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### Permalink

<https://escholarship.org/uc/item/4999m2wc>

### Journal

Molecular Therapy, 28(1)

### ISSN

1525-0016

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### Publication Date

2020

### DOI

10.1016/j.ymthe.2019.09.020

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# Improved Titer and Gene Transfer by Lentiviral Vectors Using Novel, Small $\beta$ -Globin Locus Control Region Elements

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**$\beta$ -globin lentiviral vectors ( $\beta$ -LV) have faced challenges in clinical translation for gene therapy of sickle cell disease (SCD) due to low titer and sub-optimal gene transfer to hematopoietic stem and progenitor cells (HSPCs). To overcome the challenge of preserving efficacious expression while increasing vector performance, we used published genomic and epigenomic data available through ENCODE to redefine enhancer element boundaries of the  $\beta$ -globin locus control region (LCR) to construct novel ENCODE core sequences. These novel LCR elements were used to design a  $\beta$ -LV of reduced proviral length, termed CoreGA-AS3-FB, produced at higher titers and possessing superior gene transfer to HSPCs when compared to the full-length parental  $\beta$ -LV at equal MOI. At low vector copy number, vectors containing the ENCODE core sequences were capable of reversing the sickle phenotype in a mouse model of SCD. These studies provide a  $\beta$ -LV that will be beneficial for gene therapy of SCD by significantly reducing the cost of vector production and extending the vector supply.**

## INTRODUCTION

Sickle cell disease (SCD) is caused by a single nucleotide mutation in the  $\beta$ -globin gene, which results in the formation of abnormally charged hemoglobin (HbS) protein in red blood cells (RBCs).<sup>1</sup> Vaso-occlusion is a major clinical feature of SCD and is caused by sickling of RBCs due to aggregation of HbS under low oxygen conditions.<sup>2</sup> While two Food and Drug Administration approved drug treatments for SCD exist, both Hydroxyurea and L-Glutamine only modestly improve overall health of afflicted patients even when adherence is strictly maintained.<sup>3,4</sup>

As an alternative therapy, allogeneic hematopoietic stem cell transplantation (HSCT) has the potential to cure patients afflicted with SCD; however, identification of suitable histocompatibility leukocyte antigen (HLA)-matched donors remains a significant obstacle.<sup>5</sup> Autologous HSCT combined with gene therapy is a more contempo-

rary approach that entails the genetic modification of a patient's own CD34+ hematopoietic stem and progenitor cells (HSPCs). By replacing a patient's native HSPC population with genetically modified HSPCs harboring a normal or modified  $\beta$ -globin gene (such as Lenti/ $\beta$ AS3-FB<sup>6</sup>), SCD can be permanently cured without risks of graft versus host disease or graft rejection that accompanies the more traditional strategy of allogeneic HSCT.<sup>7</sup> Preliminary data from clinical trials for hemoglobin disorders have indeed demonstrated a benefit from autologous HSCT with gene therapy.<sup>7-11</sup>

Low vector titer has represented a major barrier toward the effective advancement of therapeutic  $\beta$ -globin lentiviral vectors ( $\beta$ -LV) in clinical applications. Typically,  $\beta$ -LVs are produced at 10- to 100-fold lower titers when compared to LV with shorter genomes carrying small regulatory elements and cDNAs. In addition to the problem of low titer, clinical grade  $\beta$ -LV preparations (typically concentrated  $100\times-500\times$ )<sup>12</sup> showed reduced transduction efficiency for primary human HSPC and did not provide dose-dependent transduction of HSPCs with increasing vector concentrations (D.B.K. and R.A.M., unpublished data). The combinations of lower titer and subpar transduction efficiency mean that large volumes of vector need to be produced per patient dose, significantly adding to the clinical expense.

Poor gene transfer to HSPCs has also been a challenge in gene therapy trials and has limited the therapeutic benefit of gene therapy for patients with  $\beta$ -thalassemia and SCD.<sup>8,13</sup> Although the issues of gene transfer to HSPCs has been partially mitigated through the advent of transduction enhancers,<sup>14-17</sup> the purported use of transduction

Received 27 May 2019; accepted 19 September 2019;  
<https://doi.org/10.1016/j.ymthe.2019.09.020>.

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enhancers to achieve adequate vector copy numbers (VCNs) in clinic<sup>7,9,10</sup> does not lower the cost of vector per patient dose when clinical vectors with inherently low infectivity are used.

One predominant factor that diminishes  $\beta$ -LV titer is the relatively large size of these vector genomes (e.g., 6–9 kb) due to the use of the human  $\beta$ -globin genomic sequences (promoter, exons, introns, 3' UTR) and the  $\beta$ -globin Locus Control Region (LCR) DNase I hypersensitive site (HS) elements that augment expression. Another critical factor limiting vector performance is the inherent complexity of the human genomic elements; the  $\beta$ -globin genomic sequences and LCR are transcribed in reverse orientation relative to the vector genomic transcript (to retain intronic enhancers) and may contain problematic sequences that lead to non-functional vector genomes (e.g., cryptic polyadenylation signals).

The Globe-AS3-FB  $\beta$ -LV produced by Ferrari et al.<sup>18</sup> is ~2.7 kb smaller than the Lenti/ $\beta$ AS3-FB  $\beta$ -LV we have used<sup>19</sup> (as well as other commonly used  $\beta$ -LVs)<sup>20–23</sup> and lacks the LCR HS4 sequences, the WPRE (woodchuck hepatitis virus post transcriptional regulatory element), and possesses a smaller 3' UTR segment of the  $\beta$ -globin gene and a larger deletion of  $\beta$ -globin intron 2. It is unknown how sequence differences contribute to Globe-AS3-FB's superior packaging and transduction efficiency when compared to Lenti/ $\beta$ AS3-FB<sup>24</sup> or how these individual sequence differences influence  $\beta$ -globin expression.

Thus, we set out to determine whether the detrimental differences in titer and gene transfer of Lenti/ $\beta$ AS3-FB when compared to Globe-AS3-FB are due to proviral length or specific sequence elements. To reduce proviral size, we employed current genomic and epigenomic databases to identify and redefine functional elements present within the human genomic sequences of the LCR in Lenti/ $\beta$ AS3-FB to produce a composite LCR. A series of modified vectors were made with the aims of increasing titer and transduction efficiency while retaining high-level lineage-specific expression. Modified constructs were compared head-to-head and results were used to guide construction of a reduced length vector with superior titer and gene transfer and with sufficient expression of the anti-sickling  $\beta^{\text{AS3}}$ -globin gene to induce hematologic correction of the sickle phenotype in the "Townes" mouse model of SCD.

## RESULTS

### Proviral Length Influences Packaging and Transduction Efficiency

Previous reports have demonstrated that titer and transduction efficiency of lentiviral vectors inversely correlate with proviral length.<sup>25,26</sup> To determine whether the differences of performances between Lenti/ $\beta$ AS3-FB and Globe-AS3-FB were due to vector length (as opposed to the presence of specific adverse sequences), we cloned and tested a series of derivatives that reflect each difference between the two constructs (Figure 1A). When constructs were packaged and titered head-to-head, an inverse correlation between titer and vector genome length was observed (Figure 1B). When hu-

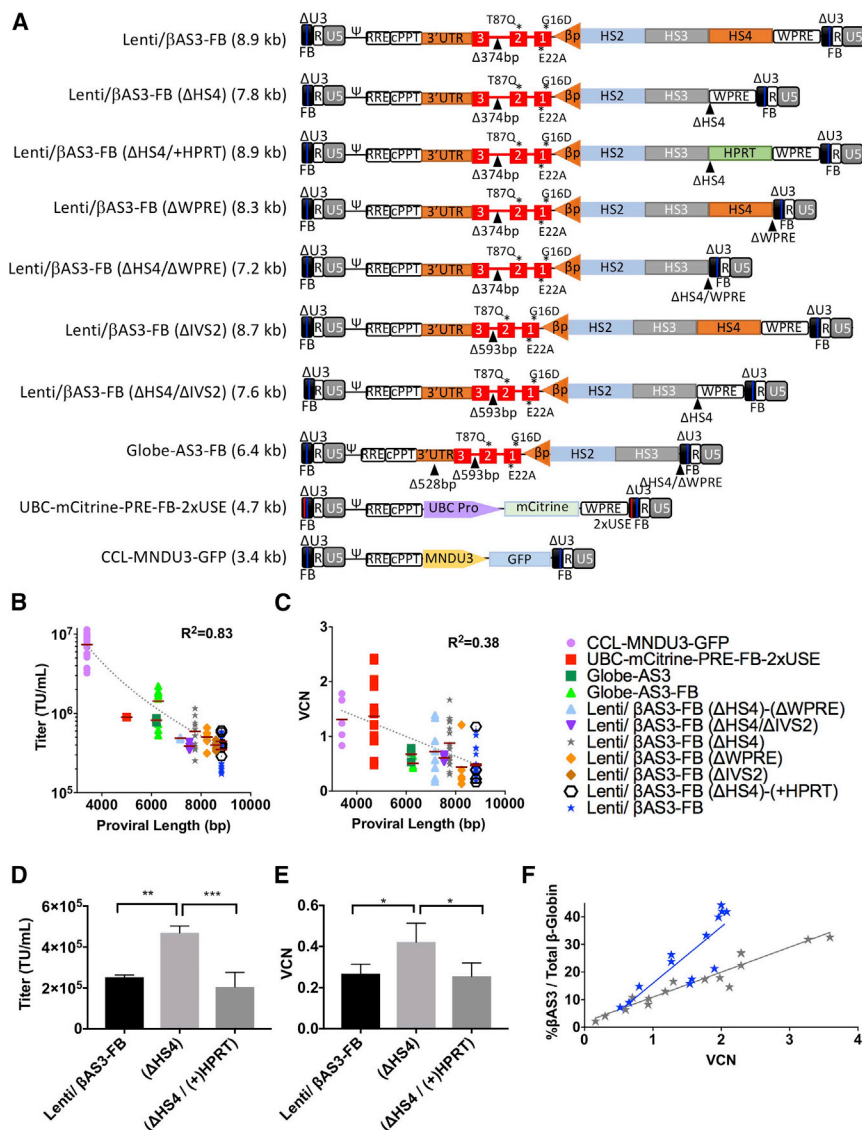
man CD34+ HSPCs were transduced at  $2 \times 10^7$  transduction units (TU)/mL (MOI 20), cultured for 2 weeks under myeloid culture conditions and VCN plotted as a function of vector genome length, an inverse relationship was also observed between genome length and VCN (Figure 1C) with donor-to-donor variation in transduction efficiency diminishing the strength of the correlation. Transduced HSPCs were cultured under myeloid culture conditions (as opposed to erythroid culture conditions) as we have shown that short-term myeloid culture more stringently reflects the VCNs seen in bone marrow after xenotransplantation immune-deficient mouse hosts.<sup>19</sup> Notably, the GLOBE-based constructs showed reduced transduction efficiency for their size, relative to the other vector.

Direct evidence that vector genome length was the major factor impacting vector performance was obtained through simply deleting the HS4 region from Lenti/ $\beta$ AS3-FB, as it was a major difference between Lenti/ $\beta$ AS3-FB and the higher titer vector Globe-AS3-FB. A 2-fold increase in both titer ( $p < 0.01$ ) and gene transduction efficiency to CD34+ HSPCs ( $p < 0.05$ ) was observed when the 1.1 kb HS4 element was removed from Lenti/ $\beta$ AS3-FB. These gains in performance were not sequence-related, as replacing HS4 with a similarly sized 1.1 kb stuffer sequence from the human *HPRT* gene intron 1 similarly reduced titer and infectivity (Figures 1D and 1E). As expected, the removal of HS4 predictably decreased expression of  $\beta^{\text{AS3}}$ -globin (Figure 1F).<sup>27,28</sup>

Additional sequence deletions (>1.1 kb) offered increasing gains in titer and infectivity suggesting that length influenced vector performance more strongly than the presence of discrete sequences (additional analyses examining derivatives of Lenti/ $\beta$ AS3-FB are provided in the Supplemental Results). Taken together, these data suggested that the titer and infectivity of Lenti/ $\beta$ AS3-FB could be improved through shortening vector length. We hypothesized that a decrease in vector length of 2–3 kb would be required to generate a construct produced at 3- to 4-fold higher raw titer that would have superior gene transfer to HSPCs when compared to Lenti/ $\beta$ AS3-FB at equivalent MOI. None of the smaller vectors were studied moving forward; we decided to focus on reducing the size of the LCR.

### Redefining the Putative Boundaries of HS Elements

Vector length reduction was achieved by redefining the boundaries of the LCR HS (2, 3, and 4) elements of Lenti/ $\beta$ AS3-FB by using published genomic and epigenomic data available through ENCODE (accessible via the UCSC Genome Browser).<sup>29</sup> To generate new boundaries for the HS sequences of Lenti/ $\beta$ AS3-FB, we looked specifically for clues to identify the Core enhancer sequences of HS 2, 3, and 4, such as histone markers of active chromatin (H3k27ac) and characteristics of open chromatin (DNase I hypersensitive and formaldehyde-assisted isolation of regulatory elements [FAIRE]), as well as TF binding (transcription factor chromatin immunoprecipitation sequencing [ChIP-seq]) and conserved TF motifs. These novel defined HS sequences, termed "ENCODE core sequences" (EC2, ~420 base pairs [bp];

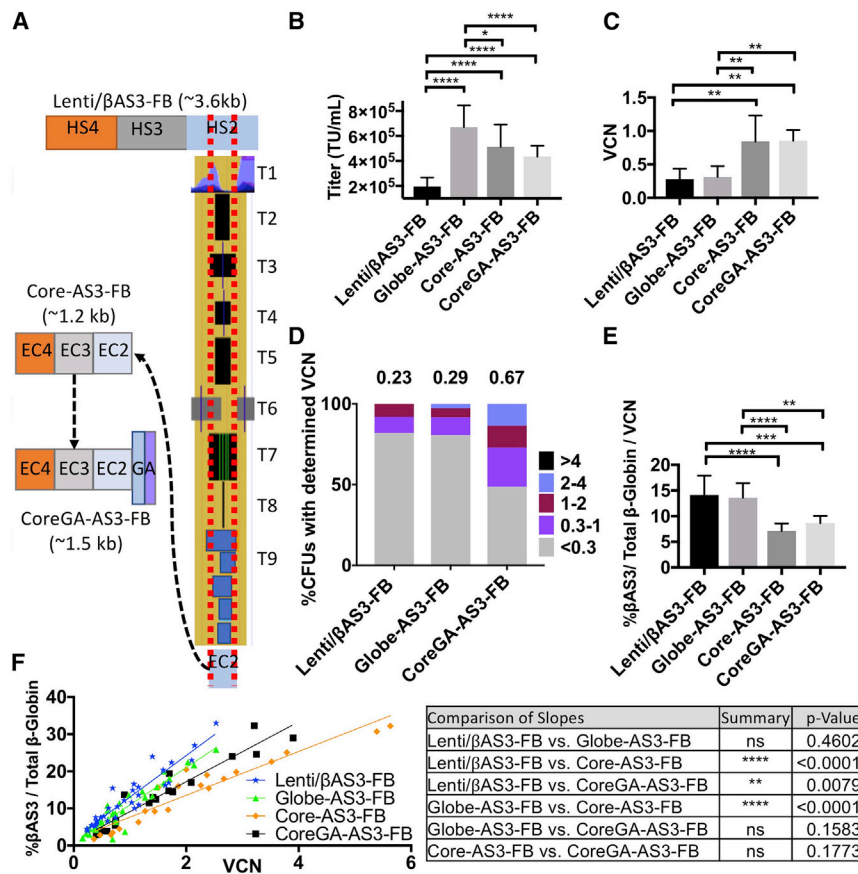


EC3, ~340 bp; EC4, ~410 bp), were used to replace the HS sequences present within Lenti/βAS3-FB (~3.6 kb reduced to ~1.2 kb) to generate a reduced length construct termed “Core-AS3-FB.” We then added a 360 bp fusion element to facilitate position-independent expression of β<sup>AS3</sup>-globin by combining a 202 bp murine Gata1 gene HS element with a 143 bp Human Ankyrin gene element<sup>30</sup> to create CoreGA-AS3-FB (Figure 2A).

The  $\beta$ -LV constructs were packaged in parallel using HEK293T cells and titrated head-to-head on HT-29 cells.<sup>12</sup> From five independent packaging experiments, titers of the Globe-AS3-FB, Core-AS3-FB, and CoreGA-AS3-FB  $\beta$ -LVs were on average about 3-fold higher than that of Lenti/ $\beta$ AS3-FB ( $p < 0.0001$ ), presumably due to their reduced proviral lengths in comparison to Lenti/ $\beta$ AS3-FB (Figure 2B).

To evaluate the efficiency of transduction of Core-AS3-FB and CoreGA-AS3-FB in comparison to Lenti/ $\beta$ AS3-FB and GLOBE-AS3-FB, we transduced human CD34+ HSPCs isolated from bone marrow (BM) of healthy donors at  $1 \times 10^7$  TU/mL (MOI 10) and cultured them for 14 days under myeloid differentiation conditions. From three independent experiments, the gene transfer efficiency of Core-AS3-FB was on average about 3-fold higher when compared to Lenti/ $\beta$ AS3-FB and Globe-AS3-FB ( $p < 0.01$ ), and the further addition of the fusion element to produce CoreGA-AS3-FB did not negate gains in gene transfer efficiency achieved by Core-AS3 ( $p < 0.01$ ) (Figure 2C).

To confirm that CoreGA-AS3-FB retained superior transduction efficiency when compared to Lenti/ $\beta$ AS3-FB and GLOBE-AS3-FB at equal MOI ( $1 \times 10^6$  TU/mL), we assessed infectivity at the



**Figure 2. Approach for Length Reduction and *In Vitro* Characterization of Reduced Length Vectors**

(A) Published genomic data available through ENCODE were used to redefine the putative HS boundaries. The track sets (T) 1–8 were used to generate new boundaries for the LCR's HS sequences. T1, H3k27ac; T2, DNase I (Encode); T3, DNase I (University Wisconsin); T4, DNase I (UNC Chapel Hill); T5, Formaldehyde-Assisted Isolation of Regulatory Elements, FAIRE (UNC Chapel Hill); T6, DNase I/FAIRE/ChIP-seq (Duke/UNC Chapel Hill/UT-Austin); T7, TF ChIP-seq; T8, conserved TF motifs; and T9, regulatory elements (Open Regulatory Annotation database). These new sequences were termed "ENCODE Cores" (EC) 2, 3, and 4 and combined to create Core β-LV. The 143 bp human Ankyrin Element was then combined with the 218 bp murine Gata1 HS4 element and added to EC 2, 3, and 4 to create CoreGA-AS3-FB β-LV. (B) Quantity of infectious particles of constructs when packaged and titered in parallel.  $n = 5$ . \* $p < 0.05$ ; \*\*\*\* $p < 0.0001$ . (C) Human CD34+ HSPCs were transduced with constructs at  $1 \times 10^7$  TU/mL and cultured under myeloid culture conditions for 14 days. VCN was determined by ddPCR.  $n = 3$ . \*\* $p < 0.01$ . (D) Human CD34+ HSPCs were transduced in parallel at  $1 \times 10^6$  TU/mL and used to seed a methylcellulose-based colony forming unit assay. Shown are stacked bar graphs representing percentages of colonies with a determined VCN range.  $n = 50$  colonies, Lenti/βAS3-FB;  $n = 36$  colonies, Globe-AS3-FB;  $n = 37$  colonies, CoreGA-AS3-FB. (E) Human CD34+ HSPCs were transduced in parallel at  $1 \times 10^7$  TU/mL and cultured under erythroid culture conditions for 14 days. Expression of different constructs is presented as percentage of βAS3-globin normalized to VCN.  $n = 9$ . \*\*\* $p < 0.001$  (F) Human CD34+ HSPCs were transduced at various MOI

and differentiated under erythroid culture conditions for 14 days. Percent βAS3-globin RNA expression was determined by RT-ddPCR and is shown in relation to VCN. Each point in the plot represents an individual transduction done in parallel with Lenti/βAS3-FB and Globe-AS3-FB.  $n = 33$ , Lenti/βAS3-FB;  $n = 36$ , Globe-AS3-FB;  $n = 21$ , Core;  $n = 18$ , CoreGA-AS3-FB. Error bars represent mean  $\pm$  SD.

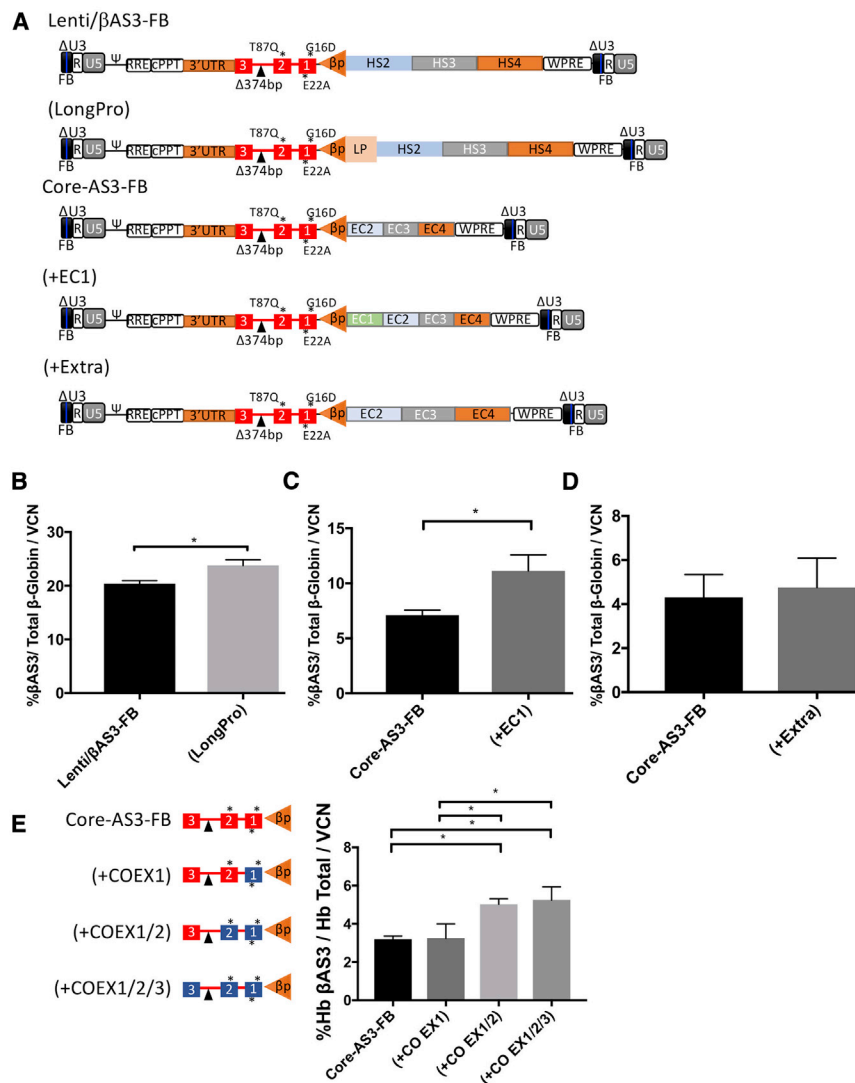
clonal level. Transduced CD34+ HSPCs were plated in a methylcellulose-based medium to allow growth of colony-forming units (CFUs). After 14 days of culture, colonies were scored manually by morphologic criteria to determine lineage differentiation (blast forming unit-erythroid [BFU-E], CFU-granulocyte-monocyte, CFU-granulocyte-erythroid-monocyte-megakaryocyte). The VCNs of bulk transduced cells used to seed the CFU assay were 0.23, 0.29, and 0.67 for Lenti/βAS3-FB, Globe-AS3-FB, and CoreGA-AS3-FB, respectively, reflecting the higher infectivity of the CoreGA-AS3-FB vector. Colonies were grouped by their VCN value as determined by droplet digital PCR (ddPCR). Colonies with VCN  $> 0.3$  were considered PCR positive for the presence of vector and further classified by VCN (0.3–1, 1–2, 2–4, or  $> 4$ ). The percentages of PCR-positive colonies seen for CoreGA-AS3-FB were significantly higher than for Lenti/βAS3-FB and Globe-AS3-FB ( $p < 0.004$ ; chi-square test) reaching 51%, 18%, and 19%, respectively (Figure 2D). Among the positive colonies, CoreGA-AS3-FB achieved greater numbers of integrations per colony when compared to Lenti/βAS3-FB (0.3–1 VCN;  $p < 0.01$ , 1–2 VCN,  $p < 0.05$ ; 2–4 VCN,  $p < 0.005$ ; chi-square test). These results demon-

strate that when assessed at comparable MOI, CoreGA-AS3-FB was able to transduce greater numbers of CD34+ HSPCs with more integrant per cells when compared to current clinical vectors.

To determine whether the newly designed "Core" constructs retained the ability to achieve therapeutic levels of β<sup>AS3</sup>-globin expression, we performed *in vitro* erythroid differentiation studies using BM CD34+ HSPCs from healthy donors. After HSPCs were transduced at equal MOI, β<sup>AS3</sup>-globin and total β-globin RNA transcript levels were measured by reverse transcription (RT)-ddPCR 14 days after transduction and *in vitro* differentiation. The percentages of β<sup>AS3</sup>-globin RNA transcripts over total β-globin RNA transcript were normalized to VCN for nine independent experiments. Expression levels normalized by VCN of Core-AS3-FB and CoreGA-AS3-FB were 0.5-fold ( $p < 0.0001$ ), and 0.4-fold ( $p < 0.001$ ) lower than that of Lenti/βAS3-FB, respectively (Figure 2E).

To examine the linear relationship between VCN and %β<sup>AS3</sup>-globin transcript levels, we transduced HSPCs derived from multiple healthy donors at different MOIs and subjected them to *in vitro* erythroid





**Table 1. *In Vitro* VCN of Gene Modified  $\beta^S/\beta^S$  Lin<sup>−</sup> BM Cells Before Transplant**

	Lenti/ $\beta$ AS3-FB	Globe-AS3-FB	CoreGA-AS3-FB
Transduction Condition (TU/mL)	$6.6 \times 10^6$	$1 \times 10^7$	$3.3 \times 10^6$
<i>In vitro</i> VCN experiment 1	2	2	2
<i>In vitro</i> VCN experiment 2	2	2	2

TU, transduction units; VCN, vector copy number.

determined from the transduced cell product 14 days after transduction (shown in Table 1).

Engraftment by flow cytometry for CD45.2<sup>+</sup> donor cells, transduction efficiency (VCN by ddPCR),  $\beta^{AS3}$ -globin expression (by RT-ddPCR), blood hemoglobin concentration, and RBC counts and hemoglobin composition by high-performance liquid chromatography (HPLC) were measured on peripheral blood (PB) samples acquired 4, 16, and 20 weeks post-transplantation. At week 20, mice were euthanized, and BM cells collected to measure engraftment, transduction efficiency, and  $\beta^{AS3}$ -globin expression. Mice with BM donor engraftment <97% at week 20 were excluded from analyses to prevent inclusion of artifactually corrected mice (as  $\geq 4\%$  residual WT recipient RBCs would mask the adverse pathophysiology induced by  $\beta^S/\beta^S$  donor cells.<sup>32</sup> At the time of euthanasia, BM cells taken from two mice from each arm were used to seed a methylcellulose-based CFU assay; after 2 weeks growth, individual primitive erythroid progenitor cell colonies (BFU-E) were isolated from the CFU dishes and assayed to measure VCN and  $\beta^{AS3}$ -globin expression. Peripheral blood cells were also collected at time of euthanasia and used to quantify the percentages of sickled erythrocytes present within the peripheral circulation.

As intended, the average gene transfer efficiency seen in PB cells did not significantly differ among experimental arms and the average PB VCNs were similar to those of *in vitro* cultured cells before transplantation. Moreover, gene marking of cells remained stable across all three time points, indicating that cells with long-term engraftment potential were stably transduced and able to contribute to hematopoiesis (Figure 4A).

Quantification of Hb $\beta^{AS3}$  tetramers in peripheral blood lysates was accomplished using HPLC. Figure S4 provides representative HPLC chromatograms of globin tetramers in PB obtained at time of euthanasia from representative mice. At 20 weeks post transplantation, the average levels of Hb $\beta^{AS3}$ /total hemoglobin tetramers were 37.4%, 28.8%, and 19.1% for Lenti/ $\beta$ AS3-FB, Globe-AS3-FB, and CoreGA-AS3-FB, respectively (Figure 4B).

To compare the difference in normalized expression between each experimental arm, we normalized and plotted %Hb $\beta^{AS3}$  to PB VCN for each mouse. An average of 18.3, 13.4, and 8.8% Hb $\beta^{AS3}$ /VCN was seen for Lenti/ $\beta$ AS3-FB, Globe-AS3-FB, and CoreGA-AS3-FB,

respectively (Figure 4C). Table S1 provides additional characterizations of globin tetramers in PB lysates generated by HPLC for weeks 4, 16, and 20.

The Hb levels seen at time of euthanasia were significantly higher when comparing recipients of BM transduced with Lenti/ $\beta$ AS3-FB ( $p < 0.0001$ ), Globe-AS3-FB ( $p < 0.05$ ), and CoreGA-AS3-FB ( $p < 0.05$ ) to recipients of mock-transduced BM cells. The Hb levels of mice that received mock-transduced cells were 8.5 g/dL on average while the Hb levels of mice that received BM transduced with Lenti/ $\beta$ AS3-FB, Globe-AS3-FB, or CoreGA-AS3-FB were 10.7, 10.2, and 9.9 g/dL on average, respectively (Figure 4D).

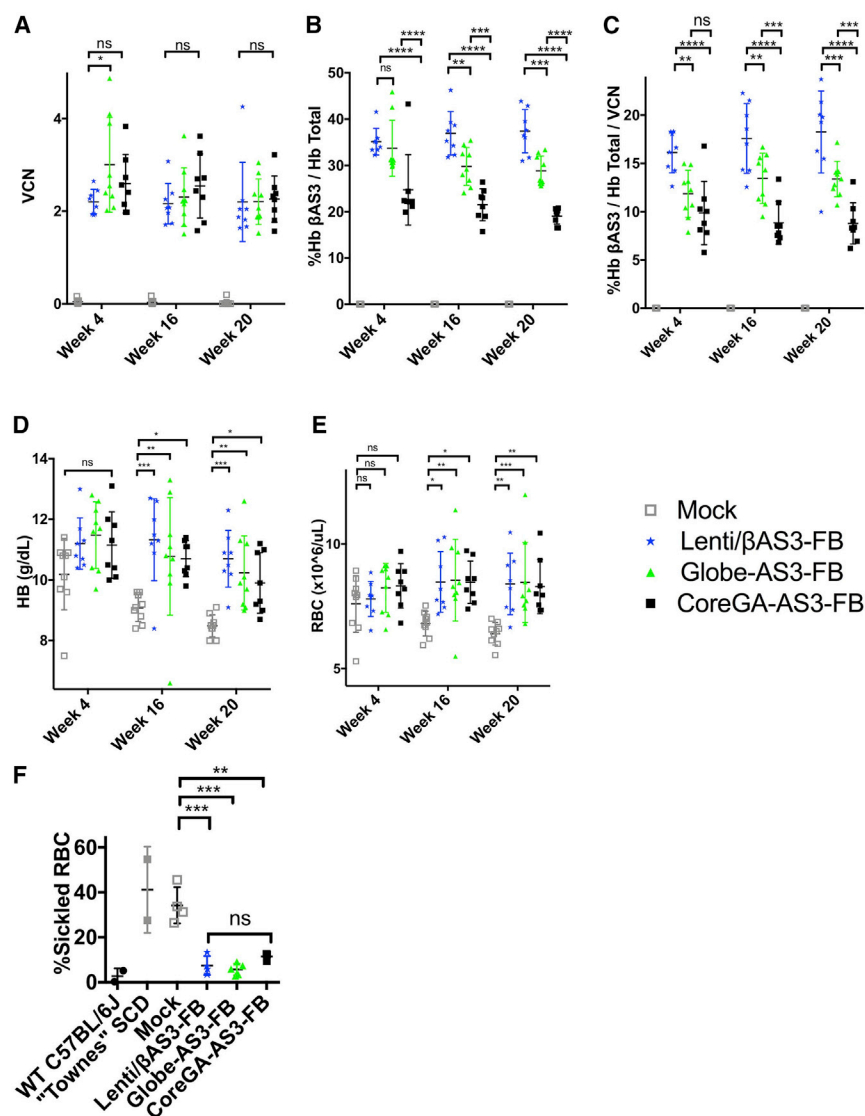
RBC counts were also significantly higher for recipients of Lenti/ $\beta$ AS3-FB ( $p < 0.001$ ), Globe-AS3-FB ( $p < 0.01$ ), and CoreGA-AS3-FB ( $p < 0.001$ ) transduced BM cells compared to recipients of mock-transduced BM cells. The RBC counts of mice that received mock-transduced cells were  $6.0 \times 10^6$  cells/ $\mu$ L on average, while the RBC counts of mice that received Lenti/ $\beta$ AS3-FB, Globe-AS3-FB, or CoreGA-AS3-FB were 8.4, 8.5, and  $8.3 \times 10^6$  cells/ $\mu$ L on average, respectively (Figure 4E). Notably, mice that received mock transduced BM cells developed progressive anemia as noted by decreasing Hb levels and RBC counts observed in PB over time.

Lastly, the quantity of sickled RBCs present within PB at time of euthanasia from a subset of representative mice was measured using the ImageStream X Mark II Imaging Flow Cytometer (ISX). Equal volumes of peripheral blood and 2% sodium metabisulfite solution were mixed together and incubated under hypoxic conditions (2% oxygen) at 37°C for 10 minutes, diluted in 2% glutaraldehyde solution, and then analyzed by ISX. Peripheral blood taken from “Townes” homozygous  $\beta^S/\beta^S$  SCD mice or mice transplanted with mock-transduced HSPCs displayed averages of 41.0% and 34.3% RBC sickling, respectively. Mice transplanted with Lenti/ $\beta$ AS3-FB, Globe-AS3-FB, or CoreGA-AS3-FB transduced HSPCs displayed a significant decrease in the percentages of sickled cells present within peripheral blood ( $p < 0.0001$ ). The average amount of sickling seen for Lenti/ $\beta$ AS3-FB, Globe-AS3-FB, and CoreGA-AS3-FB were 7.5%, 5.76%, and 11.5%, respectively (Figure 4F).

#### ***In Vivo* Analysis of Bone Marrow $\beta^{AS3}$ -Globin Expression from an SCD Mouse Model**

BM cells were collected at time of euthanasia and used to measure engraftment, transduction efficiency, and  $\beta^{AS3}$ -globin expression. As expected, average BM VCN was similar among all experimental arms and matched the average PB VCN seen at euthanasia (Figure 5A).

To determine whether CoreGA-AS3-FB retained the ability to achieve therapeutic levels of expression in BM cells, we measured  $\beta^{AS3}$ -globin and total  $\beta$ -globin RNA transcript levels by RT-ddPCR. Average levels of % $\beta^{AS3}$ -globin were 21.5%, 15.1%, and 11.4% for Lenti/ $\beta$ AS3-FB, Globe-AS3-FB, and CoreGA-AS3-FB, respectively (Figure 5B). To compare the difference in expression per vector



**Figure 4. In Vivo Analysis of Hematologic Correction of SCD Mouse Model**

Mice were bled at weeks 4, 16, and 20 and peripheral blood (PB) analyzed. Only mice with >97% BM donor engraftment were included in analysis.  $n = 9$ , Mock;  $n = 8$ , Lenti/ $\beta$ AS3-FB;  $n = 9$ , Globe-AS3-FB;  $n = 8$ , CoreGA-AS3-FB. (A) Peripheral blood VCN by ddPCR. \*\*\*\* $p < 0.0001$ . (B) Percentages of Hb  $\beta$ AS3 tetramers in PB lysates measured by HPLC. \* $p < 0.05$ . (C) Percentages of Hb  $\beta$ AS3 tetramers normalized to PB VCN. (D) Hemoglobin (Hb [g/dL]) levels. \*\* $p < 0.01$ . (E) Red blood cell (RBC) count ( $\times 10^6$ ). \*\*\* $p < 0.001$ . (F) Percentage of sickled red blood cells in PB quantified by ImageStream at 20 weeks post transplantation. "Townes" SCD (homozygous SCD mice) were included as negative control; WT C57BL/6J (Pep Boy/J [CD45.1 congenic]) mice were included as positive control. Only mice from experiment two with >97% BM donor engraftment were included in analysis.  $n = 2$ , WT C57BL/6J;  $n = 2$ , "Townes" SCD;  $n = 4$ , Mock;  $n = 4$ , Lenti/ $\beta$ AS3-FB;  $n = 5$ , Globe-AS3-FB;  $n = 3$ , CoreGA-AS3-FB. Error bars represent mean  $\pm$  SD.

onies were then grouped by their VCN value. The percentages of PCR-positive colonies seen for Lenti/ $\beta$ AS3-FB, Globe-AS3-FB, and CoreGA-AS3-FB were 86%, 87%, and 84%, respectively (Figure 5D).

Persistence of long-term  $\beta^{AS3}$ -globin expression was determined by measuring % $\beta^{AS3}$ -globin for each BFU-E. The VCNs of PCR-positive BFU-Es with detectable endogenous  $\beta$ -globin expression were plotted in Figure 5E. While all experimental arms displayed populations of BFU-Es with >20% $\beta^{AS3}$ -globin, average levels of % $\beta^{AS3}$ -globin/total  $\beta$ -globin mRNA were 22.9%, 24.2%, and 15.8% for Lenti/ $\beta$ AS3-FB, Globe-AS3-FB, and CoreGA-AS3-FB, respectively (Figure 5F). When % $\beta^{AS3}$ -globin expression values of individual CFUs were normalized to their corresponding VCNs, averages of 11.2%, 12.2%, and 7.1%  $\beta^{AS3}$ -globin/VCN were observed for Lenti/ $\beta$ AS3-FB, Globe-AS3-FB, and CoreGA-AS3-FB, respectively (Figure 5G). Taken together, these data demonstrate that long-term and persistent expression was observed in each experimental arm and differences in expression observed in bulk BM cells were reflected at the clonal level.

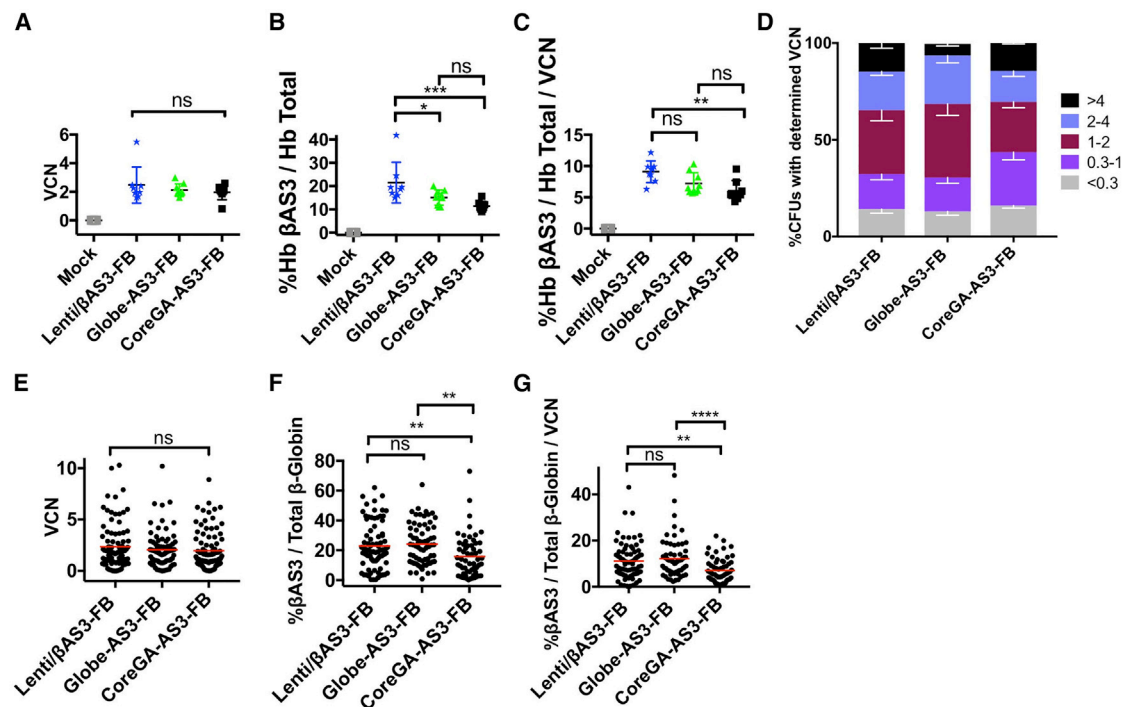
#### Incorporating ENCODE Core Sequences into a Shorter $\beta$ -LV Expression Cassette

The expression studies of CoreGA-AS3-FB were performed to ensure that exchanging the original LCR sequence for the ENCODE core sequences could still provide sufficient expression to ameliorate disease sequelae in the Towne's mouse model of SCD. Because reduction in vector size was determined to be the largest factor influencing

genome between all treatment arms, we normalized % $\beta^{AS3}$ -globin expression to BM VCN for each mouse and plotted in Figure 5C. An average of 9.1%, 7.2%, and 6.1%  $\beta^{AS3}$ -globin/total  $\beta$ -globin/VCN was seen for Lenti/ $\beta$ AS3-FB, Globe-AS3-FB, and CoreGA-AS3-FB, respectively.

Persistence of long-term  $\beta^{AS3}$ -globin expression was determined using a methylcellulose-based CFU assay. BM was taken from two mice from each treatment arm at time of euthanasia and used to seed methylcellulose supplemented with recombinant cytokines that promoted primitive erythroid progenitor cell growth. Two independent experiments were performed. The VCNs of the bulk BM cells used to seed methylcellulose at the time of euthanasia are provided in Table 2. Colonies recognized as BFU-E were plucked 14 days after seeding and both DNA and RNA were obtained from the same colony. The VCN of each colony was determined by ddPCR and col-





**Figure 5. In Vivo Analysis of Bone Marrow  $\beta$ AS3-Globin Expression from SCD Mouse Model**

Whole BM was taken from each mouse at time of euthanasia and mice with >97% donor engraftment were analyzed.  $n = 9$ , Mock;  $n = 8$ , Lenti/ $\beta$ AS3-FB;  $n = 9$ , Globe-AS3-FB;  $n = 8$ , CoreGA-AS3-FB. (A) BM VCN by ddPCR. ns = not significant. (B) Percentages  $\beta$ AS3-globin RNA expression of BM determined by RT-ddPCR. \* $p < 0.05$ ; \*\* $p < 0.01$ . (C) Percentage of BM  $\beta$ AS3-globin RNA expression normalized to BM VCN. Error bars represent mean  $\pm$  SD. (D) BM obtained at time of euthanasia was used to seed a methylcellulose based CFU assay. Individual colonies were plucked 2 weeks after plating and gDNA and RNA extracted. Shown are stacked bar graphs representing percentages of colonies with a determined VCN range. Shown are mean  $\pm$  SEM from two independent experiments ( $n = 2$  donors/arm).  $n = 189$  colonies, Lenti/ $\beta$ AS3-FB;  $n = 129$  colonies, Globe-AS3-FB;  $n = 156$  colonies, CoreGA-AS3-FB. (E) VCN of CFUs from two independent experiments ( $n = 2$  donors/arm); same data as (D). Only PCR-positive CFUs with detectable endogenous globin expression were included.  $n = 93$  colonies, Lenti/ $\beta$ AS3-FB;  $n = 84$  colonies, Globe-AS3-FB;  $n = 94$  colonies, CoreGA-AS3-FB. Each dot represents a single colony. Bar represents grand average of VCN from two independent experiments. (F) Percent  $\beta$ AS3-globin RNA expression was determined by RT-ddPCR. \*\*\*\* $p < 0.0001$ . (G) Percent  $\beta$ AS3-globin RNA expression normalized to VCN for matched colony. \*\*\* $p < 0.001$ . (H) Percentage of sickled RBCs in PB quantified by ImageStream at 20 weeks post transplantation. "Townes" SCD (homozygous SCD mice), included as negative control; WT C57BL/6J (Pep Boy/J [CD45.1 congenic]) mice are included as a positive control. Only mice from experiment two with >97% BM donor engraftment were included in analysis.  $n = 2$ , WT C57BL/6J;  $n = 1$ , "Townes" SCD;  $n = 4$ , Mock;  $n = 4$ , Lenti/ $\beta$ AS3-FB;  $n = 5$ , Globe-AS3-FB;  $n = 3$ , CoreGA-AS3-FB.

titer and gene transfer, we made further modifications to the CoreGA-AS3-FB to remove approximately 2 kb of sequence from the provirus. The main deletions were of the WPRE sequence,  $\beta$ -globin 3' UTR and IVS2 (matching those found in the Globe-AS3-FB). The resulting  $\sim 4.7$  kb UV-AS3-FB "Ultimate Vector" (shown in Figure 6A) has a titer that is 3-fold higher than CoreGA-AS3-FB (Figure 6B). Gene transfer is also improved 3-fold compared to CoreGA-AS3-FB (Figure 6C) and the expression remains identical (Figure 6D).

## DISCUSSION

The requirements to be met for a gene therapy vector to be considered as a candidate for clinical translation include high-titer, efficient, and stable proviral transmission, high-level, lineage-specific, and persistent expression, and safe integration profile. The results presented herein demonstrate that some of the major hurdles that have historically restricted clinical translation of  $\beta$ -globin gene therapy vectors for treatment of SCD can be surmounted through vector engineering.

One predominant factor that diminishes  $\beta$ -LV titer is suspected to be the relatively large size of these vectors due to the use of the human  $\beta$ -globin genomic sequences and LCR elements. Both Kumar et al.<sup>25</sup> and Barrett et al.<sup>26</sup> demonstrated that titers of LVs decrease proportionally to the length of insert irrespective of sequence. We confirmed that vector length was the major factor contributing to the difference in titer observed between Lenti/ $\beta$ AS3-FB and Globe-AS3-FB. Moreover, introduction of additional sequence deletions to Lenti/ $\beta$ AS3-FB offered increasing gains in titer and infectivity confirming that vector length strongly influenced titer and infectivity. Although the dissection revealed that vector length could be reduced by  $\sim 1$  kb by removing redundant sequences (see Supplemental Information), that level of size reduction fell short of our 2–3 kb target.

The critical minimal segments of the  $\sim 20$  kb human  $\beta$ -globin LCR needed for high-level, position-independent, erythroid expression of  $\beta$ -globin were elucidated using transgenic mouse models

**Table 2. VCNs of Bulk BM Cells Used to Seed Methylcellulose Based CFU Assay**

	Lenti/ $\beta$ AS3-FB	Globe-AS3-FB	CoreGA-AS3-FB
<i>In vitro</i> CFU experiment 1	1.6, 1.9	1.9, 1.36	1.9, 2.1
<i>In vitro</i> CFU experiment 2	2.4, 2.6	2.3, 1.9	2.0, 2.6

that, at the time, relied on a limited repertoire of restriction enzymes to introduce sequence deletions to the hypersensitive sites.<sup>34–39</sup> The findings of these studies informed the development of current first generation  $\beta$ -globin lentiviral vectors, which were engineered to contain specific LCR fragments that confer erythroid-specific expression of  $\beta$ -globin.<sup>40–44</sup> Given the methods that were deployed to define the boundaries of the LCR HS elements, we hypothesized that these fragments included core sequences that bestow erythroid specific expression, but also some extraneous sequences that could be eliminated with minimal decrease in enhancer activity. Therefore, emphasis was given to develop a strategy for reducing the length of the LCR HS elements recognizing potential risks for reducing overall expression per vector genome.

When designing the new core HS elements, several genetic and epigenetic factors were considered, including the sites binding histones associated with active genes, DNaseI hypersensitivity in erythroid lineages, and sequence conservation. Using this design approach, the overall lengths of combined HS elements 2, 3, and 4 were reduced from 3.6 kb to 1.2 kb. Regulatory elements from the erythroid-specific murine *Gata1* and human *ANK1* genes were added to aid in position-independent expression.

When compared to the standard Lenti/ $\beta$ AS3-FB design, we found that the CoreGA-AS3-FB was produced at nearly 3-fold higher titer ( $p < 0.0001$ ), possessed nearly 3-fold higher gene transfer to CD34+ HSPCs at the same MOI as for standard vectors ( $p < 0.01$ ), and expressed nearly 60% of the amount of  $\beta^{\text{AS3}}$ -globin transcripts per vector genome in CD34+ HSPCs cultured under erythroid conditions ( $p < 0.001$ ). These observations of improved titer and transduction efficiency taken together spurred the continued study of CoreGA-AS3-FB to determine whether the observed difference in expression when compared to Lenti/ $\beta$ AS3-FB was relevant to a clinical scenario.

Previous reports examining SCD patients who co-inherit the condition of hereditary persistence of fetal hemoglobin (where the distribution of HbF is thought to be pancellular) indicate that anti-sickling globin protein levels greater than 10%–15% can ameliorate the clinical sequelae of SCD.<sup>45–49</sup> When CoreGA-AS3-FB was evaluated in a mouse model of SCD at an average BM VCN = 2,  $\beta^{\text{AS3}}$ -globin RNA comprised an average of 12% of the total  $\beta$ -globin transcripts and Hb $\beta^{\text{AS3}}$  tetramers made-up 18.5% of the total hemoglobin tetramers. The finding that the percentage of Hb $\beta^{\text{AS3}}$  tetramers exceeded the relative abundance of  $\beta^{\text{AS3}}$ -globin mRNA is consistent with the increased affinity for  $\alpha$ -globin of the  $\beta^{\text{AS3}}$ -globin

protein due to the G16D amino acid substitution.<sup>32,50</sup> Thus, the *in vivo* percentages of  $\beta^{\text{AS3}}$ -globin RNA transcripts and protein seen for CoreGA-AS3-FB are at levels expected to be therapeutic, as the  $\beta^{\text{AS3}}$ -globin gene has been shown to have equivalent anti-sickling activity as  $\gamma$ -globin.<sup>33</sup>

A methylcellulose-based CFU assay using BM cells obtained from mice 20 weeks after transplantation also revealed the presence of BFU-Es with >20%  $\beta^{\text{AS3}}$ -globin in all experimental arms. This finding suggested that populations of corrected HSPCs capable of contributing to long-term erythropoiesis were established by each vector. More importantly, when murine HSPCs were transduced with CoreGA-AS3-FB at lower MOI (in comparison to those MOIs used to transduce cells with Lenti/ $\beta$ AS3-FB or Globe-AS3-FB), CoreGA-AS3-FB transduced similar percentages of colony-forming progenitors resulting in similar bulk VCNs. These data suggest that CoreGA-AS3-FB has superior infectivity in comparison to Lenti/ $\beta$ AS3-FB and Globe-AS3-FB, exemplified by its transduction efficiency even when constrained by using less vector.

The most critical pre-clinical test of any novel  $\beta$ -LV design being considered as a candidate for clinical translation is amelioration of hematologic parameters defining the pathological phenotype of a SCD mouse model. While the amounts of  $\beta^{\text{AS3}}$ -globin expressed per VCN by CoreGA-AS3-FB were lower when compared to the other vectors, the levels were sufficient to result in physiologic improvements in the signs of SCD in the mice, with significant increases in both RBC count and hemoglobin content in comparison to recipients of mock-transduced BM.

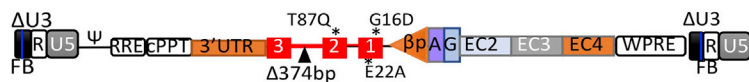
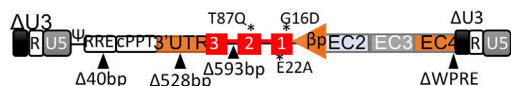
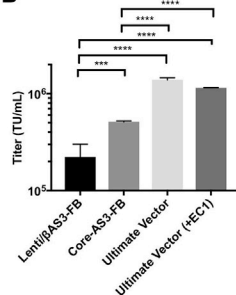
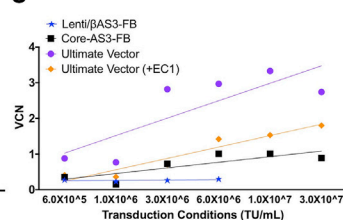
When the quantity of sickled RBCs were measured in PB at time of euthanasia from a subset of representative mice, a higher amount of sickling was seen in PB of CoreGA-AS3-FB treated mice. This observation may have resulted from transplantation of a larger number of non-transduced cells into recipient mice; a result of using less vector.

Incorporation of the ENCODE core sequences from CoreGA-AS3-FB into the approximately 2 kb shorter UV-AS3-FB resulted in a superior titer and gene transfer. The UV-AS3-FB was designed after the *in vivo* studies were complete and will be the subject of a future submission. This new lentiviral vector design with reduced sizes of the LCR components and expression cassette should have significant advantages for clinical-scale production by providing far higher level of gene transfer to HSPC from lower amounts of vector. It may extend the benefits of autologous gene therapy for SCD by significantly reducing the cost of vector production and extending the vector supply.

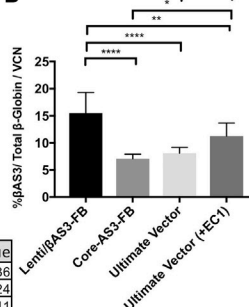
## MATERIALS AND METHODS

### Cloning and Vector Production

The Lenti/BAS3-FB vector has been described previously.<sup>19</sup> To introduce desired sequence deletions, we used sets of reverse-oriented primers to PCR amplify the Lenti/BAS3-FB plasmid backbone and then phosphorylated and ligated the resultant linearized plasmids.

**A CoreGA-AS3-FB (6.9Kb)****Ultimate Vector (4.7Kb)****B****C**

Comparison of Slopes		(*)	p-Value
Lenti/βAS3-FB vs. Core-AS3-FB	ns		0.2836
Lenti/βAS3-FB vs. Ultimate Vector	**		0.0024
Lenti/βAS3-FB vs. Ultimate Vector (+EC1)	*		0.0341
Core-AS3-FB vs. Ultimate Vector	*		0.0244
Core-AS3-FB vs. Ultimate Vector (+EC1)	ns		0.2450
Ultimate Vector vs. Ultimate Vector (+EC1)	ns		0.2194

**D**

To seamlessly introduce sequences in place of deletions, we ordered gBlock gene fragments (Integrated DNA Technologies, Skokie, IL) containing homology to the plasmid backbone and joined them to linearized plasmids using the NEBuilder HiFi DNA Assembly kit (New England Biolabs, Ipswich, MA). All plasmids were sequence verified by Sanger sequencing (Laragen, Culver City, CA). The murine Gata1 element was from bp 438–639 (GenBank: U89137.1) and the human *ANK1* element is from bp 103987–104129 (NCBI: NG\_012820.2). The UV-AS3-FB vector was created by using NEBuilder HiFi DNA Assembly kit to fuse together PCR amplified EC2 from CoreGA-AS3-FB to gBlock gene fragments for the: cPPT, β-globin expression cassette, EC3, EC4, 3' long terminal repeat (LTR) with polyadenylation enhancements (BGH polyA), and SV40 origin of replication.

Transient transfection of 293T cells using the third-generation packaging system<sup>51</sup> provided packaged virus particles. Viral supernatants were then directly used for titer determination or concentrated by tangential flow filtration, as described by Cooper et al.<sup>12</sup> Briefly, the HT-29 human colorectal carcinoma cell line was transduced with different dilutions of both raw and concentrated vectors. To calculate titers, we harvested cells and determined VCNs by ddPCR approximately 60 h post transduction.

**BM CD34+ Cell Culture and Transduction**

All BM aspirates were obtained from voluntary healthy donors supplied by AllCells (Alameda, CA), which obviated the need for Institutional Review Board review. BM mononuclear cells were isolated

**Figure 6. Size Reduction Improves Vector Performance**

(A) Diagrams of lentiviral vector constructs. All annotations are similar to those shown in Figure 1A. (B) Quantity of infectious particles of constructs when packaged and titered in parallel.  $n = 3$ . \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ . (C) Human CD34+ HSPCs were at various MOIs and cultured under myeloid culture conditions for 14 days. VCN was determined by ddPCR. (D) Human CD34+ HSPCs were transduced in parallel at  $2.0 \times 10^7$  TU/mL and cultured under erythroid culture conditions for 14 days. Expression of different constructs is presented as percentage of βAS3-globin normalized to VCN.  $n = 12$ , Lenti/βAS3-FB;  $n = 3$ , Core-AS3-FB;  $n = 9$ , Ultimate Vector;  $n = 9$ , Ultimate Vector (+EC1). Error bars represent mean  $\pm$  SD.

by Ficoll-Hypaque density gradient centrifugation. CD34+ HSPCs were enriched using CD34+ MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Enriched CD34+ HSPCs were cryopreserved in fetal bovine serum supplemented with 10% DMSO (Sigma-Aldrich, St. Louis, MO) in liquid nitrogen. Cells were thawed and plated on non-tissue culture-treated six-well plates pre-coated with Recto-Nectin (20  $\mu$ g/mL, Takara Shuzo, Otsu Japan) at  $1 \times 10^6$  cells/mL. Cells were pre-stimulated for 16–24 h in X-Vivo 15 medium (Lonza, Basel, Switzerland) supplemented with  $1 \times$  glutamine, penicillin, and streptomycin (Gemini Bio-Products, Sacramento, CA), human stem cell factor (50 ng/mL), human Flt-3 ligand (50 ng/mL), human thrombopoietin (50 ng/mL), and human interleukin-3 (20 ng/mL; all cytokines were acquired from PeproTech, Rocky Hill, NJ). Concentrated viral supernatants were used at various MOI to transduce CD34+ HSPCs for 24 h. These cells were washed, re-plated, and cultured under myeloid or erythroid culture conditions, as described by Romero et al.<sup>19</sup> On day 14 of culture, genomic DNA and/or mRNA was extracted from transduced cells.

**ddPCR for VCN and %β<sup>AS3</sup> mRNA Quantification**

Genomic DNA was extracted using PureLink Genomic DNA Mini Kit (Invitrogen, Waltham, MA). VCN was calculated by using probes SCD4 (Human Syndecan 4) as a reference and HIV-1 PSI as a target. ddPCR was carried out as described in Urbinati et al.<sup>6</sup> RNeasy Plus Mini Kit (QIAGEN, Valencia, CA) was used for RNA extraction followed by reverse transcription as described by Urbinati et al.<sup>6</sup> Probes HBB<sup>TOTAL</sup> as a reference and Hbβ<sup>AS3</sup> as a target were used to generate droplets for ddPCR, as described by Hindson et al.<sup>52</sup> Droplets were analyzed for absolute quantification of the β<sup>AS3</sup> gene expression normalized to the total B-globin gene expression.

**In Vivo Experiment in SCD Mouse Model**

All mouse work was performed under protocols approved by the UCLA Animal Care Committee. BM from 8- to 12-week-old

homozygous  $\beta^S/\beta^S$  “Townes” mice (JAX stock #013071) were lineage-depleted using the lineage cell depletion kit from Miltenyi Biotec. Lin<sup>−</sup> cells were pre-stimulated for 24 h in StemSpan (Stem Cell Technologies, Vancouver, Canada) supplemented with murine stem cell factor (100 ng/mL), human interleukin-11 (100 ng/mL), murine interleukin-3 (20 ng/mL), and human FLT-3 ligand (100 ng/mL). Pre-stimulated Lin<sup>−</sup> cells were then transduced at various MOIs to obtain similar VCNs in the bulk cell product or mock transduced. Twenty-four h later, one to two million transduced cells were delivered by retro-orbital injection after recipient mice (*Pep Boy/J* [CD45.1 congenic]) were lethally irradiated (1,075 cGy, split in two fractions). A portion of the transduced cells was cultured for 2 weeks *in vitro* under myeloid differentiation conditions to determine VCN in the cell product.

Peripheral blood samples were collected at weeks 4, 16, and 20 to measure VCN of engrafted cells by ddPCR, expression of Hb $\beta^{AS3}$  hemoglobin by HPLC, and to determine RBC indices. At week 20, mice were euthanized and BM cells were used to measure engraftment by flow cytometry (CD45.2/CD45.1), VCN, and expression and to seed a methylcellulose based CFU assay. The percentages of sickled erythrocytes present within peripheral blood were also quantified using the Amnis ImageStream Mark II Imaging Flow Cytometer (Luminex, Austin, TX) (as described below).

### HPLC

To characterize and quantify hemoglobin tetramers, including human HbS and Hb $\beta^{AS3}$ , and murine HbA and HbF, we lysed 1  $\mu$ L of murine peripheral blood in 25  $\mu$ L hemolysate and incubated at room temperature. Hemolysates were then centrifuged at 500 g for 10 minutes at 4°C to remove RBC ghost. The lysates were then stored frozen at −80°C and later thawed and processed as described by Urbinati et al.<sup>6</sup>

### CFU Progenitor Assay

To quantify VCN range and production of BAS3-globin transcripts in individual BM progenitor cells, we acquired total murine BM from transduced or mock-transduced  $\beta^S/\beta^S$  mice and plated it in a methylcellulose medium that supports murine BFU-E growth (Methocult SF M3436; Stem Cell Technologies), as described by Urbinati et al., 2017.<sup>6</sup> Cells were plated at three densities from a serial dilution: 20,000, 60,000, and 120,000 cells per 35 mm gridded plate with two plates per each density. After 14 days of culture at 37°C supplemented with 5% CO<sub>2</sub>, individual BFU-E colonies were enumerated, plucked, and separated into two portions for DNA and mRNA isolation (NucleoSpin Tissue XS; Clontech Laboratories, Mountain View, CA).

### Cell Morphology Determination and Quantification

The Amnis ImageStream Mark II Imaging Flow Cytometer was used to determine and quantify the morphologies of RBCs isolated from experimental mice at the time of euthanasia. A 2% solution of sodium metabisulfite was made fresh using ultra-distilled water (Invitrogen, Carlsbad, California). 5  $\mu$ L of blood was mixed with 5  $\mu$ L of 2% sodium metabisulfite solution (MBS) and incubated under hypoxic

conditions (2% oxygen) for 10 min at 37°C. After incubation, 5  $\mu$ L of the 1:1 blood and MBS solution was resuspended in 95  $\mu$ L of a 2% glutaraldehyde solution. The 2% glutaraldehyde solution was made by diluting 25% stock glutaraldehyde solution (Sigma-Aldrich, St. Louis, MO) in PBS. Cell images were collected on the ImageStream Flow Cytometer at 60 $\times$  magnification with bright-field set to channel four and all lasers off. About 40,000 images were collected for each sample and then analyzed using the IDEAS software (Amnis Corporation, Seattle, WA) using a custom template designed with the assistance of Amnis Corporation.

### Statistical Analysis

All data are reported as mean  $\pm$  SD unless otherwise stated. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, California, USA). The statistical significance between two averages was established using unpaired t test. When the statistical significance between three or more averages were evaluated, a one-way ANOVA was applied followed by multiple paired comparisons for normally distributed data (Tukey test). When normality assumption was violated, Mann-Whitney U test was performed for group-wise comparison instead. Chi-square tests were used to compare the frequency of transduced CFUs. Linear regression analyses were used to determine the correlation between VCN and  $\beta^{AS3}$ -globin RNA transcripts quantities. All statistical tests were two-tailed and a p value of < 0.05 was deemed significant.

### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.ymthe.2019.09.020>.

### AUTHOR CONTRIBUTIONS

R.A.M., R.P.H. and D.B.K. conceived and designed all experiments. R.A.M. executed and analyzed all experiments. M.J.U., B.A., R.O.B., K.S.O., C.K., O.B.S., S.S., B.A., D.B., M.R., J.P.Q., P.G.A., and E.M. helped execute portions of experiments. R.A.M. and R.P.H. provided research materials. R.P.H. and D.B.K. advised experiments. R.A.M. and D.B.K. provided financial and administrative support. R.A.M., R.P.H., and D.B.K. wrote the manuscript. R.A.M. and D.B.K. approved the final manuscript.

### CONFLICTS OF INTEREST

The authors declare no competing interests.

### ACKNOWLEDGMENTS

This work was supported by a Sponsored Research Agreement from Biomarin Pharmaceutical, Inc. Training grants provided support to R.A.M. (NIH/NHLBI F31 HL134313; PhRMA Foundation Paul Calabresi Predoctoral Fellowship 20174717; and UCLA MSTP T32 GM008042). The Flow Cytometry Core and the DNA Sequencing Core of the UCLA Eli & Edythe Broad Center of Regenerative Medicine and Stem Cell Research and the Jonsson Comprehensive Cancer Center were used to support studies.



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