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Brief Report

Tracking of Specific Integrant Clones in Dogs Treated with Foamy Virus Vectors

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Abstract

Vector integration can lead to proto-oncogene activation and malignancies during hematopoietic stem cell gene therapy. We previously used foamy virus vectors to deliver the *CD18* gene under the control of an internal murine stem cell virus promoter and successfully treated dogs with canine leukocyte adhesion deficiency. Here we have tracked the copy numbers of 11 specific proviruses found in these animals for 36–42 months after transplantation, including examples within or near proto-oncogenes, tumor suppressor genes, and genes unrelated to cancer. We found no evidence for clonal expansion of any of the clones, including those with proviruses in the *MECOM* gene (*MDS1-EVI1 complex*). These results suggest that although foamy virus vectors may integrate near proto-oncogenes, this does not necessarily lead to clonal expansion and malignancies. Additionally, we show that copy number estimates of these specific proviruses based on linker-mediated PCR results are different from those obtained by quantitative PCR, but can provide a qualitative assessment of provirus levels.

Introduction

Insertional mutagenesis caused by integrating vectors is a major concern in hematopoietic stem cell (HSC) gene therapy. Gammaretroviral vector integration has been associated with clonal expansion and malignancies in human clinical trials (Hacein-Bey-Abina *et al*., 2003; Howe *et al*., 2008; Ott *et al*., 2006). Alternative vector systems or designs may be less genotoxic due to their distinct integration preferences, use of internal promoters, relative enhancer activity, or potential for read-through transcription. For example, self-inactivating gammaretroviral vectors may have improved safety due to deletions in the viral long terminal repeats (LTRs) (Modlich *et al*., 2006; Zychlinski *et al*., 2008), and lentiviral (LV) vectors that use internal promoters can be less leukemogenic than gammaretroviral vectors (Montini *et al*., 2006).

Foamy virus (FV) vectors are an alternative retroviral vector system that efficiently transduces HSCs from mice, dogs, and humans (Vassilopoulos *et al*., 2001; Josephson *et al*., 2002, 2004; Leurs *et al*., 2003; Kiem *et al*., 2007; Trobridge *et al*., 2009). FV vectors may be less genotoxic than gammaretroviral or LV vectors, because they do not preferentially integrate within transcription units or actively transcribed genes (Trobridge *et al*., 2006), and they have reduced potential to activate neighboring genes due to a lack of read-through transcription and enhancer activity from the partially deleted LTRs (Hendrie *et al*., 2008). We previously used FV vectors to express the canine leukocyte integrin gene *CD18* (also known as *ITGB2*) from an internal murine stem cell virus (MSCV) promoter (Hawley *et al*., 1994) and treat dogs with canine leukocyte adhesion deficiency (CLAD) by HSC gene therapy (Bauer *et al*., 2008). This produced long-term leukocyte marking rates of 5–10% based on CD18 expression, and the animals no longer suffered from the recurrent infections characteristic of CLAD. We determined the chromosomal locations of over 800 vector proviruses isolated from the blood cells of four treated dogs, and 26 were present within 30 kb of known cellular proto-oncogenes (Bauer *et al*., 2008). This was less than that observed in CLAD dogs treated with gammaretroviral vectors (Hai *et al*., 2008), and not significantly different from the number expected for random integration, arguing against a genotoxic effect of FV vector integration. Still, because the FV vector contained an internal MSCV promoter with strong enhancer activity that could potentially activate nearby proto-oncogenes, these particular clones might preferentially expand over time. Here we have measured the copy numbers of 11 clones containing specific FV proviruses to determine if vector-induced clonal expansion occurred.

Materials and Methods

Cell samples were obtained from transplanted dogs, and their genomic DNAs were purified from peripheral blood leukocytes, lymphocytes, and neutrophils as described

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Table 1. PCR Primers and Probes Used

Dog-Integrant	*LTR primer*	*Genomic DNA primer*	*Vector probe*
FD1-1	CCTCCTTGTTTTCCCTGGTTT	CAACGGTAGAGTTCCTGACTCATG	AGTGG CATTCCACCACA
FD1-2	CACTTATCTTAAATGATGTAA CTCCTTAGGA	GCAAAGACTTAGGCAGAGGGAAA	TTGTCATGGAATTTTG
FD1-3	CCTCCTTGTTTTCCCTGGTTT	GCGCAGGTATTAGAGTCACTGGAT	AGTGG CATTCCACCACA
FD1-4	CCTCCTTGTTTTCCCTGGTTT	GTGTACGCTCCCATTTTTCACTAA	AGTGG CATTCCACCACA
FD2-1	CCTCCTTGTTTTCCCTGGTTT	TGCTTCTCCTTCTGTCTGTGTCTCT	AGTGG CATTCCACCACA
FD2-2	CCTCCTTGTTTTCCCTGGTTT	GCTCTCACCATCACTTTCACTATGA	AGTGG CATTCCACCACA
FD3-1	CCTCCTTGTTTTCCCTGGTTT	GATGAAGTGCCCATGATGGAT	AGTGG CATTCCACCACA
FD3-2	CCTCCTTGTTTTCCCTGGTTT	TCCCCAATGTTCAGGGTTCAG	AGTGG CATTCCACCACA
FD3-3	CACTTATCTTAAATGATGTAA CTCCTTAGGA	GGTACATTTACACCAGCTATTTAT ATTTCTCT	TTGTCATGGAATTTTG
FD4-1	CCTCCTTGTTTTCCCTGGTTT	GGGAATGTTCTCATCAGGAGCTA	AGTGG CATTCCACCACA
FD4-2	CACTTATCTTAAATGATGTAA CTCCTTAGGA	GCTAGGTGTTGAATCTACTTAAA AACAAAA	TTGTCATGGAATTTTG

(Bauer *et al*., 2008). Quantitative PCR (qPCR) was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using the manufacturer's recommended conditions. Each integrant-specific qPCR reaction used 200 ng of genomic DNA, an LTR-specific primer, a unique flanking genomic DNA primer for each specific junction, and a vector-specific probe (Table 1), with 45 cycles of PCR (95°C for 15 sec, 60°C for 60 sec). Copy numbers were determined by using a different set of standards for each provirus, made by diluting a plasmid containing the specific junction fragment sequence to be amplified, similar to what has been done previously (Nagy *et al*., 2004; Bozorgmehr *et al*., 2007). Each set of standards included reactions with 0, 1, 10, 100, 1,000, and 10,000 copies of the standard plasmid (corresponding to 0, 2.6×10^{-5}, 2.6×10^{-4}, 2.6×10^{-3}, 2.6×10^{-2}, and 2.6×10^{-1} copies/cell, respectively) that showed a correlation coefficient of over 0.97 when plotted against PCR cycles. The copy number was corrected for DNA quality based on the copy number obtained when amplifying the chromosomal *B2M* gene on dog chromosome 30 using primers 5′-GCACTGTGTCACTATGCTTGACCTA and 5′-AGCTTCCAGCCCCACAACTA, with probe 5′-CTG GGCCAGTAGCT.

For linker-mediated PCR (LM-PCR), 500 ng of genomic DNA from FV vector-treated dogs was digested with *Mse*I and *Pst*I in 10 μl, and the fragments were ligated to an *Mse*I linker (5′-GTAATACGACTCACTATAGGGCTCCGCTTAA GGGAC and 5′-PO_4-TAGTCCCTTAAGCGGAG). One microliter of linker-ligated genomic DNA (7.5% of the total sample, representing 37 ng of DNA) was used in each PCR reaction with a linker-specific primer (5′-GTAATACGACT CACTATAGGGC), an FV LTR-specific primer (5′-GTCTAT GAGGAGCAGGAGTA), and Platinum Taq Polymerase (Invitrogen, Carlsbad, CA) in a 50-μl volume (95°C for 2 min, then 25 cycles of 95°C for 15 sec, 55°C for 30 sec, 72°C for 60 sec). Nested PCR was performed using 1 μl (2%) of the first PCR product as template, with a linker-specific nested primer (5′-AGGGCTCCGCTTAAGGGAC) and an FV LTR-specific nested primer (5′-CCTCCTTCCCTGTAATACTC) in a 50-μl volume under the same conditions. One microliter

Table 2. Integrants and Nearby Genes Tracked in This Study

Dog-Integrant	*Nearest gene*[a]	*Chromosome: position*	*Distance to TSS*[b]	*Orientation*[c]	*Gene class*[d]	*RefSeq Name*
FD1-1	*ABI2*	37:15277461	+115096	F	TS[e]	*ABL interactor 2*
FD1-2	*MECOM*	34:37296549	+8809	F	O	*MDS1-EVI1 complex*
FD1-3	*NBN*	29:38459522	+3215	F	TS	*Nibrin*
FD1-4	*SYNJ2BP*	8:47112812	+452	R	N	*Synaptojanin 2 binding protein*
FD2-1	*FUT8*	8:43151800	+296588	R	N	*Fucosyltransferase 8*
FD2-2	*PAX5*	11:56360738	+58585	R	O	*Paired box 5*
FD3-1	*MTCP1*[f]	X:126104058	−2858	F	O	*Mature T-cell proliferation 1*
FD3-2	*GLYATL2*	18:40709511	−9135	R	N	*Glycine-N-acyltransferase-like 2*
FD3-3	*MECOM*	34:36917928	+387430	R	O	*MDS1-EVI1 complex*
FD4-1	*ALOX5AP*	25:12258290	+75071	R	N	*Arachidonate 5-lipoxygenase-activating protein*
FD4-2	*RNF38*	11:55895032	−50420	F	N	*Ring finger protein 38*

[a]Genes listed as human RefSeq gene homologs, except for canine REFSEQ gene *FUT8*.
[b]Distance in bp to transcription start site (TSS); minus and plus signs indicate upstream and downstream, respectively.
[c]F (forward) and R (reverse) refer to same and opposite orientations of provirus and gene transcription, respectively.
[d]N, not cancer-related; O, proto-oncogene; TS, tumor suppressor.
[e]Its role as a tumor suppressor remains unproven.
[f]The nearest gene is actually *BRCC3*, which is not known to be a proto-oncogene (see Fig. 1).

(2%) of these nested PCR products was then cloned into the pCR4-TOPO cloning vector and transformed into TOP10-competent cells by using the TOPO TA Cloning Kit for Sequencing (Invitrogen). Gel electrophoresis was also performed on these samples to assess the diversity of junctions that were amplified (see Fig. 3B). Sequencing of cloned junctions was performed by using the M13 Reverse primer (5′-CAGGAAACAGCTATGAC) that reads into the FV LTR and flanking genomic DNA sequences. Integration sites were determined for each cloned LM-PCR product when the sequence read included LTR sequence, canine genomic sequence, and linker sequence.

Results and Discussion

A total of 11 clones from four transplanted CLAD dogs (FD1-4) were chosen for this study (Table 2). Previously, each dog received CD34+ cells transduced with the ΔΦMscvCD18 vector (Fig. 1A) after partial myeloablation with 200 cGy of total body irradiation (Bauer *et al*., 2008). ΔΦMscvCD18 expresses canine *CD18* from an internal MSCV promoter in a foamy vector backbone with deleted, silent LTRs (Trobridge *et al*., 2002; Hendrie *et al*., 2008). One year after receiving transduced cells, 466 distinct provirus junctions were mapped from unfractionated peripheral blood leukocytes, and 2 years after transplantation 293 and 251 junctions were mapped from CD3+ T-lymphocytes and neutrophils, respectively. The specific clones tracked here were chosen from these integrants, and included examples with proviruses present near proto-oncogenes, tumor suppressor genes, or genes unrelated to cancer (Table 2). Provirus positions within or near cancer-related genes are shown in Fig. 1B.

Four of the clones contained integrations within or near known proto-oncogenes. Two of these (FD1-2 and FD3-3) were in *MECOM* (*MDS1-EVI1 complex*). This gene contains transcripts for both *MDS1* (*Myelodysplasia syndrome 1*) and *EVI1* (*Ecotropic viral integration site 1*), is involved in translocations that cause human leukemia (Morishita *et al*., 1992), and is a frequent site of gammaretrovirus integration associated with murine leukemia and primary bone marrow cell immortalization (http://rtcgd.ncifcrf.gov/) (Du *et al*., 2005). Importantly, gammaretroviral vector integrations in *MECOM* have also been shown to be genotoxic in several species. They can cause clonal dominance (Kustikova *et al*., 2005) and myeloid leukemia (Li *et al*., 2002) in mice, are overrepresented in nonhuman primates after HSC gene transfer (Calmels *et al*., 2005), and are found as common integration sites in human clinical trials (Ott *et al*., 2006; Deichmann *et al*., 2007) that may lead to expanded myelopoiesis, myelodysplasia, and leukemia (Ott *et al*., 2006; Stein *et al*., 2010).

Four other clones contained integrations in or near cancer-related genes (Table 2 and Fig. 1B). The FD3-1 integrant contained a provirus upstream of *MTCP1*, a proto-oncogene associated with T-cell leukemia in mice (Gritti *et al*., 1998) and T-cell proliferation in humans (Stern *et al*., 1993). The FD2-2 provirus was in *PAX5*, a proto-oncogene involved in human lymphoma (Busslinger *et al*., 1996; Iida *et al*., 1996; Pasqualucci *et al*., 2001) and leukemia (Cazzaniga *et al*., 2001). Overexpression of these proto-oncogenes due to enhancer and/or promoter activity of the vector provirus could potentially lead to oncogenic transformation. The FD1-3 provirus was in the tumor suppressor gene *NBN*, which encodes a component of the MRE11/RAD50 double-strand break repair complex (Carney *et al*., 1998) and, when mutated, can predispose to lymphoma and leukemia (Varon *et al*., 2001; Resnick *et al*., 2003). The FD1-1 provirus was in the *ABI2* gene, which encodes a substrate for the c-ABL tyrosine kinase that impairs ABL-dependent leukemogenesis, promotes inactivation of CDC2, and suppresses cell growth (Dai *et al*., 2001; Lin *et al*., 2004), suggesting it could function as tumor suppressor [although *Abi2-/-* mice were not reported to have a higher incidence of tumors (Grove *et al*., 2004), arguing against this role]. Inactivation of a tumor suppressor gene by provirus insertion, followed by a second mutation in the other allele, could promote tumorigenesis. The other five integrants were not in cancer-related genes (Table 2), and

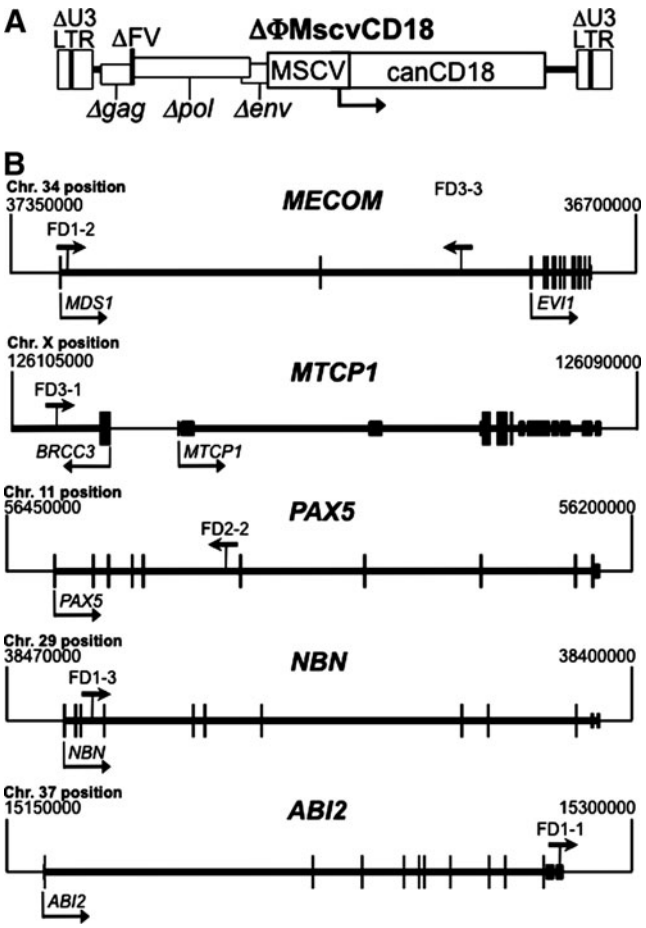

FIG. 1. Vector structure and integrant localization. (**A**) The ΔΦMscvCD18 vector used in this study is shown with the MSCV promoter and canine *CD18* gene indicated. The deleted foamy virus (ΔFV) backbone has deletions in the U3 region of the LTR (ΔU3) and in each of the remnants of the three major viral genes (Δ*gag*, Δ*pol*, and Δ*env*). (**B**) The vector proviruses found in or near cancer-related genes are shown with the arrow above each gene indicating provirus location and direction of vector transcription. Cellular genes and dog chromosomal positions are shown with transcription units in thick lines and exons as boxes (translated exons are taller boxes). Transcription start sites are indicated by arrows below each gene. Proto-oncogenes (*MECOM*, *MTCP1*, and *PAX5*) and tumor suppressor genes (*NBN* and *ABI2*) are pictured. Note that the *BRCC3* gene in the FD3-1 integrant map is not a cancer-related gene.

Table 3. Samples Used to Obtain Provirus Sequences and Their Copy Numbers

Dog-integrant	*Nearest gene*[a]	*Cell source*	*Total no. of reads*[a]	*No. of junction reads*[b]	*% CD18+ cells*	*LM-PCR copy no.*[c]	*qPCR copy no.*	*qPCR/LM-PCR*[d]
FD1-1	*ABI2*	Leukocytes	151	4	5.8	1.54×10^{-3}	1.20×10^{-3}	0.78
FD1-2	*MECOM*	Leukocytes	151	2	5.8	7.68×10^{-4}	6.80×10^{-4}	0.89
FD1-3	*NBN*	Leukocytes	151	1	5.8	3.84×10^{-4}	1.60×10^{-3}	4.2
FD1-3	*NBN*	Lymphocytes	84	2	27	6.43×10^{-3}	1.10×10^{-3}	0.17
FD1-4	*SYNJ2BP*	Leukocytes	151	7	5.8	2.69×10^{-3}	2.20×10^{-2}	8.2
FD1-4	*SYNJ2BP*	Lymphocytes	84	9	27	2.89×10^{-2}	1.80×10^{-1}	6.2
FD1-4	*SYNJ2BP*	Neutrophils	114	2	3.7	6.49×10^{-4}	1.60×10^{-2}	25
FD2-1	*FUT8*	Neutrophils	61	1	6.7	1.10×10^{-3}	1.10×10^{-3}	1.0
FD2-2	*PAX5*	Neutrophils	61	1	6.7	1.10×10^{-3}	1.20×10^{-5}	0.011
FD3-1	*MTCP1*	Leukocytes	181	2	5.1	5.64×10^{-4}	1.10×10^{-4}	0.20
FD3-1	*MTCP1*	Lymphocytes	81	1	18	2.22×10^{-3}	2.50×10^{-4}	0.11
FD3-2	*GLYATL2*	Leukocytes	181	1	5.1	2.82×10^{-4}	3.00×10^{-4}	1.1
FD3-2	*GLYATL2*	Neutrophils	60	3	4.7	2.35×10^{-3}	1.70×10^{-5}	0.0072
FD3-3	*MECOM*	Neutrophils	60	1	4.7	7.83×10^{-4}	6.00×10^{-4}	0.77
FD4-1	*ALOX5AP*	Lymphocytes	79	1	25	3.16×10^{-3}	3.40×10^{-4}	0.11
FD4-1	*ALOX5AP*	Neutrophils	95	1	2.8	2.95×10^{-4}	3.50×10^{-5}	0.12
FD4-2	*RNF38*	Neutrophils	95	1	2.8	2.95×10^{-4}	2.80×10^{-4}	0.95

[a]Number of sequence reads obtained from that cell source in that animal.
[b]Number of sequence reads obtained for that specific provirus junction.
[c]Equals [(no. of specific junction reads)/(total no. of reads with junction sequence)]×[(% CD18+ cells)/100].
[d]qPCR copy no./LM-PCR copy no.

were chosen as controls that might be present at high levels due to their recurrent identification when sequencing junctions and/or their presence in different cell samples (Table 3).

We used specific qPCR primers and probes to detect each provirus junction and track the levels of each clone in transplanted animals. Genomic DNA was purified from peripheral blood leukocytes during the 36–42-month period following transplantation, and the number of provirus copies per cell was determined for each sample. As shown in Fig. 2A, all the marking levels were initially below detection (<0.0001 copies/cell) and then increased over time. Importantly, the four clones with proviruses in or near proto-oncogenes, and the two with proviruses in potential tumor suppressor genes, did not preferentially expand over time as compared with the five control clones with proviruses near genes unrelated to cancer. The clone with a provirus in *PAX5* (FD2-2) and one of those with a provirus in *MECOM* (FD1-2) decreased in abundance during the later time points. The clone with the highest marking rate was FD1-4, with a provirus near *SYNJ2BP*, which comprised 2–4% of FD1's leukocytes over a 24-month period. *SYNJ2BP* is also known as *ARIP2*, and although there are some data that its overexpression promotes the growth of breast cancer cells (Li *et al*., 2009), it is down-regulated in renal carcinoma cells (Brito *et al*., 2008), and there is no definitive evidence for a role in cancer. In addition, the marking levels for this clone changed very little once they reached 2%, arguing against vector-induced clonal expansion.

As a further test of the accuracy of our qPCR assay, we measured the copy numbers of two of these specific junctions in the presence of increasing amounts of unrelated junction sequences, to determine if other sequences might influence the PCR reaction. The unrelated junction sequences included the same LTR as the junction being measured so that the qPCR probe and LTR primer were complementary to these sequences (see Table 2). In each case, the addition of up to 30 excess junction copies per cell (>300-fold over the total provirus copy numbers in these samples) did not have an impact on our results (Fig. 2B).

We also used qPCR to measure provirus copy numbers in the same samples originally used to isolate and sequence junctions by LM-PCR, and asked if the relative abundance of specific junction sequence reads correlated with the qPCR results. In other words, does the number of times a particular junction sequence is obtained from a sample by LM-PCR predict its copy number? This can be calculated as the proportion of sequence reads representing a particular junction times the percentage of transduced (CD18+) cells in the sample. As shown in Table 3, the LM-PCR copy number estimates were very different from those obtained by qPCR, and varied by more than 3 logs (0.0072–25; Table 3). Given that the qPCR results were obtained in a quantitative manner by comparison with known amounts of junction sequence copies present in the standards, these results underscore the inaccuracy of LM-PCR for measuring copy number. However, there was still a strong and statistically significant correlation between the qPCR and LM-PCR results (Fig. 3A), suggesting that the repeated recovery of the same junction sequence by LM-PCR is a qualitative indicator of copy number in the sample. It is possible that, with improved sequencing technologies and larger numbers of sequencing reads, this type of approach could lead to more accurate estimates of provirus copy numbers.

One factor that may influence which sequences are obtained by LM-PCR is variation in the particular junctions amplified during PCR. Figure 3B shows that this occurred. When five different LM-PCR reactions were performed on the same genomic DNA sample from dog FD3, distinct PCR products were obtained in each reaction. For some of the junctions, this may have been due to a limiting amount of template DNA, because each PCR reaction was performed

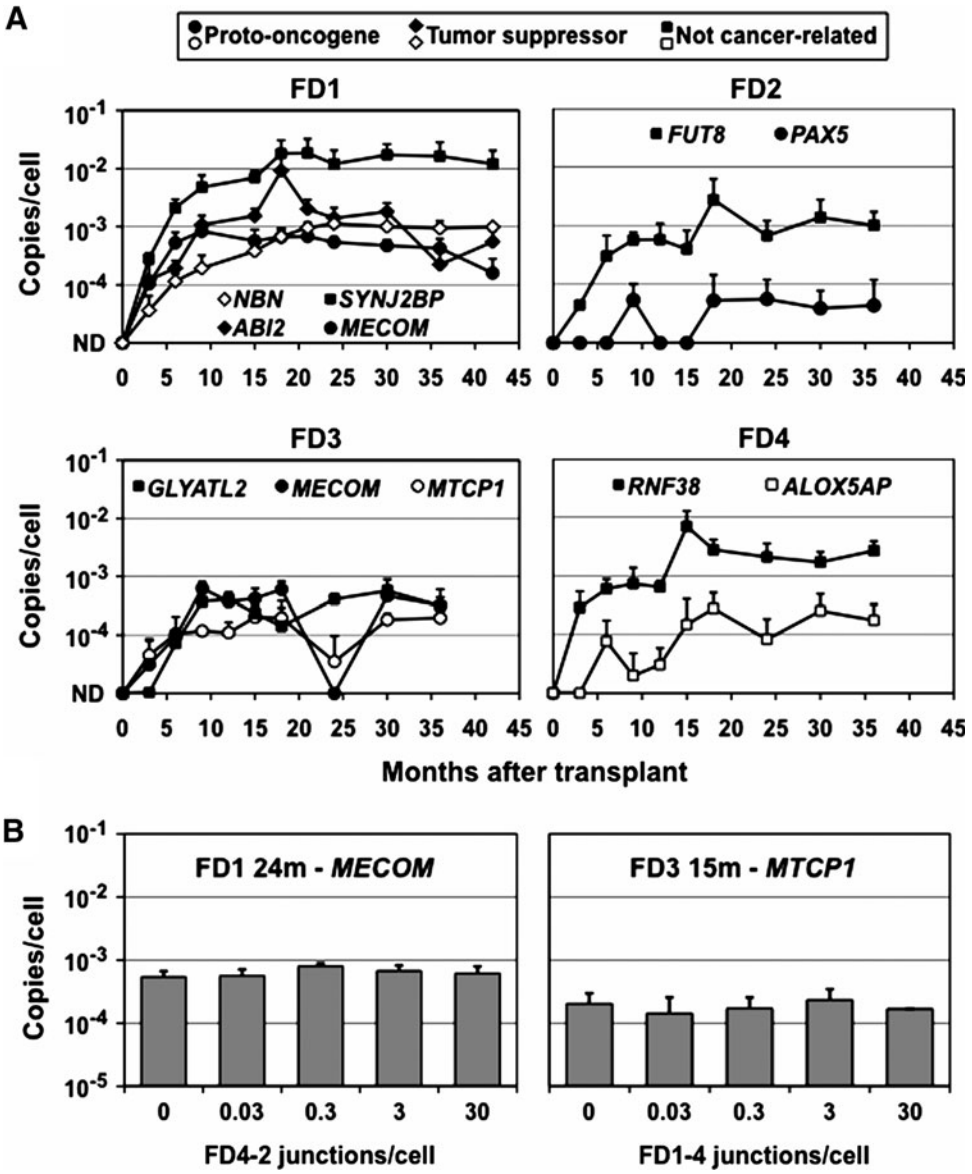

FIG. 2. Provirus copy numbers. (**A**) The number of provirus copies per cell is shown for each provirus over time. Values were determined by qPCR of peripheral blood leukocyte DNA and are means with standard deviations ($n = 3$). Each panel represents results from one animal. Proviruses near proto-oncogenes, tumor suppressor genes, and genes unrelated to cancer are shown with circle, diamond, and square symbols, respectively. ND, not detected ($<10^{-4}$ copies/cell). (**B**) Specific provirus copy numbers were determined by qPCR in the presence of increasing amounts of plasmid standards containing a different junction sequence. Samples analyzed were FD1 leukocyte DNA screened for the *MECOM* junction at 24 months and FD3 leukocyte DNA screened for the *MTCP1* junction at 15 months, in the presence of the FD4-2 or FD1-4 junctions, respectively (see Table 2). Values shown are means with standard deviations ($n = 3$).

on 37 ng of genomic DNA (see Materials and Methods), which represents approximately 7,115 cell genomes (assuming 5.2 pg/cell and 2.4 Gb/haploid genome). Thus, any junction sequence present at less than 1.4×10^{-4} copies/cell (1/7,115) may have been absent in a single PCR reaction. Although this could have prevented LM-PCR-based detection of some junctions (FD2-2 in neutrophils, FD3-1 in leukocytes, and FD3-2 in neutrophils; see Table 3), most of the junctions we analyzed were present at higher copy numbers than this and presumably available for amplification, suggesting that other variables played a role, too. For example, which PCR cycle produced the first round of amplification of a particular junction could have had a large downstream effect on the total amount of product produced, as could sequence-specific effects on polymerization rates. Limiting genomic DNA amounts were less of an issue for the qPCR assay, which was performed on 200 ng of genomic DNA in triplicate, representing 1.2×10^5 cells, and thereby increasing the sensitivity of the reaction.

In conclusion, our results show that there was no evidence for selective expansion of clones with FV vector proviruses near cancer-related genes. As with any study of this sort, it is possible that a longer follow-up or analysis of additional transduced clones could identify a provirus-related clonal

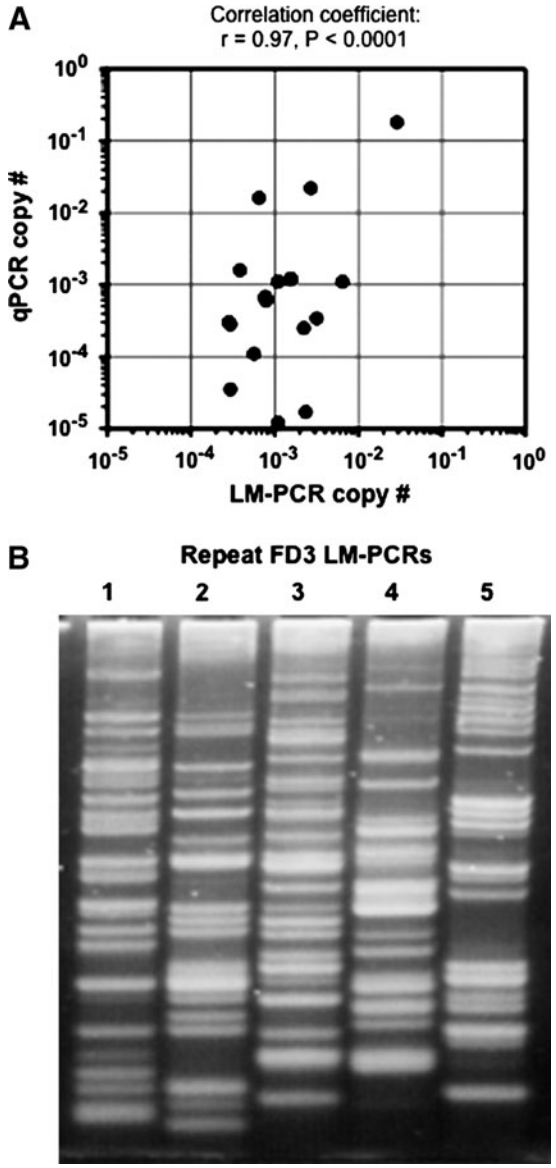

FIG. 3. Copy number estimates by LM-PCR. (**A**) Scatter plot of copy number calculations of the samples used for LM-PCR junction sequencing. The number of provirus copies per cell as determined by qPCR and by the frequency of LM-PCR sequence reads are shown for each sample listed in Table 3. The Pearson correlation coefficient *r* was statistically significant. (**B**) Gel electrophoresis of five different LM-PCR reactions performed on genomic DNA from FD3 leukocytes obtained 1 year after transplant. The reactions were performed under identical conditions, demonstrating that different junction products were obtained in each repetition.

expansion event. In other studies, specific gammaretroviral integrants in proto-oncogenes have been tracked over time without the detection of clonal expansion (Aiuti *et al*., 2007; Bozorgmehr *et al*., 2007; Metais and Dunbar, 2008; Hayakawa *et al*., 2009), despite the known genotoxicity of these vectors. However, there are several reasons to be encouraged by our results. First, the *MECOM* integrants we tracked were in a gene with well-established potential for vector-induced malignant transformation, and they were located in introns 1 and 2 (Fig. 1B), a region particularly prone to genotoxic effects (http://rtcgd.ncifcrf.gov/) (Calmels *et al*., 2005; Kustikova *et al*., 2005; Ott *et al*., 2006). Second, the CLAD model allowed us to monitor for clonal expansion for over 3 years in large animals cured of their disease by gene therapy, providing ample time and cell numbers to detect a transformation event. Third, T cells expressing CD18 have a proliferative advantage over their CLAD counterparts, based on *in vitro* proliferation assays and the selective expansion of transduced lymphocytes *in vivo* (Bauer *et al*., 2008), so the situation is analogous to X-SCID gene therapy, where the transgene itself provided an additional proliferative stimulus that cooperated with specific provirus integrations (Woods *et al*., 2006). Finally, our prior results showed that very little vector silencing occurred in these transplanted animals, based on the lack of proviruses in flow-sorted CD18– cells (Bauer *et al*., 2008). Therefore, it is likely that the proviruses tracked in this study were expressed and the internal MSCV promoter and enhancer were active despite their lack of genotoxicity. In conclusion, these results show that the presence of FV vector proviruses near proto-oncogenes does not necessarily lead to clonal expansion, supporting the use of FV vectors for HSC gene therapy.

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Author Disclosure Statement

No competing financial interests exist.

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