Superior lentiviral vectors designed for BSL-0 environment abolish vector mobilization

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Abstract

Lentiviral vector mobilization following HIV-1 infection of vector-transduced cells poses biosafety risks to vector-treated patients and their communities. The self-inactivating (SIN) vector design has reduced, however, not abolished mobilization of integrated vector genomes. Furthermore, an earlier study demonstrated the ability of the major product of reverse transcription, a circular SIN HIV-1 vector comprising a single-long terminal repeat (LTR) to support production of high vector titers. Here, we demonstrate that configuring the internal vector expression cassette in opposite orientation to the LTRs abolishes mobilization of SIN vectors. This additional SIN mechanism is in part premised on induction of host PKR response to double-stranded RNAs comprised of mRNAs transcribed from cryptic transcription initiation sites around 3’SIN-LTR’s and the vector internal promoter. As anticipated, PKR response following transfection of opposite orientation vectors, negatively affects their titers. Importantly, shRNA-mediated knockdown of PKR rendered titers of SIN HIV-1 vectors comprising opposite orientation expression cassettes comparable to titers of conventional SIN vectors. High-titer vectors carrying an expression cassette in opposite orientation to the LTRs efficiently delivered and maintained high levels of transgene expression in mouse livers. This study establishes opposite orientation expression cassettes as an additional PKR-dependent SIN mechanism that abolishes vector mobilization from integrated and episomal SIN lentiviral vectors.

Introduction

Recent successes of lentiviral vector-based gene replacement [1, 2] and immunotherapy [3, 4] at transmuting the pathologic course of human genetic and malignant proliferative diseases opened a new age in medicine. However, potential vector mobilization following infection of vector-transduced cells with HIV-1 poses biosafety risks to vector-treated patients and their communities. The development of self-inactivating (SIN) vectors significantly reduced, yet not abolished mobilization of integrated SIN vector genomes. The concept of SIN retroviral vectors is premised on deletion of the parental HIV-1 promoter and enhancer sequences from the 3’U3 in the vector cassette. The deleted 3’U3 is copied in the process of reverse transcription to the 5’U3. Consequently, reverse-transcribed SIN vector genomes devoid of the parental enhancer promoter sequences should not support transcription of vector length mRNA. Although the first SIN vectors were developed by Yu et al. [5] as a means to enhance biosafety of γ-retroviral vectors, Shinya et al. [6] were the first to describe a SIN lentiviral...
vector. The first efficient SIN lentiviral vector system was developed and successfully employed by Miyoshi et al. [7] to transduce rat retina and brain tissues. However, later studies reported on residual SIN vector mobilization, due to initiation of aberrant transcription of vector length mRNA from SIN LTRs [8–10]. This phenomenon was attributed to HIV-1 sequences within the vector’s packaging signal [9] and to host chromatin structures and regulatory elements in proximity to integrated vector genomes [8]. The majority of lentiviral vector genomes shortly after transduction comprises episomal linear and circular (containing either one or two LTRs) DNAs. However, the ability of episomal lentiviral genomes to support vector mobilization has not been studied. Furthermore, in an earlier study, Ma et al. [11] employed a circular SIN vector genome comprising a single-LTR to produce high-titer lentiviral vectors. Concerned by these data, we sought to abolish residual vector mobilization from integrated and episomal vector genomes by incorporating an additional level of safety to the currently used SIN lentiviral vector system. We reasoned that any additional biosafety measure to eliminate vector mobilization should be mechanistically independent and thus, synergistic to the current SIN vector design in preventing vector mobilization. We theorized that configuring the vector internal expression cassette in opposite orientation (ECOO) to the SIN LTRs would minimize aberrant transcription from SIN LTRs (mediated by either the vector internal promoter [11], parental HIV-1 sequences [9], or host regulatory elements [8]) and induce host Protein kinase-R (PKR) response to double-stranded RNAs comprising LTR- and internal promoter-initiated transcripts. Indeed, here, we demonstrate dramatic reduction of vector mobilization from integrated and episomal lentiviral vector genomes comprising ECOO to the LTRs. Similar to earlier publications, lentiviral vectors carrying ECOO to the LTRs exhibited low titers [12, 13]. However, stable shRNA-mediated knockdown of PKR in vector producing cells rendered titers of the novel mobilization-resistant vectors comparable to titers of their counterpart vectors, comprising expression cassettes in the same orientation to the LTRs. High-titer mobilization-resistant SIN vectors efficiently delivered and maintained firefly luciferase expression in mouse livers. Furthermore, we report here on efficient production of a lentiviral vector carrying an ECOO encoding a modified human β-globin protein under the regulation of the β-globin locus control region [13]. This study elucidates the mechanism of SIN vector mobilization, and establishes a highly efficient methodology of generating mobilization-resistant lentiviral vectors comprising expression cassettes in opposite orientation to the LTRs.

Methods and materials

Cells

SODK0 [14] and 293T cells were maintained in Dulbecco's Modified Eagle's medium (DMEM)-high glucose (Thermo Scientific, Waltham, MA) supplemented with 10% Fetal Bovine Serum (FBS) (Atlantic biologicals, Miami, FL), 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Corning Cellgro, Manassas, VA). The SODK1 cell line [14] and all its derivatives were cultured in the pre-mentioned medium with the presence of 1 μg/ml doxycycline (Dox). HepG2 cells were cultured in MEM (Gibco, Gaithersburg, MD) medium with 10% FBS and antibiotics. shRNAs targeting the hPKR and hβ-globin (HBB) genes in 293T cells were delivered by lentiviral vectors obtained from the TRC1 library. These include clones: TRCN0000196400 (pKR sh1), TRCN0000197012 (PKR sh2) and TRCN000002909 (HBB). Vector transduced 293T cells were selected with 1 μg/ml puromycin (Invivogen, San Diego, CA) for two continuous weeks. PKR knockdown was confirmed by western blot.

Plasmids

The lentiviral vector pTK485, pTK945, pTK979 and the packaging cassettes ΔNRF, pTK939 (Int-), VSV-G envelope, pMDL and the Rev plasmids, were described earlier [15–18]. The Tat plasmid was a kind gift from Dr. Thomas Hope. All other lentiviral vector plasmids were generated by standard restriction enzyme digestion and ligation (New England Biolabs, Ipswich, MA). To simplify the cloning procedure, we synthesized a plasmid pTK1866 containing BamHI-cPPT-opposite orientated BGH—multiple cloning site (MCS)—WPRE- LTR-PmeI. BGH was flanked by BstZ17I, WPRE was flanked by ClaI to make future modification feasible. The expression cassette of CMV-GFP in pTK945 was generated by EcoRI/SpeI, blunt with DNA Polymerase Klenow Fragment, and inserted into Hpal located within MCS of pTK1866 with opposite orientation. The WPRE region was removed by ClaI digestion and ligation. The BGH in the aforementioned plasmid was removed by BstZ17I, and exchanged with SV40 Polyadenylation from pCI-neo by Smal/FspI double digestion. The region (cPPT-SV40 op-GFP-CMV-WPRE-LTR) was digested by BamHI/PmeI to substitute their counterparts in pTK945. To generate pTK1940 (GenBank accession MH297436), a ClaI fragment comprising the WPRE (nt 2908-3496 in pTK1940 and pTK1956) sequence was inserted in opposite orientation to the LTRs into a BsiWI site downstream to the SV40 poly-A. pTK1956 (GenBank accession MH297437) was created by replacing CMV-GFP expression cassette (Xhol/AscI) with hAAT-luciferase (AfeI/
XhoI) from pTK979. All the unmatched overhanging enzymatic digestion sites were blunted by DNA Polymerase I, Klenow Fragment, before ligation. All plasmids were confirmed by sequencing and restriction enzyme digestion.

Viral vector production and concentration

Lentiviral vector production via three plasmids transient transfection was described earlier [16]. For animal studies, viral vectors were concentrated by sucrose ultracentrifugation as described earlier [19]. Absence of replication competent retroviruses (RCR’s) was verified by three independent safety assays (GFP rescue assay, Tat transfer assay and Gag transfer assay) as described earlier [14].

Viral vector titration

Titors of vector transducing units (TU/ml) were determined by scoring GFP-positive cell number under fluorescence microscope following serial dilutions on 293T cells. Titors of physical vector particles were determined by p24\(^{\text{PGP}}\) ELISA [20]. Vector copy number (VCN) was determined by multiplex PCR using the ABI7300 real-time PCR system. NotI794 primer/prober set (F5'-taagaccagcagca-3'; R5'-cactctcaatgtcctca-3'; Roche Universal Probe Library (UPL) #25, 4686993001, Indianapolis, IN) was used for vector detection, and paired with human RNaseP primer/probe set (Thermo Scientific 4316844) or mouse GAPDH (Roche 05190525001) as a reference gene [21].

Western blot analysis

Naive, PKR knocked-down and β-Globin knocked-down 293T cells were lysed in RIPA buffer. Protein samples were separated on 10% SDS–PAGE denaturing gels. Blots were detected with PKR antibody (1:1000, Cell signaling Technology, Cat# 3072S, Danvers, MA) or β-actin antibody (1:1000,Santa Cruz, Cat# sc-1616, Dallas, TX) followed by a polyclonal goat anti-rabbit secondary Ab labeled with horseradish peroxidase (1:10,000; Pierce, Grand Island, NY) treated with ECL (GE Health Amersham, Pittsburgh, PA), and imaged by the ChemiDoc MP Imaging System (BioRad, Hercules, CA).

FACS analysis

Target cells were harvested at 72 h post-transduction with GFP expressing vectors. Harvested cells were washed with PBS, fixed with 2% paraformaldehyde for 15 min, and re-suspended in MACS buffer (2 mM EDTA and 1% BSA in PBS). Fluorescence-activated cell sorter (FACS) analysis was performed with CyAn ADP (Becton–Dickinson). Percentage of GFP-positive cell and mean fluorescence intensity (MFI) were analyzed with the FlowJo9.3.2 (Flowjo LLC, Ashland, OR) software.

Mobilization of integration-deficient lentiviral vectors (IDLV) from the inducible packaging cell line SODK1

The inducible packaging cell line SODK1 [14] was employed to evaluate IDLV mobilization. To induce vector production, SODk1 cells were washed with PBS (X3), and passaged onto poly-lysine (Sigma, St. Louis, MO) pre-coated plates and cultured in doxycycline (Dox)-free media. At 5 days post-Dox withdrawal, 15 million cells were seeded onto 10 cm plate for IDLV mobilization analysis. On day 6 post Dox withdrawal, 2 × 10^8 TU of IDLV were employed on the induced SODK1 cells in the presence of 5 mM sodium butyrate. At 12 h post-transduction, cells were washed and cultured with Dox-free fresh media containing 5 mM sodium butyrate. Vector particles containing media were collected at 72 h after addition of sodium butyrate. IDLV transduced SODK1 cells cultured in the presence of Dox (1 µg/µl) and sodium butyrate (5 mM) media served as controls. Mobilized vector particles were employed on 2-million naive 293T target cells. To eliminate carrying over of non-mobilized IDLVs, tilters of mobilized integration competent lentiviral vector (ICLVs; generated by the induced packaging cells) were determined after five passages of the above target cells in culture. Percent of GFP-positive cells and the number target cells at the time of transduction were used to calculate vector titters.

Mobilization of ICLV from the inducible packaging cell line SODK1

SODK1 cells were transduced with ICLVs particles at an m.o. i. of 10. To dilute out episomal vector genomes, transduced SODK1 cells were passaged in culture (X5). To initiate vector production, transduced SODK1 cells were washed with PBS (X3), and passaged onto poly-lysine coated plates in Dox-free media. At 5 days post-Dox withdrawal, 15 million cells were seeded onto 10 cm plate. Sodium butyrate (5 mM) was added to culture media at day 6 post-Dox withdrawal. Vectors particles containing media were collected at 72 h after addition of sodium butyrate. IDLV transduced SODK1 cells cultured in the presence of Dox (1 µg/µl) and sodium butyrate (5 mM) media served as controls. Titters (TU/ml) of mobilized vectors were determined by scoring GFP expression following serial dilutions on 293T cells.

Luciferase assay

Vector transduced HepG2 cells were lysed at 72 h post-transduction and quantified with 1420 Multilabel Counter.
Victor 3 (PerkinElmer Waltham, MA) following the manufacturer’s instruction of Luciferase Assay system (Promega Madison, WI). Relative light units (RLU) were further normalized with protein concentration and viral copy number (VCN per cell). Protein concentration was determined by the Bradford method.

**Animal studies**

All animal cares and procedures were in accordance with the Guide for the Care and Use of Laboratory Animals and received prior approval by the UNC Animal Care and Use Committee (Animal protocol 15-123.0). C57BL/6J female mice at age 5–6 week old were obtained from Jackson Lab. One week after animal arrival, 25 µg p24gag lentiviral vectors were administrated into each mouse via intraperitoneal injection. In vivo expression of vector-delivered firefly luciferase in live animals was determined at weeks 2, 4, 6, and 8 using IVIS Lumina optical system (PerkinElmer, Waltham, MA), as described previously [21]. Shortly, mice were injected with 250 µl luciferin substrate (25 mg/ml, Regis Technology, Grove, IL) via I.P. After 10 min incubation, mice were imaged for 5 min with IVIS imagine system.

**Statistical analysis**

Student’s t-test in Excel software was used to determine the significance of in vitro assay of vector pTK979 and pTK1956.

**Results**

**A circular SIN single-LTR vector cassette supports Tat independent production of high-titer lentiviral vectors**

Mobilization of HIV-1 based vectors following infection of vector-transduced cells with HIV and consequential spread of vector particles in vector-treated patients and their communities are major biosafety concerns. Using cell lines comprising integrated SIN vector genomes, several groups demonstrated significant reduction yet not complete elimination of lentiviral vector mobilization [8, 9]. Although circular single-LTR vector genomes are a major product of lentiviral vector reverse transcription [15, 22–24], their ability to mobilize packageable vector genomes has not been evaluated. Furthermore, in an earlier study, Ma et al. [11], demonstrated that a DNA construct encoding a circular SIN single-LTR vector supported production of high vector titer. Since the precursor of the aforementioned shuttle SIN vector described by Ma et al. was isolated from lentiviral vector-transduced cells, we further studied the mechanism of SIN vector mobilization. Specifically, we characterized the role of HIV-1 Tat protein in mobilization of circular SIN single-LTR genomes pTK485 (Fig. 1a and Supplement Fig. 1), which are naturally generated in vector-transduced cells [15, 22–24]. To this end, vTK485 particles were produced by a transient four-plasmid transfection procedure, either in the presence or absence of the HIV-1 Tat protein. As shown in Fig. 1b production of high-titer (3-4E + 6 IU/ml) SIN vectors from a SIN single-LTR expression cassette (pTK485) was Tat independent. These findings are in line with an earlier report by Poon et al. [25]. Vector mobilization from integrated SIN vector genomes was attributed to aberrant transcription initiation induced by either enhancer sequences located in the vector-packaging signal or to host sequences in proximity to the vector integration site [8, 9]. However, the high titers of SIN vectors generated from pTK485 indicated that a different or additional mechanism contributes to the highly efficient Tat independent vector production from circular SIN vector genomes. Importantly, the process of vector circularization, locates the internal promoter upstream to the SIN LTRs. Thus, we hypothesize that transcriptional activity induced by the internal promoter is the major contributor to episomal SIN vector mobilization. Furthermore, we theorized that positioning the internal expression cassette in opposite orientation to the LTR’s would significantly minimize internal promoter mediated mobilization of circularized episomal vectors.

**Internal expression cassettes in opposite orientation to the LTRs reduce production of lentiviral vector particles and vector titers**

To reduce the likelihood of lentiviral vector mobilization, an internal expression cassette comprising the bovine growth hormone (BGH) polyadenylation (poly-A) site, a CMV promoter, and the green fluorescence protein (GFP) reporter gene was positioned in an opposite orientation to the SIN vector LTRs (pTK1906 in Fig. 2a). Production efficiency of pTK1906 by transient three-plasmid transfection was compared to production of its conventional counterpart vector (pTK945). As shown in Fig. 2b, c positioning an internal expression cassette in opposite orientation to the LTRs dramatically reduced transducing and physical vector titers. Furthermore, the reduction in concentration of vector particles in culture media (as determined by p24agg ELISA) suggested that the opposite orientation expression cassette in the vector construct probably induced a host PKR response that effectively inhibited synthesis of viral structural proteins encoded by the co-transfected packaging cassettes.
production of ECOO containing lentiviral vectors

Hence, we sought to characterize the effects of poly-A sites positioned upstream and in opposite orientation to conventional internal expression cassettes (in same orientation to the LTRs) on vector titers (Fig. 3a). To this end, SIN vectors comprising either internal BGH (pTK1588) or SV40 (pTK1595) poly-A sites were generated in either naive or PKR-deficient 293T cells. Vector titers were determined and compared with titers of their conventional counterpart vector (pTK945). Unexpectedly, titer analysis (Fig. 3b) indicated that the origin of the internal poly-A site had a major effect on vector titers. Specifically, regardless of PKR expression in vector producing cells, titers of SIN vectors comprising an internal SV40 poly-A site (pTK1595) were comparable to titers of their parental vector (pTK945) lacking an internal poly-A site. Per contra, when generated in naive 293T cells, titers of a vector comprising an internal BGH poly-A site (pTK1588) were more than a 100-fold lower than titers of either control vectors or same vectors generated in PKR-deficient cells. Since none of the above vectors contains an ECOO, we theorized that transcription through the parental LTR poly-A produced a BGH poly-A sequence located downstream to the 3’LTR. Consequently, the transcribed full-length vector mRNA’s comprised two complementary BGH poly-A sites, which generated an intra-molecular double-stranded RNA structure and induced a host PKR response that negatively affected vector titers. To test the above notion, we developed two SIN vectors lacking the downstream BGH poly-A site (Fig. 3a). The novel two vectors, either, comprising or lacking the internal BGH poly-A site (pTK1850 and pTK1848, respectively) either without (lane 2) or with (lane 3) the HIV-1 Tat expression cassette (pSV-Tat). Transducing unit titers (TU/ml) were determined by scoring GFP-positive cells following serial dilution on 293T cells at 72 h post-transduction. Titers are represented as average of three independent experiment ± SD (standard deviation).

PKR-deficient packaging cells support efficient production of ECOO containing lentiviral vectors

To study the effects of the host PKR response on titers of ECOO-comprising vectors, U6-shRNA expression cassettes carried by lentiviral vectors were employed to effectively knock down PKR expression in 293T cells (Fig. 2d). Conventional and ECOO-comprising vectors were generated in either PKR-deflcient 293T cells. As shown in Fig. 2b, c PKR knockdown in vector producing cells resulted in a tenfold increase in titers of ECOO-comprising vectors, with minimal effect on titers of conventional vectors. Next, we sought to delineate the mechanisms that induce PKR response following transfection of ECOO-comprising vectors.

The origin and position of poly-A sites in the vector cassette affect lentiviral vector titers

An internal poly-A site is required to facilitate efficient transgene expression from vectors comprising ECOO’s. Hence, we sought to characterize the effects of poly-A sites positioned upstream and in opposite orientation to conventional internal expression cassettes (in same orientation to the LTRs) on vector titers (Fig. 3a). To this end, SIN vectors comprising either internal BGH (pTK1588) or SV40 (pTK1595) poly-A sites were generated in either naive or PKR-deficient 293T cells. Vector titers were determined and compared with titers of their conventional counterpart vector (pTK945). Unexpectedly, titer analysis (Fig. 3b) indicated that the origin of the internal poly-A site had a major effect on vector titers. Specifically, regardless of PKR expression in vector producing cells, titers of SIN vectors comprising an internal SV40 poly-A site (pTK1595) were comparable to titers of their parental vector (pTK945) lacking an internal poly-A site. Per contra, when generated in naive 293T cells, titers of a vector comprising an internal BGH poly-A site (pTK1588) were more than a 100-fold lower than titers of either control vectors or same vectors generated in PKR-deficient cells. Since none of the above vectors contains an ECOO, we theorized that transcription through the parental LTR poly-A produced a BGH poly-A sequence located downstream to the 3’LTR. Consequently, the transcribed full-length vector mRNA’s comprised two complementary BGH poly-A sites, which generated an intra-molecular double-stranded RNA structure and induced a host PKR response that negatively affected vector titers. To test the above notion, we developed two SIN vectors lacking the downstream BGH poly-A site (Fig. 3a). The novel two vectors, either, comprising or lacking the internal BGH poly-A site (pTK1850 and pTK1848, respectively) were generated in either naive or PKR-deficient cells. As shown in Fig. 3c, d both vectors (pTK1850 and pTK1848) exhibited comparable titers, which were only marginally lower than the titers of a conventional SIN vector (pTK945) containing a BGH poly-A site downstream to the 3’LTR. These findings suggested that in the absence of a functional ECOO the mere presence of an internal poly-A site induces only a minor reduction in vector titers.
Transcription from ECOO reduces titers of SIN lentiviral vectors

Next, we sought to directly determine the effects of transcription from the ECOO at the time of vector production on vector titers. To this end, expression cassettes comprising an inducible tetracycline promoter and either a BGH or SV40 poly-A site were incorporated in opposite orientation to SIN vectors (Fig. 3a: pTK1885 and pTK1906 carrying GFP expression cassettes in different orientations to the vector LTRs. Arrows indicate the direction of transcription from the relevant internal promoters). Furthermore, to evaluate the combined effect of vector design and ECOO transcription on vector titers, a BGH poly-A site was incorporated downstream of the 3′LTR and compared to its unincorporated counterpart (Fig. 3a: pTK1927, pTK1937, and pTK1885, pTK1936, respectively). Vector particles were generated by transient transfection in naive and PKR-deficient 293T cells either in the presence or absence of a tetracycline-regulated trans-activator (tTA). Vector titers (TU/ml) were measured by scoring GFP expression following serial dilutions on 293T cells, and represented as average ± SD. c Concentration of physical vector particles in conditioned media was determined by p24 ELISA. d Western blot analysis of PKR expression in the aforementioned four vector producing cell lines including naive 293T cells (lane 1), cells expressing β-globin-directed shRNA (lane 2), cells expressing PKR-directed shRNA’s (PKR sh1-lane 3 and PKR sh2-lane 4). Probing with β-actin-directed antibody served as loading control.
minimal effect on vector titers generated in PKR-deficient 293T cells compared to the titers of a conventional SIN vector (pTK945). Note that transducing titers of vectors containing an internal SV40 poly-A site and a BGH poly-A site downstream to the 3’LTR of pTK1937 were higher than the titers of their counterpart vectors (pTK1936) that lack a BGH poly-A site downstream to the 3’LTR (Fig. 3d). These data suggest that transcription from ECOO negatively affects vector titers generated in naive 293T cells. Furthermore, the presence of a BGH poly-A site downstream to the 3’ LTR increases titers of conventional vectors, however, the presence of both an internal and an external (downstream to the 3’LTR) BGH poly-A sites significantly reduces titers of ECOO-comprising vectors.
Superior lentiviral vectors designed for BSL-0 environment abolish vector mobilization

Optimization of vector design to maximize titers of ECOO-comprising SIN vectors

To maximize titers of SIN vectors comprising ECOO, a series of vectors were developed to identify the optimal combination of poly-A sites within vector expression cassettes (Fig. 4a). Specifically, expression cassettes comprising either BHG (pTK1886 and pTK1906) or SV40 poly-A sites (pTK1907, 1909, 1908, and 1918) were cloned in opposite orientation to the LTRs in vector constructs either containing or lacking a BGH poly-A site downstream to the 3 LTR. Note that the SV40 poly-A site was cloned either in the same (pTK1908 and pTK1918) or opposite (pTK1907 and pTK1909) orientation to the LTRs. Vector particles were generated in naive and PKR-deficient 293T cells. Concentration of physical vector particles and transducing vector titers were determined by p24GS ELISA and by scoring GFP-positive cells following serial dilutions on 293T cells, respectively. Levels of transgene expression in vector-transduced 293T cells were analyzed by FACScan analysis (Supplemental Fig. 2). As shown in Fig. 4b, c when generated in PKR-deficient cells, the titers of pTK1909, a vector comprising an internal SV40 poly-A in opposite orientation to the LTRs and a BGH poly-A site downstream to the 3 LTR were comparable to the titers of a conventional SIN vector (pTK945), and more than tenfold higher than the titers of the other ECOO vectors. These data suggest that in the absence of intra-molecular double-stranded RNA structures (generated by two complementary BGH poly-A sequences), efficient shRNA-mediated knockdown of PKR facilitates production of high-titer ECOO-comprising vectors. The presence of a BGH poly-A site downstream to the 3 LTR further increases titers of vectors comprising an internal SV40 poly-A. Interestingly, internal SV40 poly-A sites positioned in opposite orientation to the internal CMV promoter (Fig. 4a, pTK1908 and pTK1918) did not reduce transgene expression levels from ECOO (Fig. 4d). Furthermore, transgene expression levels from all ECOO vectors were significantly lower than the level of expression from the conventional vector control, pTK945 (Fig. 4d) indicating that addition of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) to the internal expression cassette could improve the efficiency of ECOO-comprising vectors.

Incorporation of the WPRE to ECOOs increases levels of transgene expression

To enhance transgene expression from ECOO-comprising vectors, a DNA sequence encoding the WPRE was positioned between the GFP reporter gene and the internal poly-A site (Fig. 5a, pTK1940, GenBank accession MH297436). To address the concern that the lack of a WPRE sequence upstream to the 3 LTR would reduce vector titers, an additional WPRE sequence was incorporated between the 3 LTR and the BGH poly-A site (Fig. 5a, pTK1954). Vector particles were generated either in naive or PKR-deficient 293T cells. Concentration of physical vector particles and vector titers were determined by p24GS ELISA and by scoring GFP expression following serial dilutions on 293T cells (Fig. 5b, c). Transgene (GFP) expression levels were determined by FACScan analysis. As shown in Fig. 5a, d positioning a WPRE sequence between the transgene of interest (GFP) and the internal SV40 poly-A rendered transgene expression from ECOO (pTK1940 and pTK1954) comparable to the levels of expression obtained from a conventional vector control (pTK945). Interestingly, addition of a WPRE sequence between the 3 LTR and the BGH poly-A site (pTK1954) significantly reduced production of physical vector particles and vector titers generated in naive 293T and to a lesser extent in PKR-deficient cell lines (Fig. 5c, d). Note that positioning a single WPRE sequence between the transgene of interest and the internal SV40 poly-A site (pTK1940) had no effect on production of physical vector particles nor on vector titers generated in PKR-deficient cell lines.

Superior transgene expression from integration-deficient ECOO-comprising lentiviral vectors

Intrigued by recent successful integration-deficient lentiviral vectors (IDLV)-mediated gene delivery for vaccination and
cancer immunotherapy in preclinical studies and human clinical trials, respectively [4, 26], we sought to characterize the efficiency of transgene expression from integrase-deficient ECOO-comprising vectors. To this end, conventional and ECOO-comprising vectors (pTK945 and pTK1940, respectively) were packaged into integrase-
Several earlier studies demonstrated efficient liver transduction by lentiviral vectors carrying reporter and clinically relevant cDNAs [15, 21, 27]. Hence, we sought to test the ability of ECOO-comprising vectors to efficiently deliver and express a reporter gene under control of a liver-specific promoter. To this end, an expression cassette comprising the liver-specific promoter (hAAT), the freely luciferase cDNA, the WPRE sequence and the SV40 poly-A was cloned in opposite orientation to the LTR’s (Fig. 5f: pTK1956, GenBank accession MH297437). Vector particles of the above novel liver-specific vector and its conventional counterpart (Fig. 5f: pTK979) were generated by transient transfection [14] and employed on the HepG2 cells. Levels of luciferase expression normalized to vector copy number were analyzed as described earlier [21, 24]. As shown in Fig. 5g normalized expression levels were higher in HepG2 cells transduced with the ECOO-comprising vectors than the normalized expression levels in similar cells transduced with a conventional vector.

Efficient transgene expression from a liver-specific promoter in ECOO-comprising vectors

To further characterize the suitability of ECOO-comprising vectors for future clinical applications, we sought to compare their mobilization potential to that of conventional lentiviral vectors. ECOO-comprising vectors (pTK1940) and their conventional SIN (pTK945) counterparts (Figs 3a, 5a, and 6a) were packaged by transient transfection into vector particles comprising either functional or mutant integrase (D64E) protein. Thus, generating integration competent and integration defective lentiviral vectors (ICLV and IDLV, respectively) [15, 24, 27, 28]. To specifically evaluate mobilization of episomal forms of the above vectors, IDLVs were employed on the tetracycline-regulated, HIV-1 vector-packaging cell line SODk1 [14] either prior (in the presence of doxycycline) or after inducing (in the absence of doxycycline) production of VSV-G-pseudotyped vector particles. Titers of mobilized integrating vectors in conditioned media were determined by scoring GFP expression following serial dilutions on 293T cells. To eliminate potential carryover of the parental IDLV particles, the transduced reporter 293T cells were passaged five times prior to scoring GFP expression. As shown in Fig. 6b titers of mobilized SIN IDLV (pTK945) were only mildly lower than the titers of mobilized non-SIN IDLVs (pTK1087). These data suggest that vector mobilization from episomal vector genomes is independent of the parental HIV-1 promoter/enhancer sequences and is in line with the Tat independent production of a SIN single-LTR vector (Fig. 1b) [11]. Importantly, titers of mobilized episomal ECOO-comprising vectors (pTK1940) were more than fiftyfold lower. Mobilization efficiency of episomal SIN vector from a stable packaging cell line as described here was significantly higher than the level of mobilization of conventional integrated SIN vectors described earlier [8, 9]. To characterize mobilization of integrated ECOO-comprising vectors, the above ICLVs (pTK1087, pTK945, and pTK1940) were employed on SODk1 cells. To dilute out episomal vector forms, transduced SODk1 cells were cultured in the presence of doxycycline for more than 2 weeks (5 passages) after which production of VSV-G-pseudotyped vector particles was induced by doxycycline withdrawal as described earlier [14, 29]. Titers of integrating vector particles in conditioned media were determined by scoring GFP expression following serial dilutions on 293T cells. Vector copy number in SODk1 cells prior to doxycycline withdrawal was determined by quantitative PCR (qPCR) [30]. As shown in Fig. 6c, d titers of mobilized integrated conventional SIN vectors (pTK945) were several hundred fold lower than the titers of their non-SIN counterparts (pTK1087). These
results are in line with the data presented earlier by Logan et al. [9] and Hanawa et al. [8]. Most importantly, titers of mobilized ECOO-comprising vectors were at the limit of detection (less than 10 TU/ml) and several hundred fold lower than the titers of mobilized conventional SIN vectors (pTK945). Furthermore, normalization of vector titers for vector copy number in SODk1 cells has not affected the observed differences in mobilization efficiency of conventional and ECOO-comprising vectors (Fig. 6c). Intrigued by the low mobilization levels of ECOO-comprising SIN vectors (pTK1940), we sought to study the effects of positioning a lentiviral vector’s expression
cassette in opposite orientation to the LTRs on mobilization of non-SIN vectors. To this end, a novel ECOO-comprising non-SIN vector pTK1976 was transduced on either naive or PKR-deficient cells. In addition, conventional non-SIN and SIN vectors pTK1087 and pTK945 as well as the ECOO-comprising SIN vector pTK1940 were employed on naive 293T cells and VCN was determined by qPCR. To characterize the level of vector mobilization, the above vector-transduced cells were transiently transfected with a VSV-G envelope and a lentiviral vector-packaging cassette. Titers of mobilized vectors in conditioned media were determined by scoring GFP-positive cells following serial dilutions on 293T cells. As expected and shown in Fig. 6c, f, conventional non-SIN vector (pTK1087) exhibits the highest mobilization titers (prior and after normalization to VCN in vector producer cells). However, the mere positioning of the internal vector expression cassette in opposite orientation to the LTRs reduced VCN-normalized mobilization titers of non-SIN vectors (pTK1976) from 293T cells by more than 500-fold. These mobilization titers were only fivefold higher than the mobilization titers of conventional SIN vectors (pTK945). Interestingly, VCN-normalized mobilization titers of pTK1976 from PKR-deficient 293T cells were more than fourfold higher than the titers generated by naive 293T cells. These data suggest the reduction in mobilization-titers of non-SIN ECOO-comprising vectors (pTK1976) is mediated by PKR-dependent and independent mechanisms. Importantly, further analysis of vector mobilization-titers shows 96- and 18-fold reduction in vector mobilization titers following conversion of the parental LTR configuration in pTK1976 to a SIN one (similar to pTK1940-LTR) and after reverting the conventional orientation of the internal expression cassette in pTK945 (similar to pTK1940 vector design), respectively. Altogether, these findings indicate that three independent mechanisms (SIN vector design and the above two ECOO-related mechanisms) synergistically inhibit mobilization of ECOO-comprising integrated SIN vectors. Note that, under the above extreme conditions, mobilization of pTK1940 was not completely abolished. We speculate that some vector producing cells transcribe low levels of full-length packagable mRNA, which do not elicit a robust PKR response. This facilitates production of low yet measurable levels of mobilized vector transducing units.

**Superior hepatic gene delivery by ECOO-comprising vectors**

Minimal mobilization and efficient transgene expression render ECOO-comprising vectors better suited to in vivo gene delivery applications than their conventional counterpart. Hence, we sought to evaluate the efficacy of ECOO-comprising vectors at hepatic gene delivery by direct comparison with their conventional counterparts. To this end, ECOO-comprising and conventional lentiviral vectors carrying the firefly luciferase (Luc) expression cassettes driven by the liver-specific, human alpha 1-antitrypsin (hAAT) promoter. Arrows indicate the direction of transcription from the internal hAAT promoter. g Bar-graph describing luciferase expression from the hAAT promoter at 72 h post-transduction of HepG2 cells with conventional (pTK979) or ECOO-comprising vector pTK1956. Relative light units (RLU) were determined by luciferase assay and normalized to total protein concentration and vector copy number per cell (VCN) as determined by real-time PCR. All data were represented as average ± SD of three independent experiments. p-value (p = 0.008) was determined by two tail student’s t-test.

Minimal mobilization and efficient transgene expression render ECOO-comprising vectors better suited to in vivo gene delivery applications than their conventional counterpart. Hence, we sought to evaluate the efficacy of ECOO-comprising vectors at hepatic gene delivery by direct comparison with their conventional counterparts. To this end, ECOO-comprising and conventional lentiviral vectors carrying the firefly luciferase under control of a liver-specific (hAAT) promoter (Fig. 5f: pTK1956 and pTK979, respectively) were packaged into VSV-G pseudotyped ICLVs. Concentrated vector particles were injected intraperitonealy to C57B6 mice (25 µg p24Gag/mouse). Luciferase expression in mouse livers was periodically imaged and quantified by the IVIS Lumina imagine system. As shown in Fig. 7a, b transgene expression levels in mouse livers transduced with ECOO-comprising vectors (pTK1956) were more than fivefold higher than the levels of hepatic luciferase expression from conventional vectors (pTK979). These data underscore the superiority of the recently developed ECOO-comprising vector as a means of in vivo gene delivery vehicle.
Efficient production of ECOO-comprising vectors for clinically relevant ex-vivo applications

To further characterize the efficacy of the PKR-deficient packaging system at generating ECOO-comprising vectors, we sought to evaluate production of a lentiviral vector, which was specifically developed for hematopoietic stem cell gene therapy in sickle cell disease patients. Romero et al. [13] described the novel ECOO-comprising vector, CCL-β AS3-FB, earlier (Fig. 7c). It encodes the modified human β-globin protein HbAS3 [31] and contains the parental β-globin gene regulatory elements including its promoter, introns, poly-A site and the mini-locus control region (LCR) with hypersensitive sites (HS) 2-4. To support long-term and stable transgene expression, a 77 bp insulator was incorporated in the vector’s SIN 3’U3. Similar to other lentiviral vectors, which efficiently delivered β-globin expression cassettes, the novel CCL-β AS3-FB exhibited low titers [13, 32, 33]. We conjectured that producing the CCL-β AS3-FB vector in PKR-deficient cells would increase its titers. To test this hypothesis, CCL-β AS3-FB vector and a conventional vector (Fig. 3a: pTK945) were generated by transient transfection in either naive or PKR-deficient 293T cells. Vector titers and concentration of physical vector particles in condition media were determine by qPCR on vector-transduced 293T cells and by p24gag ELISA. As shown in Fig. 7d, e titers of CCL-β AS3-FB vector generated in PKR-deficient 293T cells were merely 2–3-fold higher than vector titers generated in naive 293T cells and yet comparable to titers of the control vector pTK945 (up to 2×10^8TU/ml). Altogether, the data presented here describe an efficient methodology of producing ECOO-comprising lentiviral vectors, which are safer and more

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**Figures:**

- **a:** Schematic representation of the packaging systems used for vector production.
- **b-f:** Graphical representation of vector production and titration in different cell lines and conditions.

**Legend:**

- IDLVs: Inverted Long Terminal Repeats
- ICLVs: Integrase-processed Long Terminal Repeats
- TU: Transduction Units

**Tables:**

- Mobilized TU/ml
- Mobilized IFU/ml/copy

**Significance:**

- *: Significant difference
- **: Very significant difference
- ***: Highly significant difference
by the induced packaging cells) were determined following over of non-mobilized IDLV butyrate (uninduced or induced, respectively). Mobilized vector par-
cline or in the absence of doxycycline and the presence of sodium mos at 72 h post IDLV transduction of the tetracycline-regulated SODk1 cells, respectively. Error bars represent standard deviation of to calculate vector titers. Black and white bars describe titers (TU/ml) lower than detection level (<10TU/mL).

induced and uninduced SODk1 cells, respectively. The results are white bars describe titers (TU/ml) of the above vectors mobilized from GFP-positive cells following serial dilutions on 293T cells. Black and employed on 293T cells and vector titers were determined by scoring induced and uninduced SODk1 cells. Conditioned media were conditioned media were employed on naïve 293T target cells. To dilute out carry-
over of non-mobilized IDLV’s, titers of mobilized ICLV’s (generated by the induced packaging cells) were determined following five pas-
sages of the above target cells in culture. Percentage of GFP-positive cells and the number target cells at the time of transduction were used to calculate vector titers. Black and white bars describe titers (TU/ml) of the above vectors mobilized from induced and uninduced SODk1 cells, respectively. Error bars represent standard deviation of three independent experiments. e Lentiviral vector mobilization from integrated vector genomes (pTK1087, pTK945, and pTK1940) in induced and uninduced SODk1 cells. Conditioned media were employed on 293T cells and vector titers were determined by scoring GFP-positive cells following serial dilutions on 293T cells. Black and white bars describe titers (TU/ml) of the above vectors mobilized from induced and uninduced SODk1 cells, respectively. The results are average of three independent experiments. Asterisk indicates titers lower than detection level (<10TU/ml). d Normalized vector titers. To minimize bias of VCN in SODk1 on mobilized vector titers, cal-
culated mobilization titers from ICLV-transduced SODk1 cells were normalized to VCN (vector producing SODk1 cells) as determined by real time PCR. Black and white bars describe normalized titers (TU/ml/copy) of the above vectors mobilized from induced and uninduced SODk1 cells, respectively. Titeres were represented as average ± SD of three independent experiments. e-f Characterizing the combined effect of SIN-LTRs and the orientation of lentiviral vector internal expression cassettes on mobilization of integrated lentiviral vectors. PKR-
deficient 293T cells transduced with integrating ECOO-comprising non-SIN vector (pTK1976) and naïve 293T cells transduced with either, conventional non-SIN and SIN vectors (pTK1087 and pTK945, respectively) or ECOO-comprising SIN and non-SIN vectors (pTK1976 and pTK1940, respectively) were analyzed for vector copy number by qPCR. The aforementioned vector-transduced 293T cell were transfected with a VSV-G envelope and the ΔNRF packaging cassettes. Mobilized vectors in conditioned media were collected at 72 h post-transfection and tittered by scoring GFP expression following serial dilutions on 293T cells. e Bar-graph describing titers of mobi-
lized lentiviral vectors from integrated vector genomes in naïve (black bars) and PKR-deficient (white bars) 293T cells. The results are average of three independent experiments. f Bar-graph showing the above titers of mobilized vectors after normalization for VCN. The results are average of three independent experiments

efficient than their conventional counterparts and thus better suited for clinical trials.

Discussion

Following numerous preclinical studies, years of promises, expectations, and early setbacks [34–37], recent accumulating successes of gene therapy clinical trials opened a new era in modern medicine. Currently, viral vectors serve as the method of choice to deliver therapeutic genetic cargos in gene therapy clinical trials. Lentiviral and AAV-based vectors have been used as a means to cure cancer as well as hereditary and acquired human diseases, most of which were not effectively treatable by conventional medicine [1, 3, 38–41]. US FDA approvals of lenti-
viral and AAV vectors-based gene therapy to treat hematopoietic and lymphoid malignancies and a hereditary retinal disease [42–44] formally established a novel class of medications. Similar to conventional medications, the scope of disease indications for gene therapy protocols is dictated by risk/benefit considerations. Findings of earlier γ-retroviral and lentiviral vector-based preclinical studies and human clinical trials raised biosafety concerns regarding potential emergence of RCR and integrating vector-mediated insertional mutagenesis [34, 45–49]. These biosafety concerns were significantly eased by a recent study summarizing biosafety test results of cancer and HIV-1 gene therapy clinical trials employing lentivirus/retroviral vectors-modified T cells [50]. Overall, in these studies, biosafety assays of vector preparations, modified T-cells and treated patients could not detect RCR and demonstrated a long-term decrease in the vector modified T-cells population to <1%. These findings are highly encouraging, and yet additional studies are needed to characterize biosafety of γ-retroviral and lentiviral vector-based gene delivery in different clinical settings, especially those involving stem cell transduction and in vivo gene delivery. Lack of clinical and experimental reports on the adverse effects associated with vector mobilization reduced attention of regulators and investigators to this potential biosafety risk. Vector mobilization is defined as the undesired packaging and transfer of vector genomes from target cells, where they are per-
foming a therapeutic objective, to other cell populations either within the host or to the community. Theoretically, vector mobilization can mediate several adverse processes including insertional mutagenesis, alteration in vector bio-
distribution, emergence of novel RCR’s, and induction of immune response to transgene products. Vector mobilization requires transcription of full-length vector genomes in a cell that concomitantly produces all viral proteins (structural and non-structural) needed to assemble productive vector particles. Genetic information encoding the above viral proteins could be provided by several mechanisms including inadvertent transferring of all or parts of the packaging cassettes to target cells, infection of target cells with par-
ental wild type viruses, expression of endogenous retroviral genomes, or by a combination of these mechanisms. Human endogenous retroviruses (HERV), which invaded the human genome via germ cell infection in the last 2–40 million years, make ~8% of the human genome [51].
Various genetic and epigenetic inactivation mechanisms rendered HERV noninfectious. However, human cell lines and tissues produce HERV proteins and noninfectious viral particles [52–55]. Furthermore, infectious HERV viruses have been successfully reconstituted from human genomes [56, 57]. The HERV-K envelope proteins, syncytin-1 and -2 are involved in human placenta formation and can pseudotype lentiviral vectors [52, 58]. Thus, the receptors to syncytin-1 and -2 envelope proteins, which are synthesized in various human tissues (including germ cells), can potentially broaden biodistribution of syncytin-pseudotyped vectors [59–62].

In contrast to AAV vector-based therapies [63–65], the relatively small number of individuals that currently are or anticipated to be infected with HIV-1 reduces the likelihood of lentiviral vector mobilization. However, the necessity to avoid vector mobilization even in the presence of all HIV-1 proteins was the impetus to the development of SIN vectors,
in which deletions of U3 sequences comprising the parental viral enhancer promoter inhibit transcription of full-length vector mRNA [5, 7, 66]. Further characterization of SIN vector mobilization demonstrated reduced yet not completely abolished mobilization from integrated vector genomes. Vector mobilization in these studies was consequent upon cryptic transcription initiation of full-length vector mRNA, which was controlled by different transcription regulatory elements within the vector and in the host genome (determined by vector integration sites) [8, 9]. The fact that most of the cryptic transcription initiation sites reported by Hanawa et al. [8] were downstream to the 5' R could not produce full-length packagable vector mRNA was in line with the relatively low titers (up to \(4 \times 10^3\) TU/ml) of SIN vectors mobilized from integrated vector genomes. Per contra, we show here that tilters of SIN vectors mobilized from episomal vector genomes (Figs. 1b and 6b) are not significantly lower than the tilters of their non-SIN counterparts. In theory, minimal if any host-genome effect on transcription of nonintegrating vectors should render episomal SIN vector less mobilized than integrating vectors.

However, vector circularization potentially positions various internal transcriptional regulatory elements upstream to the vector's single or double LTRs. We assert that this circularization event creates a new Tat independent promoter/enhancer constellation that renders the enhancer/promoter deletions of the SIN LTR ineffective at preventing transcription of full-length vector mRNA. This notion is in line with an earlier study by Poon et al. [67] showing Tat independent transcription from episomal HIV-1 genomes. The relatively high rate of vector mobilization from episomal vector genomes is in line with earlier studies describing significant differences in regulation of transgene expression from episomal and integrated lentiviral vector genomes [15, 28]. Epstein lentiviral vectors comprise the majority of reverse transcription products [15, 22, 68] and thus serve as an efficient template for transcription of mobilizable, full-length mRNAs. In dividing cells, episomal vector genomes lacking origin of replication are rapidly lost by dilution [68]. Consequently, ex-vivo gene therapy protocols including gene delivery to stem cells and genetic engineering of T-cells carry lower risk of episomal vector mobilization [1, 40]. Per contra, episomal vector genomes are highly stable in non-dividing cells; thus, in vivo administration of SIN lentiviral vector should raise biosafety concerns regarding potential vector mobilization [4].

The development of the IDLV system successfully addressed the risk of insertional mutagenesis by reducing integration of vector genomes to less than 1% [24, 69]. However, the risk of SIN vector mobilization is probably more significant following in vivo administration of IDLVS, considering more than 99% of their reverse-transcribed genomes are episomes. Recently, 3 PPT-deleted vectors have been developed as a means to minimize illegitimate, integrase-independent integration of IDLV's [24]. The newly developed vectors have been successfully employed in immunotherapy clinical trials [4]. 3 PPT-deleted vectors generate mainly single-LTR circles, which are less likely than linear double-stranded vector genomes to recombine with the host-genome, but are predicted to efficiently support vector mobilization.

To address the above biosafety concerns, we demonstrate here that positioning internal lentiviral vectors’ expression cassettes in opposite orientation to the LTRs, practically abolishes SIN vector mobilization. Premised on the fundamental differences in transcriptional regulation of gene expression from episomal and integrated vector genomes [15, 67], we speculate that ECOO-associated reduction in vector mobilization from episomal and integrated vector genomes is mediated by different mechanisms. The relatively high levels of transgene expression from ECOO-comprising IDLVS suggests that the negative effect of ECOO on episomal vector mobilization is probably secondary to silencing of cryptic transcription initiation sites in episomal vector’s SIN LTR and is not mediated by the host PKR response. Interestingly, three independent mechanisms reduce mobilization of integrated vectors. These include PKR-dependent and independent mechanisms that reduce mobilization of integrated non-SIN ECOO-comprising vectors and the SIN-LTR configuration that reduces mobilization of all integrated retroviral vectors. Consequently, ECOO-comprising vectors demonstrate the lowest level of mobilization, which renders them most suitable for a biosafety level-0 (BSL-0, the community), in which patients carrying large numbers of viral vectors genomes can potentially mobilize viral vectors and their derivatives to the community. Several research groups successfully employed ECOO-comprising vectors in hematopoietic stem cells (HSC)-based gene replacement therapy for different hemoglobinopathies [13, 31–33, 46, 70–73]. To maintain long-term therapeutic level of transgene expression, most of the disease-tailored vectors carried human globin expression cassettes comprising transcriptional and post-transcriptional regulatory elements of the human β-globin gene including its’ promoter, mini-LCR, introns and poly-A site. However, difficulties in generating high vector titers limit the usage of these efficacious vectors in clinical trials [13, 32, 33]. It is likely that the relatively large genetic payload of the above vectors negatively affect their titers. Since the parental globin promoter is not active in 293T cells, we observed merely a mild increase of ~2 fold in titers of the CCL-β AS3-FB vector in PKR-deficient cells as compared with vector titers generated by naive 293T cells. A similar increase in titers was observed for a conventional pTK945 vector. In theory, these findings could be associated with low level of antisense mRNAs, which
could be transcribed from weak promoters embedded in the parental HIV-1 sequence and in the vector cassette and stabilized by the internal poly-A signal [74–77].

Positioning the WPRE between the transgene of interest and the internal poly-A sequence significantly increased transgene expression from ECOO-comprising vectors without negatively affecting their titers. Unexpectedly, gene expression levels from ECOO-comprising vectors were comparable or higher than the levels of expression from their conventional counterparts. Interestingly, the molecular mechanism involved in this phenomenon, which was more distinct in IDLV’s has not been elucidated in this study. However, it is possible that positioning the internal promoter in opposite orientation to the LTR distanced it from negative regulatory elements in the vector’s 5′ untranslated region. This phenomenon is a significant advantage of ECOO-comprising vectors. Furthermore, in contrast to conventional vectors, ECOO-comprising vectors should facilitate delivery of intron-containing genes, which significantly increase transgene expression [78–81]. Overall, the data presented here outline an efficient methodology of producing high-titer ECOO-comprising vectors. The newly developed vector system is safer, more efficient at maintaining high levels of transgene expression and thussuites better than currently used lentiviral vectors for clinical trials.

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Compliance with ethical standards

Conflict of interest TK is an inventor of a PPT-deleted vector-based technology owned by the University of North Carolina and licensed to a commercial entity. The other authors declare that they have no conflict of interest.

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