GENE THERAPY

Liver-directed lentiviral gene therapy in a dog model of hemophilia B

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We investigated the efficacy of liver-directed gene therapy using lentiviral vectors in a large animal model of hemophilia B and evaluated the risk of insertional mutagenesis in tumor-prone mouse models. We showed that gene therapy using lentiviral vectors targeting the expression of a canine factor IX transgene in hepatocytes was well tolerated and provided a stable long-term production of coagulation factor IX in dogs with hemophilia B. By exploiting three different mouse models designed to amplify the consequences of insertional mutagenesis, we showed that no genotoxicity was detected with these lentiviral vectors. Our findings suggest that lentiviral vectors may be an attractive candidate for gene therapy targeted to the liver and may be potentially useful for the treatment of hemophilia.

INTRODUCTION

Hemophilia is a monogenic X-linked disease caused by a deficiency of coagulation factor VIII (hemophilia A) or factor IX (hemophilia B) (*1*). Bleeding, either spontaneous or posttraumatic, is the hallmark of hemophilia, which can be fatal if left untreated. According to residual factor activity, hemophilia is classified as severe (<1%), moderate (1 to 5%), or mild (6 to 30%). Patients with moderate or mild hemophilia have occasional to rare spontaneous hemorrhages; thus, rescuing factor activity to ≥1% of normal substantially benefits the clinical phenotype of severely affected patients (*2*).

Prophylactic or on-demand replacement therapy with recombinant products is the current standard of care for hemophilia in high-income countries and has improved the quality of life and life expectancy of patients with severe hemophilia (*3*). Nevertheless, this treatment has high costs, entails discomfort for patients, and has the risk of inducing neutralizing anti-factor antibodies, which complicates further treatment (*4*). Moreover, in developing countries, about 80% of people affected by hemophilia live with no or unsatisfactory treatment (*5*). Gene therapy could help to address these needs by establishing long-term endogenous production of the clotting factor at therapeutic levels after a single treatment (*2*, *6*, *7*).

Recently, long-term factor IX activity of 1 to 7% of normal has been reported long-term in adult patients with severe hemophilia B after administration of a single dose of an adeno-associated virus (AAV) vector targeting the expression of human factor IX complementary DNA (cDNA) in hepatocytes (*8*). These results establish the therapeutic potential of liver-directed gene therapy in humans and offer the prospect of a definitive treatment for hemophilia. However, there are still important hurdles to overcome before this gene therapy can be applied to most of severely affected patients (*9*). In particular, preexisting neutralizing antibodies to AAV after natural exposure to the wild-type virus may inhibit gene transfer with AAV vectors. In addition, AAV-specific cellular immune responses to the transduced hepatocytes may curtail long-term transgene expression and may require transient immune suppression to allow clearance of AAV-derived antigens (*8*). HIV-derived lentiviral vectors may complement the therapeutic reach of AAV vectors because of the low prevalence of HIV infection in humans and the capacity of the vector to accommodate larger gene inserts. Moreover, the efficient integration of lentiviral vectors into the genome of target cells may eventually make these vectors better suited for treatment of pediatric patients, in which hepatocyte turnover is high and episomal vectors may be progressively lost (*10*).

We have developed a lentiviral vector platform that achieves stable and robust transgene expression in the mouse liver and induces transgene-specific immune tolerance upon systemic administration (*11*–*15*). This lentiviral vector stringently targets transgene expression to hepatocytes through transcriptional and microRNA (miR)–mediated regulation. We and others have shown the efficacy of this vector in establishing the correction of hemophilia B and A in mouse models and of hyperbilirubinemia in rats (*12*, *16*, *17*).

Although encouraging, these results were obtained in rodents, and it is crucial to assess the feasibility and safety of scaling up lentiviral gene therapy in large animal models. In addition, whereas liver gene transfer by lentiviral vectors appeared to be safe in treated mice, concerns remain regarding the risk of insertional mutagenesis. We and others recently reported the safe and efficacious clinical testing of

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lentiviral vectors for ex vivo gene therapy with hematopoietic stem cells (*18*–*20*). The safe outcome of these trials to date supports the predictions about vector safety developed in our preclinical tumor-prone mouse models, where the consequences of insertional mutagenesis are amplified in a model species that otherwise limits the detection of low incidence, vector-induced oncogenesis (*21*, *22*). Here, we investigated liver-directed gene therapy using lentiviral vectors in dogs with hemophilia B and tested its potential for genotoxicity in mouse models prone to develop hepatocellular carcinoma (HCC).

RESULTS

Lentiviral vectors efficiently transduce and regulate transgene expression in canine cells

We generated three lentiviral vectors with self-inactivating (SIN) long terminal repeats (LTRs) expressing cDNA transgenes for canine factor IX (cFIX) under the control of an internal synthetic hepatocyte-specific enhanced transthyretin (ET) promoter and carrying four tandem repeats of miR-142 target sequences (142T; Fig. 1A). The lentiviral vectors contained the wild-type, codon-usage optimized, or codon-usage optimized hyperfunctional cFIX carrying the R338L mutation associated with human thrombophilia (cFIX, co-cFIX, and co-cFIXR338L, respectively) (*14*, *23*). All lentiviral vectors were pseudotyped with the vesicular stomatitis virus glycoprotein G (VSV-G).

We observed a two- to fivefold reduction in lentiviral vector titer after incubation with pooled and individual dog sera, possibly mediated by complement (fig. S1A) (*24*). We selected for infusion those dogs whose serum showed the lowest neutralizing potential against lentiviral vectors. To verify lentiviral vector transduction and promoter activity in canine hepatocytes, we transduced primary human and canine hepatocytes ex vivo at increasing multiplicity of infection with lentiviral vectors expressing green fluorescent protein (GFP) driven by the ubiquitously expressed phosphoglycerate kinase (PGK) promoter or the hepatocyte-specific ET promoter. We observed high levels of transgene expression in hepatocytes of both species with both promoters (fig. S1B). We assessed miR expression in DH82 cells, a cell line derived from canine macrophages, and found miR-142 to be expressed at high levels (fig. S1C). We then transduced DH82 cells with reporter lentiviral vectors encoding GFP with or without 142T, and found a ≥100-fold down-regulation of

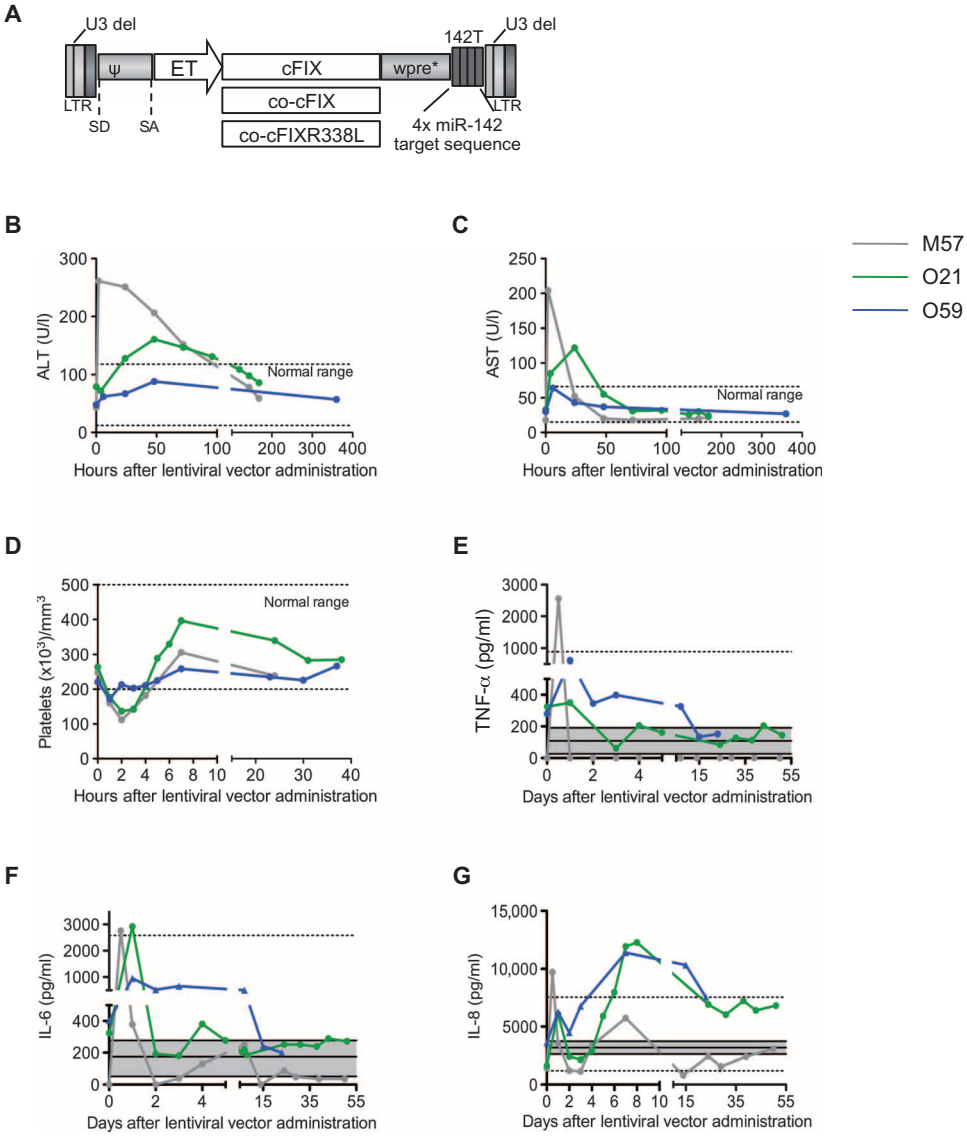

Fig. 1. Intraportal administration of lentiviral vectors to dogs with hemophilia B. (**A**) Schematic representation of the third-generation SIN lentiviral vectors (proviral form) used in this work. U3 del, deletion of the promoter/enhancer of the HIV LTR (*43*); SD, splicing donor site; SA, splicing acceptor site; ψ, packaging signal; wpre*, mutated woodchuck hepatitis virus posttranscriptional regulatory element (*44*); 142T, miR-142 target sequence made of four tandem copies of a sequence perfectly complementary to miR-142. The hepatocyte-specific ET promoter was composed of synthetic hepatocyte-specific enhancers and the transthyretin promoter (*45*). cFIX, co-cFIX, and co-cFIXR338L were used as transgenes (*14*). (**B** to **G**) Serum concentrations of alanine aminotransferase (ALT) (B) and aspartate aminotransferase (AST) (C), platelet counts (D), and serum concentrations of tumor necrosis factor–α (TNF-α) (E), interleukin-6 (IL-6) (F), and IL-8 (G) were measured in blood samples collected at the indicated time points after lentiviral vector administration to dogs M57 (gray line), O21 (green line), and O59 (blue line). Baseline values are shown as "time 0." (**B** to **D**) The normal range is shown (dashed lines). (**E** to **G**) The means ± SD (gray area) and ranges (dashed lines) of the serum concentrations of each cytokine measured in samples collected from 11 control untreated dogs are shown. Note that the lowest range for TNF-α and IL-6 is 0. Dog O59 was administered corticosteroids and antihistamine drugs before lentiviral vector infusion to reduce inflammation.

GFP expression (fig. S1D). These data indicate that the regulatory elements of our lentiviral vectors are functional in canine cells.

Intraportal lentiviral vector administration is well tolerated in dogs with hemophilia B.

We produced three large-scale batches of lentiviral vectors according to a manufacturing process previously developed for clinical-grade lentiviral vectors (*19*, *20*). Quality assessment of the vector batches (2009/D2, 2011/D13-15, and 2012/DG) is summarized in table S1. The process yielded 1.1×10^{10} to 4.5×10^{10} transducing units (TU), corresponding to 864 to 3151 μg of HIV Gag p24 equivalents (p24) of viral particles in 160 to 230 ml of saline for infusion. Lentiviral vector infectivity was 0.63×10^4 to 4.4×10^4 TU/ng p24. The lentiviral vector batches had low endotoxin content, were sterile, and were free of replication-competent lentiviral vectors. The three batches differed for the cFIX transgene: they were either wild type, co-cFIX, or co-cFIXR338L. Each lentiviral vector batch was infused into one male dog with hemophilia B by portal vein administration (Table 1).

The lentiviral vector infusion was well tolerated by the first dog (M57) except for a transient rise in body temperature (1°C above baseline). The second dog (O21) experienced acute hypotension during the infusion, attributed to an anaphylactoid reaction to an unknown component of the vector batch. This event was successfully managed by immediate administration of an antihistamine drug (Benadryl, 1 mg/kg intravenously) and corticosteroid (dexamethasone, 25 mg/kg intravenously). Lentiviral vector infusion was subsequently completed upon blood pressure recovery. We observed a transient rise in body temperature. On the basis of these events, the third dog (O59) was pretreated with corticosteroid (prednisone, 1 mg/kg orally) and antihistamine drugs [Benadryl, 1 mg/kg, and famotidine, 0.5 mg/kg, both intramuscularly] the day before, the morning before surgery, and just before vector infusion (dexamethasone, 0.2 mg/kg intravenously, and Benadryl and famotidine as above). With this regimen, there was no change in blood pressure, the infusion was uneventful, and body temperature did not increase.

In M57 and O21, serum concentrations of ALT and AST increased slightly above the normal range for the first few days after infusion, indicating minor, self-limiting hepatocellular toxicity (Fig. 1, B and C). In the third dog, both ALT and AST remained in the normal range throughout the follow-up, suggesting that antihistamine and anti-inflammatory treatment prevented acute hepatotoxicity owing to lentiviral vector infusion. In all three dogs, platelet counts fell slightly below the normal range for 2 to 3 days after lentiviral vector administration (Fig. 1D). This drop may be due to consumption at sites of surgical bleeding and the large amount of fluid infused (vector vehicle plus normal canine plasma administered as a source of factor IX on the day of surgery and the following day), as also suggested by the concurrent transient drop in hematocrit (fig. S2A). Plasma concentrations of fibrinogen and thrombin-antithrombin complex (TAT) increased in the first few days after lentiviral vector administration, with the least evident changes in O59 (fig. S2, B and C). The fibrin degradation product D-dimer increased only slightly above pretreatment values in the first two dogs and did not change in the third dog (fig. S2D). These data were consistent with the induction of an inflammatory response and the activation of the clotting system upon abdominal surgery and intraportal lentiviral vector administration. These responses were self-limiting and were effectively prevented by a 1-day anti-inflammatory pretreatment in the third dog. Indeed, in all treated dogs, we found an increase in TNF-α, IL6, and IL-8 serum concentrations after lentiviral vector administration, which declined rapidly over the following hours or days (Fig. 1, E to G). These increases were less pronounced in the dogs receiving antihistamine and anti-inflammatory treatment. Other cytokines tested (IL-2, IL-4, IL-7, IL-10, IL-15, transforming growth factor–β, and interferon-γ) were found not to be significantly different from control untreated animals ($n = 11$). All of the other blood chemistry parameters tested and cell counts were in the normal range throughout the follow-up, with minor sporadic fluctuations (tables S2 to S4)

We found very low concentrations of p24 in the serum at the first sampling, which was 3 hours after lentiviral vector administration (about 0.26% of the infused dose), indicating a rapid clearance of vector particles from the circulation (fig. S2, E and F). We did not find detectable p24 in oral, nasal, lachrymal, genital, and rectal swabs taken from days 1 to 8 after lentiviral vector administration, except for a borderline

Table 1. Gene therapy dose response in treated dogs with hemophilia B. The table shows the age and weight at treatment of three dogs with hemophilia B (M57, O21, and O59), the infused dose of lentiviral vector in TU and physical particles (p24) per weight, the follow-up (FU) time in days, the whole blood clotting time (WBCT) in minutes, and the cFIX activity [determined by activated partial thromboplastin time (aPTT)]. Also shown are the cFIX antigen (determined by ELISA), the type of transgene contained in the infused lentiviral vector: wild-type (WT) cFIX, co-cFIX, or co-cFIXR338L (see also Fig. 1). When possible, results are presented as mean values ± SEM over time (with ranges in parentheses). The values of WBCT and cFIX activity and antigen are considered valid only if measured 27 days after the last canine plasma transfusion (to ensure washout of exogenous cFIX).

	M57 (Hemil)	O21 (Valentine)	O59 (Enzo)
Age at treatment (months)	8	21	21
Weight at treatment (kg)	20	22	18
Transgene	WT cFIX	co-cFIX	co-cFIXR338L
TU/kg	5.7×10^8	2.3×10^9	1.1×10^9
μg p24/kg	44	47	174
FU (days)	1831	900	637
WBCT (min)	20.31 ± 0.91 (14.5–32)	17.36 ± 0.66 (13.5–22.5)	15.73 ± 0.5 (11–19.5)
cFIX activity (% normal)	0.08 ± 0.01 (0.01–0.25)	1.05 ± 0.12 (0.3–1.7)	1.18 ± 0.08 (0.7–1.9)
cFIX antigen (% normal)	0.05 ± 0.004 (0.01–0.09)	0.6 ± 0.06 (0.2–0.85)	0.16 ± 0.005 (0.14–0.2)

signal in the nasal secretion of O21 at day 1 (table S5). Overall, these data suggest that administration of lentiviral vectors to dogs by intraportal delivery is well tolerated provided that anti-inflammatory and antihistamine treatment is given before infusion.

Lentiviral vector gene therapy provides stable improvement in clotting time and clinical benefit in dogs with hemophilia B.

We measured the WBCT, cFIX activity (by aPTT), and cFIX antigen in blood or plasma samples collected from treated dogs at routine intervals after lentiviral vector administration (Fig. 2, A to C). The three dogs were followed up and were alive and well at 5, 2.5, and 1.75 years after lentiviral vector infusion for M57, O21, and O59, respectively. The WBCT was shortened and remained stable, albeit without reaching normal levels. The average WBCT over the follow-up time was about 20, 17, and 15.7 min for M57, O21, and O59, respectively (Table 1). In M57, cFIX activity and antigen levels averaged 0.08 and 0.05% of normal, respectively (Table 1). Although the reconstituted activity was low, this dog experienced only seven spontaneous bleedings in the 5 years after gene therapy (out of 27 expected from the bleeding frequency in the colony), whereas it had experienced six bleedings in the 3 months before gene therapy (Fig. 2D). In dog O21, cFIX activity ranged between 0.3 and 1.7% and antigen levels were between 0.2 and 0.9% of normal. This dog experienced only two bleedings over the last 2.5 years. Because this dog received a fourfold higher lentiviral vector dose and showed about 10- to 14-fold higher amounts of cFIX antigen and activity compared to dog M57, transgene codon-usage optimization may have increased cFIX expression threefold as compared with the wild-type transgene, in line with our data obtained in mice (*14*). In dog O59, cFIX activity ranged between 0.6 and 1.9% but cFIX antigen level was only between 0.1 and 0.2% of normal (Table 1). Whereas the low antigen level reflected the lower lentiviral vector dose administered compared to O21, the measured activity likely reflected the 5- to 10-fold increased activity conferred by the R338L mutation (*14*). This dog has not experienced any spontaneous bleeding to date. There is a marked difference in the monthly bleeding frequency between treated and untreated dogs from the same colony ($P < 0.0001$; Fig. 2D and table S6). Thromboelastogram values were within or close to the normal range in all treated dogs, with a shorter time to clot in dogs O21 and O59 (table S7). All treated dogs were negative for anti–factor IX inhibitory antibodies (table S8). A liver biopsy was taken from M57 and O21 at 16 and 12 months after lentiviral vector administration, respectively, and was scored normal by pathology (fig. S3). We found about 0.9 and 2.4 lentiviral vector DNA copies for every 1000 diploid genomes, respectively (fig. S4). This finding confirms the presence of the lentiviral

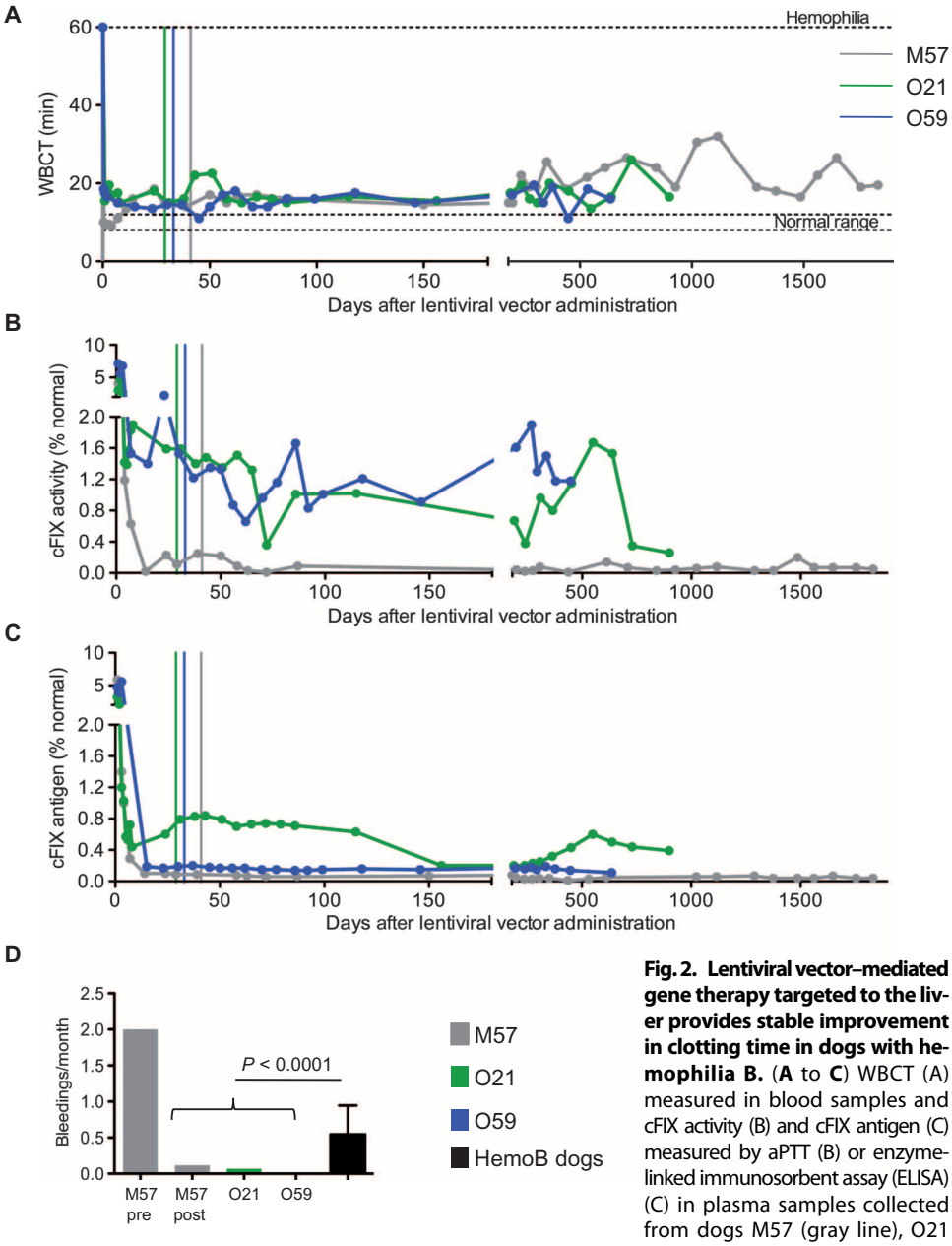

Fig. 2. Lentiviral vector–mediated gene therapy targeted to the liver provides stable improvement in clotting time in dogs with hemophilia B. (**A** to **C**) WBCT (A) measured in blood samples and cFIX activity (B) and cFIX antigen (C) measured by aPTT (B) or enzyme-linked immunosorbent assay (ELISA) (C) in plasma samples collected from dogs M57 (gray line), O21 (green line), and O59 (blue line) at the indicated times after lentiviral vector administration. The colored vertical lines indicate 27 days after the last normal plasma transfusion of the dogs at which time exogenous canine factor IX had been washed out. (**D**) Frequency of spontaneous bleedings (bleeding events per month of observation) in treated dogs after gene therapy. For M57, the frequency of spontaneous bleeding before gene therapy is shown. The mean ± SD bleeding frequency of 10 untreated dogs with hemophilia B (hemoB) in the colony is shown (black bar) (*46*). $P < 0.0001$ (two-sample test for equality of proportions; see also table S6).

vector in the dog liver in amounts consistent with the measured factor IX output. We did not find detectable lentiviral vector DNA in blood and sperm samples obtained from the treated dogs (fig. S4). These data show the long-term persistence of lentiviral vector–transduced cells in canine liver, stable reconstitution of factor IX activity up to 1% of normal, and amelioration of the clinical phenotype in three treated dogs affected by hemophilia B.

Treated mice did not show evidence of genotoxicity after lentiviral vector integration in liver

The normal blood chemistry and the stability of factor IX expression in the long-term follow-up of the treated dogs suggest a low risk for the development of neoplasia from transduced hepatocytes. To better investigate the risk of oncogenesis, we turned to mice and analyzed the safety of lentiviral integration into the liver in multiple settings of escalating stringency. In our prior studies of lentiviral vector gene therapy in mice with hemophilia B, we did not observe macroscopic liver lesions in the treated mice at necropsy (*12*, *14*, *15*). Here, we analyzed the integration site distribution in the liver of treated mice and scored for the potential enrichment of integration sites at specific genomic loci over time. Such analyses could reveal a selective growth advantage conferred on hepatocytes by lentiviral vector integration close to cancer genes before the development of overt neoplasia.

Twenty-seven adult mice with hemophilia B were administered therapeutic lentiviral vector (2.5×10^{10} to 5×10^{10} TU/kg), which carries SIN LTRs and an internal ET promoter (SIN.ET), expressing human or canine wild-type factor IX (see also Fig. 1A) in five different experiments (table S9). As expected, plasma factor IX was at 10 to 15% of normal levels and did not significantly change between sampling performed "early" (<3 months) or "late" (6 to 12 months) after lentiviral vector administration (Fig. 3A) (*12*, *14*). Nine mice were euthanized early and 18 were euthanized late to measure lentiviral vector content and integration site distribution in the liver. There was no significant difference in the average vector copies per diploid genome (vector copy number) between the early and late time points (Fig. 3B). We then deep-sequenced integration sites by linear amplification-mediated polymerase chain reaction (PCR) followed by 454 pyrosequencing or Illumina sequencing and mapped a total of 17,008 unique integration sites onto the murine genome (tables S9 and S10). We applied standard criteria to identify genomic regions recurrently hit by lentiviral vector integrations with a frequency significantly higher than random, and defined them as common insertion sites (CIS) (*25*). We identified 270 and 77 CIS in the data sets of integration sites retrieved from early or late euthanized mice, respectively, each CIS being identified by the most targeted gene in the genomic region (table S11). The higher number of CIS from the early integration site data set is likely to be the consequence of the higher number of integration sites retrieved. Nearly half of the late CIS were also found among the early data set (Fig. 3C), suggesting that they are unlikely to be the result of in vivo selection of clones carrying integration at those genes. In contrast, lentiviral vector CIS often result from intrinsic biases of viral integration that preferentially target gene-dense genomic regions (*26*). We thus used the genome-wide Grubbs' test for outliers to exclude all CIS occurring within a larger genomic cluster of lentiviral vector integrations, likely representing integration biases, as previously described (*19*, *20*). None of the late CIS passed the Grubbs' test, except for the *Sfi1* gene, which was also found among the early CIS. Overall, this analysis did not find evidence of in vivo selection of mouse hepatocytes carrying SIN.ET integrations.

SIN lentiviral vector administration did not show evidence of genotoxicity in tumor-prone mice

Mice can only be administered a limited dose of vector and followed up for a short time given their short life span compared to humans. For this reason, insertions leading to gain or loss of function in cancer genes that have a delayed effect on hepatocyte proliferation and/or selection may have escaped detection in the previous analysis (Fig. 3).

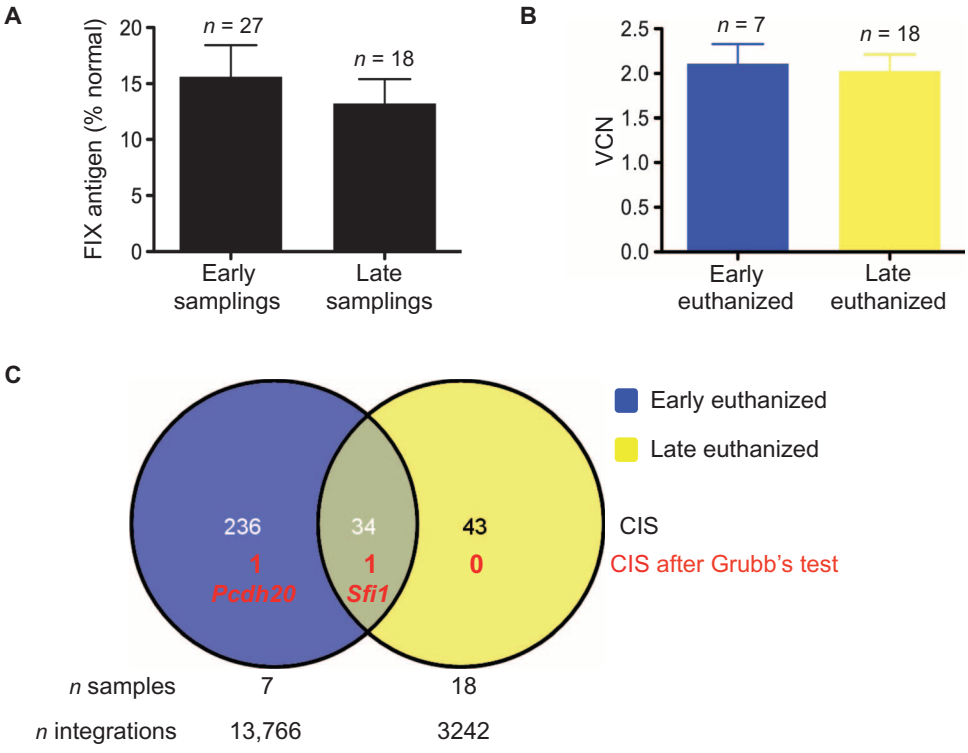

Fig. 3. No evidence of genotoxicity after lentiviral vector integration into the liver of mice. (**A**) Factor IX (FIX) antigen measured by ELISA in the plasma collected from mice early (<3 months) or late (6 to 12 months) after lentiviral vector administration. $P = 0.391$, Student's t test. (**B**) Vector copy number (VCN) in liver DNA collected from mice euthanized early or late after lentiviral vector administration. $P = 0.806$, Student's t test. (**A** and **B**) Data are means ± SEM. (**C**) Venn diagram representing CIS identified in liver DNA of mice euthanized early or late after lentiviral vector administration. The overlap is calculated considering the gene associated with each CIS; the number of CIS that passed the Grubbs' test is shown along with the gene name. The number of samples analyzed and integration sites retrieved are indicated for the two data sets.

To increase the sensitivity of our analysis, we administered the SIN.ET lentiviral vector to newborn tumor-prone mice or wild-type mice given a tumor-promoting regimen (*27*). We used a previously described genotoxic lentiviral vector carrying transcriptionally active LTRs containing the ET promoter (ET.LTR lentiviral vector) as a positive control for genotoxicity (Fig. 4A). Matched doses of ET.LTR or SIN.ET lentiviral vector (2.5×10^{10} TU/kg) were administered to newborn *Cdkn2a*$^{-/-}$*Ifnar1*$^{-/-}$ mice or newborn wild-type mice that were then given a regimen of carbon tetrachloride (CCl_4); 125 new experimental mice and 29 historical controls (*27*) were analyzed (table S12). *Cdkn2a*$^{-/-}$*Ifnar1*$^{-/-}$ mice were euthanized if they displayed signs of illness or at 30 weeks of age. Wild-type mice given the CCl_4 regimen were euthanized at 1 year of age. By visual inspection at necropsy and histopathological analysis of serial liver sections from multiple lobes, we detected two microscopic HCCs in 62 tumor-prone or CCl_4-treated wild-type mice administered SIN.ET, an incidence overlapping with that observed in nontransduced mice. On the contrary, ET.LTR induced a significantly higher incidence of HCC (22 HCCs, $P = 0.0007$ for *Cdkn2a*$^{-/-}$*Ifnar1*$^{-/-}$ and $P < 0.0001$ for CCl_4-treated wild-type mice), most of which were visible at necropsy (Fig. 4, B and C, and fig. S5, A to D). We measured the vector copy number in nontumoral mouse liver and in the HCCs collected from the experimental groups at the end of the experiment and from four cohorts of mice (*Cdkn2a*$^{-/-}$*Ifnar1*$^{-/-}$ or wild-type) euthanized 2 weeks after neonatal administration of SIN.ET or ET.LTR lentiviral vectors. The SIN.ET and ET.LTR vector copy numbers were comparable in mice euthanized at 2 weeks of age, indicating comparable in vivo transduction. However, the ET.LTR vector copy number was higher in livers harvested at the end of the experiment and showed an even greater increase in HCCs, indicating expansion of transduced hepatocytes ($P = 0.0394$; Fig. 4, D and E). This increased vector copy number over time was not observed in SIN.ET-treated mice. Moreover, we did not detect any significant change in circulating factor IX over time in either *Cdkn2a*$^{-/-}$*Ifnar1*$^{-/-}$ mice or wild-type mice transduced with SIN.ET, confirming the stability of gene transfer

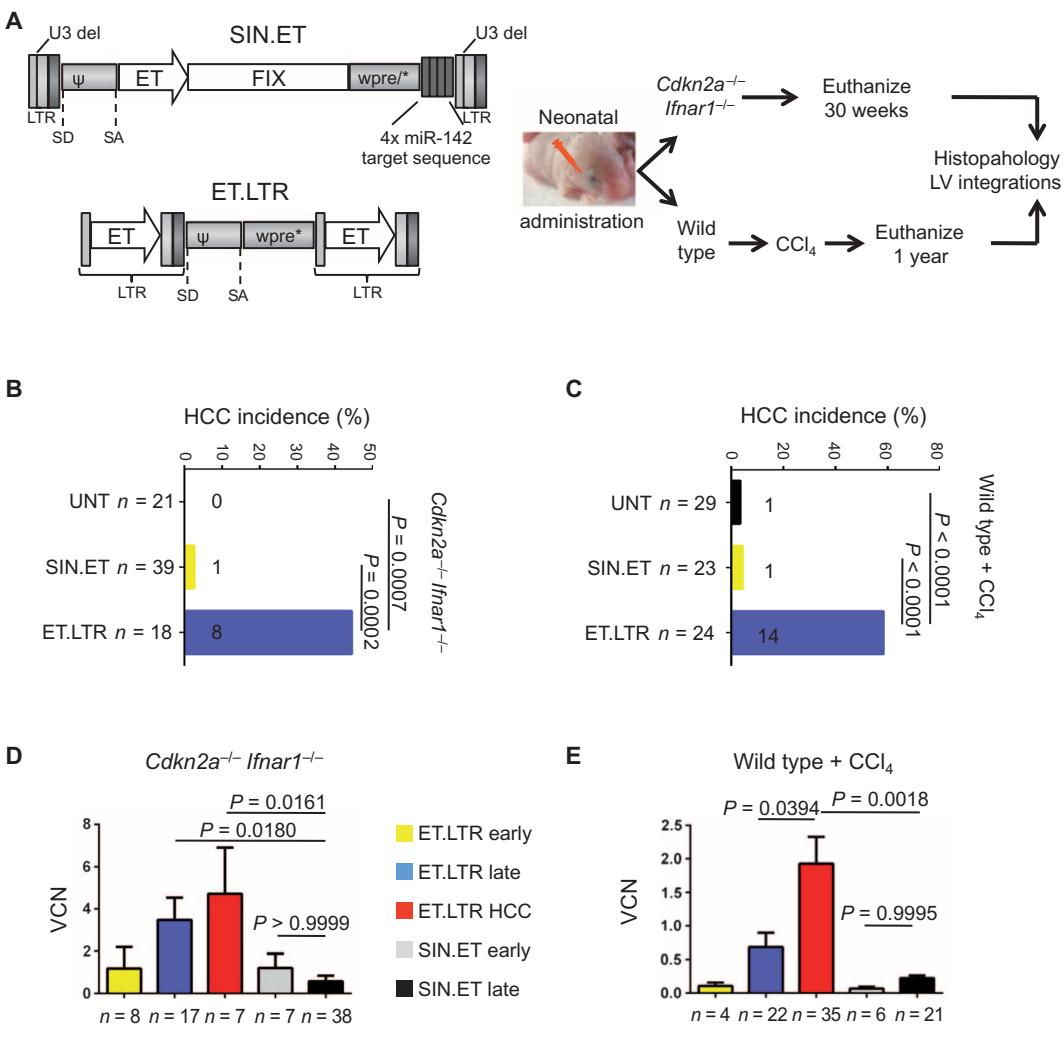

Fig. 4. SIN.ET lentiviral vectors do not induce HCC in tumor-prone mice. (**A**) Experimental outline of the in vivo biosafety study in mice. Left: Schematic representations of the lentiviral vectors used. Either SIN.ET (gene therapy lentiviral vector with SIN LTRs and an internal ET promoter) or ET.LTR (oncogenic lentiviral vector with transcriptionally active LTRs containing the ET promoter) was administered at matched doses to newborn *Cdkn2a*$^{-/-}$*Ifnar1*$^{-/-}$ tumor-prone mice or wild-type mice resulting in four experimental groups. Wild-type mice then were given a CCl_4-based tumor-promoting regimen. Mice were euthanized at the indicated time points or earlier if sick. Necropsy was performed and samples were collected for DNA extraction (for determination of vector copy number and the retrieval of integration sites) and for histopathological analysis. (**B** and **C**) Shown is the incidence of HCC in *Cdkn2a*$^{-/-}$*Ifnar1*$^{-/-}$ mice (B) or wild-type mice (C) transduced with the two different lentiviral vectors (SIN.ET or ET.LTR) or untransduced (UNT). Untransduced mice include historical controls ($n = 20$ *Cdkn2a*$^{-/-}$*Ifnar1*$^{-/-}$ and $n = 9$ wild-type mice) (*27*). *P* values were calculated by two-tailed Fisher's exact test. Numbers on the histograms indicate the number of mice that developed HCC. (**D** and **E**) Vector copy number in liver DNA from *Cdkn2a*$^{-/-}$*Ifnar1*$^{-/-}$ mice (D) or wild-type mice (E) collected 2 weeks after lentiviral vector administration (early) or at necropsy (late). Data are means ± SEM. *P* values were calculated by one-way analysis of variance (ANOVA) and Bonferroni's multiple correction test. All vector copy numbers were measured in nontumoral liver tissue except for ET.LTR-induced HCCs.

after administration to neonatal mice (fig. S5, E and F). These data suggest that there was no transformation or expansion of SIN.ET-transduced hepatocytes even in sensitive mouse models administered lentiviral vectors as neonates.

We then retrieved lentiviral vector integrations from 83 liver samples (by linear amplification–mediated PCR and 454 pyrosequencing) and identified 9615 unique integration sites (table S13). We identified 60 and 12 CIS in the data sets of integration sites from SIN.ET- and ET.LTR-treated mice, respectively (table S13). There was almost no overlap between SIN.ET and ET.LTR CIS (Fig. 5A), and the latter CIS were also different from the CIS identified in SIN.ET-transduced mice with hemophilia B reported in Fig. 3C (fig. S6A). Moreover, when we applied the Grubbs' test, the only significant CIS of SIN.ET was *Sfi1*, already found in the integration sites of mice with hemophilia B euthanized early and late after lentiviral vector administration, and was thus most likely due to an intrinsic lentiviral vector integration bias (table S14). Conversely, five of the ET.LTR CIS passed the Grubbs' test for outliers (table S14), among which were found the genes *Braf*, *Rtl1*, and *Fign* that are three previously validated liver oncogenes (*27*). ET.LTR integration sites in these CIS were clustered in narrow regions and were almost always in the same orientation of transcription as the targeted gene, consistent with a previously described mechanism of insertional mutagenesis. This mechanism involves transcription from the inserted active LTR and splicing into the oncogene, leading to the up-regulation of expression of a truncated or full-length oncogenic transcript and the in vivo expansion of cells harboring that integration site (Fig. 5B and fig. S6, B and C) (*27*). These data confirm the genotoxic features of the positive control lentiviral vector and the sensitivity of the mice to insertional oncogenesis. In contrast, integration sites for SIN.ET CIS had a lower integration density without orientation bias (Fig. 5C and fig. S6D). Additionally, we found that the power and density of SIN.ET CIS, two measures of the extent of enrichment over random occurrence, were significantly lower ($P = 0.0128$ and $P < 0.0001$, respectively) as compared to those of the ET.LTR (Fig. 5D and fig. S6E). We also compared the relative sequence counts

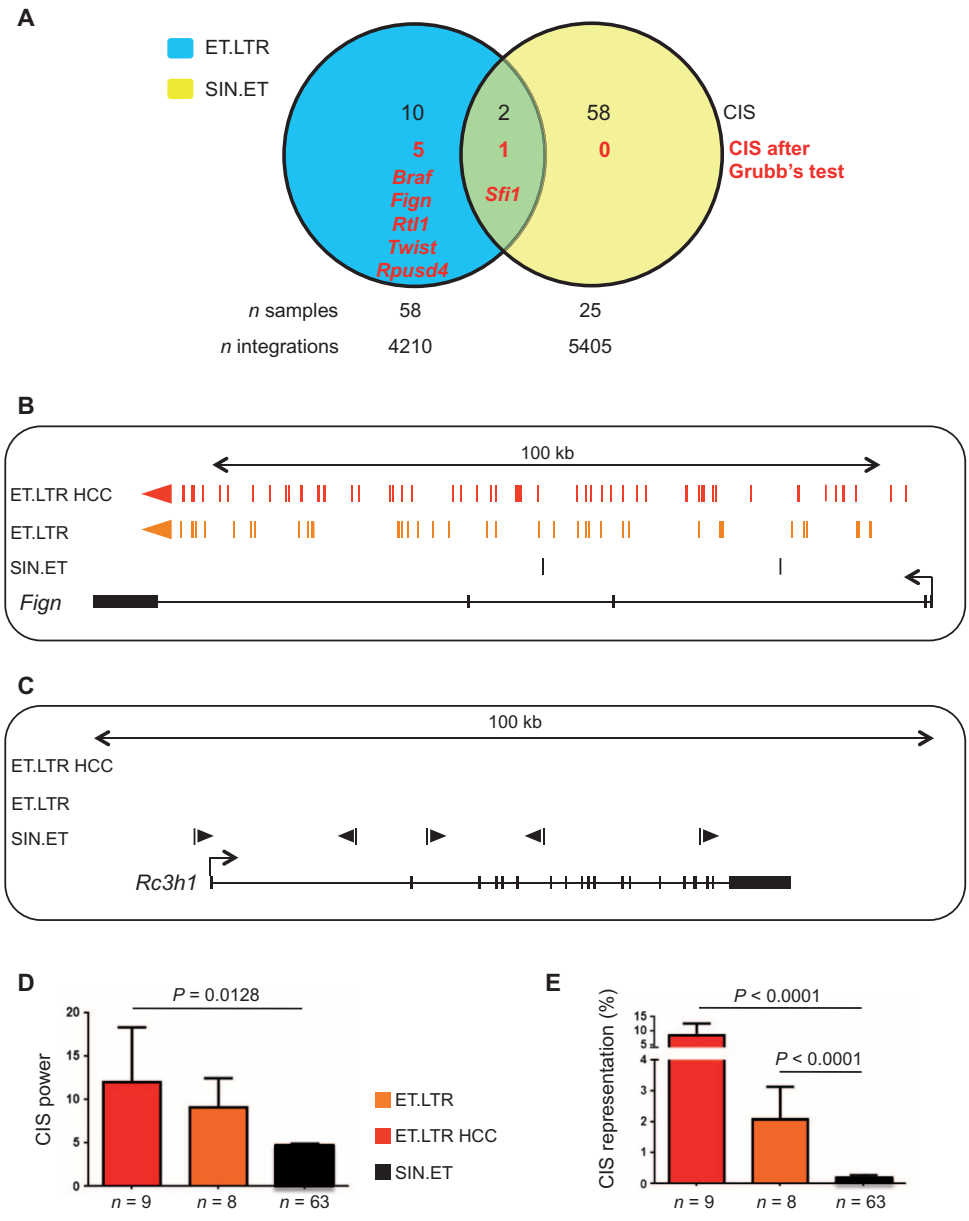

Fig. 5. Integration site analysis does not reveal genotoxicity of SIN.ET lentiviral vectors in tumor-prone mice. (**A**) Venn diagram representing CIS identified in liver DNA of SIN.ET-transduced and ET.LTR-transduced mice. The overlap is calculated considering the gene associated with each CIS. The number of CIS that passed the Grubbs' test is shown with the gene name (red). The number of samples analyzed and the total number of integration sites are indicated for the two data sets. (**B** and **C**) Schematic drawing of two representative CIS of ET.LTR (B) and SIN.ET (C) lentiviral vectors. Each colored bar represents an integration site (red, from ET.LTR-induced HCCs; orange, from nontumoral liver of mice transduced with ET.LTR; black, from liver of mice transduced with SIN.ET). Colored arrows indicate the orientation of the integration site. The gene within the region is represented below, with black boxes indicating exons and arrows indicating transcription orientation. The span of the outlined genomic region is indicated on top. (**D**) CIS power calculated as the number of different integration sites targeting each CIS. (**E**) CIS representation calculated as percent of sequencing reads from all integration sites comprised within a CIS over the total number of reads within an experimental data set. (**D** and **E**) Data are means ± SEM. *P* values were calculated by one-way ANOVA and Bonferroni's multiple correction test. For all the analyses, integration sites from the two mouse models were merged.

of all the integration sites in a CIS as a surrogate readout of the relative abundance of cell clones harboring integration at that CIS within the sampled liver tissues of that experimental group (Fig. 5E). The relative sequence counts of SIN.ET CIS were significantly lower than those of ET.LTR CIS retrieved from nontumoral or tumoral samples (Fig. 5E; $P < 0.0001$). These data further indicate that SIN.ET CIS were not the result of in vivo clonal selection or expansion. Accordingly, the analysis of molecular pathways enriched in the CIS data sets showed that ET.LTR-targeted genes frequently act in pathways associated with cancer and transformation, whereas SIN.ET-targeted genes did not (fig. S6F). Overall, we did not detect evidence of SIN.ET-induced genotoxicity, even after investigation of early molecular readouts of transformation in highly permissive tumor-prone mice or mice in which tumors were chemically promoted.

DISCUSSION

Here, we show that lentiviral vector–mediated gene therapy in the liver of dogs with hemophilia B is feasible and provides stable long-term factor IX activity up to 1% of normal with therapeutic benefit. The treatment was associated with manageable self-limiting acute toxicity without any detectable long-term toxicity or development of antitransgene immune responses.

We produced three large-scale batches of lentiviral vectors for in vivo administration and analyzed them using a panel of tests for identity, potency, and purity according to methods and specifications previously used for clinical trials (*20*). Upon portal vein administration of these lentiviral vectors carrying a factor IX transgene to dogs with hemophilia B, we observed a mild transient fever and hepatocellular toxicity accompanied by a transient rise in circulating TNF-α, IL-6, IL-8, fibrinogen, and TAT. This acute inflammatory response may be a consequence of abdominal surgery and type I interferon release by hepatosplenic plasmacytoid dendritic cells, triggered by nucleic acids contaminating or associated with the infused vector particles, as has been observed in previous mouse studies (*28*). This response was alleviated in one dog pretreated with anti-inflammatory and antihistamine drugs 1 day before lentiviral vector infusion, a mild regimen that could also be translated to the clinical setting. In one dog, we observed a hypotensive anaphylactoid reaction to an unknown component during lentiviral vector infusion. Given that dogs with hemophilia B receive frequent plasma transfusions in response to spontaneous bleedings, they may be presensitized to developing allergic reactions.

Our prior studies in mice with hemophilia B indicated that an optimal lentiviral vector dose to achieve >5% of normal factor IX activity is 2.5×10^{10} TU/kg. According to manufacturing capacity at the beginning of this study and precautions dictated by the testing of lentiviral vectors in large animals, we administered a 45-fold lower dose, 5.7×10^{8} TU/kg, to the first dog (M57). Given that canine factor IX activity approached 0.1% of normal, this outcome suggests that predictions based on dose response in the mouse are reliable in the canine model. Through improvements in large-scale vector production and transgene codon-usage optimization, we could administer a fourfold higher dose to a second dog (O21). This dog achieved about 1% of normal canine factor IX activity, which was more than 10-fold higher than that for the first dog. This can be explained by the two- to threefold gain in potency of the codon-optimized canine factor IX, as had been previously observed in mice (*14*). By exploiting the Padua mutation that confers sevenfold higher activity on activated factor IX (*14*, *23*), we could reconstitute 1% of normal canine factor IX activity in a third dog, even though it was administered a twofold lower dose compared to dog O21. Overall, these results show that by delivering a codon-optimized hyperfunctional factor IX transgene, one can reduce the effective lentiviral vector dose by more than 1 log. All dogs experienced a long-lasting therapeutic benefit from the single treatment, as shown by a marked decrease in spontaneous bleedings recorded throughout the follow-up period of several years. Moreover, the treatment did not induce anti–factor IX inhibitory antibodies, consistent with the observed stable long-term factor IX activity in plasma. However, it should be noted that the dogs treated in this study, although totally devoid of circulating factor IX (*29*), do bear a missense mutation in their *F9* gene and thus are not prone to developing anti–factor IX antibodies.

There are a number of limitations to our study. The main limitation is the small number of dogs treated, with each being treated with a different transgene and vector dose. In addition, relatively low doses of lentiviral vector were administered, giving rise to transgene expression at the threshold of therapeutic activity. Thus, further dose-escalating studies in dogs as well as in nonhuman primates are warranted to investigate the potential occurrence of dose-limiting acute toxicity and lentiviral vector stability in blood and biodistribution in multiple tissues. These further studies will be crucial to establish the safety of in vivo administration of lentiviral vectors to large animal models and to establish that there is no immune response to the transgene.

If we extrapolate the observed dose response in dogs to humans, the current lentiviral vector manufacturing capacity (*20*) could support the treatment of adult patients with hemophilia, provided that a hyperfunctional factor IX transgene is used. The manufacturing of lentiviral vectors for clinical use should become less onerous through further improvements in procedures and scale-up and the availability of stable packaging cell lines.

The occurrence of vector-induced oncogenesis in hematopoietic stem cell gene therapy trials based on γ-retroviral vectors necessitates stringent preclinical assessment of potential vector genotoxicity (*30*–*33*). This caution also applies to liver gene therapy because hepatocytes are susceptible to insertional mutagenesis (*27*, *34*–*37*). Accumulating evidence from nonclinical studies and recent clinical trials supports the view that lentiviral vectors entail a lower genotoxic risk compared to γ-retroviral vectors (*19*, *21*, *22*, *38*). Given that most studies of vector-induced genotoxicity have focused on hematopoietic stem and progenitor cells, we undertook a preclinical analysis of lentiviral vector–induced genotoxicity in the mouse liver. We did not find evidence of genotoxicity of our therapeutic lentiviral vectors in mice with hemophilia B and in two ad hoc mouse models with enhanced sensitivity to hepatocellular carcinogenesis (>100 mice and >9000 integration sites analyzed).

The distribution of lentiviral vector integrations in liver DNA of mice with hemophilia showed that deviations from random were already evident early after gene therapy, thus most likely representing the intrinsic integration biases of the vector and not the outcome of in vivo selection. This contention was supported by the substantial overlap between the CIS identified in early and late liver harvests and the lack of new CIS that passed the Grubbs' test for outliers in the late data set. Note that our sampling and depth of analysis were not designed to attain saturation of CIS; thus, we did not expect full overlap between early and late CIS.

The low genotoxicity of the SIN.ET lentiviral vector design was further demonstrated by administration to tumor-prone mice, where this vector did not increase the spontaneous occurrence of HCC. This was at variance with an aptly designed genotoxic lentiviral vector serving as a positive control. Lentiviral vectors were administered to newborn mice, thus increasing the likelihood that proliferating hepatocytes would accumulate additional mutations complementing an eventual oncogenic lentiviral insertion resulting in the induction of hepatocyte transformation. The observation that SIN.ET CIS have lower integration power, integration density, and representation in the whole data set compared to ET.LTR CIS indicates that SIN.ET integration sites are not subject to the same process of in vivo selection as ET.LTR integration sites. We found three previously validated liver oncogenes (*Braf*, *Fign*, and *Rtl1*) as significant CIS after the Grubbs' test in the ET.LTR data set. Moreover, for some oncogenes such as *Fign*, we could observe clustering of ET.LTR integration sites in a significant CIS even in nontumoral tissues (Fig. 5B), indicating that our experimental design could detect the occurrence of clonal selection due to an oncogenic vector even before overt neoplasia. Therefore, it is unlikely that the lack of evidence for in vivo selection of SIN.ET insertions was due to a limited sensitivity of our assay. Rather, our studies did not uncover any biological or molecular evidence of SIN.ET-induced genotoxicity even in the "worst-case scenario" of transduction of newborn tumor-prone mice.

In conclusion, our study positions liver-directed lentiviral gene therapy for further preclinical development. Lentiviral vectors may complement other available vectors to address some of the outstanding challenges posed by liver gene therapy for the treatment of hemophilia and conceivably other diseases.

MATERIALS AND METHODS

Study design

Dog studies were necessarily limited in sample size for feasibility and ethical reasons. The sample size of mouse studies was chosen according to the previously observed tumor incidence in the positive control group (*27*). No sample or animal administered the intended dose was excluded from the analysis. Mice were randomly assigned to each experimental group. Investigators involved in histopathological analysis and initial integration site mapping were blinded. Investigators performing mouse handling, sampling, euthanasia, and raw data analysis were not blinded.

Large-scale lentiviral vector production

Large-scale lentiviral vector production for dog studies was outsourced to MolMed S.p.A. or Généthon. The vector batches were produced by using a large-scale validated process (*19*, *20*) and following pre-GMP (Good Manufacturing Practice) guidelines. Briefly, lentiviral vectors were produced by transient four-plasmid transfection of 293T cells in 10-tray cell factories by calcium phosphate precipitation. Twenty-four hours after removal of the transfection medium, the cell supernatant was harvested and stored at 4°C. The culture medium was replaced, and after further 24 hours, a second harvest was performed. The media collected from the two harvests were pooled and filtered through 5/0.45-μm filters to discard cell debris. The downstream purification process included a benzonase treatment overnight at 4°C, followed by a diethylamino anion exchange chromatography step, concentration, and gel filtration in phosphate-buffered saline or 5% dimethyl sulfoxide. The resulting lentiviral vector preparation underwent 0.2- or 0.45-μm filtration and aseptic filling. The purified vector preparation was stored at −80°C.

Dog experiments

Hemophilia B dogs (carrying a E379G single amino acid substitution in the factor IX protein) were maintained at the Francis Owen Blood Research Laboratory, which provides for breeding, whelping, housing, treating, and performing the experiments in the dogs on site. Complete blood counts and platelet counts were performed on EDTA-anticoagulated blood with a cell counter (Heska) calibrated for canine cells. Serum liver enzymes were performed by Antech Diagnostics, a commercial veterinarian diagnostic laboratory.

Mouse experiments

Founder C57BL/6 *F9* knockout mice were obtained from the laboratory of I. Verma at the Salk Institute (*39*). *Cdkn2a*$^{-/-}$*Ifnar1*$^{-/-}$ mice were generated to couple the sensitivity to genotoxic mutations conferred by the *Cdkn2a* deficiency (*21*, *22*) to the increased permissiveness to liver gene transfer by lentiviral vectors conferred by the *Ifnar1* deficiency (*28*). Additionally, this noninflammatory tumor-prone mouse model has a clinical relevance because *CDKN2A* and its targets—pRB (retinoblastoma protein) and p53—are frequently inactivated or silenced in HCC (*40*). *Cdkn2a*$^{-/-}$ (C57BL6/J) mice were obtained from the National Cancer Institute–Frederick Mouse Models of Human Cancers Consortium Repository, whereas *Ifnar1*$^{-/-}$ (129/SvEv) mice were obtained from B&K Universal Limited. Wild-type C57BL/6 mice were purchased from Charles River Laboratories. Eight-week-old wild-type mice, transduced or not with ET.LTR at neonatal stage, were administered CCl_4 (1 mg/kg) twice weekly for 6 weeks in a 10% mineral oil solution (Sigma-Aldrich). CCl_4 administration results in waves of hepatocyte necrosis and regeneration that cause liver damage (*41*). Both mouse models were previously described (*27*).

All mice were maintained under specific pathogen–free conditions. Vector administration was carried out by tail vein injections in adult (7 to 10 weeks old) mice and by temporal vein injection in newborns (1 to 2 days old). Mice were bled from the retro-orbital plexus using capillary tubes, and blood was collected into 0.38% sodium citrate buffer (pH 7.4). Mice were anesthetized with tribromoethanol (Avertin) and euthanized by CO_2 inhalation. All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee. At necropsy, masses in the liver parenchyma as well as nontumoral liver were collected for microscopic and molecular analyses.

Statistical analysis

Statistical analyses were performed upon consulting with professional statisticians. When data were in percent, log odds were calculated to perform tests assuming normal distribution (*42*). Standard statistical tests were performed using Student's *t* test (two experimental groups) or ANOVA with Bonferroni's multiple comparison posttest (more than two experimental groups) at $\alpha = 0.05$ level of confidence.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Lentiviral vectors efficiently transduce and regulate transgene expression in canine cells.

Fig. S2. Hematocrit, plasma fibrinogen, TAT, D-dimer, and clearance of lentiviral vector particles

from blood after portal vein infusion in dogs with hemophilia B.
Fig. S3. Histology of liver biopsies from treated dogs with hemophilia B.
Fig. S4. Lentiviral vector content in liver biopsies and blood and sperm samples from treated dogs with hemophilia B.
Fig. S5. Factor IX expression and liver histology of tumor-prone mice or mice treated with a tumor promoter transduced with SIN.ET or ET.LTR lentiviral vectors.
Fig. S6. Comparison of the ET.LTR and SIN.ET CIS under different experimental conditions.
Table S1. Large-scale batches of lentiviral vectors.
Table S2. Blood cell counts and blood chemistry in M57.
Table S3. Blood cell counts and blood chemistry in O21.
Table S4. Blood cell counts and blood chemistry in O59.
Table S5. Lentiviral vector particles in swabs from dogs O21 and O59.
Table S6. Bleeding frequency in treated and untreated dogs with hemophilia B.
Table S7. Thromboelastography in treated dogs with hemophilia B.
Table S8. Inhibitor screen in treated dogs with hemophilia B.
Table S9. Mice with hemophilia B transduced with SIN.ET as adults.
Table S10. Integration sites retrieved from mice with hemophilia B transduced with SIN.ET as adults.
Table S11. CIS identified in the data set of integration sites retrieved from mice with hemophilia B transduced as adults.
Table S12. *Cdkn2a*$^{-/-}$*Ifnar1*$^{-/-}$ and wild-type mice transduced with SIN.ET or ET.LTR as neonates.
Table S13. Integration sites retrieved from tumor-prone mice transduced as neonates.
Table S14. CIS identified in the data set of integration sites retrieved from tumor-prone mice transduced as neonates.
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Liver-directed lentiviral gene therapy in a dog model of hemophilia B

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Advancing gene therapy for hemophilia

Hemophilia is an inherited bleeding disorder caused by a deficiency in a blood clotting factor. The current treatment requires lifelong intravenous administration of the missing clotting factor every few days, a costly and demanding regimen for patients with hemophilia. Gene therapy has the potential to provide a single-shot treatment option by replacing a functional gene in liver cells that naturally produce the factor. Cantore *et al.* now report a study of the efficacy and safety of liver-directed in vivo gene therapy in large and small animal models using lentiviral vectors. This gene therapy strategy with lentiviral vectors may complement the use of other gene therapy vectors for treating hemophilia.

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