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ORIGINAL RESEARCH PAPER

Integration pattern of HIV-1 based lentiviral vector carrying recombinant coagulation factor VIII in Sk-Hep and 293T cells

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Abstract 293T and Sk-Hep-1 cells were transduced with a replication-defective self-inactivating HIV-1 derived vector carrying FVIII cDNA. The genomic DNA was sequenced to reveal LTR/human genome junctions and integration sites. One hundred and thirty-two sequences matched human sequences, with an identity of at least 98%. The integration sites in 293T-FVIIIDB and in Sk-Hep-FVIIIDB cells were preferentially located in gene regions. The integrations in both cell lines were distant from the CpG islands and from the transcription start sites. A comparison between the two cell lines showed that the lentiviral-transduced DNA had the same preferred regions in the two different cell lines.

Keywords Coagulation factor VIII · Lentiviral vector · Integration sites

Purpose of work We have examined lentiviral integration site selection in hepatocyte and kidney cell lines to see whether there are determinants of integration tendency other than the structure and biological characteristics of lentiviruses.

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Introduction

Lentiviral vectors are at the forefront of gene delivery systems for gene therapy due to some of their capacity to transduce slowly dividing and non-dividing cells, to insert large genetic payloads in the host chromatin, and to sustain stable long-term transgene expression (Picanço-Castro et al. 2008). However, gene therapy treatments through lentiviral vector integration can lead to tumor formation. Retroviruses that do not carry oncogenes usually induce tumors in the host by insertional mutagenesis. In a mouse experimental model, mice developed leukemia as a consequence of the high expression levels of the evi-1 proto-oncogene, caused by a nearby integrated proviral genome (Li et al. 2002). In clinical trials for X-chromosome-severe-combined immunodeficiency (X1-SCID) (Cavazzana-Calvo et al. 2000), integration of the retroviral vector in the LMO-2 proto-oncogene resulted in insertional mutagenesis and development of T-cell leukemia in children (Hacein-Bey-Abina et al. 2003a, b).

Integration events of retroviruses were believed to be random, and the chance of accidentally disrupting or activating a gene was considered remote. Early studies of retroviral integration proposed that condensed chromatin from inactive DNA regions was not prone to retroviral integration; integration would occur predominantly in more open and active regions (Rohdewohld et al. 1987). Various studies have shown that most of the host genome is accessible to retroviral integration and also that target site selection is not random (Mitchell et al. 2004; Wu et al. 2003). Many researchers have addressed the viral integration profile of viruses to better understand virus integration behavior and to assess the risk of gene therapy. Whether transduction of different cell lines with the same lentiviral vector produces different or similar patterns of integration remains to be elucidated.

We showed that a lentiviral-based vector carrying B-domain-deleted FVIII (FVIIIDB) can generate human hepatic and kidney cell lines that produce FVIIIDB in vitro (Picanco et al. 2007). These two cell types have a similar number of lentiviral copies/cell, though their levels of FVIII expression are quite different. We examined lentiviral integration site selection in hepatocyte and kidney cell lines to see whether there are determinants of integration tendency other than the structure and biological characteristics of lentiviruses.

Materials and methods

Lentiviral vector and cell lines

Human embryonic kidney epithelial cells Hek 293T (DSMZ ACC 305, www.dsmz.de) and human liver adenocarcinoma cell line Sk-Hep-1 (DSMZ ACC 141, www.dsmz.de) cells were transduced with a replication-defective self-inactivating HIV-1 derived vector containing the coagulation factor FVIII gene (cPPT-C (FVIIIDB)IGWS). The vector cPPT-C(FVIIIDB) IGWS is a derivative of the bicistronic vector C(FVIIIDB)IGWS coding for human FVIIIDB and EGFP(Tonn et al. 2002). EGFP was used as a marker for transduction efficiency and selection. Infected cells were selected by sorting on a FACSVantage SE flow cytometer. We were interested in cells expressing high levels of FVIIIDB. Cells with a high level of EGFP expression were sorted and expanded in culture. The FVIIIDB-expressing cells were named 293T-FVIIIDB and Sk-Hep-FVIIIDB. The two cell lines that we used had a similar number of integrated lentiviral copies/cell. The 293T-FVIIIDB line contains 1.5 lentiviral copies/cell and 10^6 cells express 2 IU of FVIIIDB/ml and the Sk-Hep-FVIIIDB line contains 1.3 lentiviral copies/cell and 10^6 cells express 5 IU of FVIIIDB/ml (Picanco et al. 2007). The transduced cell lines were analyzed at passage nine. Genomic DNA was isolated from these cells to analyze the lentiviral integration pattern.

Ligation-mediated PCR

Genomic DNA was extracted from 10^6 transduced 293T-FVIIIDB cells and Sk-Hep-FVIIIDB cells. Ligation-mediated polymerase chain reaction (LM-PCR) was performed as previously described (Wu et al. 2003). The genomic DNA was digested with the restriction enzymes MseI and SacI to create a ligation site to the oligonucleotide linker and to avoid amplification of an internal viral fragment from 5′-LTR, respectively. The fragments of genomic DNA were then subjected to ligation of the MseI double strand linker (linker for: 5′-GTAATACGACTCACTAT AGGGCTCCGCTTAAGGGAC-3′ and linker rev:

5′PO_4-TAGTCCCTTAAGCGGAG–NH_2-3′); the ligation reaction tube was incubated at 16°C overnight in the presence of T4 ligase.

The LM-PCR was performed with a pair of primers; one primer was specific to the vector's LTR and the other to the synthetic linker. In the first PCR, we used as a template the DNA digested and bonded to the linker and primers to the 3′LTR junction (HIV 3′LTR-1:AGTGCTTCAAGTAGTGT GTGCC) and to the linker (AP-1: 5′GTAATACGAC TCACTATAGGGC 3′). After denaturation for 2 min at 95°C, PCR was run at 95°C for 25 s, 55°C for 30 s and 72°C for 1 min, for 35 cycles. A 4 min extension at 72°C completed the protocol. A 1 µl aliquot of the PCR product was amplified by nested PCR using primers specific to the proviral 3′ LTR (HIV 3′LTR-2: GTCTGTTGTGTGACTCTGGTAAC) and to the MseI linker (AP-2: 5′-AGGGCTCCGCTTAAGGG AC-3′), using the same PCR protocol.

PCR cloning product and bacterial colony screening

Nested-PCR products were cloned into the pCR2.1-plasmid vector and they were used to transform DH10β bacteria to form libraries of integration junction fragments. Some 154 colonies were screened by direct PCR of the bacterial colonies with standard-vector primers (M13 forward/reverse). The reaction mixture was incubated at 95°C for 3 min, then 35 cycles run at 95°C for 40 s, 60°C for 40 s and 72°C for 55 s. A 5 min extension at 72°C completed the protocol.

Mapping and analysis of the integration sites

The nucleotide sequence of the LM-PCR amplicons was determined using a DYEnamic ET Dye Terminator Kit in a MegaBACE automatic sequencer. The sequences were first analyzed using Chromas 2.23 software. Genomic sequences were considered authentic if flanked by HIV 3′-LTR and linker-specific sequences. The true sequences were analyzed by NCBI BLAST optimized for high similarity using nucleotide query; only high-quality sequences with $\geq$98% of identity to the human genome were further analyzed and included in our study. GTSG/Vector Integration Site Mapping and Analyses (www.gtsg.org) was used to enrich the information about integration sites of the vector in the two cell lines. In addition to the true sequences, we also detected integrations of LTRs back to back; these were not included in the analyses. Four sequences were not long enough to be mapped in the human genome.

Cell growth assays

To analyze cell growth, Sk-Hep and 293T cells (transduced and non-transduced) were seeded into T150 tissue culture flasks, in duplicate, at day 0. Cells were maintained in culture until day 7, to guarantee that maximum confluence had been reached. Confluent cells were removed using trypsin, diluted in buffered Trypan Blue solution. The viable cells were manually counted in a Neubauer hemocytometer. The cells were reseeded and this process was repeated seven times. These assays were done in duplicate to minimize effects such as differences in plating, sensitivity to trypsinization and accuracy in cell counting. Population doubling was calculated as: $x = \log(N/N_0)/\log 2$, where N is the total number of viable cells harvested from culture, N_0 is the number of viable cells seeded and x is the number of times that the cell population had doubled. The doubling time was calculated by dividing the total time elapsed by the number of generations (population doubling). Statistical analyses of the differences in cell growth between non-transduced and transduced cells were performed using Student's-t test.

Statistical analysis

The Chi-square test with Yates' correction was used to analyze whether the observed integration number (oi) arose from a multinomial distribution with specified expected integrations (ei) for the 24 chromosomes (22 autosomes, X and Y). The expected integration was calculated assuming a discrete uniform distribution, correcting for the chromosome size distribution [ei = (chromosome bases/genome bases) $\times$ total number of integrations] The α level for significance was set at 0.05. A cut-off of $(\text{oi} - \text{ei})^2/\text{ei} \geq 3$ was used for detection of preferred integration sites.

Fisher's exact test was used to determine if there is an association between the integrations and the TSS or CpG island regions in the two cell lines.

Results

Analyses of integration sites

We sequenced 154 integration junctions: 83 derived from 293T-FVIIIDB and 71 from Sk-Hep-FVIIIDB cells. Among these, 132 (86%) sequences matched human genome sequences deposited in DNA databases with an identity of at least 98% (60 sequences from Sk-Hep-FVIIIDB and 72 from 293T-FVIIIDB cell lines). Twenty-two of the sequences had no match in the human genome. The integration sites were sequenced to saturation; subsequent to the integrations described here the integration sites began to repeat. We emphasize that we analyzed a population that expressed high levels of GFP and recombinant FVIII. We did further analyses, considering only sequences that matched the human genome. The 72 sequences of 293T-FVIIIDB cells and the 60 from Sk-Hep-FVIIIDB were set at 100%.

The chromosomes that displayed relatively high frequencies (≥5 copies) of integration events in the 293T-FVIIIDB cell genome were chromosomes 1 (20.8%), X (13.9%), 2 (12.5%), 6 (9.7%) and 19 (6.9%). In the Sk-Hep-FVIIIDB cell line, the highest frequencies of integrations were in chromosomes 6 (21.7%), X (18.3%), 1 (11.7%) and 2 (10%). We noted that some chromosomes (1, 2 and X) were targeted by the lentiviral vector in both cell lines in the same position (Fig. 1 and Table 1).

The statistical analysis of the observed integration number versus the expected integrations did not show that integration of this lentiviral vector is a random phenomenon (Fig. 2).

Distance of integration to the transcription start site

When analyzing the distance of the integrants that occurred within 60 kb of a transcription start site (TSS), we found that in cell line 293T-FVIIIDB, 27 integrations were associated with 85 TSS and in cell line Sk-Hep-FVIIIDB 12 integrations were associated with 35 TSS. Among these integrations, the percentage of mapped integrations in the Sk-Hep-FVIIIDB that landed within a 10 kb region (upstream or downstream) was 17%, whereas in 293T-FVIIIDB it was 28%. The percentage integration landing 30–60 kb downstream or upstream of target genes was 83 and 72% for Sk-Hep-FVIIIDB and 293T-FVIIIDB, respectively (Fig. 3a). Fisher's exact test did not show association of the TSS and integration among the two cells ($P = 0.250$), indicating that in the both cell lines the lentiviral vector prefers regions that are distant from the transcription start sites. (Supplementary Table S1)

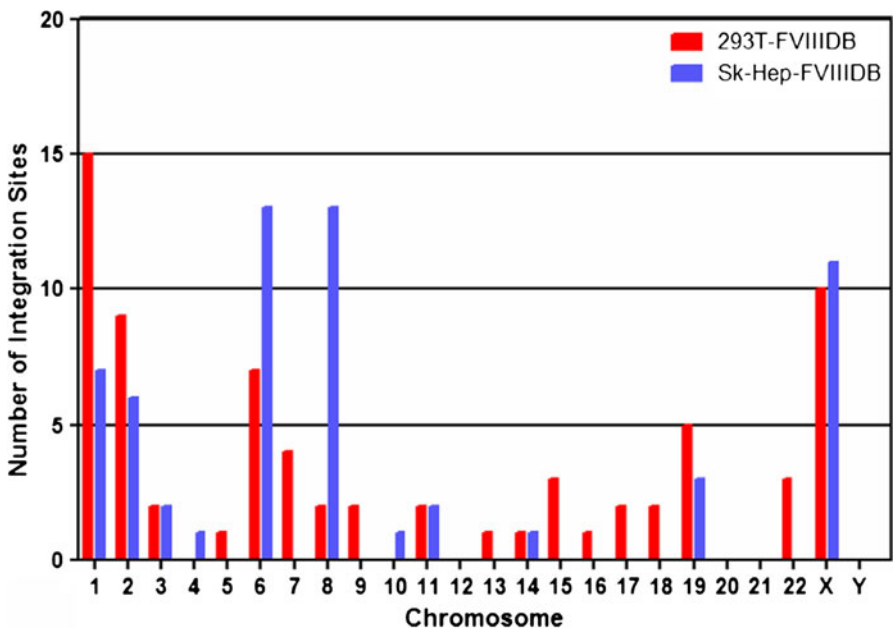

Fig. 1 Distribution of the lentiviral vector integrations (in numbers) into chromosomes of the two cell lines. *Blue bars* represent the number of integrations in each Sk-HepFVIIIDB's chromosome and *red bars* the number of integrations in the 293T-FVIIIDB cells' chromosomes

Table 1 Human genes targeted by the lentiviral vector in both cell lines

	293T integrations	SK-Hep integrations	Gene function	Biological process
CDC42BPA, intron	2	0	Kinase activity, nucleotide binding,	Actin cytoskeleton reorganization, microtubule cytoskeleton organization, intracellular signaling cascade
				Nuclear migration, protein amino acid phosphorylation
MEIS2, intron	3	0	Transcription factor activity	Eye development, regulation of transcription, DNA-dependent
DNASE1L1, intron	2	0	DNA binding	DNA catabolic process
Q14677-2, intron	1	0	Without described function	
ZNF77 e ZNF57, intron	1	0	DNA binding	Regulation of transcription
PIGS, intron	1	0	Protein binding	Attachment of GPI anchor to protein
SPEN, intron	2	0	Nucleic acid binding, transcription factor activity	Notch signaling pathway
EIF3C, intron	1	0	Translation initiation factor activity	Regulation of translational initiation
MCF2L2, intron	1	0	Rho guanyl-nucleotide exchange factor activity	Regulation of Rho protein signal transduction
FRK, intron	1	0	Nucleotide binding	Negative regulation of cell proliferation
IMMP2L, intron	1	0	Peptidase activity	Proteolysis
GOLGB1, intron	1	0	Protein binding	Golgi organization
DNAJC11, intron	1	0		Heat shock protein binding
ZKSCAN1,, intron	1	0	Transcription factor activity	Regulation of transcription, DNA-dependent
MAPK1, intron	1	0	Protein binding	Cell cycle, induction of apoptosis, negative regulation of cell differentiation
PSIP1, intron	1	0	DNA binding	Provirus integration, initiation of viral infection
A2A2Z9_HUMAN, intron	1			
BRWD3, exon	6	11	Cellular cycle; cell-division cycle	
RERE, intron	1	5	Transcription factor activity	Chromatin remodeling, transcription
TTC27, intron	5	5	Protein binding	
RPS6KA4, intron	1	2	ATP binding	Regulation of transcription, DNA-dependent
CARD8, exon	2	0	Caspase activator	Regulation of apoptosis
PHACTR4, intron	5	0		Actin binding
CCBE1, intron	2	0		Calcium ion binding
DNM3, intron	1	0		
Ref Seq peptide	1	0		
Q8NDA8_HUMAN, intron	0	2		
ATAD3B, intron	0	2	Protein folding	
ERC2, intron	0	2	Protein binding	

The hit genes showed in gray are common for both cell lines

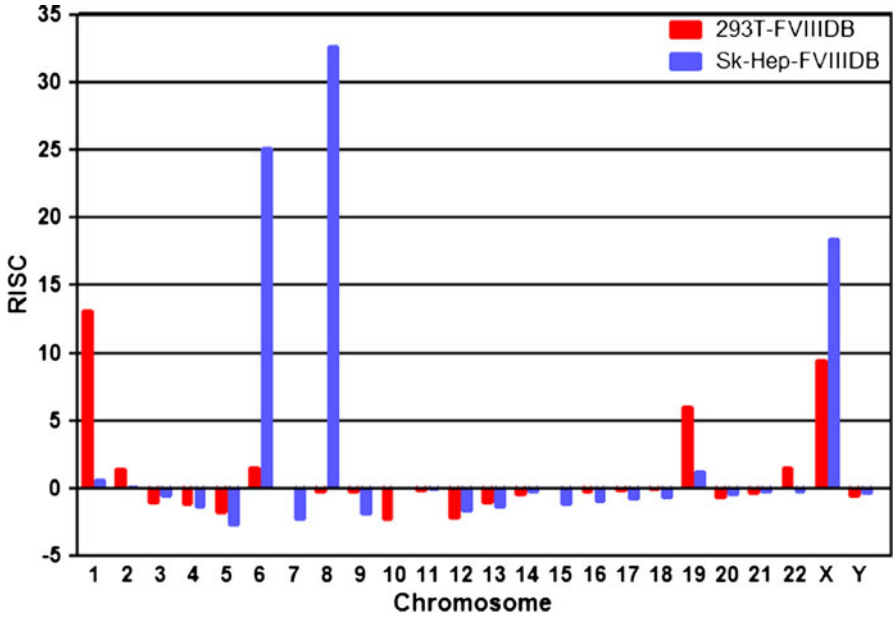

Fig. 2 Schematic distribution of lentiviral vector integrations into chromosomes, showing the RISC score (retroviral insertion estimate into chromosome). Highly significant differences are evidenced by $(oi - ei)^2/ei \geq 3$. In the case of nonpreferential integration, a RISC score of 0 would be expected. The statistics were done with the Chi-square test, followed by the Yates' correction. *Blue bars* Sk-HepFVIIIDB; *red bars* the 293T-FVIIIDB

Distance of integration from CpGs island

CpG islands are associated with promoters that are transcriptionally active at different stages of development and can also be associated with replication origins (Antequera and Bird 1999). We analyzed the distance of the integrants from the next CpG island up- and down-stream (Fig. 3b). In the cell line 293T-FVIIIDB, 44 integrations affected 63 CpG regions located within 50 kb of the location of integration. Among these integrations, the percentage occurring upstream of the CpG islands was 57% for 293T-FVIIIDB and 63% for Sk-Hep-FVIIIDB. The frequency of downstream integrations was 43% in 293T-FVIIIDB and 37% in Sk-Hep-FVIIIDB cells. In both cell lines, integration was generally distant from the CpG islands (>5 kb), accounting for 82.5% in 293T-FVIIIDB and 86% in Sk-Hep-FVIIIDB. Only 17.4% of 293T-FVIIIDB and 14.3% of Sk-Hep-FVIIIDB integrations occurred within 5 kb of the CpG islands. The two cell lines have a similar integration profile in CpG islands (Supplementary Table S2) (with no significant difference; Fisher's exact test $P = 0.781$).

We found that 36% of the integration sites in 293T-FVIIIDB cells and 40% in Sk-Hep-FVIIIDB cells were located in repetitive genomic elements, such as SINES and LINES (Table 2). Among the repetitive sequences, 293T-FVIIIDB cells showed integration in SINES and LINES, while in the Sk-Hep-FVIIIDB cells the most frequent repetitive regions were LINES. We also analyzed the integration frequency near the Alu sequences. We found that in 293T-FVIIIDB, 18% of all mapped integrations landed in these sequences, while in Sk-Hep-FVIIIDB there was no integration in this region (Table 2).

In 293T-FVIIIDB, 68% of the integrations were in RefSeq genes, whereas in Sk-Hep-FVIIIDB, only 48% were in these genes. Interestingly, we found four identical RefSeq for the two cell lines, corresponding to the genes BRWD3, RERE, TTC27, and RPS6K4A (Table 1). Amongst these RefSeq integrations, 84% in 293T-FVIIIDB and 62% in Sk-Hep-FVIIIDB were in intronic regions of the genes.

Analyses of cell growth

Transduced cells had a faster proliferation rate than non-transduced cells (Table 3). The population doubling time in Sk-Hep-FVIIIDB and 293T-FVIIIDB (51.7 h; 49.57 h) was consistently shorter than in non-transduced cells (46 and 37 h, respectively; Student-*t* test—$P < 0.05$).

Discussion

We report the integration profile of the lentiviral vector cPPT-C(FVIIIDB)IGWS in kidney and hepatic

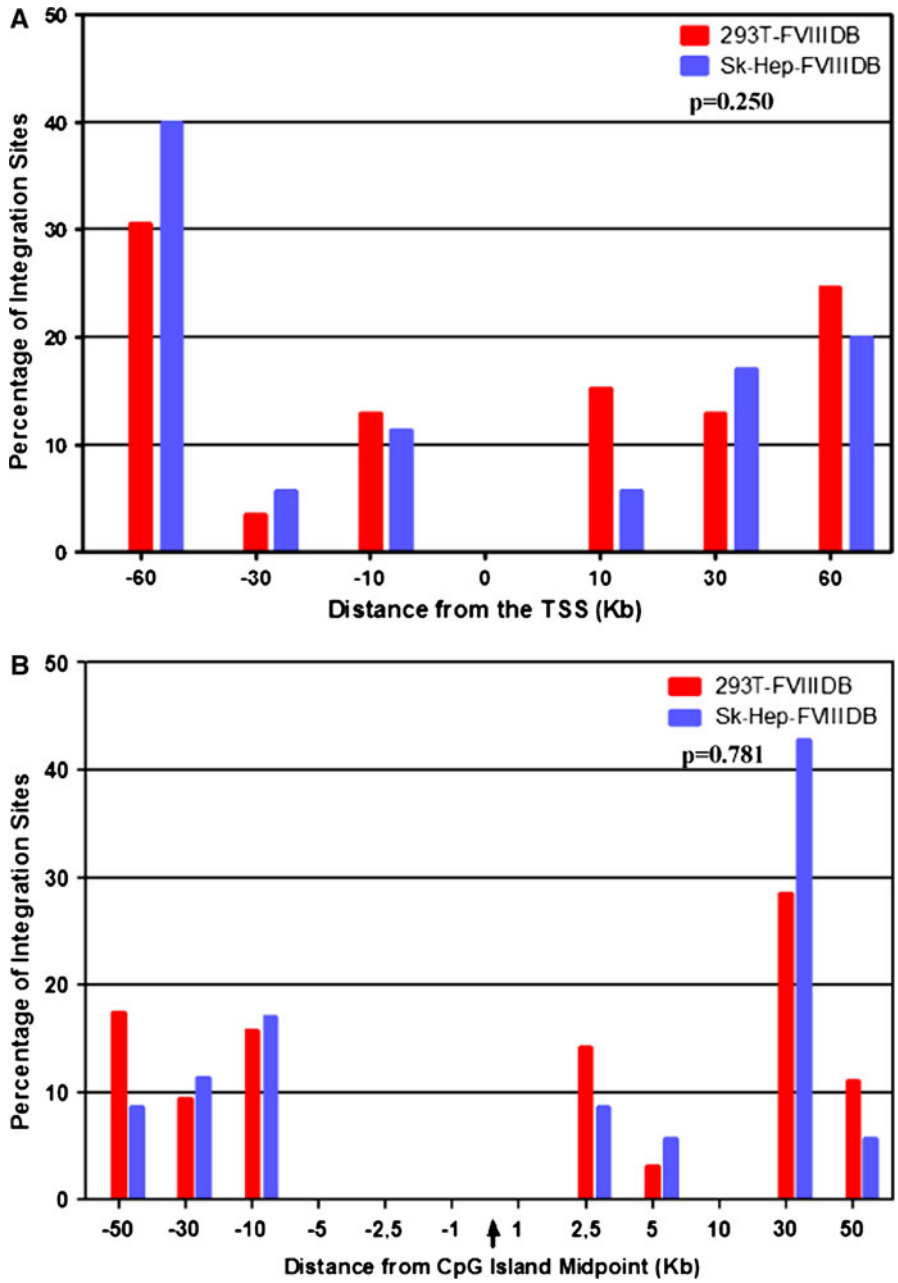

Fig. 3 Frequency of integration sites near transcription start sites (TSS) and CpG islands. **a** The percentage integration within each kb interval is shown for TSS islands, and in **b** for CpG islands. Fisher's exact test did not show statistical association between the integrations and the TSS or CpG island regions in the two cell lines. *Blue bars* Sk-HepFVIIIDB; *red bars* the 293T-FVIIIDB

human cell lines; they produce moderate and high levels of FVIIIDB coagulant protein, respectively. These cells did not have a Y chromosome. Our data did not support the hypothesis that lentiviral integration is a random phenomenon, since the number of integrations was not proportional to the number of base pairs in the chromosomes (Fig. 2). Hacker et al. (2006) did not find a strict relationship between chromosome length and number of integrations. The distribution of the integration sites may have been influenced by some characteristics of the population, since the cells were selected by flow cytometry and the karyotypes were difficult to define, with chromosomes over and underrepresented. To what extent cell selection based on proviral gene expression affects the integration site distribution is uncertain; though, a previous study showed that selecting for proviral expression can influence integration sites (Lewinski

Table 2 Distribution of the lentiviral vector integration sites in the genome of the 293T-FVIIIDB and cell lines sites Sk-Hep-FVIIIDB

The human genome column represents the percentage of chromosomal elements throughout the human genome (Venter et al. 2001)

* *e* expected percentage

Chromosomal feature	Human genome (%)	Integration sites 293T-FVIIIDB (%)	Integration sites Sk-Hep-FVIIIDB (%)
Transcriptional units	31.6	68	48.3
Exon	1.2	11.11 (e:2.7)	18.3 (e = 1.92)
Intron	30.4	56.89 (e:65.3)	30 (e:46.38)
SINES			
Alu	10.6	18.05	0
Mir	2.2	5.55	0
LINE	20	12.5	40

Table 3 Characteristics of the growth of the human cell lines transduced or not with lentiviral vector

	Population doubling (generation)	Population doubling time (h)
Sk-Hep	3.28 ± 0.36	51.7 ± 4.93
Sk-Hep-FVIIIDB	3.71 ± 0.32	45.26 ± 3.76
293T	3.41 ± 0.257	49.57 ± 3.8
293T-FVIIIDB	4.58 ± 0.23	36.75 ± 1.81

Presented data is a mean of cultures in replicate

et al. 2006). Our results do not represent all possible lentiviral integrations; they represent the preferences of this vector in the populations that we analyzed.

The percentage of integrations of the lentiviral vector that landed in RefSeq in both cell lines was significantly higher than that deduced from a computer-simulated random data set, in which 22% of 10,000 simulated integrations landed in RefSeq genes (Wu et al. 2003). This shows that the lentiviral vector also has preference for the RefSeq region, as previously described (Laufs et al. 2006; Beard et al. 2007). Most of the integrations in the RefSeq regions landed in intron sequences. None of the targeted genes are tumor suppressor genes or oncogenes, as defined by the Sanger Cancer Gene Census, different from the findings of Beard et al. (2007). In both cell lines, there was a preference for RefSeq, but there were differences in intron frequency integration in the two populations (293T 84% and Sk-Hep 62%). Evaluation of preference for RefSeq is important because although HIV vectors are self-inactivating, reducing the potential to activate genes by promoter activation from LTR, there is a potential for enhancer activation from the internal promoter.

In chromosomes 1, 2 and X, integrations were found in both cell lines and in the same gene region. These appear to be preferential sites for lentiviral integration. The most commonly affected genes were BRWD3, RERE, TTC27 and RPS6K4A. The work of Laufs et al. (2006) indicated a gene that we also found to be integrated: the bromodomain and WD repeat domain (BRWD3), though they found the integration site in an intron and ours was in an exon in both cell lines.

Cell line Sk-Hep-FVIIIDB produced higher levels of recombinant FVIIIDB in culture and showed 40% of the integrations in LINE regions, compared to only 12.5% in 293T cells. LINE 5′UTR has strong promoter activity in both directions (Lavie et al. 2004); this may explain the high FVIIIDB levels produced by this cell line.

The proportion of integration within 10 kb of the transcript start site and near CpG islands was low, showing no preference for these genome regions. The higher integration preference of some retroviruses, such as MLV, near transcription start sites and CpG islands has been attributed to be a contributing factor to their higher genotoxicity (Kim et al. 2008; Montini et al. 2006). The low preference of the HIV-derived vector integrants for the TSS and CpG islands indicates that it would be safer to use lentiviruses than MLV (Hacker et al. 2006). Integration near transcription start sites can transactivate host genes due to promoter/enhancer effects, leading to malignant transformation (Kustikova et al. 2005).

A comparison between the integration profiles of the two cell lines showed that the lentivirus incorporated into the same preferred regions, with no significant differences in the main DNA regions between the two cell lines. There were some differences between the cell lines that could be due to molecular characteristics of some regions of these cells; as a consequence integration can differ from

tissue to tissue due to cell-specific transcription (Mitchell et al. 2004).

We found a characteristic profile of lentiviral integration in two cell lines, indicating that the vector targets some regions preferentially; an understanding of lentiviral behavior in human cell lines could be exploited for safer gene therapy.

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References

Antequera F, Bird A (1999) CpG islands as genomic footprints of promoters that are associated with replication origins. Curr Biol 9:R661–R667

Beard BC, Dickerson D, Beebe K, Gooch C, Fletcher J, Okbinoglu T, Miller DG, Jacobs MA, Kaul R, Kiem HP, Trobridge GD (2007) Comparison of HIV-derived lentiviral and MLV-based gammaretroviral vector integration sites in primate repopulating cells. Mol Ther 15:1356–1365

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, Bousso P, Deist FL, Fischer A (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 288:669–672

Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M, Fischer A (2003a) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 348:255–256

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N et al (2003b) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 302:415–419

Hacker CV, Vink CA, Wardell TW, Lee S, Treasure P, Kingsman SM, Mitrophanous KA, Miskin JE (2006) The integration profile of EIAV-based vectors. Mol Ther 14:536–545

Kim S, Kim N, Dong B, Boren D, Lee SA, Das Gupta J, Gaughan C, Klein EA, Lee C, Silverman RH, Chow SA (2008) Integration site preference of xenotropic murine leukemia virus-related virus, a new human retrovirus associated with prostate cancer. J Virol 82:9964–9977

Kustikova O, Fehse B, Modlich U, Yang M, Dullmann J, Kamino K, von Neuhoff N, Schlegelberger B, Li Z, Baum C (2005) Clonal dominance of hematopoietic stem cells triggered by retroviral gene marking. Science 308: 1171–1174

Laufs S, Guenechea G, Gonzalez-Murillo A, Zsuzsanna Nagy K, Luz Lozano M, del Val C, Jonnakuty S, Hotz-Wagenblatt A, Jens Zeller W, Bueren JA, Fruehauf S (2006) Lentiviral vector integration sites in human NOD/SCID repopulating cells. J Gene Med 8:1197–1207

Lavie L, Maldener E, Brouha B, Meese EU, Mayer J (2004) The human L1 promoter: variable transcription initiation sites and a major impact of upstream flanking sequence on promoter activity. Genome Res 14:2253–2260

Lewinski MK, Yamashita M, Emerman M, Ciuffi A, Marshall H, Crawford G, Collins F, Shinn P, Leipzig J, Hannenhalli S, Berry CC, Ecker JR, Bushman FD (2006) Retroviral DNA integration: viral and cellular determinants of target-site selection. PLoS Pathog 2:e60

Li Z, Dullmann J, Schiedlmeier B, Schmidt M, von Kalle C, Meyer J, Forster M, Stocking C, Wahlers A, Frank O, Ostertag W, Kuhlcke K, Eckert HG, Fehse B, Baum C (2002) Murine leukemia induced by retroviral gene marking. Science 296:497

Mitchell RS, Beitzel BF, Schroder AR, Shinn P, Chen H, Berry CC, Ecker JR, Bushman FD (2004) Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. PLoS Biol 2:E234

Montini E, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C, Sergi Sergi L, Benedicenti F, Ambrosi A, Di Serio C, Doglioni C, von Kalle C, Naldini L (2006) Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. Nat Biotechnol 24:687–696

Picanco V, Heinz S, Bott D, Behrmann M, Covas DT, Seifried E, Tonn T (2007) Recombinant expression of coagulation factor VIII in hepatic and non-hepatic cell lines stably transduced with third generation lentiviral vectors comprising the minimal factor VIII promoter. Cytotherapy 9:785–794

Picanço-Castro V, Fontes AM, Russo-Carbolante EMS, Covas DT (2008) Lentiviral-mediated gene transfer—a patent review. Exp Opin Therap Patents 18(5):525–539

Rohdewohld H, Weiher H, Reik W, Jaenisch R, Breindl M (1987) Retrovirus integration and chromatin structure: Moloney murine leukemia proviral integration sites map near DNase I-hypersensitive sites. J Virol 61:336–343

Tonn T, Herder C, Becker S, Seifried E, Grez M (2002) Generation and characterization of human hematopoietic cell lines expressing factor VIII. J Hematother Stem Cell Res 11:695–704

Venter JC, Adams MD, Meyers E, Li PW, Mural RJ et al. (2001) The Sequence of the Human Genome. Science 1304–1351

Wu X, Li Y, Crise B, Burgess SM (2003) Transcription start regions in the human genome are favored targets for MLV integration. Science 300:1749–1751