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Effect of Insulators on the Expression of betaAS3 in Lentiviral Gene Therapy for Sickle Cell Disease

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Effect of Insulators on the Expression of betaAS3 in Lentiviral Gene Therapy for Sickle Cell Disease

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Microbiology, Immunology, and Molecular Genetics

by

Jennifer Patricia Wherley

2013
ABSTRACT OF THE THESIS

Effect of Insulators on the Expression of betaAS3 in Lentiviral Gene Therapy for Sickle Cell Disease

by

Jennifer Patricia Wherley

Master of Science in Microbiology, Immunology, and Molecular Genetics
University of California, Los Angeles, 2013

Professor Donald B. Kohn, Chair

Insulators can be used to increase the therapeutic potential of gene therapy for sickle cell disease (SCD) by improving safety and resisting transgene silencing. Four experimental lentiviral vectors (LVs) carrying the betaAS3 transgene were engineered to contain the FB and Ank small insulator elements. When evaluated in single vector copy MEL cell clones, the Ank R LV demonstrated barrier activity that most closely resembled that observed with the positive control 1.2kb cHS4 insulator. Erythrocytes derived from human SCD hematopoietic
stem/progenitor cells (HSPCs) transduced with the Ank R LV did not yield higher betaAS3 expression or phenotypic correction of the sickle shape than levels produced without a barrier insulator. Since the benefits of the Ank insulator in the betaAS3 LV are yet to be realized in primary cells, future experiments using serial transplants of murine HSPCs are needed to determine the extent of the barrier activity in the Ank R LV.
The thesis of Jennifer Patricia Wherley is approved.

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2013
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Introduction

Sickle cell disease (SCD) is a debilitating monogenic disorder caused by a point mutation that changes the amino acid at codon six of the beta-globin gene. This results in the production of hemoglobin tetramers containing the sickle beta-globin chain (HbS) that polymerize when exposed to low oxygen conditions. The polymer chains cause the normally flexible red blood cell (RBC) membrane to become rigid, leading to vaso-occlusive crises that obstruct blood flow through the capillaries. Consequently, patients experience anemia as well as acute and chronic episodes of pain in addition to serious splenic, neurologic, pulmonary, and renal injury. The condition can be managed with frequent blood transfusions to reduce HbS content (Stamatoyannopoulos, 2000) and induction of fetal hemoglobin (HbF) with hydroxyurea treatments to impede RBC sickling (Voskaridou et al., 2010), but lacks a universal curative approach. Allogeneic bone marrow (BM) transplant is potentially curative but is only feasible for the few patients who have an HLA-matched related or unrelated donor available, and is complicated by the risks of graft rejection and graft versus host disease.

Autologous gene therapy has the potential to be an effective and curative option for many SCD patients. Preclinical studies have demonstrated the feasibility and efficacy of engineering lentiviral vectors (LVs) to carry a gamma-globin (Pestina et al., 2009) or beta-globin (Pawliuk et al., 2001) gene in murine models to ameliorate the symptoms of SCD with transgenic globin expression. Although naturally increased expression of HbF in patients with hereditary persistence of fetal hemoglobin has decreased the severity of disease symptoms (Conley et al., 1963), the benefits of using gamma-globin-based therapies are limited by the endogenous regulatory mechanisms in adult RBCs that impair the expression of HbF at this stage (Sankaran et al., 2010); attempts to alter this regulation have shown encouraging results in a SCD murine model (Xu et al., 2011). To incorporate the sickling-resistant properties of gamma-globin with the high transcriptional activity of beta-globin, a beta-globin-like chain was
developed with three “anti-sickling” amino acid substitutions, betaAS3, by Levasseur et al. An LV used to deliver the betaAS3 gene was shown to efficiently transduce murine SCD BM, which resulted in betaAS3 protein expression that restored normal RBC physiology and prevented SCD manifestations after transplantation (Levasseur et al., 2004).

While the results of gene transfer studies are promising, there are concerns associated with current gene therapy approaches. Retroviral and lentiviral gene delivery vectors have the inherent potential for insertional oncogenesis. The need for safety modifications was inspired by the development of leukemia in patients who were treated for X-linked severe combined immunodeficiency (Hacein-Bey-Abina et al., 2010) and chronic granulomatous disease (Ott et al., 2006) with retroviral gene therapy. As a result, the gene therapy field began working with self-inactivating (SIN) vectors that contain deletions of the endogenous enhancer/promoter sequences in the viral long terminal repeats (LTRs) and allow for the use of tissue-specific promoters to reduce the risk of trans-activation by integrated vectors (Iwakuma et al., 1999), but further precautions are still needed. In addition to safety risks, the efficacy of gene therapy is limited by silencing of therapeutic transgene expression by DNA methylation (Challita and Kohn, 1994; Stein et al., 2010) and the encroachment of heterochromatin due to the genomic environment around the vector integration site (Emery et al., 2000). For gene therapy to be successful as a long-term treatment, gene delivery vectors should be equipped with elements that improve safety and prevent transgene silencing.

Chromatin insulators can be used to improve the safety profile and preserve transgene expression when included in gene therapy vectors. Insulators regulate gene expression by maintaining and/or creating boundaries between active and silenced chromatin regions. Insulators and their associated binding proteins possess enhancer-blocking and/or barrier functions (Gazner and Felsenfeld, 2006). In the context of gene therapy, the enhancer-blocking activity prevents exogenous enhancers in the viral vector from interacting with
endogenous promoters of genes neighboring the vector integration site. By hindering this interaction, insulators reduce the risk of insertional mutagenesis that may activate proto-oncogenes and lead to severe adverse events in patients treated with gene therapy. The **barrier function** diminishes the consequences of chromosomal position effect (expression dependent on integration site) and position effect variegation (different levels of transgene expression from the same vector integration). Without an insulator, the expression of a transgene is dependent on the chromatin structure at the site of vector integration. Barrier insulators reduce the spread of heterochromatin and maintain accessible chromatin structures around the inserted LV, which allows for consistent transgene expression independent of integration site (Emery, 2011).

The 1.2kb DNase I hypersensitive site 4 at the 5' end of the chicken beta-globin locus (cHS4) was identified as an insulator that possesses both enhancer-blocking and barrier activity (Chung *et al.*, 1993). Since its discovery, the cHS4 1.2kb region has been the prototypic vertebrate insulator used to define the sequences and corresponding transcription factors responsible for the insulator enhancer-blocking and barrier functions. Inclusion of the full-length 1.2kb insulator in the LTRs of LVs lowers viral titers, making it impractical for use in human LV gene therapy for SCD (Urbinati *et al.*, 2009). Studies identified a smaller 250bp “core” that demonstrated lower levels of insulator activity when compared to the full-length 1.2kb sequence, although including two copies in tandem restored equivalent insulator function (Chung *et al.*, 1997). Extending the “core” to 400bp by the addition of the 3’ flanking sequence restored insulator activity to that seen with the full-length cHS4 insulator (Aker *et al.*, 2007) and is currently included in a gamma-globin vector used in preclinical gene therapy studies for the treatment of beta-thalassemia (Wilber *et al.*, 2011). Further characterization of the chicken beta-globin locus identified a 400bp sequence at the 3’ end of the gene cluster that exhibits insulator activity; this was combined with the existing 250bp “core” to create a 650bp sequence
that achieved insulator capacity comparable to the 1.2kb cHS4 (Arumugam et al., 2009). While full functionality of the cHS4 insulator was harnessed in smaller fragments, these are still large enough to decrease viral titer (Urbinati et al., 2009), requiring the examination of other insulator elements to be used in the betaAS3 LV.

In an effort to improve the safety of gene delivery vectors, Ramezani et al. developed a small 77bp element, termed FB, which is capable of enhancer-blocking activity with the same efficiency as the 1.2kb cHS4 insulator. Since previous studies showed the CCCTC-binding factor (CTCF) was responsible for the enhancer-blocking function of vertebrate insulators (Bell et al., 1999), the FB element was designed to include the minimal CTCF binding sequence from the footprint II of the cHS4 250bp "core" and the homologous sequence found in the T-cell receptor BEAD-1 insulator. When tested in vitro in enhancer-blocking assays, the FB insulator demonstrated the ability to block the interaction between an enhancer and a promoter at levels comparable to the 1.2kb cHS4 insulator (Ramezani et al., 2008). The FB insulator is a safer alternative to other cHS4 derivatives because the duplicate CTCF binding sites are stable and do not recombine when incorporated into the viral LTRs following reverse transcription and integration into the host genome (Romero and Urbinati et al., in press), unlike tandem repeats of the larger 250bp “core” (Cavazzana-Calvo et al., 2010). The FB enhancer-blocking insulator reduces the risk of trans-activation after LV integration, but has not been shown to possess barrier activity.

Studies conducted by Gallagher et al. identified a region within the promoter of the human ankyrin-1 gene that exhibits barrier insulator activity, but does not demonstrate enhancer-blocking insulator function. Variegated expression of the ankyrin-1 gene in a transgenic murine model for hereditary spherocytosis revealed that mutations in the promoter region perturbed protein expression, implying the loss of barrier function (Gallagher et al., 2001). Further analysis found an element within the promoter that was sensitive to DNase I
digestion, indicating open chromatin as a probable binding site for transcription factors. Extensive characterization of this element using in vitro assays and chromatin immunoprecipitation (ChIP) confirmed its function as a barrier insulator with the USF1/2 binding capabilities typical of barrier elements (Gallagher et al., 2010).

The ankyrin-1 (Ank) element used in the experiments presented here is 159bp of the promoter region that possesses the barrier activity described by Gallagher et al. A mutation was introduced into the GATA-1 binding site to abolish any residual promoter activity, allowing the Ank element to be included in the betaAS3 LV as a barrier insulator. The relatively small size of the FB and Ank elements presents the opportunity to combine the enhancer-blocking and barrier insulator activity in the betaAS3 LV, while potentially avoiding the titer reduction seen with larger elements (Urbinati et al., 2009).

Considering these factors, we hypothesized that combining the enhancer-blocking activity of the FB element and the barrier function of the Ank element in the betaAS3 LV would yield more consistent therapeutic levels of betaAS3 protein expression, resulting in safer and more effective gene therapy for SCD. To test this, the betaAS3 expression conferred by experimental LVs insulated by the FB element alone, or the FB insulator together with either one or two copies of the Ank element, was compared to levels produced from a non-insulated LV, or an LV insulated by the 1.2kb cHS4 element. The betaAS3 expression measured in single vector copy murine erythroleukemia (MEL) cell clones was most stable over time in cells transduced with the LV containing the FB insulator and one copy of the Ank element, indicating the presence of barrier activity in this LV design. These results prompted the analysis of transduction efficiency, betaAS3 mRNA and protein expression, and phenotypic correction in RBCs derived from primary SCD human BM hematopoietic stem/progenitor cells (HSPCs) transduced with the FB and Ank dually insulated LV. When compared to HSPCs transduced with LVs that contained only the FB element or the 1.2kb cHS4 insulator, there was no clear
evidence of barrier activity conferred by the Ank element in vitro. Although the combination of the FB and Ank elements in the same LV showed evidence of barrier activity in long-term cultures using a leukemic cell line, the therapeutic efficacy of the Ank barrier insulator in the context of betaAS3 LV gene therapy in primary cells remains to be seen.

Results

Design and production of experimental LVs

The LVs evaluated in this study share the same core components, with variations only in the inclusion of insulators with different orientations and combinations. The non-insulated AS3 LV was engineered by Romero and Urbinati et al. (Romero and Urbinati et al., in press) using a pCCL backbone that contains SIN LTRs to carry the betaAS3 expression cassette driven by the endogenous beta-globin promoter with the potent enhancer activity of the locus control region (LCR) (Figure 1A). The AS3 vector was modified further by the addition of insulators to study which elements possess barrier activity.

To construct the experimental LVs, the FB element was included in the AS3 LV. The enhancer-blocking FB insulator was cloned into the U3 region of the lentiviral 3' LTR so it would be present in both proviral LTRs following reverse transcription and integration into the genome (FB LV) (Figure 1A). Incorporating the FB element in the AS3 LV did not affect the titer of the vector, with only a 1.1-fold change seen in two independent LV packaging experiments (Figures 1B and 1C). Since the combination of the enhancer-blocking and barrier activity of insulators will optimize both safety and transgene expression, the Ank element was added to the FB LV.

The Ank sequence was incorporated into the FB vector in multiple locations and orientations to determine which design generated the most consistent betaAS3 expression. The Ank R LV contains one copy of the Ank element upstream of the betaAS3 expression cassette, oriented in the LV to mirror its endogenous arrangement upstream of the gene it regulates.
(Figure 1A). The addition of the single Ank element had a negligible effect on viral titer with only a 1.1-fold decrease observed when compared to the non-insulated AS3 LV, and no change in comparison to the FB LV (Figures 1B and 1C).

Previous studies revealed that flanking an expression cassette with barrier insulator elements would most effectively protect the transgene from position effect variegation and chromosomal position effects (Pannell and Ellis, 2001). The other Ank-containing LVs have the Ank element in the same orientation and position as the Ank R LV, plus an additional copy flanking the betaAS3 cassette in either the forward (Ank F-R) or reverse (Ank R-R) direction (Figure 1A). Incorporating a second copy of the Ank insulator had deleterious effects on LV titer. When compared to the AS3 LV, the Ank F-R LV and Ank R-R LV had a 3.6-fold and 2.4-fold lower viral titer, respectively (Figures 1B and 1C).

As a positive control for subsequent experiments, an LV containing the full-length cHS4 1.2kb insulator with known enhancer-blocking and barrier activity (Chung et al., 1993) was generated. The 1.2kb LV was constructed similarly to the FB LV, by incorporating the insulator into the U3 region of the 3′ LTR so it would be present in both proviral LTRs (Figure 1A). The inclusion of large elements in viral LTRs, such as the cHS4 1.2kb insulator, has been shown to reduce viral titer (Urbinati et al., 2009), as was evidenced by an 8.7-fold decrease in titer when the 1.2kb LV was compared to the non-insulated AS3 LV (Figures 1B and 1C). While the 1.2kb cHS4 insulator possesses the functional properties ideal to enhance safety and efficacy of betaAS3 LV gene therapy, its detrimental effects on viral titer make it inadequate for use in primary human cells.

**Generation of single vector copy MEL cell clones**

MEL cells were used to measure betaAS3 expression over time because of their lineage and species of origin. An erythroid cell line was needed to detect betaAS3 expression due to
the erythroid-restricted expression by the LCR (Li et al., 2002) and beta-globin promoter. MEL cells are naturally maintained at an erythroid progenitor stage but can be induced to differentiate into more mature erythroid cells in the presence of N,N'-hexamethylene bisacetamide (HMBA), which results in the upregulation of hemoglobin expression (Marks et al., 1987). Human hemoglobin tetramers containing either the transgenic betaAS3 or endogenous human beta-globin chains cannot be distinguished by available antibodies. Using a murine, rather than human, cell line ensured specific measurement of transgenic betaAS3 expression by flow cytometry. The betaAS3 protein produced by the LV pairs with the murine alpha-globin chains to generate hemoglobin tetramers (h-betaAS3/m-alpha) in vitro that can be detected by antibodies against the human beta-globin chain.

Single vector copy MEL cell clones were generated to evaluate the presence of barrier activity in the different insulators included in each LV. Analysis of betaAS3 expression over 4 months allowed assessment of each insulator’s ability to protect transgene expression from stochastic silencing and integration site-specific effects. MEL cells were transduced with each LV (Figure 1A) at a range of multiplicities of infection (MOIs) to determine which transduction conditions yielded 8-10% betaAS3 positive cells as determined by FACS. Considering Poisson distribution statistics, a population that is expressing the transgene at levels of 8-10% is predicted to have the majority of transduced cells containing one copy of the LV. Portions of the transduced populations were differentiated with HMBA, and then stained with an antibody against human beta-globin for detection of betaAS3 expression. The undifferentiated portion from the pools of cells that were 8-10% betaAS3-positive for each vector were then sorted (unstained) using the FACS automated cell deposition unit (ACDU) technique, which placed one cell per well in a 96 well plate.

After sorting, the MEL cell clones were characterized before beginning the analysis of betaAS3 expression in single vector copy clones. The clones grew for 1 week before the
original plate was duplicated (2 weeks post-transduction). One plate was maintained in culture, while genomic DNA was obtained from cells in the duplicate plate by a single-step method (Charrier et al., 2011) for PCR detection of the psi region in the integrated vectors. The psi-positive clones were expanded and a more pure genomic DNA isolation was performed (1 month post-transduction) to analyze the number of vector copies contained in each psi-positive clone by quantitative PCR (qPCR) (Figure 2). The efficiency of generating MEL cell clones from pilot experiments done to optimize transduction and differentiation conditions is shown in Table 1.

**BetaAS3 expression in single vector copy MEL cell clones**

Ten to 15 MEL cell clones with one vector copy from each LV group were differentiated in HMBA and then analyzed for betaAS3 expression by FACS every other week for 2 months (Figure 2). The results follow betaAS3 expression levels from 2 to 4 months post-transduction with each experimental LV.

A pool of mock-transduced MEL cells was maintained in culture and differentiated in parallel with the single vector copy clones as a negative control for the FACS analysis of transgene expression. The differentiated mock sample was used to set flow cytometry parameters after staining with the mixture used to evaluate experimental samples: AQUA for viability determination and an anti-human beta-globin antibody to detect intracellular betaAS3 expression (Figure 3A). The distribution and background staining observed in the mock sample served to control for the stability of the cytometer settings and gating limits to allow for the accurate analysis of betaAS3 expression in the experimental samples over time.

The flow plots of the single vector copy MEL cell clones revealed a variety of patterns in betaAS3 expression. Some clones demonstrated considerable **intraclonal** variation with a broad, bimodal distribution of transgene expression, indicative of position effect variegation.
(Figure 3B, clones AS3 1G7, FB 2C9, Ank F-R 2H9). Other clones displayed a more narrow distribution with a single expression peak; the majority of the clonal population either expressed betaAS3 (Figure 3B, clones 1.2kb 3H10, Ank R 1G9), or did not express betaAS3 as a result of transgene silencing (Figure 3B, clone Ank R-R 4C1). Although these patterns were observed in clones transduced with each LV, the Ank R and 1.2kb conditions had more clones with homogeneous expression profiles and less intraclonal variation, indicating the presence of barrier insulator activity.

When the level of betaAS3 measured in each MEL cell clone is organized according to LV, more consistent transgene expression was evident over time in the clones transduced with the Ank R and 1.2kb LVs in comparison to the other conditions (Figure 3C). The tightly clustered pattern in the Ank R and 1.2kb conditions is most pronounced at 2 months post-transduction. While this pattern persists over time, transgene silencing appears to occur in clones transduced with each LV (Figure 3D). It is possible that maintaining the MEL cells in a long-term (over 4 months) in vitro culture impairs their ability to fully differentiate with HMBA, leading to the detection of lower hemoglobin levels by flow cytometry overall, rather than universal silencing.

Low interclonal variability is a characteristic of barrier insulator activity that indicates protection against chromosomal position effects. The MEL cell clones containing the Ank R LV maintained lower interclonal variability that most closely resembles the positive control group containing the 1.2kb known barrier insulator (Chung et al., 1993). On the other hand, the FB, Ank F-R, and Ank R-R LVs, demonstrated a wide range of betaAS3 expression among clones, similar to that seen in the MEL cell clones containing the non-insulated AS3 LV. Even though betaAS3 levels decreased over time in the MEL cell clones transduced with each of the LVs, the interclonal consistency was still most apparent in the clones transduced with the Ank R LV when measured at 4 months post-transduction (Figure 3C and 3E).
The stability of betaAS3 expression observed over time in clones transduced with each experimental LV is presented in Figure 3E and Table 2. A lower percentage of the coefficient of variation (%CV) indicates more consistent transgene expression among clones within the same group; a lower %CV is descriptive of low interclonal variability. For each group, there is an increasing trend in the %CV over time; however, the increase in %CV for the clones transduced with the Ank R LV is less severe than the other groups (Figure 3E, solid black bar). Notably, the Ank R LV maintains low interclonal variability over time better than the positive control 1.2kb insulator. These results demonstrate the ability of the Ank element to exert barrier insulator activity in its position and orientation within the Ank R LV.

*In vitro erythroid differentiation of SCD BM CD34+ HSPCs*

After studies in MEL cell clones revealed the barrier function of the Ank element in the Ank R LV, the effect of this insulator activity was examined in a population of transduced primary human SCD BM CD34+ HSPCs differentiated into mature erythrocytes. The Ank R LV was compared to the FB LV, which has enhancer-blocking activity but is not expected to possess barrier activity (Ramezani et al., 2008), and the 1.2kb LV that has full insulator properties (Chung et al., 1993) (Figures 3C and 3D). BM-derived CD34+ cells from multiple SCD donors were either mock-transduced or transduced with 2x10^7 TU/mL (MOI= 40) of the FB, Ank R, and 1.2kb LVs. After transduction, the cells were expanded in medium containing erythroid-specific growth factors (Giarratana et al., 2005) for 7 days, then transferred into coculture with MS-5 murine stromal cells to promote erythroid differentiation into enucleated RBCs (Figure 4A).

The erythroid cultures were evaluated to determine whether transduction with the LVs affected the expansion or differentiation capacity of the HSPCs. While there was slight variability observed between donors, LV transduction did not impair the expansion ability of
differentiating HSPCs where a 40-50-fold overall expansion was achieved (Figure 4B). The erythroid differentiation progress was closely monitored by flow cytometry to follow the changes in expression of hematopoietic (CD34 and CD45) and erythroid lineage (transferrin receptor [CD71] and Glycophorin A [GpA]) cell surface proteins. The HSPC marker CD34 was present before (day 0) and immediately after mock or LV-transduction (day 3), but quickly disappeared when the cells were grown in erythroid-specific conditions. Expression of the pan-leukocyte marker CD45 exhibited a similar expression pattern as CD34, with high levels detected in the pre-stimulation and transduction conditions (day 0 and day 3), followed by a rapid decline after cells were transferred to the erythroid expansion medium. Conversely, levels of the erythroid markers CD71 and GpA sharply increased after the cells were exposed to erythroid-specific growth factors. CD71 expression quickly increased on day 3 and remained high until day 21 when levels decreased dramatically, indicating successful differentiation into reticulocytes and mature erythrocytes (Migliaccio et al., 2002). GpA expression increased during the differentiation stage of the *in vitro* culture, with high levels sustained until the conclusion of the erythroid differentiation at day 21 (Figure 4C). The proportion of enucleated erythrocytes present at the end of the culture was measured by flow cytometry using an antibody to detect GpA expression and the DNA dye, DRAQ5. The *in vitro* erythroid differentiation process achieved up to 86-95% enucleated RBCs, defined as GpA+DRAQ5-, in all conditions evaluated. LV transduction did not impair RBC differentiation when compared to mock-transduced cells from the same SCD donor and levels reached in healthy donor (HD) samples (Figure 4C and 4D).

*Comparison of betaAS3 expression in erythrocytes derived from SCD HSPCs transduced with the FB, Ank R, and 1.2kb LVs*
The erythroid cells generated by the in vitro differentiation were used to examine the transduction efficiency achieved by the FB, Ank R, and 1.2kb LVs in HSPCs from SCD donors. At day 14 of the erythroid differentiation, cells were harvested for genomic DNA isolation and subsequent vector copy number (VCN) analysis by qPCR. The average VCN was comparable between the FB (average VCN 1.2 ± 0.5) and Ank R LVs (average VCN 1 ± 0.5). While there was a range of VCN achieved in both the FB and Ank R-transduced conditions, the cells from the same donor yielded a similar VCN for both vectors. The variability in the VCN can be attributed to the differences in permissibility to LV transduction of each SCD donor, rather than a true difference in the transduction efficiency between the FB and Ank R vectors. The VCN was significantly reduced in cells transduced with the 1.2kb LV (average VCN 0.1 ± 0.1; p≤0.2) when compared to that reached by the FB and Ank R LVs (Figure 5A and Table 3). As expected based on previous work by Urbinati et al. (Urbinati et al., 2009), the cHS4 insulator’s large size and location in the lentiviral LTRs are responsible for the lower viral titer of the 1.2kb LV in comparison to the FB and Ank R vectors. Because of this, the 1.2kb LV does not efficiently transduce primary human cells (Figures 1B, 1C, and 5A).

Cells were taken from the in vitro erythroid culture at day 14 for analysis of betaAS3 mRNA production in cells transduced with each LV. RNA was isolated from each sample, reverse transcribed into cDNA, and then quantified using digital droplet PCR. The level of betaAS3 mRNA was calculated as a percentage of the total mRNA from beta-globin-like chains (normal beta-globin, sickle beta-globin, and betaAS3-globin) and then normalized to VCN. The average amount of betaAS3 produced per VCN was nearly equivalent in cells transduced with the FB LV (average 14.7 ± 3.5 betaAS3 mRNA / VCN) and the Ank R LV (average 14.5 ± 3.8 betaAS3 mRNA / VCN); however there was a slight difference observed between the two conditions in transduced cells from SCD donors 2 and 3. The Ank R LV provided higher betaAS3 mRNA expression in donor 2, while the FB LV produced higher levels of betaAS3
mRNA in donor 3. Since the variability was present in both directions, either favoring higher expression in the FB or Ank R-transduced cells, this difference may be donor-specific rather than due to the ability of either vector to produce more betaAS3 mRNA than the other. Although erythrocytes containing the 1.2kb vector have low levels of betaAS3 mRNA measured, they appeared to have a slightly larger, but not statistically significant, amount of mRNA produced per VCN (average 18.8 ± 2.2 betaAS3 mRNA / VCN; p=0.159), potentially due to the barrier activity exhibited by the cHS4 insulator (Figure 5B and Table 3).

Expression of the betaAS3 protein in differentiated RBCs grown from mock and LV-transduced SCD BM HSPCs was analyzed by isoelectric focusing (IEF) and quantified using densitometry. The three amino acid changes present in the betaAS3-globin chain alter the charge of hemoglobin tetramers containing the transgenic globin, HbAS3, allowing it to be distinguished from HbS by IEF. A representative IEF cellulose acetate membrane in Figure 5C shows the abundance of HbAS3 as a percentage of the total protein present in each lane. The amount of HbAS3 was normalized to the VCN of the sample to determine if including the Ank element in the LV resulted in higher therapeutic protein expression in primary human cells. In the samples analyzed from two SCD donors, the levels of HbAS3 in erythrocytes transduced with the FB and Ank R LVs were comparable, with the FB LV demonstrating a slightly higher average (15.8 HbAS3 / VCN) than the Ank R LV (13.1 HbAS3 / VCN). Given the similarity of HbAS3 expression between the FB LV and Ank R LV transduced conditions, there was no evidence of barrier insulator activity conferred by the Ank element. Little HbAS3 expression was seen in the RBCs containing the 1.2kb vector due to the low transduction efficiency achieved. As a result, the average HbAS3 produced per VCN in the 1.2kb-transduced samples (7.9 HbAS3 / VCN) was lower than erythrocytes containing the FB or Ank R LVs (Figure 5C).

The phenotypic benefits of HbAS3 expression were examined by analyzing the morphology of RBCs derived from LV-transduced SCD BM CD34+ cells compared to RBCs
generated in culture from mock-transduced SCD BM and mock-transduced HD BM HSPCs. The erythrocytes were deoxygenated with sodium metabisulfite to induce sickling of the cells with high HbS levels, imaged, and then classified according to RBC morphology (Figure 5D, left). The number of normal and sickle RBCs was quantified in each condition, allowing the percentage of phenotypic correction due to HbAS3 expression to be calculated as (% Normal RBC in transduced SCD samples) – (% Normal RBC in SCD mock sample). When the percentage of phenotypic correction from two SCD donors was averaged together, there was a negligible difference between the FB-transduced (average 27.1% correction) and the Ank R-transduced (average 26.8% correction) samples. Similarly, when the phenotypic correction was normalized to average VCN, there was no difference observed between the two conditions: 16.1% correction with FB LV, and 16.9% correction with Ank R LV. The amount of normal, spherical RBCs was lower in the erythrocytes containing the 1.2kb LV (average 9.7% correction), than in the other LV-transduced samples. Since the VCN for the 1.2kb samples was very low, the phenotypic correction normalized to VCN was 33.7%, nearly double what was seen in the FB and Ank R conditions (Figure 5D and Table 4). This discrepancy is indicative of either barrier activity of the cHS4 insulator or slightly skewed results due to low VCN measurements.

Taken together, these data obtained from SCD BM CD34+ HSPCs transduced with the FB, Ank R, and 1.2kb LVs revealed no evidence of the Ank element barrier activity in a bulk population of primary human cells following a short-term (2-3 weeks) in vitro culture.

Discussion

For gene therapy to be clinically applicable as a treatment for SCD, gene delivery vectors should include elements that promote safety by preventing vector-mediated genotoxicity as well as maintain long-term, consistent transgene expression. The prototypic cHS4 1.2kb
insulator possesses both enhancer-blocking activity to address the safety concerns of transactivation as well as barrier activity to prevent transgene silencing (Chung et al., 1993). Due to size constraints of the complex betaAS3 LV, it is not feasible to include the full 1.2kb insulator without deleteriously affecting viral titer, making it impractical for the transduction of human primary cells. Romero and Urbinati et al. equipped the betaAS3 LV with a favorable safety profile by including the 77bp FB element to incorporate the enhancer-blocking activity seen in the much larger 1.2kb cHS4 insulator (Ramezani et al., 2008) without lowering vector titer (Romero and Urbinati et al., in press). Transduction efficiency of primary human HSPCs with the FB insulated betaAS3 LV has been restricted to approximately one VCN in previous preclinical studies performed by Romero and Urbinati et al. Increasing the MOI and/or vector concentration has not yielded substantial improvements to overcome this limitation (Romero and Urbinati et al., in press). Because adding more LV during transduction will not improve transgene expression, including a barrier insulator in the betaAS3 LV has the potential to increase and maintain consistent betaAS3 expression from a single vector copy. While having many cells transduced with a low VCN is ideal for safety purposes (Ramezani et al., 2008; Urbinati et al., 2009), expression from this single vector copy must resist silencing for gene therapy to be successful.

The FB insulator is not predicted to exhibit barrier activity as a result of the sequence specificity used to design the element, necessitating the inclusion of one or more small barrier insulator fragments in the betaAS3 LV to preserve transgene expression and protect the integrated vector from silencing and heterochromatin encroachment. The small Ank element from the upstream promoter region of the human ankyrin-1 gene has been shown to possess insulator barrier activity in vitro and in vivo (Gallagher et al., 2001; Gallagher et al. 2010; Breda et al., 2012). The Ank barrier insulator was included in multiple locations and orientations within the betaAS3 LV (Ank R, Ank R-R, and Ank F-R) and analyzed for its effect on viral titer.
Since globin LVs are inherently limited in their transduction efficiency due to suboptimal viral titers (Papanikolaou and Anagnou, 2010), it is imperative to maintain the highest titer possible to achieve therapeutically relevant levels of gene transfer. Only the Ank R LV was produced at titers comparable to the non-insulated AS3 LV and the FB-only insulated FB LV (Figure 1). Although increasing the length of the insert in a LV above 6kb has deleterious effects on viral titer (Kumar et al., 2001), the small 159bp size of a second Ank element alone should not cause the up to 4-fold decrease in LV titer observed with the Ank F-R and Ank R-R LVs (Figure 1C). Rather, the decreased titers of the Ank F-R and Ank R-R LVs are more likely due to vector instability caused by the increased potential for recombination as a result of repetitive sequences within the vector (Hu et al., 1997).

To determine which LV design conferred the most robust barrier insulator activity, the Ank-containing LVs were examined for their ability to protect against position effect variegation and chromosomal position effects in single vector copy MEL cell clones. The use of single vector copy MEL cell clones has been well-established as a suitable in vitro model system for the study of insulator effects on beta-globin transgene expression (Arumugam et al., 2007). The MEL cell clones transduced with the Ank R LV demonstrated higher levels and more consistent betaAS3 expression over time when compared to the non-insulated AS3 LV control, the FB LV, and the other Ank-insulated vectors (Figures 3C, 3D, and 3E). Similar to the pattern observed in the positive control clones transduced with the 1.2kb LV, the Ank R-transduced clones demonstrated low interclonal variability in betaAS3 expression over 4 months in culture. Long-term maintenance of transgene expression independent of vector integration site signifies the barrier activity of the Ank element within the Ank R LV (Figure 3C and 3E).

Remarkably, further analysis of the intraclonal variation in betaAS3 levels over time revealed a general decrease in expression from each clone, although this effect was least pronounced with clones containing the Ank R LV in comparison to the other experimental LVs.
(Figures 3C and 3D). Two explanations for this observation are: (1) silencing is occurring in clones transduced with each LV, resulting in a lower level of transgene expression, or (2) maintaining MEL cells in culture for 4 months impairs their ability to differentiate in the presence of HMBA, leading to lower levels of detectable betaAS3-globin. Future studies using ChIP to evaluate the integrated vector for the presence of histone marks associated with heterochromatin (e.g. H3K27me3 and H3K9me2) and DNA methylation will reveal whether or not silencing of the transgene has occurred. On the other hand, if the detection of endogenous alpha-globin levels by flow cytometry decreases as MEL cells are grown in culture for long periods of time, the suboptimal differentiation process is more likely responsible for this universal effect.

It has been postulated that barrier insulators may be more effective if they surround the expression cassette (Pannell and Ellis, 2001). In studies using MEL cell clones to investigate the barrier activity of two copies of the Ank element flanking a beta-globin expression cassette, Breda et al. found an increase in transgenic beta-globin expression from the clones containing a single copy of their Ank-insulated vector. Interestingly, our results show that a single copy of the Ank element within the vector had comparable barrier effects as when two copies were present (one in each proviral LTR) surrounding the expression cassette (Breda et al., 2012). Furthermore, the LVs tested that contain multiple copies of the Ank element had lower levels and more variable betaAS3 expression when compared to the Ank R MEL cell clones.

The Ank R LV had the highest viral titer of the Ank vectors and exhibited the most prominent barrier activity of the LV constructs evaluated in MEL cell clones, making it the most favorable Ank-containing vector for its potential to efficiently transduce primary human cells and provide the benefits of a barrier insulator in the betaAS3 LV (Figure 5A). While the Ank R LV displayed barrier activity in MEL cell clones, there was no evidence of barrier function observed in a short-term in vitro erythroid culture of transduced primary human cells when compared to
controls. The Ank R LV was able to transduce SCD HSPCs with efficiency comparable to that achieved by the FB LV and much higher than the 1.2kb LV. Transduction did not impair the expansion or differentiation capacity of the cells, as was predicted from the results reported by Romero and Urbinati et al. and Breda et al. (Figures 4 and 5A) (Breda et al., 2012; Romero and Urbinati et al., in press). Due to the low transduction efficiency achieved by the 1.2kb LV, it is difficult to compare the HbAS3 protein and phenotypic correction between the Ank R LV and 1.2kb LV as a result of the low betaAS3 levels measured in the positive control 1.2kb samples. The Ank R-transduced cells showed no clear difference in the amount of betaAS3 mRNA, HbAS3 protein expression, or phenotypic correction in primary human erythroid cells when compared to those transduced with the FB LV (Figure 4B, 4C, and 4D).

These results contrast with the findings of in vivo experiments done by Breda et al. in a murine model of beta-thalassemia where a higher level of beta-globin expression was produced from primary cells transduced with the Ank-insulated vector when compared to the non-insulated control (Breda et al., 2012). The differences in beta-globin expression detected by Breda et al. were evident at 3 months post-transplant of gene-modified murine HSCs, whereas our results only capture a 3 week in vitro culture using human SCD primary cells. The short-term in vitro erythroid differentiation culture may be too brief for sufficient silencing to occur, thus preventing the detection of expression differences resulting from the presence of the barrier insulator in the Ank R samples in contrast to its absence in the FB samples.

Although we did not identify a robust increase in betaAS3 production and expression in primary human SCD cells transduced with the Ank R LV, future in vivo studies using serial HSC transplants will reveal the effects of the transgene silencing on betaAS3 expression and allow for the appreciation of the Ank element’s barrier activity in primary cells. As was demonstrated by Breda et al., the use of gene-modified murine HSCs provides an appropriate model system for examining the effects of the Ank barrier insulator in long-term in vivo studies. If the Ank
element provides protection against silencing as was observed in the MEL cell clones, betaAS3 expression is predicted to be more consistent and at higher levels over time in murine erythrocytes containing the Ank R LV than those transduced with the FB LV.

Including both the FB enhancer-blocking insulator and the Ank barrier insulator in the betaAS3 LV improved the consistency and level of betaAS3 expression in MEL cells, but evidence for its benefits in primary human HSPCs is yet to be realized. The studies presented here provide the initial indication for incorporating the Ank element in the betaAS3 LV for use in gene therapy to treat SCD.
Figure 1

A

AS3

FB

Ank R

Ank F-R

Ank R-R

1.2kb

B

LV titers

10^7

10^6

10^5

10^4

10^3

10^2

10^1

AS3

FB

Ank R

Ank F-R

Ank R-R

1.2kb

GFP

TUmL
**Figure 1.** Lentiviral proviruses and production titers

(A) Proviral maps of the six LVs compared. All LVs contain SIN LTRs, the Woodchuck Hepatitis Virus post-transcriptional regulatory element (WPRE), and the betaAS3 expression cassette (introns labeled 1,2,3) driven by the endogenous beta-globin promoter (beta-p) under the control of the beta-globin locus control region (HS2,HS3,HS4). The original non-insulated AS3 LV was modified by the inclusion of insulator elements. For the FB LV, one copy of the FB insulator was cloned into the U3 region of the 3' LTR, and is transferred during reverse transcription to the 5' LTR of the proviral DNA. The Ank R, Ank F-R, and Ank R-R LVs include the FB insulator element as well as one copy of the Ank insulator element in the reverse orientation, or two copies flanking the expression cassette, one forward and one reverse, or two reverse, respectively. The 1.2kb LV contains one copy of the full length cHS4 insulator in the 3' LTR that is copied to 5' LTR during reverse transcription. (B) Titers of LVs in unconcentrated viral supernatant as determined using HT29 cells. The values represent the average and standard deviation of two vector productions and titers of each production performed in triplicate. (C) Fold decrease in titer of insulated LVs compared to the non-insulated AS3 LV. The titers for each insulated LV (B) were compared to the titer achieved by the AS3 LV to determine the fold decrease in LV titer as a result of incorporating the FB, Ank, and 1.2kb insulator elements.
Figure 2. Generation of single vector copy MEL cell clones

MEL cells were transduced with each LV at an MOI of 0.1, 0.2, 0.5, 1, 2, and 5. A portion of cells from each condition was differentiated with HMBA, and then screened for betaAS3 expression using flow cytometry. Cells from the transduction condition that yielded 8-10% betaAS3 expression were ACDU sorted so that a single cell was placed in each well of a 96 well plate. Clones grew over 1 week in culture before they were split; half of the cells continued to expand in culture while the other half was lysed to screen for presence of the vector (psi+ as determined by PCR). After 2 weeks of further expansion, a purified genomic DNA sample was obtained from each psi+ clone for qPCR analysis of vector copy number (VCN). Only clones that contained 1 copy of vector were maintained in culture. BetaAS3 expression in single vector copy clones was evaluated every 2 weeks after differentiation with HMBA beginning at 2 months post-transduction and continued for 2 months (until 4 months-post-transduction).
Figure 3

A

B

C

2 months post-transduction

3 months post-transduction

4 months post-transduction
Figure 3. Analysis of single vector copy MEL cell clones

(A) Representative FACS plot of the mock-transduced sample to set population parameters. The target population was identified and gated on the FSC vs. SSC plot (left). Live cells from this gate were negative for the Aqua viability dye (middle), and were subsequently evaluated for betaAS3 expression. (B) FACS plots from single vector copy MEL cell clones transduced with each LV. Plots presented here are representative of the betaAS3 distribution observed in the clonal populations. While only one clone transduced with each LV is shown above, clones from all LV conditions displayed the above variations. (C) BetaAS3 expression in MEL cell clones over time. BetaAS3 expression was analyzed by flow cytometry after differentiations performed 2, 3, and 4 months post-transduction. Each point represents one single vector copy clone (AS3 n=15, FB n=10, Ank F-R n=11, Ank R-R n=13, Ank R n=11, 1.2kb n=12). The background betaAS3 expression measured in mock-transduced samples was subtracted from the expression measured in the transduced clones; in some cases the background staining in the mock sample was higher than in the clone analyzed, leading to a betaAS3 expression value lower than 0. (D) Mean betaAS3 expression ± SEM. The average and standard error of the betaAS3 expression
measured in each group of clones at 2, 3, and 4 months post-transduction is shown. (E) Percentage of the coefficient of variation (%CV) of betaAS3 expression in MEL cell clones over time. The %CV at each time point was calculated using the mean and standard deviation of betaAS3 levels from the clones in each group at the time analyzed. A lower %CV is representative of more consistent betaAS3 expression among the clones within a group.
Figure 4

A

B

C

D

% Enucleated RBC

% Marker Positive

% Enucleated RBC

Fold Expansion

Day of Culture

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100
Figure 4. *In vitro* erythroid differentiation

(A) *In vitro* erythroid differentiation schema. BM CD34+ HSPCs were pre-stimulated overnight in culture with cytokines, and then transduced for 24 hours with the LV at $2 \times 10^7$ TU/mL (MOI=40). After transduction, the cells were stimulated to expand for 7 days in erythroid-specific growth factors. The cells were then plated on an MS-5 stromal layer for 14 days of differentiation into mature erythrocytes. Samples for molecular and phenotypic analysis were taken at the indicated time points. (B) Fold expansion of BM CD34+ HSPCs during *in vitro* erythroid differentiation. The growth curves for two SCD donor samples are shown (SCD donor 3, black; SCD donor 4, gray). Cells from the same donor that were mock-transduced, or transduced with one of the LVs (FB, Ank R, or 1.2kb) are represented. (C) Presence of hematopoietic and erythroid lineage markers present during the stages of erythroid differentiation. Mock-transduced and LV-transduced samples from the *in vitro* erythroid culture were evaluated for hematopoietic (CD34 and CD45) and erythroid markers (CD71 and Glycophorin A [GpA]) on days 0 (light gray), 3 (gray), 14 (dark gray), and 21 (black). The mean and standard deviation of the mock and LV-transduced conditions from the same SCD donor are shown. (D) Extent of RBC enucleation at the end of *in vitro* erythroid differentiation. A representative FACS analysis of enucleated RBC (94% GpA+DRAQ5-) present at day 21 of erythroid culture is shown (left). The average percentage of enucleated RBC present after *in vitro* differentiation of SCD BM CD34+ HSPCs that were mock-transduced or transduced with one of the LVs (FB, Ank R, or 1.2kb) is compared to the enucleation of mock-transduced HD BM CD34+ cells (right).
Figure 5

A. Vector Copy Number

B. % betaAS3 mRNA / VCN

C. % HbAS3 / VCN

D. Phenotypic Correction
Figure 5. Comparison of LVs in erythrocytes derived in vitro from human SCD BM CD34+ HSPCs

(A) VCN in transduced SCD BM CD34+ samples cultured in erythroid differentiation conditions. The VCN achieved by transduction with the FB, Ank R, and 1.2kb LVs in 4 independent transductions of different SCD donors was measured by qPCR (SCD donor 1, circle; SCD donor 2, square; SCD donor 3, triangle; SCD donor 4, diamond). Statistically significant differences are represented by (*) for p≤0.02. (B) Amount of betaAS3 mRNA produced per VCN. The amount of sickle beta, normal beta, and betaAS3-globin transcripts was analyzed using digital droplet PCR. The amount of betaAS3 mRNA is calculated as the percentage of betaAS3 transcripts of the total beta-globin-like transcripts (sickle beta-globin + normal beta-globin + betaAS3-globin), then normalized to the VCN of the sample (n=4). The differences in betaAS3 mRNA production by each LV were not statistically significant (p=0.159). (C) Amount of HbAS3 produced per VCN. IEF (left) was used to quantify the amount of hemoglobin tetramers containing transgenic betaAS3 chains. Bands on the cellulose acetate were quantified by densitometry and reported as a percentage of the total. The percentage of HbAS3 was normalized to VCN. The average and standard deviation of HbAS3 produced per VCN from two SCD donors is shown (right). (D) Correction of the sickle phenotype. The RBCs derived from mock-transduced HD BM CD34+ and SCD BM CD34+ cells mock-transduced or transduced with each LV (FB, Ank R, 1.2kb) were deoxygenated, imaged, and then characterized based on cell morphology (left). The percentage of normal round, non-sickled cells present in the transduced SCD samples (above that present in the mock-transduced SCD sample) signifies the phenotypic correction conferred by betaAS3 expression from each LV. The graph represents the average and standard deviation (SD only shown for % Normal RBC) of experiments done with two SCD donors (% Normal RBC, dark gray; % Sickle RBC, light gray; % N/A, black).
### Table 1. Clonogenicity of transduced MEL cells after ADCU sorting

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<th>No. of clones</th>
<th>% of wells that grew clones</th>
<th>No. of psi+ clones</th>
<th>% of psi+ clones</th>
<th>No. of single copy clones</th>
<th>% single copy clones / psi+ clones</th>
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### Table 2. Analysis of betaAS3 expression over time in MEL cell clones

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Table 3. Raw data for VCN and mRNA analysis in LV transduced SCD BM

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Table 4. Phenotypic correction observed in sickle cell morphology assay normalized to VCN

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<th>Average % Correction / VCN</th>
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Materials and Methods

LV packaging and titration

The LV packaging and titration assay and analysis was performed as described by Romero and Urbinati et al. (Romero and Urbinati et al., in press).

MEL cell culture and differentiation

MEL cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 1X Glutamine, Penicillin, and Streptomycin (both from Cellgro, Manassas, VA) and 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA). Every 2 weeks, MEL cells were induced to differentiate in the presence of N,N’-hexamethylene bisacetamide (H MBA) (Sigma-Aldrich, St. Louis, MO) as previously described (Papayannopoulou and Brice, 1992). For the differentiation process, 2.5x10^5 cells were placed in 1 well of a 12-well tissue culture plate in 1 mL of the differentiation medium: DMEM, 1X Glutamine, Penicillin, and Streptomycin, 20% FBS, with 5mM HMBA. The MEL cells remained in the differentiation medium for 5 days before analysis for betaAS3 expression was performed.

MEL cell LV transduction

For the generation of single vector copy MEL cell clones, 1x10^5 cells were transduced with the AS3, FB, Ank R, Ank F-R, Ank R-R, and 1.2kb LVs using a range of multiplicities of infection (MOIs): 0.1, 0.2, 0.5, 1, 2, 5. The transduction was performed in DMEM supplemented with 1X Glutamine, Penicillin, and Streptomycin (both from Cellgro, Manassas, VA) and 10% FBS (Gemini Bio-Products) in the presence of 4 ug/mL polybrene (Sigma-Aldrich, St. Louis, MO). After 24 hours in the presence of the virus-containing medium, the cells were harvested, washed to remove the virus, then cultured and differentiated as described above.
**Intracellular betaAS3 staining and flow cytometry analysis**

After 5 days of differentiation with HMBA, 1-3x10^6 MEL cells were harvested for intracellular staining with an anti-human beta-globin antibody to assess the level of betaAS3 expression. The cells were washed, and then re-suspended in PBS (Cellgro, Manassas, VA) for viability staining using the Aqua Dead Cell Stain (Life Technologies, Grand Island, NY). The viability stain was removed after 15 minutes, and the cells were fixed with 4% paraformaldehyde (USB Corporation, Cleveland, OH) for 1 hour, then subsequently with 100% methanol for 5 minutes (Sigma-Aldrich, St. Louis, MO). Following fixation, the cells were washed before blocking with 5% non-fat milk (Santa Cruz Biotechnology, Dallas, TX). The MEL cells were stained with a PE-labeled anti-human beta-globin antibody (Santa Cruz Biotechnology, Dallas, TX) in the presence of Fix&Perm Solution B (Life Technologies, Grand Island, NY) for 30 minutes. The staining solution was removed and the cells were re-suspended in PBS for analysis by flow cytometry. All of the flow cytometry analyses were performed on an LSR Fortessa cell analyzer (BD Biosciences, San Jose, CA).

**PCR for the psi region of the LVs**

Two weeks after transduction, a portion of the cells from each MEL cell clone was lysed to obtain a genomic DNA solution as described by Charrier et al. (Charrier et al., 2011). DNA from each clone was interrogated by PCR using primers specific to the HIV-1 packaging signal (psi) region of the proviral LV to determine which clones contained at least one integrated copy of the LV. The PCR reaction was started at 94°C for 1 min., followed by 35 cycles of: 98°C for 5 sec., 62°C for 5 sec., and 72°C for 5 sec., using an Eppendorf Mastercycler Pro S (Eppendorf, Hamburg, Germany). The resulting PCR product was loaded in a 2% agarose (BioExpress, Kaysville, UT) gel and run in 1x TAE (Cellgro, Manassas, VA) for qualitative analysis of transduced MEL cell clones.
Quantitative PCR for VCN in MEL cell clones

Four weeks post-transduction, cells from each MEL cell clone containing at least one copy of an LV (as determined by PCR for psi) were harvested for genomic DNA isolation using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA). The average VCN was determined by two separate qPCR reactions: one specific for the HIV-1 packaging signal sequence (psi) in the LV provirus, and the other specific for the housekeeping gene UC483 present at consistent levels in the MEL cell line. The psi signal was normalized to the amount of UC483 amplified to calculate the average number of vector copies per cell in each transduced MEL cell clone based on the a standard curve method described by Cooper et al. (Cooper et al., 2011).

BM CD34+ cell isolation, pre-stimulation, and LV transduction

The BM CD34+ cell isolation, pre-stimulation, and LV transduction was performed as described by Romero and Urbinati et al. (Romero and Urbinati et al., in press).

In vitro erythroid differentiation culture

The in vitro erythroid differentiation culture was adapted from Giarratana et al. (Giarratana et al., 2005) and performed as described by Romero and Urbinati et al. (Romero and Urbinati et al., in press).

Flow cytometry during erythroid differentiation

The cell staining and flow cytometry analysis during the erythroid differentiation was performed as described by Romero and Urbinati et al. (Romero and Urbinati et al., in press).

Quantitative PCR for vector copy number in human cells
The qPCR assay and analysis was performed as described by Romero and Urbinati et al. (Romero and Urbinati et al., in press).

**BetaAS3 mRNA quantification by digital droplet PCR**

As described by Romero and Urbinati et al. (Romero and Urbinati et al., in press), 1.5 x 10^5 cells were harvested on day 14 of erythroid differentiation to determine betaAS3-globin mRNA expression. RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The genomic DNA elimination columns contained in the kit were used to remove 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, Carlsbad, CA) according to the manufacturer’s protocol.

Droplet digital PCR (ddPCR) was employed to reliably quantitate betaAS3 mRNA expression levels. Reaction mixtures of 20 μl volume comprising 1× ddPCR Master Mix (Bio-Rad, Hercules, California), relevant primers and probe (500nM and 100nM for ddHBBAS3 primers and probe), and 1μl of cDNA were prepared. The primer and probe sets used were: 5’ GGA GAA GTC TGC CGT TAC TG 3’ (forward primer); 5’ CAC TAA AGG CAC CGA GCA CT 3’ (reverse primer); 5’ FAM-ACA AGG TGA -ZEN- ACG TGG ATG CCG TTG-3IABkFQ 3’ (AS3 probe); 5’ HEX-AAC CTC TGG -ZEN- GTC CA A GGG TAG ACC ACC AGC AG-3IABkFQ 3’ (Total beta-globin-like probe).

Droplet generation was performed as described in Hindson et al. (Hindson et al., 2011). The droplet emulsion was then transferred with a multichannel pipet to a 96-well propylene plate (Eppendorf, Hamburg, Germany), heat sealed with foil, and amplified in a conventional thermal cycler (T100 Thermal Cycler, Bio-Rad). Thermal cycling conditions consisted of 95°C 10 min, 94°C 30 sec., and 60°C 1 min (55 cycles), 98°C 10 min (1 cycle), and 12°C hold. After PCR, the
96-well plate was transferred to a droplet reader (Bio-Rad). Acquisition and analysis of the ddPCR data was performed with the QuantaSoft software (Bio-Rad), provided with the droplet reader. BetaAS3 expression levels were calculated as a percentage of all beta-globin-like expression levels and then normalized to the VCN.

**HbAS3 tetramer quantification by IEF**

The IEF assay and analysis was performed as described by Romero and Urbinati *et al.* (Romero and Urbinati *et al.*, in press).

**Sickle cell phenotypic correction assay**

The sickle cell phenotypic correction assay and analysis was performed as described by Romero and Urbinati *et al.* (Romero and Urbinati *et al.*, in press).
References


Blood, 100(9):3077–86.


Stein, S., Ott, M. G., Schultze-Strasser, S., Jauch, A., Burwinkel, B., Kinner, A., Schmidt, M., et al. (2010). Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. Nature Medicine,

