β-globin gene transfer to human bone marrow for sickle cell disease

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Autologous hematopoietic stem cell gene therapy is an approach to treating sickle cell disease (SCD) patients that may result in lower morbidity than allogeneic transplantation. We examined the potential of a lentiviral vector (LV) (CCL-βAS3-FB) encoding a human hemoglobin (HBB) gene engineered to impede sickle hemoglobin polymerization (HBBAS3) to transduce human BM CD34+ cells from SCD donors and prevent sickling of red blood cells produced by in vitro differentiation. The CCL-βAS3-FB LV transduced BM CD34+ cells from either healthy or SCD donors at similar levels, based on quantitative PCR and colony-forming unit progenitor analysis. Consistent expression of HBBAS3 mRNA and HbAS3 protein compromised a fourth of the total β-globin–like transcripts and hemoglobin (Hb) tetramers. Upon deoxygenation, a lower percentage of HBBAS3-transduced red blood cells exhibited sickling compared with mock-transduced cells from sickle donors. Transduced BM CD34+ cells were transplanted into immunodeficient mice, and the human cells recovered after 2–3 months were cultured for erythroid differentiation, which showed levels of HBBAS3 mRNA similar to those seen in the CD34+ cells that were directly differentiated in vitro. These results demonstrate that the CCL-βAS3-FB LV is capable of efficient transfer and consistent expression of an effective anti-sickling β-globin gene in human SCD BM CD34+ progenitor cells, improving physiologic parameters of the resulting red blood cells.

Introduction

Sickle cell disease (SCD) is one of the most common monogenic disorders worldwide and is a major cause of morbidity and early mortality (1). Although SCD is well characterized, there is still no ideal long-term treatment. Current therapies are based on induction of fetal hemoglobin (Hbf) to inhibit polymerization of sickle hemoglobin (Hbs) (2) and cell dehydration (3) or reduction of the percentage of Hbs by transfusions (4). Allogeneic HSC transplantation (HSCT) from BM or umbilical cord blood (UCB) is a potentially curative therapy, although only a small percentage of patients have undergone this procedure, mostly children with severe symptoms who had HLA-matched sibling donors (5–7). Transplantation of allogeneic cells carries the risk of graft-versus-host disease (GvHD), which can be a cause of extensive morbidity. HSCT using UCB from matched unrelated donors holds reduced risk of acute or chronic GvHD compared with using BM; however, there is a higher probability of engraftment failure using UCB as a result of its lower cell dose and immunologic immaturity (8, 9).

Gene therapy with autologous HSCs is an alternative to allogeneic HSCT, since it avoids the limitations of finding a matched donor and the risks of GvHD and graft rejection. For gene therapy application in SCD patients, the safest source for autologous HSC would be BM, due to the complications previously described when G-CSF was used to collect autologous peripheral blood stem cells (PBSCs) in SCD patients (10–12). Although general anesthesia imposes a risk for SCD patients as well, current best medical practices can minimize these (13).

The development of integrating vectors for β-globin gene transfer has been challenging due to the complex regulatory elements needed for high-level, erythroid-specific expression (14). γ-Retroviral vectors were unable to transfer these β-globin expression cassettes intact (15, 16); in contrast, lentiviral vectors (LV) can transfer β-globin cassettes intact with relatively high efficiency, although the titers of these vectors are reduced compared with those of vectors bearing simpler cassettes (17, 18). In the last decade, many groups have developed different β-globin LV for targeting β-hemoglobinopathies, with successful therapeutic results following transplantation of ex vivo–modified HSC in mouse models (17–23).

Sickle patients with hereditary persistence of Hbf (HPFH) have improved survival and amelioration of clinical symptoms, with maximal clinical benefits observed when the Hbf is elevated above threshold values (e.g., 8%–15% of the total cellular Hb) (2, 24). Therefore, some gene therapy strategies have employed viral vectors carrying the human γ-globin gene (HGB1/2). However, these constructs expressed Hbf poorly in adult erythroid cells,
since fetal-specific transcription factors are required for high-level expression of the γ-globin gene (25, 26). These limitations have been overcome by embedding the exons encoding human γ-globin within the human β-globin gene 5′ promoter and 3′ enhancer elements (20, 27, 28). Breda et al. (29) used an LV vector encoding the human hemoglobin (HBB) gene to increase the expression of normal HbA in CD34+ derived erythroid cells from SCD patients; however, the expression level needed when the HBB gene is used would be higher than would be required for HBG1/2 gene expression to achieve therapeutic benefits in SCD patients.

Another approach is to modify β-globin genes to confer anti-sickling activity by substituting key amino acids from γ-globin; the modified β-globin cassette should yield the necessary high-level, erythroid-specific expression in adult erythroid cells. Pavliuk et al. (18) designed an LV carrying a human β-globin gene with the amino acid modification T87Q; the glutamine at position 87 of γ-globin has been implicated in the anti-sickling activity of HbF (30). This anti-sickling construct corrected SCD in 2 murine models of the disease, and a similar LV has been used in a clinical trial for β-thalassemia and SCD in France (31).

Townes and colleagues have taken a similar approach, developing a recombinant human anti-sickling β-globin gene (HBBAS3) encoding a β-globin protein (HbAS3) that has 3 amino substitutions compared with the original (Hba): T87Q; the glutamate at position 87 of γ-globin has been implicated in the anti-sickling activity of HbF (30). This anti-sickling construct corrected SCD in 2 murine models of the disease, and a similar LV has been used in a clinical trial for β-thalassemia and SCD in France (31).

The goal of this study was to characterize the capacity of a β-AS3 LV (CCL-βAS3-FB) to transduce human BM-derived CD34+ cells from SCD donors for potential use in a clinical trial of gene therapy for SCD. This vector achieved efficient transduction of BM CD34+ cells from healthy or SCD donors. To assess the erythroid-specific expression of the HBBAS3 gene and its anti-sickling properties, we used an in vitro model of erythroid differentiation to produce mature erythroid cells from human BM CD34+ cells (34). We assessed the gene expression activity of the CCL-βAS3-FB at the mRNA and protein levels, characterized the effects of HBBAS3 expression on sickling of deoxygenated rbc, and performed an in vitro assay to detect potential genotoxicity. Transduced BM CD34+ cells were also xenografted into immunodeficient mice, and human hematopoietic progenitor cells were reisolated from the marrow of the mice after 2 to 3 months, subjected to in vitro erythroid differentiation, and found to continue to express the anti-sickling HBBAS3 gene. These results demonstrate the capability of the CCL-βAS3-FB LV to efficiently transduce BM CD34+ progenitor cells and produce sufficient levels of an anti-sickling Hb protein to improve the physiological parameters of the rbc that may be applied for clinical gene therapy of SCD.

**Results**

The CCL-βAS3-FB LV vector carrying the HBBAS3 cassette. The original LV produced by Levasseur et al. (19) to carry the HBBAS3 cassette (DL-βAS3) contained the intact HIV 5′ LTR, which engenders dependence on the HIV TAT protein for production of high-titer vector. To eliminate the need for TAT during packaging, we moved the HBBAS3 cassette plus the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) to the pCL LV backbone (35), which is a SIN vector with the CMV enhancer/promoter substituted in the 5′ LTR, eliminating the need for TAT. This pCL backbone was further modified to have a compact (77 bp) insulator in the U3 region of the 3′ LTR, denominated FB, which contains the minimal CTCF binding site (FII) of the 250-bp core of the 1.2-kb chicken β-globin HS4 (cHS4) insulator and the analogous region of the human T cell receptor δ/α BEAD-1 insulator (36). The resulting SIN-LV was named CCL-βAS3-FB, and the proviral form is shown in Figure 1A.

In 3 independent experiments, we packaged preparations of the CCL-βAS3-FB vector as well as a version lacking the FB insulator (CCL-βAS3), the parental DL-βAS3 vector, and a vector expressing the enhanced GFP (CCL-MND-GFP) as a positive control. The vector preparations were made with and without inclusion of a plasmid that expressed the HIV-1 TAT protein. The titers were determined by transducing a permissive cell line (HT29 human colon carcinoma) and measuring vector copies (VC)/cell using quantitative PCR (qPCR) with primers to the HIV packaging signal (PsI) of the vector proviruses (ref. 37 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JC167930D1). The CCL-βAS3-FB vector as well as the noninsulated version could be produced in the absence of TAT to a 10-fold higher titer than the original DL-βAS3 vector (P = 0.017, 2-tailed t test; CCL-βAS3 and CCL-βAS3-FB combined compared with the DL-βAS3), and inclusion of the FB insulator did not decrease vector titer.

The stability of the FB insulator was evaluated by PCR analysis of the FB-containing fragment size in bulk populations of transduced BM CD34+ cells (Figure 1B) and at a clonal level (a total of 32 single CFU colonies; data not shown). All samples showed the expected sizes of single bands after PCR analysis, demonstrating intact passage of the FB insulator. Additionally, Southern blot analysis of CCL-βAS3-FB–transduced cells showed the presence of a single band of the size expected for full-length vector provirus (Supplemental Figure 2).

To evaluate the functional activity of the FB insulator, binding of the CTCF protein to the LTRs of the CCL-βAS3-FB was assessed by ChIP in transduced KS62 cells (Figure 1C). ChIP indicated a 12-fold enrichment of CTCF binding in the CCL-βAS3-FB LTR when compared with the input control; no enrichment was found with the CCL-βAS3 vector lacking the FB insulator, indicating the specific binding of the CTCF to the FB sequence. The association with CTCF to the CCL-βAS3-FB LTR was at least as high as with other sequences known to bind CTCF, such as the 1.2-kb cHS4 insulator (38), the c-Myc promoter (39), or the H19 imprinting control region (40).

Assessment of transduction and hematopoietic potential of BM CD34+ cells. Preliminary dose-response experiments were performed to determine the most efficient concentration of the CCL-βAS3-FB vector to transduce human BM CD34+ cells, using a range of vector concentrations during transduction from 2 × 106 to 2 × 107 transduction units/ml (TU/ml) (MOI = 4–400). A dose-related increase in gene transfer achieved (the average VC/cell measured by qPCR) was found only for vector concentrations below 2 × 107 TU/ml. Higher vector concentrations did not increase the transduction efficacy and, in fact, often had a negative effect on
the extent of transduction (data not shown). Based on these findings, the CCL-βAS3-FB vector was used at a standard concentration of $2 \times 10^7$ TU/ml (MOI = 40) for all subsequent studies.

The colony-forming capacities of BM CD34+ cells were similar for samples from SCD donors or healthy donor (HD) controls, whether transduced with the CCL-βAS3-FB vector or not, with approximately 10% of cells forming colonies when plated in methylcellulose, without significant differences between groups (in all the groups compared, $P > 0.1$, by 2-way ANOVA) (Figure 2A). We noted higher percentages of burst-forming unit erythroid (BFU-E) (erythroid) colonies in SCD samples (41.34% ± 19.87% in SCD-mock and 42.33% ± 17.79% in SCD-βAS3-FB) compared with HD samples (30.67% ± 17.06% in HD-mock and 28.62% ± 12.91% in HD-βAS3-FB) ($P = 0.048$, by 2-way ANOVA) (Figure 2B). Similar erythroid skewing of progenitor cells from the BM of SCD patients has been reported (41) and may reflect the increased level of erythropoiesis in SCD patients due to the underlying hemolytic anemia.

qPCR of individual CFU to detect the CCL-βAS3-FB vector sequences demonstrated the percentage of transduced colony-forming progenitor cells from SCD donor BM. Fifty-seven of 191 colonies contained the CCL-βAS3-FB vector (29.84% ± 16.68% positive colonies in 5 independent experiments) with an average of $0.92 \pm 0.57$ VC/cell in the bulk population cultured in vitro in erythroid differentiation conditions. Most of the vector-positive colonies analyzed had 1 to 2 VC/cell (88%), while 11% had 3 to 6 VC/cell and 2% had 7 to 9 VC/cell (no colony had more than 9 copies) (Figure 2C).

After 2 weeks of culture under in vitro erythroid differentiation conditions, transduction of CD34+ cells from HD ($n = 11$) led to $1.28 \pm 0.51$ VC/cell compared with $0.93 \pm 0.37$ for SCD donors ($n = 15$), which was borderline significantly different ($P = 0.05$, Wilcoxon rank sum test) (Figure 2D).

**Figure 1**
The CCL-βAS3-FB LV provirus carrying the HBBAS3 cassette. (A) The CCL-βAS3-FB LV provirus has the HBBAS3 expression cassette with the human β-globin gene exons (arrowheads) with the 3 substitutions to encode the HbAS3 protein, introns, the 3′ and 5′ flanking regions, and the β-globin mini-locus control region (LCR) with hypersensitive sites 2–4. The 3′ LTR contains the SIN deletion and FB insulator, both transferred during RT to the 5′ LTR of the proviral DNA. (B) To test FB insulator stability, PCR reactions were performed using DNA from cells collected at day 14 of in vitro culture of BM CD34+ cells: mock transduced (lane 1), transduced with the CCL-βAS3 LV (lane 2), and transduced with the CCL-βAS3-FB LV (lane 3). Primers amplified either the 5′ LTR (A to B) or the 3′ LTR (C to D) or the FB insertion sites in both LTRs (A to D) of the provirus. The expected sizes of the PCR products with these primer pairs are indicated for the CCL-βAS3 LV and the CCL-βAS3-FB LV. NTC, no template control. (C) CTCF-binding protein ChIP. Chromatin was isolated from K562 cells transduced with the CCL-βAS3-FB LV (FB), the CCL-βAS3-1.2 kb cHS4 LV (cHS4), or the CCL-βAS3 vector lacking the insulator (U3). qPCR amplification was done using primers to the HIV SIN LTR (U3, cHS4, and FB) and to the HIV RRE region of the vector backbone (RRE) as negative control or the cellular c-Myc and H19/ICR sites, known to bind CTCF. *$P = 0.006$. Values shown are mean ± SD.
transduced with the CCL-βAS3-FB LV or mock transduced (Figure 3A shows a representative experiment). Expansion of cell numbers up to 700-fold was reached by the end of the culture.

Flow cytometry was performed during erythroid differentiation culture to analyze the changes in markers of hematopoietic progenitors (CD34 and CD45) and erythroid progenitors (glycophorin A [GpA] and CD71). The percentages of CD34+ cells was analyzed after isolation, showing an average of 76.74% ± 3.01% of CD34+ cells. High variability in CD34 expression was observed after 3 days in culture between the different donors, with a sharp decline of CD34 expression between days 3 and 14 in all the samples (Figure 3B). The pan-leukocyte marker CD45 was expressed by the entire cell population at day 3 and became essentially undetectable between days 14 and 21, as expected for reticulocytes and mature rbc (43). CD71 (transferrin receptor) was expressed during the early part of the culture period (days 3 to 14), but decreased by the end of culture period as expected (day 21). GpA expression was detected on more than 90% of the cells by day 14 and persisted until the end of the culture.

Enucleated rbc were identified at the end of the differentiation (days 18 to 21) by double staining with an antibody to the erythroid membrane glycoprotein GpA and the fluorescent dye DRAQ5, which labels DNA; enucleated rbc were defined as being GpA+DRAQ5-. The frequency of enucleated rbc among multiple cultures ranged from 65% to 85%: 67.61% ± 17.68% in SCD-mock (n = 7), 69.69% ± 18.11% in SCD-βAS3-FB (n = 7) (Figure 3B), 83.40% ± 10.07% in HD-mock (n = 7) and 79.04% ± 10.19% in HD-βAS3-FB (n = 3), without significant differences between mock-transduced
and LV-transduced samples (SCD mock vs. βAS3-FB, P = 0.80; HD mock vs. βAS3-FB, P = 0.69, by 2-way ANOVA). The large-cell expansion and robust erythroid differentiation with high levels of enucleation (Figure 3, C and D) supported the further analyses to characterize the activity of the HBBAS3 transgene.

**HBBAS3 mRNA expression after in vitro erythroid differentiation of BM CD34+ cells.** The successful production of rbc from BM CD34+ cells plus the confirmation of efficient gene transfer allowed us to evaluate the function of the HBBAS3 cassette. HBBAS3 mRNA expression levels in cells collected on day 14 from in vitro erythroid differentiation cultures of SCD donor and HD BM CD34+ cells, either transduced with the CCL-βAS3-FB LV or mock transduced, were assessed by a qRT-PCR assay and compared with mRNA levels from the endogenous HBB and HBB5 (HBB gene carrying the sickle mutation) genes. HBBAS3 mRNA levels made up 15.73% ± 8.36% and 17.12% ± 7.25% of total β-globin–like mRNA in erythroid cells from cultures of SCD and HD BM CD34+ cells, respectively. For each CCL-βAS3-FB LV-transduced BM sample analyzed (SCD and HD), the percentage of HBBAS3 mRNA detected was compared with the VC/cell obtained by qPCR from that sample. There was a strong positive correlation between VC/cell and the percentage of HBBAS3 mRNA (Pearson correlation = 0.73, P = 0.0003), indicating consistent expression (Figure 4A). When normalized to VC/cell to adjust for variable gene transfer, the average HBBAS3 mRNA expression per VC/cell, was 26.22% ± 10.71% in SCD and 17.84% ± 11.60% in HD cells. On average, from all the samples studied (n = 20, 16 samples for SCD and 4 for HD) HBBAS3 mRNA comprised 24.55% ± 11.03% per VC/cell.

Finally, we assessed the erythroid specificity of expression of the HBBAS3 cassette by analyzing HBBAS3 mRNA expression in CCL-βAS3-FB LV-transduced BM CD34+ cells divided into parallel cultures under myeloid and erythroid differentiation conditions. We found a higher expression of HBBAS3 mRNA in cells produced under erythroid conditions compared with myeloid conditions, which was essentially unmeasurable (Supplemental Figure 3).

**HbAS3 protein expression after in vitro erythroid-differentiation of BM CD34+ cells.** We used isoelectric focusing (IEF) to examine the Hb tetramers present in erythroid cells produced in vitro from BM CD34+ cells transduced with the CCL-βAS3-FB LV. Despite the 3 amino acid differences, HbAS3 tetramers cannot be distinguished...
from HbA by IEF because of their identical net charge. However, HbAS3 production can be readily distinguished from HbS, as the Glu6Val substitution introduced by the canonical sickle mutation deletes a negative charge in the protein, resulting in a more positive relative net charge of HbS. Therefore, only cells from SCD donors were analyzed for HbAS3 expression by IEF.

An IEF membrane from a representative experiment is shown with 5 independent transductions of SCD BM CD34+ cells with the CCL-βAS3-FB LV, plus a mock-transduced sample (Figure 4B). In total, 10 SCD samples were analyzed after erythroid differentiation. There was a strong correlation between the percentage of HbAS3 present in each sample and the extent of transduction measured by the VC (Pearson correlation = 0.88, P = 0.001) (Figure 4C). A concomitant analysis of the same erythroid cell samples was performed by HPLC and IEF and showed similar results by both methods (Supplemental Table 1).

We then compared HBBAS3 mRNA and protein expression levels normalized per VC/cell (Figure 4D). While there was greater variability for HBBAS3 mRNA per VC/cell values compared with protein per VC/cell, the 2 methods indicated similar values of HBBAS3 expression (24.55% ± 11.03% HBBAS3 mRNA per VC/cell and 17.96% ± 3.09% HbAS3 protein per VC/cell), again indicating consistent expression.

In 4 independent transductions, we compared the expression (mRNA and protein) from the HBBAS3 cassette in the presence or absence of the FB insulator (Supplemental Figure 4). We found that the addition of the FB insulator did not alter the expression of the HBBAS3 cassette when compared with the noninsulated LV.

SCD phenotypic correction. To assess the functional effects of HBBAS3 expression on the sickling of rbc produced in vitro from SCD BM CD34+ cells, we adapted and optimized an assay used in clinical laboratories to diagnose SCD: exposure of cells to the reducing agent sodium metabisulfite to induce HbS polymerization. rbc were harvested at the end of the erythroid culture (day 21) and incubated in sealed chambers of glass slides with sodium metabisulfite. After incubation, the morphology and shapes of the individual rbc were analyzed using phase-contrast microscopy to quantify the percentages of sickled-appearing rbc (srbc) and round, discoid nonsickled normal rbc (nrbc) (Figure 5, A and B). In each experiment, 200–900 cells were analyzed for each sample.
rbc from HD controls did not sickle in the presence of sodium metabisulfite, with more than 98% retaining their round morphology. In contrast, rbc produced in vitro from SCD BM CD34+ cells underwent sickling to a high extent in sodium metabisulfite, with averages of 88% ± 9% srbc and 12% ± 9% nrbc. In SCD samples transduced with the CCL-βAS3-FB LV, there was an increase in the percentage of rbc that did not undergo sickling, with 69% ± 16% srbc and 31% ± 16% nrbc, representing 19% ± 8% more nrbc compared with the nontransduced samples. These results demonstrated that expression by the CCL-βAS3-FB LV reduced rbc sickling during deoxygenation. The percentage of corrected sickle cells was positively correlated with the VC present (Spearman correlation = 0.77, P = 0.04) (Figure 5C and Table 1).

In vivo assessment of CCL-βAS3-FB LV transduction of BM CD34+ cells. To characterize the gene transfer and expression by the CCL-βAS3-FB LV in more primitive human hematopoietic stem and progenitor cells (HSPC), βAS3-FB–transduced BM CD34+ cells from SCD donors and HD controls were xeno-transplanted into immunodeficient NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice. Transduction conditions were the same as used for the in vitro analyses, and the cells were transplanted immediately after an overnight transduction. The transplanted cell doses ranged from 10^5 to 10^6 cells per mouse, depending on cell availability (BM source, cell dose, and number of mock- and βAS3-transduced mice used in each transplant are provided in Table 2). Eight to twelve weeks after transplant, the mice were euthanized and the BM was harvested for FACS analysis. Human cells recovered from the NSG BM were cultured under erythroid differentiation for further analysis.

FACS analyses were performed to determine the engraftment of human cells in murine BM, defined as the percentage of human CD45+ cells of the total CD45+ population (murine CD45+ plus human CD45+). Engraftment values were variable among different transplants (up to 78%) (Figure 6A). There were not consistent differences in engraftment using BM CD34+ cells from SCD donors or HD controls (P = 0.6, by 2-way ANOVA) or between cells transduced with the βAS3-FB LV or mock-transduced (P = 0.8, by 2-way ANOVA).

The human CD45+ populations from the transplanted mice were further analyzed for expression of markers for B-lymphoid cells (CD19), myeloid progenitors (CD33), hematopoietic progenitors (CD34), and erythroid cells (CD71). There were no differences in the relative proportions of the different types of human cells between mice engrafted with mock-transduced or CCL-βAS3-FB LV-transduced BM CD34+ cells, with the majority of human cells being B lymphoid cells (Figure 6B), demonstrating that the transduction did not alter the differentiation potential of the cells.
BM was harvested from NSG mice, and human cells were enriched by depletion of murine CD45+ cells using immunomagnetic beads. The cells were then grown under in vitro erythroid differentiation conditions to induce terminal erythroid differentiation to allow the assessment of HBBAS3 mRNA expression by the CCL-βAS3-FB LV vector using qRTPCR.

The VC/cell measured in cells grown from mice engrafted with human CD45+ cells ranged from 0.05 to 0.91 (Figure 6C). Similar levels of gene marking were seen in samples from mice transplanted with BM CD34+ cells from SCD donors and HD (P = 0.3, by 2-sample, 2-tailed t test). Overall, the VC/cell values assessed by qPCR were highest in cells grown in vitro under erythroid differentiation conditions (1.18 ± 0.64 VC/cell), were lower in CFU (0.71 ± 0.75 VC/cell) and cells produced by in vitro myeloid differentiation cultures (0.46 ± 0.33 VC/cell), and were lowest in the human cells recovered from the NSG mice (0.34 ± 0.31 VC/cell) (Supplemental Figure 5).

Quantification of HBBAS3 mRNA expressed by the human erythroid cells produced by in vitro erythroid differentiation of the cells isolated from the NSG mice was done using qRTPCR. Expression of vector transcripts was correlated with VC/cells, with a mean value of 21.69% ± 8.35% of the total β-globin–like mRNA/VC (Pearson correlation = 0.89, P = 0.0004) (Figure 6D). Thus, expression by CCL-βAS3-FB LV was at a level in erythroid cells differentiated from the human cells engrafted in the NSG mice similar to that in transduced BM CD34+ cells that were directly differentiated in vitro.

Genotoxicity assessment of the CCL-βAS3-FB LV. To evaluate the potential genotoxicity of the CCL-βAS3-FB LV, which contained strong erythroid enhancer elements as part of the lineage-specific β-globin expression cassette, 2 evaluations were performed: vector integration site (IS) analysis and in an in vitro immortalization (IVIM) assay.

The vector IS in transduced human BM CD34+ cells were identified using nonrestrictive ligation-amplified PCR (nrLAM-PCR) and mapping of the flanking sequences to the human genome with bioinformatic analyses. Comparisons were made between the patterns of the vector integration in the transduced BM CD34+ cells after a brief in vitro expansion versus after engrafment in NSG mice to look for evidence of preferential in vivo selection of clones containing integrants near cancer-associated genes (44) or transcriptional start sites (TSS) as evidence of vector-related genotoxicity.

There were no increases in the percentages of vectors in proximity to cancer-associated genes following in vivo growth (binomial test, P = 0.32; P value was determined using the binomial test, taking the proportion of cancer gene–proximal IS in the in vitro condition as an estimate of the probability of observing such an IS in engrafted mice) (Figure 7A). There also was not an increased frequency of cells with vector integrations in proximity to TSS of genes (Supplemental Table 3) compared with a random data set; in contrast, a comparative vector IS data set from a clinical trial using a γ-retroviral vector (45) did show higher than random integrations near TSS (Figure 7B).

To further assess the risk of insertional transformation by the βAS3-globin LV vectors, we performed genotoxicity studies using the IVIM assay that quantifies the immortalizing events by insertional transformation of murine lineage-negative BM cells grown in limiting dilution (46). The immortalizing capacities of the LV vectors CCL-βAS3, CCL-βAS3-FB, and CCL-βAS3-CHS4 were compared with those of the γ-retroviral RSF91-GFP-wPRE as a positive control and with mock-transduced cells as a negative control. RSF91-GFP-wPRE carries the spleen focus-forming virus (SFFV) LTRs and is known to transform primary murine cells by insertional mutagenesis with a high probability in this assay.

Consistent with previous reports, the SFFV LTR–driven RSF91-GFP vector frequently generated clones (in 8 out of 14 transductions) with high replating frequencies of up to 5.26 × 10−2 (or 1 in 20 cells). In contrast, we found that in a total of 22 independent transductions (CCL-βAS3, n = 4; CCL-βAS3-FB, n = 14; and CCL-βAS3-CHS4, n = 4), the βAS3-globin LV vectors did not give rise to any clones after the replating step (Figure 7C and Supplemental Table 2). In this in vitro setting, CCL-βAS3-FB was significantly less genotoxic than the SFFV LTR–driven γ-retroviral vector RSF91-GFP (P = 0.002, by 2-sided Fisher’s exact test) (Figure 7C).

### Table 1

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<td>58.3</td>
<td>32.6</td>
</tr>
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</table>

*Multiple SCD-BM samples were pooled for these experiments. NA, not analyzed.*

### Table 2

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<th>4</th>
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<td>SCD</td>
<td>HD</td>
<td>HD</td>
<td>SCD</td>
<td>HD</td>
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<tr>
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<td>3 × 10⁴</td>
<td>10⁴</td>
<td>5 × 10⁴</td>
<td>10⁴</td>
<td>6.3 × 10⁵</td>
</tr>
<tr>
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<td>2</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>No. βAS3-FB mice</td>
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<td>2</td>
<td>6</td>
<td>6</td>
<td>4</td>
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We performed studies using human BM CD34+ cells from SCD donors to assess the potential suitability of the CCL-βAS3-FB LV to achieve the requisite levels of transfer and expression of the anti-sickling HBBAS3 gene to inhibit sickling in rbc. BM is the likely autologous HSC source that would be used clinically for gene therapy in SCD because of the increased risks from mobilization of PBSC with G-CSF in SCD patients (10–12).

In allogeneic HSCT for SCD, stable donor HSC chimerism of 10%–30% can lead to significant hematologic and clinical improvement due to a selective survival advantage of the normal donor–derived rbc compared with the shortened survival of the HbS-containing recipient-derived rbc (47–50). In SCD patients with HPHH, levels of HbF of 8%–15% or more (24, 51) ameliorate the severity and frequency of clinical symptoms. These clinical findings define the minimum threshold for autologous transplant of gene-corrected HSC to benefit SCD because it is unknown whether rbc expressing the HBBAS3 gene will be as beneficial as rbc expressing only HBB from an HD. Hence, at least 10%–30% engrafted gene-corrected HSC producing rbc expressing at least 8%–15% HbAS3 would be needed to potentially achieve the same therapeutic effect as a similar level of allogeneic donor engraftment. Human CD34+ cells are relatively resistant to gene transfer by LV vectors compared with permissive cell lines, and this is accentuated when the vector titers are low. Thus, a key challenge is transducing a sufficient percentage of the CD34+ cells to lead to engraftment of gene-corrected HSC at the needed frequencies (e.g., 10%–30%). Stable engraftment of 10%–20% gene-modified autologous HSC has been demonstrated in clinical trials for X-ALD and β-thalassemia using LV vectors and fully cytoablatve conditioning, indicating that it should be achievable in the setting of SCD as well (31, 52). In our study, the CFU assay demonstrated that 30% of the colony-forming progenitors were transduced; transduction of this percentage of engrafting HSC would be within the target range for a clinical trial, but it is not known how the frequency of lentiviral transduction measured in CFU assay correlates with the transduction frequency of HSC.

**Figure 6**

In vivo assessment of CCL-βAS3-FB LV transduction of BM CD34+ cells. (A) Engraftment of human cells in NSG mice. BM cells isolated from each transplant group (nos. 1–6) were analyzed by flow cytometry to measure the percentage of human CD45+ cells among all CD45+ cells in the marrow (human and murine) as a measurement of engraftment. Mock transduced, white triangles; CCL-βAS3-FB transduced, black triangles. BM samples from HD were used in transplants 3, 4, and 6 and from SCD donors in transplants 1, 2, and 5. (B) Immunophenotypic analysis of human cells isolated from NSG mice transplanted with transduced BM CD34+ cells. Flow cytometry was used to enumerate the percentage of the human CD45+ cells that were positive for the markers of B-lymphoid cells (CD19, white), myeloid progenitors (CD33, light gray), hematopoietic progenitors (CD34, dark gray), and erythroid cells (CD71, black). Mean ± SD are shown of 3 independent experiments. Mock, n = 4; βAS3-FB, n = 8 mice. (C) VC/cell in human cells cultured from NSG mice transplanted with transduced BM CD34+ cells. Black circles represent samples from mice transplanted with HD BM, and white squares represent mice transplanted with SCD BM. All the human cells examined from mock-transduced mice were negative for VC analysis by qPCR. (D) HBBAS3 mRNA expression measured by qRT-PCR from cells transduced to different VC/cell. Five independent transductions are shown. HD, black circles (n = 6); SCD, white squares (n = 4).
The anti-sickling activity of the **HBBAS3** gene was shown to be equivalent to HbF in vitro (33), so production of HbAS3 at greater than 8%–15% of total Hb levels may inhibit sickling in a clinically beneficial manner. In a murine model of SCD, the parental LV DL-βAS3 expressed HbAS3 at 20%–25% of the total Hb, with the remainder coming from the human HBBS transgene (19). These prior results suggest that LV-mediated transfer of the **HBBAS3** gene could be clinically efficacious in gene therapy. In our study, the expression and functional activity of the CCL-βAS3-FB LV was remarkably consistent and effective. There was a very reproducible level of expression of the **HBBAS3** gene by the vector in primary human erythroid cells produced from transduced BM CD34+ cells, making up 15%–25% of the total β-globin–like mRNA transcripts and Hb tetramers. Expression of the HbAS3 protein consistently increased the percentage of rbc produced from CCL-βAS3-FB–transduced SCD CD34+ cells that did not sickle upon deoxygenation, indicating a functional protection similar to the effect of γ-globin expression. These results are consistent with the initial studies with the **HBBAS3** gene by Townes and colleagues, in which the parental DL-βAS3 LV corrected abnormal rbc morphology and hematologic parameters in BM-transplanted SCD mice (19).

We have achieved vector transduction levels and HbAS3 protein production within the target range; however, a high-

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**Figure 7**

Assessment of genotoxicity of the CCL-βAS3-FB LV vector. (A) Frequency of vector IS in and near cancer-associated genes. The bars represent the frequencies of integrations in transcribed regions or within 50 kb of promoters of cancer-associated genes (in vitro, 32.1%; in vivo, 34.3%), as defined in Higgins et al. (44). (B) Integration frequency around TSS. The frequencies of vector IS in the four 5-kb bins in a 20-kb window centered at gene TSS are plotted. The IS are shown for the following: BM CD34+ cells analyzed after 2 weeks growth in vitro (lenti in vitro, n = 2091; gray bars) and 2–3 months in vivo engraftment in NSG mice (lenti in vivo, n = 414; black bars) along with an MLV γ-retroviral vector data set from a clinical gene therapy trial (MLV in vitro, n = 828; white bars) (45) and a random data set generated in silico and analyzed by identical methods (random, n = 12,837; black line). (C) IVIM assay. The replating frequencies for murine lineage-negative cells transduced with the different vectors are shown, calculated based on Poisson statistics using L-Calc software corrected for the bulk VC/cell measured by qPCR on day 8 pTD. The fractions presented across the lower portion of the figure represent the number of negative assays in which no clones were formed divided by the total number of assays performed for that vector. The horizontal bar represents the mean replating frequency of all positive assays. *P = 0.002, by 2-sided Fisher’s exact test.
er percentage of HSC bearing the \(HBBAS3\) transgene would likely provide a larger population of rbc containing the anti-sickling HbAS3 and therefore may provide greater clinical benefit. Attempts to improve \(\beta\)-globin LV vectors have shown that removing \(\beta\)-globin regulatory elements increased titer and transduction efficiency; however, this compromised expression levels (53). Further efforts to improve the transduction efficiency of \(\beta\)-globin vectors without compromising their transgene expression would be an important advance in the field.

We developed and tested a derivative of the original DL-\(\beta\)-AS3 LV (19), named CCL-\(\beta\)-AS3-FB, replacing the HIV promoter in the 5’ LTR with the CMV enhancer/promoter to eliminate the need for expressing the HIV TAT protein during the packaging process (35). This modification in the original LV backbone may improve the biosafety of the vector by eliminating the TAT gene from the packaging step. It also led to at least a 10-fold increase of the vector titers when compared with the original. However, despite this improvement, the large amount of regulatory elements needed for high-level expression of the \(\beta\)-globin gene makes this type of LV complex and lowers the achievable titers when compared with vectors with simpler gene cassettes.

In some gene therapy settings in which strong enhancers and other regulatory elements are needed for sufficient expression of a transferred gene (e.g., chronic granulomatous disease, \(\beta\)-thalassemia), the genotoxic potential of these elements may be diminished when insulator elements are added (54). Insulators are DNA sequences that act as boundary elements to inhibit interactions between adjacent chromatin domains, which can manifest as either enhancer-blocking activity, heterochromatin barrier activity, or both. The enhancer-blocking activity of insulators would reduce trans-activation of transcription from promoters of adjacent cellular genes. The barrier activity of insulators would decrease transgene silencing caused by spreading of surrounding heterochromatin into the vector provirus (55).

The major DNA-binding protein associated with enhancer-blocking activity of insulators in vertebrates is the CTCF (CCCTC-binding factor) protein (38), a highly conserved and ubiquitous zinc finger protein (56–58). The FB insulator used in the CCL-\(\beta\)-AS3-FB LV was previously shown to have enhancer-blocking activity similar to the full 1.2-kb CHS4 insulator in a reporter plasmid transfection assay and exceeding that of the 250-bp core CHS4 insulator fragment (36).

In the CCL-\(\beta\)-AS3-FB LV, the relatively small FB insulator (77 bp) did not lower the titers of the parental CCL-\(\beta\)-AS3 LV when inserted into the U3 region of the 3’ LTR. It was transmitted faithfully to the 5’ LTR during reverse transcription, with no detectable deletion or losses in the vector provirus by Southern blot analysis or by PCR analysis of the FB insulator region from pools of transduced human CD34+ cells and from clonal CFUs grown in vitro. We could not assess the functional ability of the FB insulator to decrease risks for genotoxicity in the IVIM assay because neither the parental vector lacking the FB insulator nor the CCL-\(\beta\)-AS3-FB LV caused any clonal outgrowth. However, we did observe evidence of in vitro activity of the FB insulator based on the greatly enriched binding of CTCF protein to LTR regions of the CCL-\(\beta\)-AS3-FB, as assessed by ChIP analysis from K562 cells.

In light of the recent report of aberrant splicing into the 250-bp CHS4 insulator element in an LV vector used for transduction of BM CD34+ cells in a trial for \(\beta\)-thalassemia (31), we performed an in silico splice site analysis of the FB insulator sequences. Whereas the NetGene2 server (59) identified the cryptic splicing site seen in the CHS4 insulator by Cavazzana-Calvo et al. (31), it did not predict splicing signals in an FB-containing SIN LTR. These studies indicate that the FB insulator does not lower vector titers, is transmitted intact, binds the major cellular factor responsible for producing enhancer-blocking activity, and is not predicted to serve as a cryptic splice site; however, it is unknown whether the presence of the FB insulator in the vector will increase safety in clinical applications.

Safety assessments using the IVIM assay with CCL-\(\beta\)-AS3-FB-transduced murine BM cells and vector IS analyses of human BM CD34+ cells transplanted in vivo to NSG mice did not reveal any evidence of genotoxicity, although the sensitivity of these surrogate assays may be relatively low. The observed pattern of vector IS for the LV was consistent with those described previously for HIV-1-based LV vectors, with preferential integration into genes and no preference for integrations near TSS (60). This contrasted with a recently published \(\gamma\)-retroviral IS data set (45).

In all, these studies provide preclinical data for sufficiently effective transduction of human BM CD34+ progenitor from SCD patients to support translation to a clinical trial of gene therapy for SCD using the CCL-\(\beta\)-AS3-FB LV. Subsequent steps will involve defining components of a clinical trial, such as treatment plan, subject eligibility, end points, and other study parameters to support regulatory submissions, performing cell processing scale-up, further assessing toxicology, and developing the clinical reagents. Outcomes from autologous transplants of gene-modified HSC will need to be compared with those from allogeneic transplant approaches, which continue to advance, to define the clinical utility of gene therapy for SCD.

**Methods**

**BM CD34+ cell LV transduction.** For transduction, BM CD34+ cell samples from SCD and HD were thawed and plated at 1 × 10^6 cells/ml in tissue culture plates precoated with RetroNectin (20 μg/ml, Takara Shuzo Co.). Premutation was performed for 18–24 hours in X-Vivo 15 medium (Lonza) containing 1x glutamine, penicillin, and streptomycin (Gemini Bio-Products). Cytokines were added at the following concentrations: 50 ng/ml human SCF (hSCF) (StemGent), 50 ng/ml human hFlt3 ligand (hFlt3-L) (PeproTech), 50 ng/ml human thrombopoietin (hTPO), and 20 ng/ml human IL-3 (hIL-3) (both from R&D Systems). Cells were transduced with concentrated viral supernatants of the CCL-\(\beta\)-AS3-FB LV at a final concentration of 2 × 10^6 TU/ml (MOI ~ 40, based on titers on HT29 cells) for all experiments done. Twenty-four hours after transduction, the cells were plated in methylcellulose for CFU assay and were also plated in in vitro erythroid differentiation culture and used for xeno-transplant into NSG mice.

**In vitro erythroid differentiation culture.** The in vitro erythroid differentiation technique used is based on a 3-phase protocol adapted from Giarratana et al. (42). After 2 days of culture, for prestimulation and transduction, cells were transferred into erythroid culture. The basic erythroid medium was Iscove’s Modified Dulbecco’s Medium (IMDM; Life Technologies) (1x glutamine, penicillin, and streptomycin) supplemented with 10% BSA, 40 μg/ml inositol, 10 μg/ml folic acid, 1.6 μM monothioglycerol, 120 μg/ml transferrin, and 10 μg/ml insulin (all from Sigma-Aldrich). During the first phase (6 days), the cells were cultured in the presence of 10^-6 M hydrocortisone (Sigma-Aldrich), 100 ng/ml hSCF, 5 ng/ml hIL-3, and 3 IU/ml erythropoietin (Epo) (Janssen Pharmaceuticals). In the second phase (3 days), the cells were transferred onto a stromal cell layer (MS-5, murine stromal cell line (61) (provided by Gay Crooks, UCLA) with the addition of only Epo (3 IU/ml) to basic erythroid medium. At day 11, all the cytokines were removed from the medium and the cells were cocultured on the MS-5 stromal layer until days 18 to 21.
The samples were incubated at 5% CO2, 37°C for 25–40 minutes. Images were loaded onto a glass microscope slide, covered, and sealed at the edges. Sodium Metabisulfite (Sigma-Aldrich) was added to each sample. This mixture was incubated for 5 minutes, and the resulting signal was used for quantification by qRT-PCR. To determine HBBAS3 mRNA expression, 1 to 2 × 10⁵ cells were harvested on day 21 of erythroid differentiation. RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer’s instructions. The genomic DNA elimination columns contained in the kit were used to eliminate possible DNA contamination during the extraction. First-strand cDNA was synthesized using random primers, M-MLV reverse transcriptase, and RNAseOUT Recombinant Ribonuclease Inhibitor (all from Invitrogen) according to the manufacturer’s protocol. SYBR Green qPCR amplification of cDNAs was performed using Platinum Taq DNA Polymerase (Platinum Green PCR SuperMix; Invitrogen) on a ViiA7 Real-Time PCR System (Applied Biosystems).

To specifically detect mRNA transcripts originating from the vector CCL-pBS-FB (HBBAS3 mRNA) in differentiated rbc and compare them with the levels of endogenous β-globin–like mRNA (HBB in HD samples and HBBS in SCD samples, respectively), 2 sets of allele-specific primers were designed (HBB/HBB and HBB; Supplemental Table 4). The percentage of HBBAS3 transcripts (%HBBAS3) among all β-globin–like transcripts was determined from the relative expression of HBBAS3 vs. HBB and HBBS transcripts, respectively, as the mean and SD by experimental conditions are presented in figures. HBBAS3 tetramer quantification by IEF. Hb IEF was performed using the Hemoglobin Electrophoresis Procedure (Helena Laboratories) according to the manufacturer’s instructions. Briefly, a minimum of 3 × 10⁵ cells were harvested on day 21 of erythroid differentiation. The cells were lysed with Hemolysate Reagent (Helena Laboratories) per instructions and incubated overnight at 4°C. If necessary, lysates were concentrated the next day using Micron Centrifugal Filters (Ultracel YM-30; Millipore); 5 μl of the samples were loaded onto a Titan III cellulose acetate plate (Helena Laboratories) and electrophoresed for 25 minutes at 350 volts. The plate was stained with Ponceau S (Sigma-Aldrich) for visualization of the Hb tetramers, cleared using Clear Aid solution (Helena Laboratories), and dried. The Hb bands were identified by comparison with a Helena Hemo Controls and quantified by densitometry using ImageQuant TL software (GE Healthcare).

SCD phenotypic correction assay. At day 21 of the erythroid differentiation, 2.5 × 10⁵ cells per condition were harvested for SCD phenotypic correction assay. The samples were spun down (500 g for 5 minutes), and the resulting pellets were harvested in 10 μl of the supernatant; 10 μl of 20 μg/ml Sodium Metabisulfite (Sigma-Aldrich) was added to each sample. This mix was loaded onto a glass microscope slide, covered, and sealed at the edges. The samples were incubated at 5% CO2, 37°C for 25–40 minutes. Images of the cells were then captured by inverse microscopy with a Nikon DS-Fi1 camera, from consecutive fields at ×10 magnification. Computer vision was utilized to isolate cells within each field and then individually present them to the user for visual analysis of normal or sickle morphology in a randomized and unbiased fashion across treatment groups.
HbAS3 mRNA and correlation of VC/cell and percentage of HbAS3; Spearman correlation (67) was used to evaluate the correlation of the VC/cell with the percentage of corrected sickle cell. For binary outcome, such as the rejecting condition in the IVIM assay (positive vs. negative), Fisher’s exact test (68) was used to compare CCL-βAS3-FB vector with RSF9-GFP vector. To compare the proportions of IS near cancer-related genes in cells grown in vitro with cells engrafted in mice, a binomial test was performed using the proportion of cancer gene–proximal IS in the in vitro condition as an estimate of the probability of observing such an IS in engrafted mice. For all statistical investigations, tests for significance were 2 tailed. P < 0.05 was considered to be statistically significant. All statistical analyses were carried out using SAS version 9.3 (69), GraphPad Prism version 5.0d (GraphPad Software Inc.), and MATLAB version 7.12.0.635 (MathWorks Inc.).

Study approval. All human samples have been following UCLA IRB protocol #10-001399. Written informed consent was obtained from the subjects used in these studies. All work with mice was done under protocols approved by the UCLA Animal Care Committee.

Acknowledgments

This work was supported by a Disease Team Award (DR1-01452) from the California Institute for Regenerative Medicine (ICRM) and by the UCLA Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research and the UCLA Jonsson Comprehensive Cancer Center. A.R. Cooper was supported by the Ruth L. Kirschstein National Research Service Award GM007185. Tim Townes generously provided the parental βAS3 LV and technical advice. Beatriz Campo-Fernández assisted with molecular assays and integrity. Fernando Oliviera Raya assisted with image formatting. Sohel Talib (CIRM), Gay Crooks (UCLA), Bobby Parkman (Children’s Hospital Los Angeles), and Elliott Vichinsky (Children’s Hospital & Research Center Oakland) provided valuable advice and guidance. The Broad Stem Cell Research Center Microscopy, Flow Cytometry and High-Throughput Sequencing Core Resources were essential to the performance of these studies. Most importantly, we thank the donors who provided BM samples for these studies.

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Received for publication November 25, 2012, and accepted in revised form May 2, 2013.


