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Rapid establishment of stable retroviral packaging cells and recombinant susceptible target cell lines employing novel transposon vectors derived from *Sleeping Beauty*

Karen Berg[a,b], Vanessa Nicole Schäfer[a], Natalie Bartnicki[a], Reto Eggenschwiler[b], Tobias Cantz[b], Jörn Stitz[a,*]

[a] Research Group Pharmaceutical Biotechnology, Faculty of Applied Natural Sciences, STEPs Institute, TH Köln – University of Applied Sciences, Chempark Leverkusen E28, Kaiser-Wilhelm-Allee, 51368 Leverkusen, Germany

[b] Research Group Translational Hepatology and Stem Cell Biology, Cluster of Excellence REBIRTH, Department of Gastroenterology, Hepatology, and Endocrinology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

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ABSTRACT

Viral vector particles derived from murine leukemia virus (MLV) mediate highly efficient stable gene transfer used in gene therapeutic approaches and in the generation of transgenic cell lines. However, the establishment of stable viral packaging cells (VPCs) is a time-consuming challenge. To overcome this limitation, we successfully generated novel *Sleeping Beauty*-derived transposon vectors entailing envelope and packaging expression cassettes as well as a transfer vector. Upon multiplexed transposition in human cells, VPC bulk populations yielding titers of over 1×10^6 transduction-competent vectors were established within three weeks. In contrast, conventional plasmid-based establishment of VPCs, conducted in parallel, took much longer and yielded significantly lower vector productivity and vector fitness. The generated MLV vectors decorated with the envelope proteins of ecotropic MLV PVC-211mc mediated efficient transduction of Chinese hamster ovary (CHO) cells. Cell susceptibility was further elevated upon recombinant expression of the murine ecotropic receptor mCAT employing a transposon vector.

1. Introduction

Viral vectors derived from a variety of different members of the retrovirus family such as murine leukemia virus (MLV), human and simian immunodeficiency virus (HIV and SIV) mediate highly efficient stable gene transduction and insertion into the target cell genome and subsequent sustainable expression in gene therapeutic approaches (for review see Lundstrom, 2018). The host cell tropism can be altered by pseudotyping - i.e. the employment of envelope proteins of heterologous parental donor viruses. The host cell range is defined by the surface-expression of the respective cognate viral receptor recruited to allow for cell-entry upon engagement with the envelope displayed on the vector particle (Schnierle et al., 1997; Stitz et al., 2000a,2000b; King and Daly, 2014). Besides their utility in gene therapy, viral vectors of retroviral origin are also valuable tools facilitating stable modification of the cellular geno-, and thus phenotype, e.g. in cell differentiation and development studies (Koch et al., 2006) and the rapid establishment of recombinant mammalian cells for the high-yield production of biologics following multiple rounds of transduction resulting in multiple integration and high expression levels of the gene of interest encoding the therapeutic protein (Oberbek et al., 2011; Stitz, 2011).

Chinese hamster ovary (CHO) cells are the gold standard in industrial manufacture of biologics. To transduce these cells viral vectors are most frequently pseudotyped with glycoproteins of vesicular stomatitis virus (VSV-G; Oberbek et al., 2011). The resulting MLV(VSV-G) pseudotype vector particles are pantropic as the cognate LDL-receptor is expressed ubiquitously on a wide array of cell-types of different donor organisms, which requires the experimental work being conducted under biosafety level 2 (BSL-2) conditions in most countries (Mosier, 2004; Koch et al., 2006; Finkelshtein et al., 2013). Widely used ecotropic vectors derived from Moloney MLV (MoMLV) – revealing a host cell tropism restricted to rodent cells and consequently being produced under biosafety level 1 (BSL-1) conditions - fail to mediate efficient transduction in CHO cells as they apparently insufficiently recruit the hamster homologue of the murine ecotropic receptor (murine cationic aminoacid transporter; mCAT; Albritton et al., 1989) possibly due to its

* Corresponding author.
E-mail address: joern.stitz@th-koeln.de (J. Stitz).

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variant glycosylation (Eiden et al., 1994). An exception to this is the molecular clone PVC-211 of Friend MLV harboring two characteristic amino acid residue deviations in the receptor-binding domain of the envelope surface protein gp71-SU, distinguishing it from the MoMLV envelope ((Env); Remington et al., 1992; Masuda et al., 1996, 1997; Jinno-Oue et al., 2001; Stitz, 2011). We thus intended to generate stable MLV(PVC-211) producing packaging cells based on human fibro-sarcoma HT-1080 cells known to enable high titer yielding vector preparations (Cosset et al., 1995).

However, the establishment of high-titer stable viral packaging cell lines (VPCs) is very time consuming as cells have to be serially transfected and selected for the expression of the plasmid-based vector constructs. The packaging construct encodes the structural viral proteins Gag/Pol, which are required to assemble and mature the viral core upon processing of the Gag precursor protein to matrix, capsid (p30-CA) and nucleocapsid protein mediated by the viral protease. The protease as well as all other viral enzymes (reverse transcriptase (RT), integrase, RNAse) originate from the Pol precursor protein. The envelope construct facilitates expression of the Env proteins gp71-SU and the transmembrane protein p15-TM, which renders fusogenicity upon proteolytic cleavage of the cytoplasmatically located p2-R peptide. Trimers of the resultant mature heterodimer of gp71-SU and p15-TMΔR mediate cell-entry via receptor recruitment. The transfer vector encompasses minimally the packaging signal (Ψ) and the flanking long terminal repeats (LTRs). The viral promoter is located in the 5′-LTR whereas the 3′-LTR harbors the polyadenylation signal (p(A)). Additional sequences e.g. reporter genes or further promoters can be inserted downstream of Ψ (Miller, 1990).

Transposon vector systems have been reported to enable efficient stable gene transfer into human cells (Grabundzija et al., 2010). To drastically shorten the time-investment needed for the establishment of stable VPCs we thus generated three transposon vectors derived from *Sleeping Beauty* (SB) encompassing expression cassettes for Env, Gag/Pol and the transfer vector, respectively. The transposon vectors are characterized by inverted terminal repeats (ITRs) of the Tc-mariner transposon family found in the genome of salmonid fish. Upon co-transfection of a transposase construct, the transposase cuts out the ITR-flanked retroviral vector component expression cassettes from the plasmid backbone and inserts them into the host cell genome at the target sequence TA. The transposase of *Sleeping Beauty* has been optimized for its activity by molecular evolution (SB100x; Mátés et al., 2009). SB100x was demonstrated to integrate multiple copies of transposon vectors into host cell genomes (Izsvák et al., 2009; Grabundzija et al., 2010). Consequently, we sought to establish VPCs by multiplex transfection of HT-1080 cells employing our novel transposon vectors and a SB100x transposase construct followed by subsequent stringent selection. We compared this strategy with classical serial transfection of conventional plasmid-based vector constructs. In addition, we aimed at the generation of susceptible target cells upon stable recombinant overexpression of the ecotropic receptor mCAT using transposon vectors. Such target cells could be used for the establishment of highly efficient producer cell lines for biologics upon retroviral vector-mediated gene transduction.

2. Materials and methods

2.1. Cells

Human fibro-sarcoma HT-1080 cells (ATCC CCL-121), murine fibroblast NIH-3T3 cells (ATCC CRL-1658), CHO-K1 cells (ATCC CCL-61) and their recombinant derivatives were all grown in high glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-Gln and 10% FBS (Gibco BRL, Eggenstein, Germany). Cells were cultured at 37 °C in an humidified atmosphere at 5% CO_2. For passaging, cells were detached using 1 mM EDTA in PBS.

2.2. Construction of expression and transposon vectors

In all constructs used in this study a CMV promoter/enhancer element (P_{CMV}) drove the expression of the respective transgenes. A synthetic intron (In), an internal ribosomal entry side (IRES), antibiotic-resistance genes and a woodchuck hepatitis posttranscriptional regulatory element (WPRE; nucleotides 1093 – 1684; GenBank accession no. J04514; Donello et al., 1998; Zufferey et al., 1999) and the polyadenylation signal (p(A)) of the bovine growth hormone gene (bgh) completed the expression cassettes. With the exception of WPRE, all other genetic elements were provided by the parental vectors pIRESpuro3 and pIRESyhg3 (Clontech, USA). The WPRE was inserted in the *XbaI*-site just upstream of p(A). The retroviral transfer vector constructs were derived from LEGFP-N1 (Clontech, USA). The flanking ITRs within the respective transposon vectors were synthesized (GenScript; NY, USA) originating from the Tc-like element (GenBank accession no. L48685) found in *Tanichthys albonubes* as described by Ivics et al. (1997) flanking a P_{CMV}, a multiple cloning site and a bgh-p(A) framed by *FseI*-restricition motifs. Both ITRs were modified to enhance transposition efficiency by the addition of outer flanking TA dinucleotide as previously reported by Cui et al. (2002). In addition, a mutation of A to T at in the right inner direct repeat (DRri) was introduced to reconstitute the consensus sequence TTT found in other Tc-elements of salmonid fish within the minimal core binding-site of the SB transposase required for efficient transposition. The MLV *gag/pol* genes were amplified from the template pKA1558 (GenBank accession no. JO2255; Skou & Anderson 1993) using the oligonucleotides 5'-TTTAAA GAAT TCACCATGGGCCAGACTGTTACCACTCCTG-3 ´and 5′-TTTAAAGC GGCCGCTTAGGGGGCCTCGCGGGTTAAC-3`. The amplicon was inserted into the vectors IpW and SB-IpW, respectively, previously opened using *EcoRV* to generate the packaging constructs. The human codon-optimized *env* gene of PVC-211mc (GenBank accession no. AAA46478.1) was obtained by gene synthesis (GenScript, NY, USA) flanked by the restriction side motifs of *EcoR*V and *BstX*I and inserted accordingly into IhW and SB-IhW, respectively, to obtain the envelope constructs. SB-LEGFP-N1 encompassing the entire transfer vector LEGFP-N1 was generated employing PCR using 5´- and 3`LTR-matching primers encompassing the restriction side motifs for *FseI* (5′-AAAAGGCCGGCCG CTTTT GAAAGACCCCACCCGTAGGTGGCAAG-3′; 5′-AAAAGGCC GGC CCCCAAATGAAAGACCCCCGCTGACGGGTAG −3′). The resultant DNA fragment was inserted into a transposon vector using *FseI*-mediated restriction. The SB-mCATIpW construct was generated by insertion of the synthesized human codon-optimized *mCAT* gene (GenBank accession no. NM 007513) flanked with restriction motifs of *EcoRV* and *BstXI* into SB-IpW. The construct CMV-SB100x was generated by synthesis of P_{CMV}, the human codon-optimized sequence of SB100x transposase (Mátés et. al, 2009) and bgh-p(A) and inserted into pUC57. Cloning strategy details as well as the novel vectors are available upon request.

2.3. Establishment of VPCs and recombinant target cells

For stable VPCs generation HT-1080 cells were transfected using the TransIT-LT1 transfection reagent (Mirus, USA) according to the manufactures instructions. One day prior to transfection, 1.5×10^5 cells were seeded per well in 2 mL using six-well plates (Nunc, Wiesbaden, Germany). To establish SB-VPC cells were co-transfected with the viral vector component harboring plasmids (0.75 µg SB-gpIpW; 0.75 µg SB-eIhW and 1.5 µg SB-LEGFP-N1) and 0.3 µg of the transposase construct CMV-SB100x. Two days post transfection, cells were transferred to a 10 cm culture dish and subjected to selection on the following day. Initially, concentrations of 0.5 µg/mL of puromycin, 50 µg/mL of hygromycin and 100 µg/mL of G418 (all InvivoGen, Toulouse, France) were used and escalated with each passage to reach the final concentrations reported in results. For the establishment of PL-VPC HT-1080 cells were transfected with 1 µg of each plasmid but serially with individual selection for each construct. First, gpIpW was transfected and selection was performed using escalating puromycin concentration until cells could not survive higher selection pressure anymore. In the next step, eIhW was transfected into the puromycin resistant cells followed by selection in the presence of the maximal puromycin concentration and escalating hygromycin concentrations. Lastly, LEGFP-N1 was transfected and selected using maximal concentrations of puromycin and hygromycin and increasing concentrations of G418.

To establish HT-1080 and CHO-K1 cells recombinantly expressing the ecotropic receptor mCAT cells were seeded as described above and subsequently co-transfected with 2.25 µg SB-mCATIpW and 0.25 µg CMV-SB100x. Three days post transfection, cells were subjected to selection using 1 and 10 µg/mL of puromycin, respectively. Resistant cells were obtained after four days of selection and survived passaging in the presence of the respective antibiotic concentrations.

2.4. Viral vector titration and gene transfer analysis

To assess the viral vector titers produced by PL- and SB-VPCs 1.5×10^5 NIH-3T3 target cells per well were seeded in six-well dishes (Nunc, Wiesbaden, Germany). In parallel, confluent VPC populations in T75 flasks were overlaid with 6 mL of fresh medium without antibiotics and incubated for 8 h. Supernatants harvested from VPCs were passaged through a 0.45 µm filter to remove contaminating cells. Different dilutions of supernatants in total volumes of 1 mL were subjected to transduction of target cells after removal of the medium used for their seeding. One day post transduction, 1 mL of fresh medium was added to transduced cells. Three days post transduction, cells were detached and analyzed employing flow cytometry (S3e, Bio Rad) to determine the percentage of EGFP-positive cells. Vector titers were calculated as described previously (Salmon and Trono, 2006) using supernatant dilutions, which yielded transduction efficiencies between 1.0% and 10.0% EGFP-positive cells.

2.5. Western blot-analysis, reverse transcriptase (RT)-assay and microscopy

Cell-free vector particle-containing supernatants were harvested from VPCs as described and subjected to ultracentrifugation at 4 °C and 25,000 rpm for 1.5 h using a SW28 swing-out rotor and a Optima XE centrifuge (Beckman Coulter; IN, USA). Pellets were re-suspended in equal volumes of Laemmli-Buffer containing 50 mM Dithiothreitol (DTT) and incubated for 10 min at 95 °C. Equal volumes of the lysed vector pellets were subjected to SDS-PAGE using a 15% acrylamide gel and blotted on a PVDF membrane (Roth, Germany). TBS-buffer supplemented with 0.05% tween 20% and 2% milk powder was used for blocking. Polyclonal goat antibodies specific for MLV p30-CA (ATCC VR-1564AS-Gt) were used at a dilution of 1:2000. To detect p15-TM monoclonal antibodies of hybridoma clone 42-114 (Merck Millipore, Darmstadt, Germany) were used at a dilution of 1:500. Horse relish peroxidase (HRP)-labeled secondary antibodies against goat-IgG (Abcam, Cambridge, UK) and HRP-Protein G (Invitrogen, CA, USA) diluted 1:10,000 and 1:2000, respectively, were employed for protein detection. Substrate ECLplus (Thermo Scientific, Bremen, Germany) and a ChemiDoc XRS + imager (BioRad, Hercules, USA) enabled luminescence signal visualization.

Activity of viral reverse transcriptase (RT) present in vector particles was detected using cell-free supernatants at different dilutions of both VPCs and HT-1080 cells serving as negative controls. Samples were prepared as described above employing the C-type RT Activity Kit (Cavidi, Uppsala, Sweden) according to the manufacture´s instructions. RT-activities were examined using a Multiskan FC mikrotiterplate-photometer (Thermo Scientific).

An Axio Vert. A1 (Zeiss, Jena, Germany) was used for all microscopic analysis described here.

2.6. Rescue assay for the detection of replication-competent retroviruses (RCRs)

To ensure that viral vector preparations did not contain contaminating RCRs potentially originating from recombinations of vector component transcripts rescue assays were conducted. Susceptible NIH-3T3 target cells previously transduced with the transfer vector LEGFP-N1 (Clontech, USA) and subsequently selected using 1000 µg/mL G418 were seeded at 50% confluency in T75 flasks and exposed to undiluted supernatants of VPCs and, in parallel, co-cultivated with VPCs. Upon expansion of cells for at least five days, supernatants were harvested and contaminating cells were removed by passage through a 0.45 µm filter. Naïve NIH-3T3 cells were exposed to these supernatant preparations and expanded for at least three days. Cells were then examined using flow cytometry and fluorescence microscopy to detect EGFP-expressing cells resulting from the mobilization of the transfer vector by potentially generated RCRs.

2.7. RNA-isolation from vector particles, RT-PCR and semi-quantitative PCR

To examine the amount of packaged transfer vector mRNA cell-free supernatants of naïve HT-1080 cells, serving as negative controls, and both VPCs were harvested and subjected to ultracentrifugation as described above. Vector particle pellets were suspended in 350 µL TRIzol Reagent (Thermo Scientific) and mRNA was isolated according to the manufacturer's instructions yielding samples of RNAse-free solutions with a volume of 10 µL. To eliminate potential DNA contamination 8 µL of the mRNA samples were subjected to DNAse I-treatment (Invitrogen) following the instructions of the manufacturer. RT-PCR was performed using the entire amount of isolated mRNA employing 1 µL of 50 µM random hexamer primers and 1 µL of 10 mM dNTPs (Invitrogen). After a five minute incubation at 65 °C followed by storage on ice first-strand cDNA synthesis was initiated by the addition of SuperScript III reverse transcriptase (200 units) and incubation at 25 °C for five minutes, 50 °C for 60 min and stopped by exposure to 70 °C for 15 min. Samples were stored at −20 °C.

Different amounts of volumes (0.3 µL; 0.6 µL; 1.2 µL; 2.5 µL) of thawed cDNA samples served as templates for a semi-quantitative PCR. Oligonucleotides specifically binding to the 5′- and 3′-ends of the *egfp* reporter gene (GFP_sqPCR_for: 5′-ATGGTGAGCAAGGGCGAGGAGCTG TTC-3′; GFP_sqPCR_rev: 5′-GTCTTTGCTCAGGGCGGACTGGGTGC-3′) and the DreamTaq (NEB) in a reaction mixture supplemented with 5% DMSO were instrumental in the amplification of DNA-fragments of 633 bp. In the first step samples were heated up to 95 °C for three minutes. The twenty cycles of 95 °C for 30 s, 68 °C for 15 s and 72 °C for two minutes were followed by a final extension at 72 °C for 10 min.

2.8. Assessment of vector copy numbers (VCN) integrated into cellular genomic DNA

The average vector copy numbers (VCN) of the packaging construct, the envelope construct and the EGFP encoding transfer vector in PL-VPC and SB-VPC bulk populations were analyzed using qPCR as described before (Maetzig et al., 2010). Genomic DNA was isolated using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. Custom TaqMan assays (Thermo Scientific) were designed detecting *puro*^{R} (Assay-ID: AR9HJ4N) in the packaging construct, *hyg*^{R} (Assay-ID: AR7DRJR) in the envelope construct and *egfp* (Assay-ID: AII1MR7) in the transfer vector construct. A primer/probe set detecting the endogenous genomic locus of the PTBP2 gene was used as an internal reference (PTBP2 for: 5′-TCTCCATTCCCTATGTTCATGC-3′; PTBP2 rev: 5′-GTTCCCGCAGAATGGTGAGGTG-3′; PTBP2 probe: JOE-ATGTTCCTCGGACCAACTTG-BHQ1; Eggenschwiler et al., 2016). 10-fold serial dilutions with known concentrations of plasmids containing *puro*^{R}, *hyg*^{R}, *egfp* or PTBP2 sequences were used for standardization. The

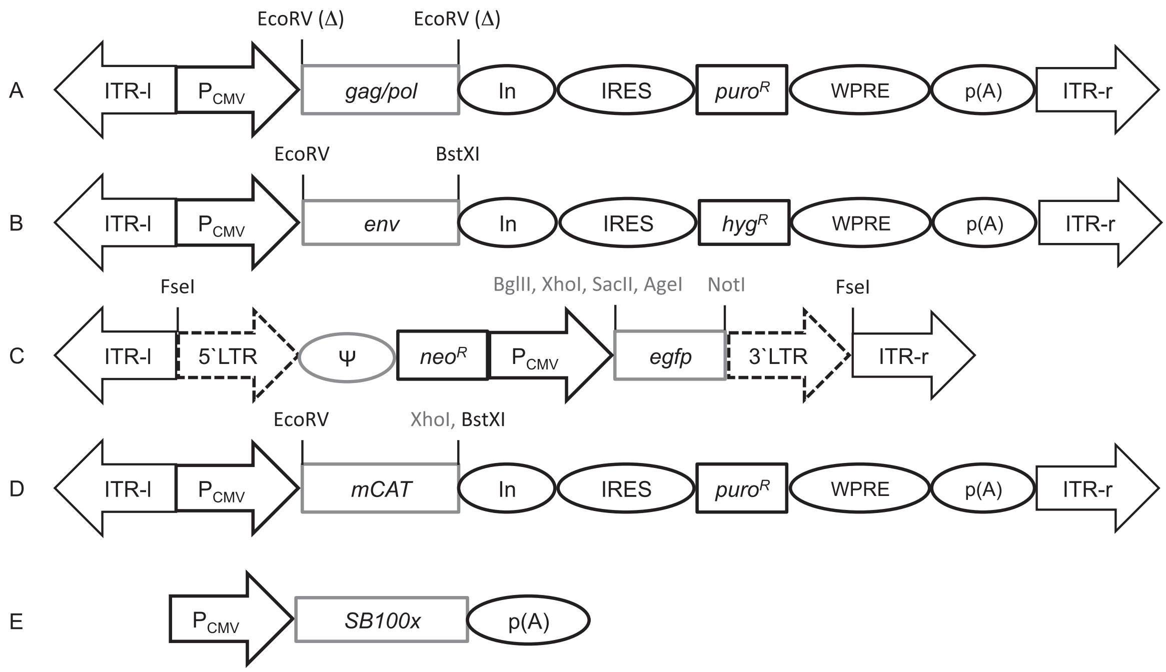


Fig. 1. Schematic illustration of the genetic elements in transposon vectors and the transposase construct. Insertion sites of the transgenes or expression cassettes into transposon vectors are shown in black (Δ indicates that the sites are not recleavable). Other single cutter sites are shown in grey. (A) In the packaging construct SB-gpIpW, a CMV promoter/enhancer element (P_{CMV}) drives expression of MLV genes *gag/pol* followed by an synthetic intron (In), an internal ribosome entry signal (IRES), a puromycin-resistance gene (*puro*^{R}), the Woodchuck hepatitis virus post transcriptional element (WPRE) and the polyadenylation signal (p(A)) of the bovine growth hormone gene. The inverted terminal repeats (ITRs) flank the expression cassette at the 5`-end (left, ITR-l) and 3′-end (right, ITR-r). (B) The envelope construct SB-eIhW encodes for the human codon-optimized ecotropic envelope gene *env* derived from the Friend MLV molecular clone PVC-211 and the hygromycin-resistance gene (*hyg*$^R$). (C) SB-LEGFP-N1 entails the 5`- and 3′-LTRs of MLV and the respective packaging signal Ψ. The MLV promoter in the 5′-LTR drives the expression of the neomycin-resistance gene (*neo*^{R}) and the transcription of the full-length transfer vector. In addition, P_{CMV} mediates the expression of the reporter gene *egfp*. (D) SB-mCATIpW encompasses the human codon-optimized coding sequence of the ecotropic receptor coupled to the expression of *puro*^{R}. (E) The transposase construct CMV-SB100x harbors the human codon-optimized sequence encoding the hyper-active *Sleeping Beauty* transposase variant SB100x.

StepOnePlus Real-Time PCR System (Thermo Scientific) was employed to conduct qPCR. Further experimental details are available upon request.

3. Results

3.1. Multiplexed transposition of Sleeping Beauty transposon vectors encoding the vector components facilitates the rapid establishment of high vector titer producing VPCs

To establish stable VPCs, human HT-1080 cells were co-transfected with the respective *Sleeping Beauty*-derived vector constructs: the packaging construct SB-gpIpW, the envelope construct SB-eIhW, the transfer vector SB-LEGFP-N1 and the transposase construct CMV-SB100x as illustrated in Fig. 1 A–C and E. Three days post transfection, cells were subjected to the selection process by expansion in the presence of three antibiotics, namely puromycin, hygromycin and G418. After only three weeks under escalating selection pressure a bulk VPC population was established – named SB-VPC - surviving cultivation in the presence of maximal concentrations of all three antibiotics (5 µg/mL puromycin, 500 µg/mL hygromycin; 800 µg/mL G418).

In parallel, cells were triple co-transfected with plasmids harboring the exact same expression cassettes (gpIpW; eIhW; LEGFP-N1) representing the conventional approach for VPC establishment using stable transfection. As expected, no cell survival was observed conducting triple-selection in the presence of all three antibiotics. Consequently, cells were again transfected but this time only with the packaging construct gpIpW. After four weeks of slowly escalating the puromycin concentration utilized for selection, resistant cells growing in the presence of 3 µg/mL puromycin were established and subsequently transfected with the envelope construct eIhW followed by increasingly stringent selection with hygromycin. Cells were obtained after again four weeks growing in the presence of 3 µg/mL puromycin and 300 µg/mL hygromycin. These cells were then transfected with the transfer vector LEGFP-N1 and again carefully selected to finally survive a maximum selection pressure of 3 µg/mL puromycin, 300 µg/mL hygromycin and 400 µg/mL G418. After three months the resultant VPC bulk population was established and named PL-VPC.

Both VPCs were expanded under the highest applicable selection pressure. To examine VPC productivity cell-free vector-containing supernatants were harvested and titrated using NIH-3T3 target cells as described in methods. Three days post transduction, cells were analyzed using fluorescence microscopy and flow cytometry to detect successfully transduced EGFP-positive cells. As shown in Table 1, using three titration experiments, SB-VPC was demonstrated to advance PL-VPC yield of transduction-competent vector particles by more than 20- and

Table 1
Transduction-competent vector particles harvested from viral packaging cells established by serial stable transfection of plasmid DNA-based constructs (PL-VPC) and by multiplexed transposition of *Sleeping Beauty*-derived transposon vectors (SB-VPC), respectively. MLV vectors were titrated on NIH-3T3 target cells as indicated. Titers are reported as transducing units per mL (TU/mL). Experiments 1, 2 (both n = 1) and 3 (n = 3) were conducted independently. For experiment 3 mean values from titrations performed in triplicate are shown together with the respective standard deviations (SD).

VPC	Titer [TU/mL]		
	Exp. 1	Exp. 2	Exp. 3 (Mean ± SD)
PL-VPC	5.1×10^4	6.9×10^4	$2.2 \times 10^4 \pm 0.20 \times 10^4$
SB-VPC	1.2×10^6	2.3×10^6	$1.1 \times 10^6 \pm 0.28 \times 10^6$

even 30-fold, reaching 1.1×10^6, 1.2×10^6 and 2.3×10^6 transducing units per mL (TU/mL) as compared to 2.2×10^4, 5.1×10^4 and 6.9×10^4 TU/mL, respectively. The third titration experiment was conducted in triplicate to assess the reliability of the data. A standard deviation of 9–26% demonstrated sufficient precision.

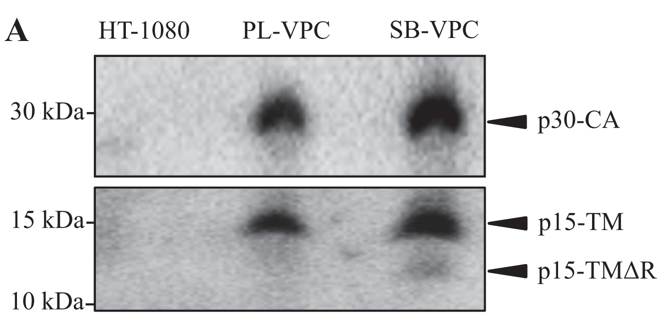
In order to excluded the possibility that the gene transfer efficiencies observed with vector harvests from both VPCs were, at least partially, due to the unintended generation of replication-competent retroviruses (RCRs) - as a result of recombination events between the transfer vector, the packaging and the envelope construct - we performed a rescue assay as described in materials and methods and previously reported by Cosset et al. (1995). Briefly, we did not detect mobilization of the transfer vector and thus, no generation of RCRs (data not shown). Consequently, the above-described gene transfer efficiencies were exclusively a result of replication-incompetent viral vector particle-mediated gene transductions.

3.2. Higher productivity of transduction-competent vector particles of SB-VPC results from elevated particle count and enhanced vector fitness

Cell-free vector-containing supernatants of confluent VPCs and naïve HT-1080 cells serving as negative controls were in parallel subjected to ultracentrifugation and resultant pellets were examined for viral protein quantities using Western blot-analysis and antibodies specific for the core protein p30-CA and the envelope protein p15-TM. As depicted in Fig. 2 A, respective viral proteins were readily detected in samples of both VPCs but not in negative controls. Vector pellets generated from SB-VPC supernatants revealed visibly higher amounts of p30-CA protein as compared to samples from PL-VPC. This demonstrated that more vector particles were produced by SB-VPC. Immature p15-TM was readily detected in particles of both VPCs. Noteworthy, the R-peptide-deficient and thus, mature and fusogenic form p15-TMΔR with an apparent molecular weight of under 15 kDa was only detectable in particles harvested from SB-VPC. This could also further partially explain the higher vector titers obtained from SB-VPC. As shown in Fig. 2 B, an RT-assay performed on vector particle pellets revealed approximately two-fold higher amounts of the enzyme for SB-VPC (216 mU/mL) as compared to PL-VPC (102 mU/mL). This further substantiated the advanced productivity of SB-VPC.

However, we deemed it unlikely that the discriminative vector titers of 20- to 30-fold between both VPCs was sufficiently explainable by the observed differences in vector particle numbers and potentially more efficient maturation of the envelope proteins. We thus analyzed both VPCs - and again naïve HT-1080 cells serving as a negative control – using fluorescence microscopy to assess the expression levels of the transfer vectors encoding the reporter gene *egfp*. As visualized in Fig. 3 A, EGFP-positive cells were readily detected in both VPCs, while naïve HT-1080 cells did not reveal any fluorescence. The SB-VPC bulk population was demonstrated to express much higher levels of the reporter gene product as compared to PL-VPC. Analysis of EGFP-expression employing flow cytometry confirmed an over three-fold elevated median fluorescence intensity (MFI) of SB-VPC (225,370) as compared to PL-VPC (68,860; data not shown). This suggested also a much higher amount and abundance of transfer vector transcripts in SB-VPC available for packaging into vector particles rendering them transduction-competent. To examine this, supernatants were harvested from both VPCs and parental HT-1080 cells serving as negative controls and subjected to ultracentrifugation. RNA was isolated from pelleted vector particles and served as templates for RT-PCR yielding cDNA samples of 20 µL total volume. As shown in Fig. 3 B, different amounts of cDNA templates were employed to perform a semi-quantitative PCR amplifying the reporter gene *egfp* encoded on the transfer vector. No *egfp*-amplicons were detected using samples of negative control HT-1080 cells. As expected SB-VPC derived vector particles were demonstrated to have packaged considerably higher amounts of transfer vector transcripts as compared to PL-VPC. Densitometric analysis was used to quantify amounts of amplified DNA-fragments. The maximum signal intensity (sample 2.5 µL of SB-VPC) was set as a relative value equal 100%. The relative intensity for the other reactions with decreasing cDNA template amounts reached values of 98, 73 and 46 for SB-VPC and 49, 30, 14, 5 for PL-VPC. Thus, the values of 46 for 0.3 µL cDNA of SB-VPC samples and alike 49 for 2.5 µL of PL-VPC indicated an about 8-fold higher amount of transfer vector mRNA in SB-VPC produced vector particles.

We consequently assumed that the higher amount of particles produced by SB-VPC, the potentially elevated particle decoration with fusogenic p15-TMΔR as well as the more efficient packaging of the transfer vector transcripts contributed to the much higher vector titers as a result of advanced vector fitness.



B **RT [mU/mL]** (Mean +/- SD)

HT-1080	PL-VPC	SB-VPC
0	103 +/- 0.010	216 +/- 0.007

Fig. 2. Detection of viral proteins in vector particles. (A) Supernatants harvested from both VPCs, and naïve HT-1080 cells serving as negative controls, were filtered and ultracentrifuged to pellet viral particles. Western blot-analysis of viral pellets was performed using polyclonal antibodies directed against MLV p30-CA (upper panel) and monoclonal antibodies specific for MLV p15-TM (lower panel). The positions of the molecular weight markers are depicted on the left. (B) An reverse transcriptase (RT)-assay was performed in triplicate employing pellets upon ultracentrifugation of supernatants of naïve HT-1080, PL- and SB-VPC cells. RT-activity determined for the samples of HT-1080 cells serving as negative controls were set to a value of null and were subtracted as a background signal from the other RT-amounts shown in milliunit (mU). Standard deviations (SD) are indicated.

3.3. Superior productivity of SB-VPC results from higher vector copy number (VCN) per cell

We assumed that the advanced productivity of SB-VPC was a result of higher vector copy numbers per cell upon efficient integration mediated by transposition for each individual construct. To examine this, genomic DNA of both, SB- and PL-VPCs was isolated and subjected to qPCR using primer pairs and probes specific for the $puro^R$, hyg^R and *egfp* present in the packaging, envelope and transfer vector, respectively. The amplification of the cellular gene PTBP2 served as an internal reference. As illustrated in Fig. 4, SB-VPC showed visibly higher VCN as compared to PL-VPC. Namely, SB-VPC and PL-VPC revealed VCN values of 6.9 and 4.3 for the packaging construct, 2.1 and 1.3 for the envelope construct and 8.2 and 1.6 for the transfer vector, respectively. In summary, the VCN values ranged from approximately 1.5-fold for packaging and envelope construct and about 5-fold for the transfer vector. This mirrored the previous findings showing higher gene expression levels of SB-VPC as observed in higher productivity for Gag/Pol and Env proteins and transfer vector transcripts assembling to transduction-competent retroviral vector particles.

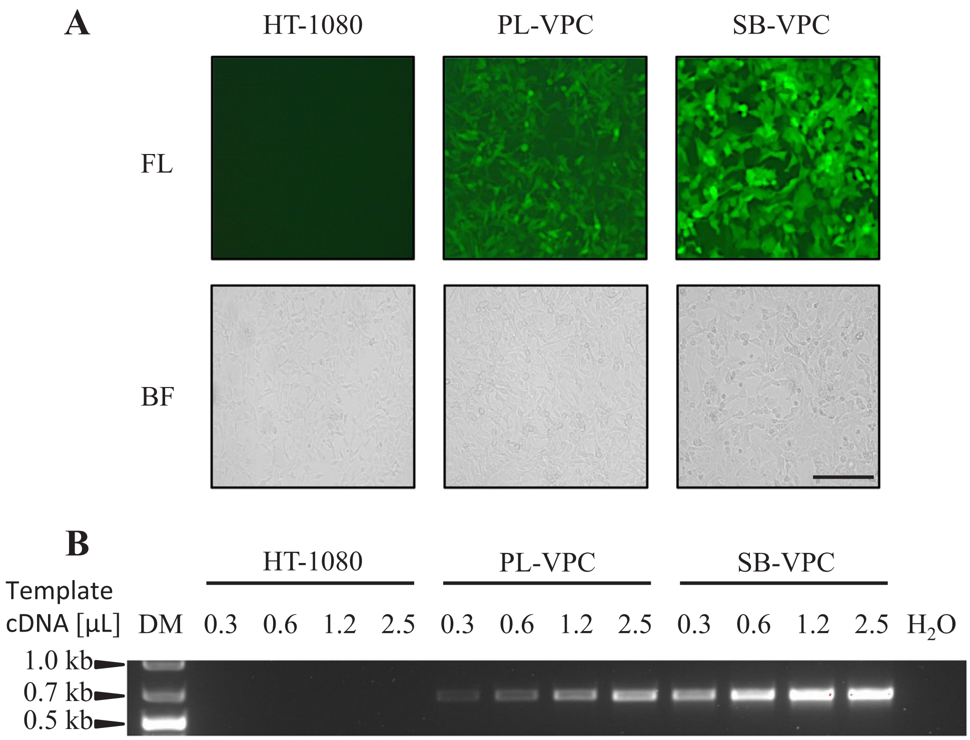


Fig. 3. Detection of transfer vector expression in packaging cells and transcripts packaged in viral vector particles. (A) Images of negative control HT-1080 cells and both VPCs indicated using fluorescence (FL; upper panel) and bright-field (BF; lower panel) microscopy. The scale bar (lower panel, right) represents a distance of 100 µm for all images shown. (B) Semi-quantitative PCR on template cDNA reverse transcribed from transfer vector mRNA isolated from vectors harvested from indicated cells and pelleted upon ultracentrifugation. Different volumes of template cDNA were used as shown. Employing oligonucleotides specifically binding to transfer vector reporter gene *egfp* DNA-fragments of 0.633 kb were amplified. DM, DNA marker.

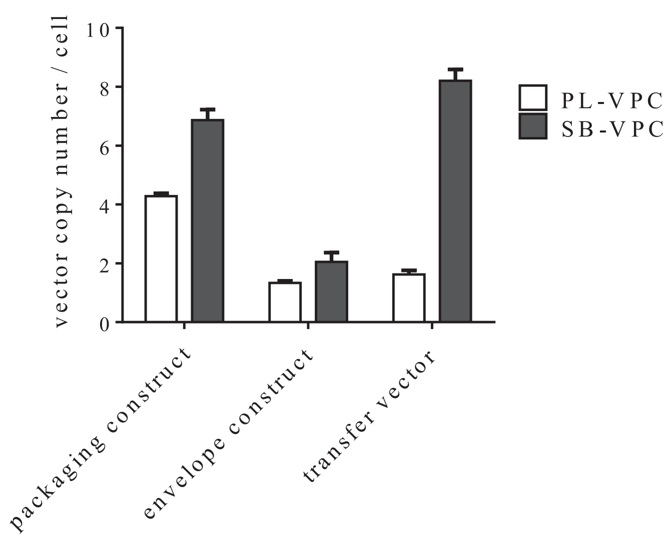


Fig. 4. Quantification of vector copy numbers (VCN) per cell genome. Genomic DNA of cell lines indicated served as templates for qPCR-analysis using oligonucleotides allowing for the amplification of DNA-fragments of $puro^R$ present in the packaging constructs, hyg^R (envelope construct) and *egfp* (transfer vector). An additional primer/probe set detecting the endogenous genomic locus of the PTBP2 gene was used as an internal reference. Error bars indicate standard deviations resulting from technical replicates (n = 3).

3.4. Target cells recombinantly expressing mCAT are rapidly established using transposon vectors and reveal high susceptibility to transduction mediated by MLV(PVC-211) vectors

We next established HT-1080 and CHO-K1 cells recombinantly overexpressing the ecotropic receptor mCAT on the cell-surface by co-transfection with the transposon vector SB-mCATIpW illustrated in Fig. 1D and the transposase construct CMV-SB100x. Three days post transfection, cells were subjected to selection using 1 and 10 µg/mL of puromycin, respectively. Resistant recombinant cell populations CHO-K1/mCAT and HT-1080/mCAT were established within a week. To assess their susceptibility to MLV(PVC-211) vectors, cell-free particle preparations harvested from SB-VPC were stored at −80 °C and, upon re-thawing, titrated in triplicate on NIH-3T3 target cells. Subsequently, recombinant mCAT expressing cells as well as their naïve parental cell lines and NIH-3T3 cells were transduced employing an multiplicity of infection (MOI) of 1.0 and analyzed three days later for reporter gene product EGFP expression using fluorescence microscopy and flow cytometric analysis. Untransduced cells served as negative controls and did not show any detectable EGFP expression. As illustrated in Table 2, two independent transduction experiments were conducted. Experiment 1 was performed in duplicate to assess the accuracy of the assay and variability of transduction efficiencies using one vector preparation. The NIH-3T3 target cell population revealed 56.3–58.7% of EGFP-positive cells. As expected using MLV(PVC-211) vector particles, CHO-K1 cells were also efficiently transduced, yet showing only 7.2–10.7% genetically modified cells. Strikingly and upon recombinant expression of the murine ecotropic receptor, CHO-K1/mCAT cells were transduced with efficiencies of 63.7–78.6% and thus, exceeded the susceptibility of murine NIH-3T3 fibroblasts. Naïve human HT-1080 cells did not reveal any susceptibility to ecotropic MLV(PVC-211) vector particles over the

Table 2
Transduction efficiencies of MLV(PVC-211) vector particles in different target cells using an identical multiplicity of infection (MOI). Vector preparations pre-titrated on NIH-3T3 were used to transduce the target cells indicated at an MOI of 1.0. Transduction efficiencies are shown in the percentage of EGFP-positive cells. Experiment 1 and 2 were conducted independently. Experiment 1 was performed in duplicate (A and B).

Target cell line	Transduced $EGFP^+$ cells [%]		
	Exp. 1		Exp. 2
	A	B	
NIH-3T3	58.7	56.3	57.2
CHO-K1	8.2	7.2	10.7
CHO-K1/mCAT	63.7	72.9	78.6
HT-1080	> 0.1	> 0.1	> 0.1
HT-1080/mCAT	78.6	77.4	93.4

detection limit of 0.1%. However, HT-1080/mCAT cells showed the highest susceptibility amongst the target cells examined with transduction efficiencies of 78.6–93.4%. In summary, this demonstrated the potential of the SB-mCATIpW transposon vector co-transfected with the transposase construct for genetic engineering of susceptible target cells. MLV(PVC-211) particles were instrumental in the efficient transduction of naïve CHO cells which could be further advanced upon the overexpression of mCAT.

4. Discussion

In this study, we show for the first time that multiplexed co-transfection of a *Sleeping Beauty*-derived transposase construct and three transposon vectors entailing a packaging, envelope and transfer vector cassette required to generate retroviral vectors allows for the rapid establishment of highly productive VPCs. This strategy was superior over the conventional approach of sequential stable transfection and selection of plasmid-based constructs harboring the identical expression cassettes. As a prove of concept we used human HT-1080 cells previously reported to allow for the establishment of stable high-titer VPCs (Cosset et al., 1995) and aimed at the generation of ecotropic MLV (PVC-211) vector particles (Stitz, 2011).

Conventional triple co-transfection of the plasmids gpIpW, eIpW and LEGFP-N1 and subsequent triple-selection using the respective antibiotics even at low concentrations did not result the establishment of triple-resistant stable VPCs. Consequently, a PL-VPC bulk population had to be established using serial transfection of single constructs and subsequent selection over a total time of three months. In contrast, triple-resistant SB-VPC cells were rabidly established within three weeks employing the respective SB-transposon vectors co-transfected with the transposase construct. This was assumed to be a result of superior integration efficiencies of transposon vectors into the host cell genome at multiple copies per cell as previously reported by Grabundzija et al. (2010). In both, SB-transposon and plasmid-based panels of constructs, the antibiotic-resistance genes were geno-phenotype coupled to the expression of the viral structural genes *gag/pol*, *env* and the transfer vector. It appears feasible to assume that thus the higher integration efficiency upon transposition enabled SB-VPC to survive higher selection pressures of 5 μg/mL puromycin, 500 μg/mL hygromycin and 800 μg/mL G418 as compared to 3 μg/mL, 300 μg/mL and 400 μg/mL reached by PL-VPC, respectively. Consequently, we expected higher expression levels of the viral vector components in SB-VPC and thus, the detection of higher vector particle titers. Noteworthy, SB-VPC and PL-VPC were established as bulk populations to ensure comparability. Remarkably, differences in vector titers of 20- to 30-fold were observed between SB-VPC ($> 1 \times 10^6$ TU/mL) and PL-VPC ($< 1 \times 10^5$ TU/mL). Rescue assays conducted did not detect any generation of RCRs demonstrating that observed gene transfer efficiencies were mediated by retroviral vector-mediated transduction.

Western blot-analysis confirmed higher productivity of SB-VPC detecting more Gag p30-CA, immature Env p15-TM and fusogenic p15-TMΔR proteins in pelleted particles but could not satisfyingly explain the enormous differences in vector titer. We thus examined the expression of the reporter gene product EGFP encoded on the transfer vectors. SB-VPC showed much higher expression of EGFP as compared to PL-VPC suggesting a much larger amount of transfer vector mRNA available for packaging into particles. This was confirmed using a semi-quantitative PCR performed on cDNA templates generated using RT-PCR and mRNA isolated from vector particles. The about eight-fold higher amount of transfer vector transcripts packaged in SB-VPC produced particles resulted in an enhanced vector fitness. This is also supported by the data of Troyanovsky et al. (2015) reporting on a transfer vector imbedded into a transposon vector derived from *PiggyBac*. High-titer vector preparations could be generated employing transposition-mediated integration of a hybrid vector into the genomic DNA of previously established clonal Phoenix-A VPCs stably expressing Gag/Pol and Env. In addition, we demonstrated notable superior vector copy numbers (VCN) in SB-VPC for all three constructs employing qPCR. Thus, we were able to confirm our assumption that highly efficient integration mediated by transposition enabled elevated VCN per cell as compared to stable transfection.

To show the utility of SB-transposon vectors to the rapid establishment of target cells susceptible to ecotropic vectors under BSL-1 conditions we co-transfected CHO-K1 and HT-1080 with the transposon vector SB-mCATIpW and the transposase construct and subsequently selected stable recombinant cells within one week. To demonstrate their advanced susceptibility target cells were transduced with MLV (PVC-211) vectors at an MOI of 1.0 determined by titration on NIH-3T3 cells. Naïve CHO-K1 cells were readily transduced but at lower efficiency than murine NIH-3T3 cells. However, recombinant CHO-K1/mCAT exceeded the susceptibility of NIH-3T3 cells resulting to more than a doubling of the percentage of transduced cells. Maybe even more strikingly, human HT-1080/mCAT cells were transduced with more than three-fold higher efficiency than NIH-3T3 cells. This underscores the potential of receptor-encoding transposon vectors for the rapid establishment of susceptible recombinant target cells.

We anticipate this novel transposon vector-based strategy to be of utility in the rapid establishment of VPCs for the production of retroviral vector particles pseudotyped with envelope proteins of other retroviruses such as amphotropic MLV, MLV 10A1mc, gibbon ape leukemia virus (GaLV), feline endogenous retrovirus RD114 (Porter et al., 1996) and vectors derived from other parental viruses such as lentiviruses like HIV and SIV (Sanber et al., 2015) as well as for example adenovirus-associated virus (AAV; Yuan et al., 2011; Martin et al., 2013). Our novel SB-based panel of constructs was demonstrated to be instrumental in the production of MLV(PVC-211) vector particles at high titers. These ecotropic vectors transduced naïve CHO cells and recombinantely mCAT overexpressing CHO cells at high efficiency. In future and upon retroviral vector-mediated transduction employing high MOIs, multiple copies of transfer vectors encoding proteins of interest (POIs) – e.g. cytokines or heavy and light chains of antibodies – could be stably integrated into genome of CHO cells. This should prove useful for the rapid establishment of high-yield CHO-derived protein producer cells at BSL-1. We believe this represents a competitive alternative to currently used HIV(VSV-G) pseudotype vectors requiring laboratories operated under BSL-2 (Oberbek et al., 2011).

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