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Authors
Romero, Zulema
Campo-Fernandez, Beatriz
Wherley, Jennifer
et al.

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ARTICLE

The human ankyrin 1 promoter insulator sustains gene expression in a β-globin lentiviral vector in hematopoietic stem cells

Zulema Romero1, Beatriz Campo-Fernandez2, Jennifer Wherley1, Michael L Kaufman1, Fabrizia Uribinat1, Aaron R Cooper2, Megan D Hoban1, Kismet M Baldwin1, Dianne Lumaquin1, Xiaoyan Wang3, Shantha Senadheera1, Roger P Hollis1 and Donald B Kohn1,4

INTRODUCTION

Sickle cell disease (SCD) is one of the most common monogenic disorders, caused by a point mutation in the sixth codon of the human β-globin gene.1 This multisystem disease is associated with severe episodes of acute illness and progressive organ damage.2 While allogeneic hematopoietic stem cell transplantation (HSCT) may ameliorate SCD, it requires identification of an HLA-compatible donor and carries the risks of immunological complications of graft rejection and graft-versus-host disease.3 Autologous stem cell gene therapy is an alternative treatment without the limitations of allogeic HSCT.

To date, several lentiviral vectors (LV) have been designed and successfully used to target β-hemoglobinopathies in ex vivo modified hematopoietic stem cells (HSC) showing phenotypic correction of in vitro preclinical human models4-6 and in vivo murine models.5,7-12 However, LV developed for the treatment of β-hemoglobinopathies require the presence of the β-globin introns13,14 as well as the DNase hypersensitive sites (HS) of the locus control region (LCR),15,16 which contain strong enhancers and regulatory elements to achieve sufficient expression of the β-globin transgene. The combination of these elements yields a complex vector payload that may have a deleterious effect on the titer and transduction efficiency of the LV.

Moreover, the efficacy of these vectors may be still limited by transgene silencing due to DNA methylation and heterochromatini- zation, as a consequence of chromosomal positional effects, which causes a lack of uniform and stable transgene expression. There are silencer sequences in the LV themselves, mainly located in the long terminal repeats (LTR), which also contribute to transgene silencing. Part of this problem was overcome by the deletions made in the 3’LTR of the self-inactivating (SIN)-LV;17 however, there still may be residual silencing even from SIN-LTR.18,19

The inclusion of insulator sequences in LV has improved the expression problems. The addition of the 5’DNase I HS 4 of the chicken β-globin locus (cHS4, 1.2 kb) in the LTR of a β-globin LV was able to rescue chromosomal position effect, as shown by correction of the thalassemia phenotype in in vivo murine models.20 However, the strategy of placing relatively long insulator elements in both LTR still adversely affects titers and stability of the LV.21,22 Alternatively to the prototypic cHS4, other insulator elements with barrier activity have been identified, such as the DNase I HS immediately 5’ to the promoter of the human ankyrin-1 gene (ANK1). Gallagher et al.23 demonstrated that this promoter region had structural and functional characteristics of a barrier element in erythroid cells, and was able to prevent gene silencing in vivo.

1Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, California, USA; 2Molecular Biology Interdepartmental PhD Program, University of California, Los Angeles, California, USA; 3Department of Internal Medicine and Health Services Research, University of California, Los Angeles, California, USA; 4Department of Pediatrics, UCLA Children’s Discovery and Innovation Institute David Geffen School of Medicine, University of California, Los Angeles, California, USA.

Correspondence: DB Kohn (dkohn@mednet.ucla.edu)
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Here we analyzed the potential barrier activity of a 159 bp fragment of the 5′HS promoter region of the ANK1 (described from now on as “Ank insulator”) when placed in different orientations and sites within the previously described CCL-βAS3-FB LV. This promoter region included a point mutation in GATA-1 (ΔGATA-1) binding site to remove the enhancer activity; this point mutation was not expected to affect the barrier activity as described by Gallagher et al.23 In vitro clonal analysis of these constructs showed that a vector with a single copy of the Ank insulator in reverse orientation with respect to the LV genome (Ank-R) was shielded from chromosomal position effect as effectively as by the cHS4 insulator. This Ank-R LV construct was then compared in vivo with the parental vector, showing a more consistent expression of the transgene in clonal assays and a significantly higher expression of the transgene and lack of silencing in long-term vector-transduced murine HSC.

RESULTS
Ankyrin-insulated lentiviral vector designs
We have shown that the CCL-AS3-FB LV (modified from CCL-βAS3, Figure 1a) is capable of efficient transduction of human SCD bone marrow (BM) CD34+ progenitor cells, and provides consistent expression of an antisickling β-globin gene reducing the percentage of sickling red blood cells (RBCs).6

To test the potential barrier activity of the 159 bp 5′HS region of the promoter of the ANK1 gene, the parental CCL-βAS3-FB LV (FB LV) (Figure 1b) was modified to include the Ank element. All of the constructs designed contained one copy of the synthetic FB insulator placed in the 3′LTR of the parental FB LV. The FB element has been shown to exhibit the CTCF binding7 and functional activity of an enhancer-blocking insulator element, but does not have the barrier activity.24 One construct was designed to carry one copy of the Ank sequence upstream of the β-globin LCR cassette (near the 3′ end of the vector), which is in reverse orientation relative to LV transcription (Figure 1c) (Ank-R LV). In other constructs, a second copy of the Ank sequence was placed downstream of the βAS3-globin cassette (near the 5′ end of the vector), either in forward (Figure 1d) or reverse orientation (Figure 1e), (Ank-FR and Ank-RR LV, respectively). The noninsulated version of the CCL-βAS3-FB LV was used as a negative control (Figure 1a) (AS3 LV) and a sixth vector including the large prototypic cHS4 insulator (Figure 1e) (cHS4 LV) was used as a positive control for a known insulator barrier activity.

The inclusion of the FB insulator in the 3′LTR did not modify the titer when compared with its noninsulated counterpart,6 neither did the addition of only one copy of the Ank insulator in reverse orientation (Ank-R). Instead, the inclusion of two copies of the Ank insulator, independently of their orientation (Ank-FR and Ank-RR), had a deleterious effect on the LV titers. As expected, the insertion of the full-length chS4 insulator made the titers drop more than 10-fold21 (Supplementary Figure S1).

In vitro assessment of the insulator activity in clonal erythroid cells
To test for protection against chromosomal-position effects, individual clones carrying only one copy of the vector were generated from pools of murine erythroleukemia (MEL) cells with each of the LV previously described. The parental CCL-βAS3-globin vector showed substantial variegation of expression, with 54.19 ± 17.02% of cells expressing the vector (Figure 2a). The clones carrying the Ank-R LV had a significantly higher percentage of cells expressing

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**Figure 1** The proviral maps of the βAS3-globin vectors. The AS3 provirus has the βAS3-globin expression cassette including the human β-globin gene exons (arrow heads) with three amino acid substitutions to encode the βAS3-globin protein, introns and 3′ and 5′ flanking regions, the β-globin mini-LCR with hypersensitive sites 2–4, the mutated Woodchuck hepatitis virus post-transcriptional regulatory element (ΔWPRE) and the 3′SIN-LTR containing the FB insulator which is copied into the 5′LTR after reverse transcription. (a) AS3 LV, noninsulated version lacking the FB element; (b) CCL-βAS3-FB parental vector (FB LV); (c) Ank-R LV includes one copy of the Ank insulator in the reverse orientation; (d) Ank-FR LV with two copies of the Ank insulator element flanking the expression cassette, one in forward and one in reverse orientation; (e) Ank-RR LV with two copies of the Ank insulator flanking the expression cassette, both in reverse orientation (all of the Ank-insulated LV retain the FB element); and (f) a positive control vector that includes a full-length copy (1.2 kb) of the chS4 insulator in the 3′LTR that is copied to 5′LTR during reverse transcription.
the βAS3-globin cassette (63.14 ± 10.99%) than: (i) those transduced with LV carrying two copies of the Ank insulator independently of their orientation (Ank-R versus Ank-FR, \( P = 0.019 \)) (Ank-R versus Ank-RR, \( P = 0.028 \)) (one-way analysis of the variance (ANOVA)) or (ii) those transduced with the parental FB LV (\( P = 0.022 \)) (one-way ANOVA). The average percentage of expressing clones transduced with the Ank-R LV carrying one copy of the Ank insulator was equivalent to the positive-control clones transduced with the chS4 insulator. The differences in expression between clones transduced with the LV insulated by the FB and noninsulated LV were not significant (one-way ANOVA).

No significant increase in the level of expression of the βAS3-globin transgene was observed, when the Ank-insulated LV were compared with the AS3, FB, and chS4 LV, (Figure 2b) (one-way ANOVA with multiple comparisons); the Ank element was expected to protect from chromosomal-position effects, but not to enhance the level of transgene expression. Further analyses were performed 3 and 4 months post-transduction showing that, overall, a general silencing was observed in the clones of all the groups including the positive control (chS4LV) and no significant differences were observed among groups (Figure 2c) (repeated-measure ANOVA).

The coefficient of variation (CV) of the βAS3-globin expression reflects the degree of expressing cells among clones with different integrations, and a lower CV indicates protection from chromosomal-position effect. A higher percentage of expressing cells along with more homogenous expressions was observed in the clones transduced with the Ank-R LV and chS4 LV, as indicated by their lower CV after 2 months in culture (Figure 2d). Moreover, the Ank-R LV maintained a lower CV with respect to all of the other LV analyzed, including the chS4 LV (Figure 2d) demonstrating the barrier activity of the Ank insulator.

The instability of insulator elements in LV vectors has been previously reported;\(^1\) to address this possible concern and evaluate the insulator stability, PCR in 10 MEL cell clones with each of the LV constructs carrying this element were performed. In the case of the Ank-FR and Ank-RR, 30–50% of the clones analyzed did not show amplification for one or both copies of the Ank insulator (top and middle rows in Supplementary Figure S2a). However, when only

**Figure 2** Consistent expression of the βAS3-globin cassette in MEL cell clones carrying one copy of the Ank insulator. (a) The percentage of cells expressing βAS3-globin transgene was analyzed by flow cytometry after in vitro erythroid differentiation 2 months post-transduction. Each point represents one single vector copy/cell clone (AS3, \( n = 15 \); FB, \( n = 10 \); Ank-R, \( n = 11 \); Ank-FR, \( n = 11 \); Ank-RR, \( n = 8 \); and chS4, \( n = 12 \). Bars represent average values for each group. One-way ANOVA with pairwise comparisons was used to compare the percentage of cells expressing βAS3-globin transgene between groups. The significance threshold used for \( P \)-value was 0.05. (b) Geometric mean of βAS3-globin expression of the samples shown in (a), and at the same time point. Bars represent average values for each group. No significant increase in the level of expression of the βAS3-globin transgene was observed due to the addition of the Ank insulator (one-way ANOVA with multiple comparisons, \( P > 0.05 \)). (c) Mean values of the percentage of cells expressing βAS3-globin transgene. The average and SE of the mean (SEM) of the percentage of cells expressing βAS3-globin transgene measured in each group of clones at 2, 3, and 4 months post-transduction are summarized. No significant differences were observed among groups over time (repeated-measure ANOVA). (d) Percentage of the CV (%CV) of βAS3-globin expressing MEL cell clones over time. The %CV at each time point was calculated as the ratio of the SD to the average percentage of expressing cells of the clones of each group at each time point.
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one copy of the Ank insulator was present, it was transferred intact in all of the clones screened (bottom row in Supplementary Figure S2a). All the clones analyzed were positive for the PCR used to measure transduction, amplifying the packaging signal sequence (PsI) region common for all the constructs (Supplementary Figure S2b). The ch54-insulator stability was analyzed in both LTR, showing that 20% of the clones analyzed did not display amplification of the ch54 insulator in the 5′LTR (first panel in Supplementary Figure S2c).

**In vitro** assessment of the Ank-R LV activity in primary human CD34+ cells

The effect of the Ank insulator on βAS3-globin expression from the LV was examined in transduced primary human SCD BM CD34+ HSC differentiated into mature erythrocytes.6–8 BM-derived CD34+ cells from multiple SCD donors were either mock-transduced or transduced with 2 × 10^6 TU/ml (multiplicity of infection (MOI) of 40) of the FB, Ank-R, and ch54 LV. The average vector copy number (VCN) was comparable between the FB (1.2 ± 0.5) and Ank-R LV (1.0 ± 0.5) (P > 0.01); however, the VCN was significantly reduced in cells transduced with the ch54 LV (0.1 ± 0.1; P ≤ 0.02), (Supplementary Figure S3a). The average percentage of βAS3-globin mRNA per VCN was equivalent between the three LV (P > 0.05) (Supplementary Figure S3b). These data revealed no evidence of the Ank-element barrier activity in a bulk population of primary human cells in a short-term (3 weeks) in vivo culture.

Transgene expression in clonal progeny from murine HSC

**In vivo** colony-forming unit from spleen assay (CFU-S) was performed to examine the expression of βAS3-globin transgene at a clonal level. BM from long-term primary recipients (at least five different mice per vector) were transplanted into each secondary recipient mouse (FB LV, n = 19; Ank-R LV, n = 11; and Mock, n = 5) for CFU-S analysis. CFU-S were harvested 10 days post-transplant (FB LV, 13 mice-produced colonies: n = 47; Ank-R LV, 7 mice-produced colonies: n = 36; and Mock, 2 mice-produced colonies: n = 5) for the VCN and gene-expression assessment. In the total secondary CFU-S analyzed, 91.5% (43/47 colonies) contained the FB LV sequences and 97.2% (35/36 colonies) were positive for the Ank-R LV. The FB LV-treated colonies showed a greater variability and almost a twofold higher CV (62.9%) compared with the Ank-R colonies (36.7%), which showed a more homogeneous and consistent expression of the βAS3-globin transgene (Figure 3 and Table 1).

**Table 1** Summary of the percentage of expression of βAS3-globin mRNA per VCN in clonal progeny of secondary and tertiary mice shown in Figure 3

|        | FB LV | Ank-R LV |
|--------|-------|----------|
| Average % | 3.04  | 3.38     |
| SD     | 1.9   | 1.24     |
| %CV    | 62.9  | 36.7     |

Figure 3  Transgene expression in clonal progeny from murine HSC. Percentage of βAS3-globin mRNA normalized per VCN from CFU-S in secondary and tertiary recipients harvested 10 days post-transplant. Bars represent average values for each group and SD. The percentage of CV (%CV) shown at the bottom of the graph was calculated as the ratio of the SD to the mean of βAS3-globin levels per VCN from the clones in each group.

analysis of the clonal progeny, BM cells from long-term secondary mice were transplanted into tertiary recipients (FB LV, n = 14; Ank-R LV, n = 15; and Mock, n = 1). Tertiary CFU-S were harvested 10 days post-transplant (FB LV, 9 mice-produced colonies: n = 47; Ank-R LV, 15 mice-produced colonies, n = 42; and Mock, 1 mouse-produced colony, n = 5), and samples were analyzed for VCN and βAS3-globin mRNA production. In the colonies analyzed from the tertiary transplant recipients, 70.21 (33/47 colonies) and 66.67% (28/42 colonies) were positive for the FB and Ank-R LV proviruses, respectively.

Both LV presented a lower CV in tertiary CFU-S compared with the secondary ones: for the FB LV, the CV decreased from 62.9% in the secondary to 44.7% in the tertiary mice; and for the Ank-R LV, the CV dropped from 36.7% in the secondary mice to 23.7% in the tertiary mice (Figure 3 and Table 1). However, as with the secondary CFU-S, the tertiary CFU-S from the FB LV-treated mice had a wider distribution of the percentage of transgene expression, resulting in more than a twofold higher SD, and therefore a CV that was 1.8 times greater than in the Ank-R LV mice. These results demonstrate the propensity of the Ank-R LV to protect from position-effect variation, providing a more stable and consistent expression of the βAS3-globin transgene.

**In vivo** expression of the βAS3-globin transgene by FB versus Ank-R LV

With the aim of analyzing gene expression in long-term murine HSC, primary and secondary transplants were carried out. Lineage negative cells from BM from CD45.1 (+) B6.SJL-Ptprc a Pep3b/BoyJ male donor mice were transduced with the Ank-R LV and transplanted into congenic CD45.2 (+) C57bl/6J female mice and compared with mice transplanted with the cells transduced with the parental FB LV. VCN from transduced BM cells cultured in vitro for 2 weeks was 2.4 ± 0.2 for the FB LV and 2.6 ± 0.1 for the Ank-R LV, from two independent transductions.

Engraftment in blood was above 90% at 10 weeks post-transplant in all primary mice (Supplementary Figure S4a). VCN in blood samples was measured periodically every 3 weeks up to 20 weeks, showing stable and efficient gene transfer, without differences between vectors (repeated-measure ANOVA) (Supplementary Figure S5a). βAS3-globin mRNA expression was analyzed simultaneously from blood samples obtained for VCN analysis. The percentage of the βAS3-globin mRNA transcripts relative to the murine β-globin-like mRNA transcripts (“percentage of the βAS3-globin mRNA”) per VCN in blood of the primary recipients did not differ between groups (Supplementary Figure S5b) at any of the time points analyzed (repeated-measure ANOVA). Primary mice were euthanized 20 weeks post-transplant to perform secondary transplants. No differences were observed in engraftment, VCN, and βAS3-globin mRNA expression in the BM from the primary mice, as was observed in
blood samples. The engraftment of donor cells in BM from primary mice ranged from 70 to 99% (Supplementary Figure S4b), and the VCN values were 2.03 ± 1.03 and 2.09 ± 1.14 for the FB LV and Ank-R LV, respectively; and the average expression of the βAS3-globin mRNA normalized to the VCN was 3.45 ± 1.47% and 4.85 ± 1.97% for the FB-LV and Ank-R LV, respectively (Supplementary Figure S6).

Secondary mice were euthanized 11 weeks post-transplant. High levels of engraftment were observed in blood and BM in the three groups of mice (Supplementary Figure S7). The blood samples were analyzed to determine gene marking in the two groups FB LV (0.96 ± 0.64) and Ank-R LV (0.84 ± 0.95) and no difference was seen in the two groups (P > 0.05, two-sample t-test) (Figure 4a). However, the gene-expression values [βAS3-globin mRNA/VCN] were statistically higher in the Ank-R LV group compared with the FB LV group (P = 0.001; two-sample t-test) (Figure 4b). We observed that the βAS3-globin transgene expression per VCN declined in the FB LV group significantly (P = 0.01; two-sample t-test), from 24.76 ± 25.30% [βAS3-globin mRNA/VCN] in primary mice to 6.92 ± 6.05% [βAS3-globin mRNA/VCN] in secondary mice. In contrast, no significant differences were observed in the βAS3-globin transgene expression per VCN between primary (28.13 ± 14.64% [βAS3-globin mRNA/VCN]) and secondary mice (32.68 ± 31.09% [βAS3-globin mRNA/VCN]) for the Ank-R LV-treated group (P = 0.7, two-sample t-test). These results suggest that the inclusion of the Ank insulator protected long-term expression of the βAS3-globin cassette from silencing (Figure 4c).

Similar results were obtained with BM samples from the secondary mice. There were no differences in gene transfer between the FB and Ank-R LV-treated groups (Figure 5a). However, the expression levels of the FB LV were significantly lower than the expression levels of the Ank-R LV (P < 0.001, two-sample t-test) (Figure 5b). When the transgene expression was compared between BM of primary and secondary mice, the same pattern as in blood was observed. The expression of the βAS3-globin transgene was maintained between primary (4.85 ± 1.97% [βAS3-globin mRNA/VCN]) and secondary mice (3.74 ± 2.06% [βAS3-globin mRNA/VCN]) in the Ank-R LV group (showing no significant differences P = 0.2, two-sample t-test), but was significantly lower in the secondary mice of the FB LV group (1.43 ± 1.36% [βAS3-globin mRNA/VCN]), compared with the primary mice (3.45 ± 1.47% [βAS3-globin mRNA/VCN]) (P = 0.002; two-sample t-test) (Figure 5c).

The expression of the vectors was also analyzed at the protein level by measuring AS3 hemoglobin (HbAS3) production in blood, by high-performance liquid chromatography (HPLC). The higher percentage of βAS3-globin mRNA produced per VCN by the Ank-R LV-treated group (P = 0.7, two-sample t-test).
β-correction in -thalassemic mice, at least to the same levels as its moter, when included in both LTR, was able to maintain phenotypic expression in the provirus, leading to a more stable and consistent expression of the transgene of interest can be negatively affected. These results confirm that one copy of the Ank insulator in reverse orientation in the CCL-β-ANK1 gene to provide a more 5'HS promoter region of the human expression. Barrier insulators are defined as heterochromatin boundaries as they block the spread of the heterochromatin; addition of barrier elements to LV constructs may protect from heterochromatin formation in the provirus, leading to a more stable and consistent expression from silencing in the long-term transduced HCS.

DISCUSSION
Chromosomal-position effects on gene expression have a critical impact in LV-based gene therapy due to the semirandom pattern of integration of these vectors. If the integration of the vectors happens within or near heterochromatin areas, the level and pattern of expression of the transgene of interest can be negatively affected. Barrier insulators are defined as heterochromatin boundaries as they block the spread of the heterochromatin; addition of barrier elements to LV constructs may protect from heterochromatin formation in the provirus, leading to a more stable and consistent expression.

We have analyzed the potential capacity of the insulator from the 5'HS promoter region of the human ANK1 gene to provide a more stable and homogenous expression from the parental CCL-βAS3-FB LV previously described by our group. Breda et al. showed that in the context of a β-globin LV, a 190bp fragment of the ANK1 promoter, when included in both LTR, was able to maintain phenotypic correction in β-thalassemic mice, at least to the same levels as its counterpart noninsulated LV. However, in all of the new constructs we designed, the Ank insulator was placed within the βAS3-globin cassette with the aim of keeping the Ank element flanked by the enhancer-blocking FB insulator, included in both LTR of the provirus. With this design, the genes surrounding the proviral integration site would be protected by the FB insulator from any residual enhancer activity from the Ank fragment. The stability of the Ank sequence in the different constructs was analyzed, indicating that the presence of more than one copy of the insulator led to the loss of one or both of these sequences. A potential explanation for the loss of the insulator would be deletion during reverse transcription or homologous recombination after the reverse transcription; however further analysis would be necessary to fully understand these events.

Protection against position-effect variegation was observed in MEL clones only when a single copy of the Ank insulator was used. But, after 4 months in culture, the clones from all of the LV analyzed, including the ones containing the full ch54 insulator, showed a dramatic loss of expression. This could be a result of the loss of differentiation capacity of the erythroleukemia cell line used for this assay after extended culture. Therefore, the Ank-R LV was compared with the FB and ch54 LV in human primary cells. However, the in vitro assessment of the barrier activity of the Ank-R LV in cells from SCD BM donors was not conclusive, as a result of the short duration of the erythroid differentiation of the bulk population analyzed.

Figure 5 Long-term assessment of VCN and βAS3-globin mRNA expression in BM from secondary mice. (a) VCN analyzed in BM samples 11 weeks post-transplant. No differences in VCN were found between mice treated with the two different LV (P = 0.50, two-sample t-test). (b) Percentage of βAS3-globin mRNA/VCN in BM from secondary mice. (c) The percentages of βAS3-globin mRNA expressed in the primary and secondary mice at week 20 and 11 post-transplant, respectively, were analyzed for each LV (two-sample t-test). FB-LV treated mice: primary mice, n = 9 and secondary mice, n = 18; Ank-R LV-treated mice: primary, n = 8 and secondary, n = 21. The data shown in this graph at week 20 for the primary mice correspond to the average values shown in Supplementary Figure S6, (right y-axis); and the data shown at week 11 for the secondary mice are the same as graphed in this panel in 5(b). Bars represent average and SD values.
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Chromosomal-position-effect variation therefore was evaluated in vivo in CFU-S from secondary and tertiary mice, which provides a more stringent assay for expression in the products of long-term HSC than short-term cultures and primary transplants do. The consistent level of expression of the βAS3-globin transgene per VCN in the Ank-R LV group, compared with the wide range of expression of the transgene per VCN in the FB LV group, indicates that the Ank insulator is able to provide a more position-independent expression of the βAS3-globin transgene. The HS3 fragment of the β-globin gene LCR has been shown to be able to provide an open chromatin environment appropriate to confer copy-number-dependent gene expression in transgenic mice;28,29 however, only the whole LCR is able to provide the optimal chromatin architecture necessary to confer position independence. Therefore, the addition of the Ank sequence upstream of the mini-LCR in the FB-LV might be responsible for recruiting transcription factors such as USF1/2 (ref. 23) that directly interacts with HAT and HMT maintaining high levels of H3 acetylation and K4 methylation as shown with the chS4 insulator.30

The protection against position-effect variation observed at a clonal level in the Ank-R LV group correlated with a lack of silencing in long-term vector-transduced HSC in serial transplants in the Ank-R LV-treated mice. The main difference between the vectors with and without the Ank insulator was observed in the transgene expression in blood in secondary mice, where the Ank-R LV provided a significantly higher percentage of expression of the βAS3-globin transgene per VCN, at both the mRNA and protein levels. Additionally, the average percentages of βAS3-globin mRNA per VCN in both primary and secondary mice from the Ank-R LV group were very consistent and sustained, showing no silencing of the βAS3-globin cassette. In contrast, the expression levels of the βAS3-globin transgene in the secondary mice treated with the FB LV were significantly lower compared with the primary mice of this group. The same pattern of mRNA expression per VCN was observed in the BM samples from secondary mice.

When initially describing the Ank element, Gallagher et al.31 demonstrated the enrichment for USF1/2 transcription factors and histone markers associated with open chromatin, thus following a model for barrier insulator activity in which proteins are recruited to block the spread of heterochromatin. Our data follow this model as the addition of the Ank insulator to the CCL-AS3-FB LV promoted in vitro and in vivo protection from chromosomal-effect variation with a lower CV of the βAS3-globin transgene expression per VCN. In addition, the Ank insulator helped to maintain the expression of the βAS3-globin transgene per VCN at the RNA level, which was correlated with higher HbAS3 protein production in long-term secondary mice.

These data together indicate that the addition of the Ank insulator protects the βAS3-globin expression cassette from positional-effect variation and long-term silencing after transplantation of primitive murine HSC. Further analyses in murine SCD models will allow assessment of whether the addition of the Ank element to the CCL-AS3-FB LV significantly improves inhibition of sickling in a clinically beneficial manner.

MATERIALS AND METHODS

βAS3-globin lentiviral vectors construct, packaging, and titer determination

The CCL-βAS3, CCL-βAS3-FB, and CCL-βAS3-chS4 LV were previously described. Briefly, the LV carrying the Ank insulator were cloned as follows. Ank-dGATA1-top and bottom decoy oligonucleotides (see sequences below) were annealed and made double stranded with Pfu Ultra II HS Polymerase (Agilent, Santa Clara, CA). For Ank insertion at the 3’ end of the gldobin cassette next to the 3’ genomic enhancer, pCCL-AS3-dWPRE-FB was linearized with EcoRV and dephosphorylated with Antarctic phosphatase (New England Biolabs, Ipswich, MA). Blunt-end ligation was carried out with this linearized vector and the annealed, double-stranded Ank cassette, and clones were screened for single-cassette insertion and proper orientation. For Ank insertion at the 5’ end of the gldobin cassette next to the HS4 sequence of the mini-LCR, pCCL-AS3-dWPRE-FB was cut with MfeI (New England Biolabs, Ipswich, MA). The large and small fragments were gel purified. The large fragment was religated, and the resulting plasmid was then digested with EcoRI (New England Biolabs), blunted, and dephosphorylated. The blunt-end ligation was carried out with this linear vector and the Ank cassette, and clones were screened for single-cassette insertion and proper orientation. The smallest of the gel-purified MfeI fragments was then reinserted to generate the full AS3 vector with desired Ank sequences. Ank-dGATA1-top /5Phos/TGGGGAGCCGCTCTCCTGTGGGTATTTCACCCCCGCTGCTGGCAGGGCTAGGAGGTGCTCTTGTAATCTGCGGT7 (HindIII) and 7 GGGGGCCTCCTGGGGTTGGGGGAGGAGGTGCTCTTGTAATCTGCGGT (BamHI) were ligated to generate the full plasmid. The protection against position-effect variation observed at a clonal level in the Ank-R LV group correlated with a lack of silencing in long-term vector-transduced HSC in serial transplants in the Ank-R LV-treated mice. The main difference between the vectors with and without the Ank insulator was observed in the transgene expression in blood in secondary mice, where the Ank-R LV provided a significantly higher percentage of expression of the βAS3-globin transgene per VCN, at both the mRNA and protein levels. Additionally, the average percentages of βAS3-globin mRNA per VCN in both primary and secondary mice from the Ank-R LV group were very consistent and sustained, showing no silencing of the βAS3-globin cassette. In contrast, the expression levels of the βAS3-globin transgene in the secondary mice treated with the FB LV were significantly lower compared with the primary mice of this group. The same pattern of mRNA expression per VCN was observed in the BM samples from secondary mice.

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The CCL-βAS3, CCL-βAS3-FB, and CCL-βAS3-chS4 LV were previously described. Briefly, the LV carrying the Ank insulator were cloned as follows. Ank-dGATA1-top and bottom decoy oligonucleotides (see sequences below) were annealed and made double stranded with Pfu Ultra II HS Polymerase (Agilent, Santa Clara, CA). For Ank insertion at the 3’ end of the globin cassette

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using the PureLink Genomic DNA Mini Kit (Invitrogen). Two sets of primers were designed to amplify the Ank sequence upstream of the β-globin LCR cassette (near the 5′ end of the vector), one set to amplify the Ank insulator in forward orientation (Ank-F: Forward: 5′-AGT TTG GGT CGA GGA TTG GGG AGC-3′ and Reverse: 5′-GAA TTC CGA TCC GAT GTG CCG GGG C-3′, with an expected band of 190 bp) and a second set to amplify the Ank insulator in reverse orientation (Ank-R5′: Forward: 5′-GAG TGG AGG ATG AGC TGG GCG CCG ACG-3′ and Reverse: 5′-GAG AAT TGA CCT CCG ATT GGG GAG C-3′, with an expected band of 187 bp). A third set of primers was designed to amplify the Ank insulator located downstream of the β-globin LCR cassette (near the 3′ end of the vector), and placed in reverse orientation (Ank-R3′: Forward: 5′-CCA GGA GAT TGA CGT CCG and Reverse: 5′-TTG ATT ATC GGA ATT TGG GGA GGC GGC C-3′, in this case the corresponding amplicon had a length of 183 bp). The stability of the ch54 insulator was tested as well by PCR in NEL clones that were transduced with the LV carrying this insulator. In this case, the primers used were the following ones. To amplify the ch54 sequence in the 5′LTR of the provirus: F-5LTR-FB: 5′-GGCTAATTCACTCCTCCACAAGACAACAG-3′ and R-5LTR-FB: 5′-CTT CAG CAA GCC GAC TCC TGC-3′. To amplify the ch54 sequence in the 3′LTR of the provirus: F: 3′-LTRCH54: 5′-CTG CAG ATA TCC ATC ACA CTA CGT GGC-3′ and R-3LTR-FB: 5′-CAG AGA GAC CCA GTA CAA GCA AAA AG-3′. PCR were executed using Taq DNA Polymerase, Native (Invitrogen) on an Eppendorf (Hamburg, Germany) thermocycler. The PCR products were visualized by GelGreen (Phenix Research Products, Candler, NC) on 2% agarose gels.

Human CD34+ cell isolation, transduction, erythroid differentiation, and VCN and mRNA analysis

Isolation of human CD34+ cells from BM, transduction, and in vitro erythroid differentiation were performed as described by Romero et al.6 Bone marrow aspirates from volunteer donors with SCD were obtained under UCLA IRB # 010399.

Isolation, transduction, and transplantation of murine HSCs

BM cells from females and females from untreated 8-week-old male CD45.1 (+) B6C3F1-Pep3+/BoyJ (Jackson Laboratories, Bar Harbor, ME) donor mice were harvested, lineage depleted using the Lineage Cell Depletion Kit (Miltenyi Biotech, Bergisch Gladbach, Germany) and lineage negative (lin) cells were cryopreserved. Forty hours prior to transplant, lin cells were thawed and prestimulated in StemSpan SFEM serum-free expansion medium (STEMCELL Technologies, Vancouver, British Columbia, Canada) containing 50 ng/ml mSCF, 100 ng/ml human interleukin (IL)-1, 20 ng/ml IL-3, 100 ng/ml hFlt3-L (Celldex Therapeutics, Needham, MA), and 1x glutamine, penicillin and streptomycin in plates coated with retronectin (20 μg/ml; Clontech, Mountain View, CA). After 16 hours of prestimulation, transductions with the FB and Ank-R-LV were performed at the concentration of 2 x 10^5 TU/ml (MOI ~80). Twenty-four hours post-transduction, the cells were harvested, counted, and 10^7 lin cells were transplanted into 8-week-old female CD45.2 (+) C57Bl/6J (Jackson Laboratories) after total body irradiation (10.25 Gy; split into two doses separated by 3 hours). Primary mice were maintained up to 4 months and retro-orbital bleeds were performed periodically for VCN and β-globin mRNA analysis.

Mice harvest and secondary transplants

Four months after transplants, BM cells from females and females of primary recipients were harvested. Portions of the marrow were used to determine engraftment by FACS, and for VCN and mRNA analyses. Prior to euthanasia, retro-orbital bleeds were performed as well for engraftment, VCN, and mRNA from blood analysis at that last time point. For long-term analyses of gene expression and silencing, 20 x 10^5 cells of whole female BM cells were transplanted into each second recipient. Secondary mice were euthanized after 11 weeks for final analysis of engraftment, VCN, and [βAS3-globin mRNA in blood and BM. All work with mice was done under protocols approved by the UCLA Animal Research Committee.

Spleen colony-forming units (CFU-S)

For vector expression analyses in clonal progeny from murine HSC, 1 x 10^6 whole BM cells from primary and secondary mice were transplanted in multiple CD45.2 female recipients. Individual CFU-S (secondary and tertiary) were isolated by dissection from spleens collected 10 days after transplants. Each CFU-S was divided in two halves, for genomic DNA isolation (PureLink Genomic DNA Mini Kit, Invitrogen) for VCN determination and for RNA isolation (RNeasy Plus Mini Kit, Qiagen, Valencia, CA) for βAS3-globin mRNA expression assay.

Engraftment assessment

Donor-recipient chimerism was determined in blood and BM samples from primary and secondary recipients by FACS. Samples were incubated at 4°C for 5 minutes with rat anti-mouse CD16/CD32 antibody to block Fc receptors, prior to addition of APC-conjugated mouse anti-mouse CD45.2 and V450-conjugated mouse anti-mouse CD45.1 (all antibodies from BD Biosciences) followed by incubation at 4°C for 30 minutes. Antibody excess was washed out with PBS and samples were analyzed with a LSR Fortessa cell analyzer (BD Biosciences). The percentage of engrafted donor cells was defined as follows: %CD45.1+/ (%CD45.1+ + %CD45.2+).

DNA analysis

Genomic DNA isolations from blood samples and BM samples were performed using the NucleoSpin Tissue XS kit (Clontech) and the PureLink Genomic DNA Mini Kit (Invitrogen), respectively, and according to manufacturer's instructions.

For VCN determination, primers and probes were designed to detect a conserved LV sequence (HIV-1 Psi region). Forward-HIV-US: 5′-AAATGATGTTGCGCTCCGTCG-3′; Reverse-HIV-Psi: 5′-CCCTGTTCTCTCTTTGCCGCT-3′ and probe: 5′-SHEX/CCTCTCA/GAC/ZN/CCTT/GATC/TGTTGGAA AATCTTCAA/31A/BQF/3′ and Y-chromosome-specific primers and probe (Mm00527143_en) (published as a 20x premix of primers and FAM-MGBNFQ probe; Applied Biosystems, San Francisco, CA) were used for normalization. Reaction mixtures of 20 μl volume comprising 1× ddPCR Master Mix (Bio-Rad, Hercules, CA), primers, and probe (900 nmol/l and 250 nmol/l for Y-chromosome primers and probe respectively; 500 nmol/l and 100 nmol/l for Psi primers and probe, respectively) were used. Droplet generation was performed as described in Hindson et al.12 PCR amplification of droplet emulsion, droplet read, and ddPCR analysis were performed as previously described. VCN of donor cells was calculated by dividing the concentration (copies/μl) of PSI by the concentration of Y-chromosome.

RNA analysis

RNA from BM samples was extracted using the RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer's instructions. Blood samples for RNA analysis were collected in RNAProtect Animal Blood Tubes (QIAGEN) and incubated at room temperature for 2 hours, prior to RNA isolation with RNeasy Protect Animal Blood Kit (QIAGEN). In both cases, first-strand cDNA was synthesized using random primers, M-MLV reverse transcriptase, and RNAOUT Recombinant Ribonuclease Inhibitor (all from Invitrogen) according to the manufacturer's protocol.

To specifically detect βAS3-globin mRNA transcripts originating from the vector CCL-βAS3-FB, a set of primers and probe, previously described (HBBAS3),2 were used together with a second set of primers and probe described for amplification of murine single haplotype β-globin-like transcripts. For reverse-murine β-globin-like transcripts, Forward-murine β-globin probe: 5′-GTCTGTGGTGTTCCTACCTGGAC-3′; Reverse-murine β-globin probe: 5′-GTCTTAAAGGACGATGATCACCTTTCCTG-3′ and probe: 5′-SHEX/CCTATCC/TCC/ZEN/TGCCTCTGATCCGGATATTGCAAA/31A/BFx (all primers were used at a final concentration of 500 nmol/l and probes at 100 nmol/l); ddPCR was performed as described2 and the relative expression of βAS3-globin/murine β-globin-like was calculated by dividing the concentration (copies/μl) of βAS3-globin by the concentration of murine β-globin-like, and normalizing to the VC/cell measured for each sample.

High-performance liquid chromatography (HPLC)

Secondary mice were bled 11 weeks post-transplant. One microliter of peripheral blood was lysed as follows: RBCs were pelleted and lysed in Hemolytase reagent (Helena Laboratories, Beaumont, TX) for 5–10 minutes at room temperature. After centrifugation at 20,800g for 10 minutes at 4°C to remove cellular debris, RBC lysates were stored frozen at −80°C. Upon thawing, cell lysates were diluted 1:40 in mobile phase A and characterized by HPLC (Infinity 1260, Agilent) using a weak cation-exchange column (PolyCAT A, PolyLC, Columbia, MD). FASC Reference Material (Trinity Biotech, Wicklow, Ireland) was used to define the elution time of common human hemoglobin forms (HbF, HbA, HbS, and HbC). Analysis and peak integration were performed using OpenLAB CDS Chemstation software (Agilent, Santa Clara, CA). The relative percentage of HbAS3 produced for each sample was calculated based on the sum total
of areas under the curve for each of the primary hemoglobin peaks, which included β-major and β-minor.

Statistical analysis

Descriptive statistics such as number of observations, mean and SD were reported and presented graphically for quantitative measurements. Normality assumption was checked for outcomes before statistical testing. For both primary and secondary mice, comparisons of expression (i.e., VCN, percentage of βAS3-globin mRNA/VCN, and percentage of HbAS3/VCN) and engraftment in BM and blood between experimental groups were performed by either two-sample t-tests (Figure 4a–d; Figure 5a–c and Supplementary Figure S5e) or one-way ANOVA with pairwise comparisons (Figure 2a,b; Supplementary Figure S3a,b; Supplementary Figure S4a,b; Supplementary Figure S5a,b). Repeated-measure ANOVA was used to assess if there was treatment group difference in AS3-globin expression in MEL cell clones (Figure 2c) or in blood over time (Supplementary Figure S5a,b). All hypothesis testing was two-sided and a significance threshold of 0.05 for P-value was used. Analyses were carried out using SAS version 9.3 (SAS Institute, Cary, NC).35

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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11. Miccio, A, Cesari, R, Lotti, F, Rossi, C, Sanvito, F, Ponzozi, M et al. (2008). In vivo selection of genetically modified erythroid progenitors leads to long-term correction of beta-thalassemia. Proc Natl Acad Sci USA 105: 10547–10552.

12. Pay, D, Gray, JT, Boyd, KM and Persons, DA (2009). Correction of murine sickle cell disease using gamma-globin lentiviral vectors to mediate high-level expression of fetal hemoglobin. Mol Ther 17: 245–252.

13. Antoniou, M, Geraghty, F, Hurst, J and Grosved, F (1998). Efficient 3′-end formation of human beta-globin mRNA in vivo requires sequences within the last intron but occurs independently of the splicing machinery. Nucleic Acids Res 26: 721–729.

14. Custódio, N, Carmo-Fonseca, M, Geraghty, F, Pereira, HS, Grosved, F and Antoniou, M (1999). Inefficient processing impairs release of RNA from the site of transcription. EMBO J 18: 2855–2866.

15. Grosved, F, de Boer, E, Dillon, N, Fraser, P, Gribnau, J, Milot, E et al. (1998). The dynamics of globin gene expression and gene therapy vectors. Semin Hematol 35: 105–111.

16. Li, Q, Peterson, KR, Fang, X and Stamatoyannopoulos, G (2002). Locus control regions. Nature 467: 318–322.

17. Zufferey, R, Dull, T, Mandel, RJ, Bukovsky, A, Quioz, D, Naldini, L et al. (1998). Self-inactivating lentivector virus for safe and efficient in vivo gene delivery. J Virol 72: 9873–9880.

18. Ellis, J and Pannell, D (2001). The beta-locus control region versus gene therapy vectors: a struggle for expression. Clin Genet 59: 17–24.

19. Ellis, J (2005). Silencing and variegation of gammaretrovirus and lentivirus vectors. Hum Gene Ther 16: 1241–1246.

20. Arumugam, PI, Scholes, J, Perelman, N, Xia, P, Yee, JK and Malik, P (2007). Improved human beta-globin expression from self-inactivating lentiviral vectors carrying the chicken hypersensitive site-4 (cHS4) insulator element. Mol Ther 15: 1863–14 (cHS4) insulator element. Mol Ther 15: 1863–1871.

21. Urbinati, F, Arumugam, P, Higashimoto, T, Perumbeti, A, Mitts, K, Xia, P et al. (2009). Mechanism of reduction in titters from lentivirus vectors carrying large inserts in the 3′LTR. Mol Ther 17: 1527–1536.

22. Breda, L, Casu, C, Gardenghi, S, Bianchi, N, Cartegni, L, Narla, M et al. (2012). Therapeutic hemoglobin levels after gene transfer in β-thalassemia mice and in hematopoietic cells of β-thalassemia and sickle cell disease patients. PLoS One 7: e46746.

23. Lu, Q and Stamatoyannopoulos, JA (1994). Position independence and proper developmental control of gamma-globin gene expression require both a 5′-locus control region and a downstream sequence element. Mol Cell Biol 14: 6087–6096.

24. Ellis, J, Tan-Oh, KC, Harper, A, Michalovich, D, Yamnoutos, N, Philipson, S et al. (1996). A dominant chromatin-opening activity in 5′-hypersensitive site 3 of the human beta-globin locus control region. EMBO J 15: 562–568.

25. Douay, L and Giarratana, MC (2009). Ex vivo generation of human red blood cells: a new advance in stem cell engineering. In: Walker, JM, Audet, J and Stanford, WL (eds). Methods in Molecular Biology, vol. 482. Humana Press/Totowa, NJ, 127–140.

26. Emery, DW (2011). The use of chromatin insulators to improve the expression and safety of integrating gene transfer vectors. Hum Gene Ther 22: 761–774.

27. Maitani, K, Leblanc, S, Salvarin, M, Maitani, Y, Rhee, EN et al. (2012). Therapeutic human hemoglobin synthesis in beta-thalassemic mice expressing lentivirus-encoded human-beta-globin. Nature 460: 86–82.

28. Pawliuk, R, Westerman, KA, Fabry, ME, Payen, E, Tighe, R, Bouhassira, EE et al. (2001). Correction of sickle cell disease in transgenic mouse models by gene therapy. Science 294: 2368–2371.

29. Levassor, DN, Ryan, TM, Pawlik, KM and Townes, TM (2003). Correction of a mouse model of sickle cell disease: lentiviral/antiscissint beta-globin gene transduction of unmobilized, purified hematopoietic stem cells. Blood 102: 4312–4319.

30. Hanawa, H, Hargrove, PW, Kepse, S, Srivastava, DK, Nienhuis, AW and Persons, DA (2004). Extended beta-globin locus control region elements promote consistent therapeutic expression of a gamma-globin lentiviral vector in murine beta-thalassemia. Blood 104: 2281–2289.

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