCTGCCGTCCTCGAT	-	250nM
	Mouse β-actin	mmACTB F	CAACGAGCGGTTCCGATG	-	250nM
		mmACTB R	TACCCAAGAAGGAAGGCTGGA	-	250nM
shGFP expression (SYBR Green)	shGFP	shGFP F	GCGTATAGTTGTACTCCAGC	-	250nM
		shRNA R	GTGCAGGGTCCGAGGT	-	250nM
		shGFP-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCACAA	Reverse transcription	
		shGFP-3p	TAGTTGTACTCCAGCTTGTGC	Oligo control	
α/(β+γ) ratio (TaqMan)	α1/α2 -globin	HBA1/ HBA2	Hs00361191_g1*	5'FAM 3'NFQ-MGB	1X
	β-globin	HBB	Hs00747223_g1*	5'FAM 3'NFQ-MGB	1X
	Aγ/Gγ -globin	HBG1/ HBG2	Hs00361131_g1*	5'FAM 3'NFQ-MGB	1X
α1-globin expression (SYBR Green)	α1-globin	HBA1 F	CTCGGTGGCCATGCTTCT	-	200nM
		HBA1 R	GGGTACGGGTGCAGGA	-	200nM
α2-globin expression (SYBR Green)	α2-globin	HBA2 F	CTCGGTAGCCGTTCCTCC	-	200nM
		HBA2 R	GAAGGGCCGGTGCAAGG	-	200nM
β-globin expression (SYBR Green)	β-globin	HBB F	GCACGTGGATCCTGAGAACT	-	250nM
		HBB R	ACCAGCCACCACTTTCTGAT	-	250nM
Reference gene	Human β-actin	hsACTB F	AGGCACCAGGGCGTGAT	-	250nM
		hsACTB R	TCGCCCACATAGGAATCCTT	-	250nM
βA-T87Q-globin mRNA splicing	βA-T87Q -globin	HBBT87Q F	TCAAGGGCACCTTTGCCCAG	-	500nM
		HBB R	ACCAGCCACCACTTTCTGAT	-	500nM

FAM: 6-carboxyfluorescein ester, NFQ: non-fluorescent quencher, MGB: minor groove binder, Conc: Concentration, *ThermoFisher Scientific

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Antibodies	Assay	Supplier (Cat. No.)
Fixable Viability Dye eFluorTM 780	Flow cytometry	eBioscience (65-0865-14)
Propidium Iodide	Flow cytometry	Sigma Aldrich (P4864)
Hemoglobin α (D4) Alexa Fluor® 647	Flow cytometry	Santa Cruz Biotechnology (sc-514378 AF647)
Hemoglobin β (37-8) PE	Flow cytometry	Santa Cruz Biotechnology (sc-21757 PE)
PE mouse anti-human CD36 (Ac106)	Flow cytometry	Miltenyi (130-095-472)
APC mouse anti-human CD71 (Ac102)	Flow cytometry	Miltenyi (130-091-727)
FITC mouse anti-human CD235a (HIR2)	Flow cytometry	eBiosciences (11-9987)
Hemoglobin α (D-4);	Western blot	Santa Cruz Biotechnology (sc-514378)
Hemoglobin β (37-8)	Western blot (K562)	Santa Cruz Biotechnology (sc-21757)
β-actin (C4)-HRP	Western blot (K562)	Santa Cruz Biotechnology (sc-47778)
Goat anti-mouse IgG-HRP:	Western blot (K562)	Santa Cruz Biotechnology (sc-2005)
HBB monoclonal antibody (M02) clone 7B12	Western blot (HUDEP2)	Abnova (H00003043-M02)
HBA monoclonal antibody (MO2) clone 4F9	Western blot (HUDEP2)	Abnova (H00003039-M02)
Goat anti-mouse IgG-HRP	Western blot	Jackson ImmunoResearch (115-035-006)

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