Development of transposon and retrovirus-derived vectors for the rapid establishment of efficient producer cell lines

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Abstract

With the growing demand for therapeutic proteins such as antibodies or vaccines, manufactures are in need to deliver products at high quality and constant productivity. For the biotechnological production of these products, mammalian cells are predominantly used. The productivity of producer cells is, amongst others, dependent on the number of integrated transgene copies per cell, and thus elevating stable gene transfer efficiencies to ensure sustained expression of the gene of interest is paramount. In this study the *Sleeping Beauty* transposon two-component vector system, consisting of a transposon donor plasmid and a transposase expression plasmid, was used to generate stable cell pools. However, using sensitive genomic PCR and reverse transcriptase PCR (RT-PCR), the transposase gene integration and expression was demonstrated for a time period of 48 days post transfection. To provide an alternative to the employment of plasmid-based transposase expression circumventing potential re-mobilization events of the already stably transposed transgenes, the transposase gene was transcribed *in vitro* into mRNA. After co-transfection of transposase transcripts at different ratios to the donor vector efficient transposition was obtained. This study demonstrated that this technical approach mediated high copy numbers and expression levels of transgenes in recombinant cells without the risk of undesired extended transposase expression.

Besides transposons, retroviral vectors are frequently used to introduce foreign genetic material into mammalian cells aiming for the establishment of producer cells. Such viral vectors are commonly pseudotyped with VSV-G achieving high vector copy numbers in mammalian cells. However, this requires handling under BSL-2 conditions. To circumvent this, viral vectors were equipped with the ecotropic envelope protein $PVC211_{mc}$, a molecular clone of Friend *murine leukemia virus* (MLV), enabling transduction of CHO cells and murine hematopoietic stem cells but allowing experiments in BSL- 1 laboratories. The aim of this work was to optimize gene transfer efficiencies by generating PVC211-derived envelope protein (Env) variants lacking the R-peptide and thus rendering the Env proteins fusogenic. All generated variants failed to efficiently pseudotype MLV vectors but two variants successfully pseudotyped HIV-1 particles. The HIV-1 vectors pseudotyped with the envelope variant eMLV-GaLVΔR mediated superior infectivity as compared to wild-type Env. HIV(eMLV-GaLVΔR) should prove useful as a tool for the establishment of productive producer cells.

Keywords: *Sleeping Beauty* transposon, mRNA, retroviral vector, PVC211, pseudotype vector

Kurzfassung

Angesichts der wachsenden Nachfrage nach therapeutischen Proteinen wie Antikörpern oder Impfstoffen müssen die Hersteller Produkte mit hoher Qualität und konstanter Produktivität liefern. Für die biotechnologische Herstellung dieser Produkte werden überwiegend Säugetierzellen verwendet. Die Produktivität der Produktionszellen hängt unter anderem von der Anzahl der integrierten Transgen-Kopien pro Zelle ab. Daher ist es von größter Bedeutung, die Effizienz des stabilen Gentransfers zu erhöhen, um eine dauerhafte Expression des gewünschten Gens zu gewährleisten. In dieser Studie wurde das *Sleeping Beauty* Transposon Zweikomponenten-Vektorsystem, bestehend aus einem Transposon-Donor-Plasmid und einem Transposase-Expressions-Plasmid, zur Erzeugung stabiler Zellpools verwendet. Mittels sensitiver genomischer PCR und reverser Transkriptase-PCR (RT-PCR) wurde die Integration und Expression des Transposase Gens über einen Zeitraum von 48 Tagen nach Transfektion nachgewiesen. Als Alternative zur plasmidbasierten Transposase Expression, bei der eine mögliche Remobilisierung der bereits stabil transponierten Transgene umgangen wird, wurde das Transposase Gen *in vitro* in mRNA umgeschrieben. Nach Ko-Transfektion von Transposase Transkripten in unterschiedlichen Verhältnissen zum Donorvektor wurde eine effiziente Transposition erreicht. Diese Studie zeigte, dass dieser technische Ansatz hohe Kopienzahlen und Expressionsniveaus von Transgenen in rekombinanten Zellen vermittelt, ohne das Risiko einer unerwünschten, andauernden Transposase Expression.

Neben Transposons werden häufig retrovirale Vektoren verwendet, um fremdes genetisches Material in Säugetierzellen einzuschleusen, mit dem Ziel der Etablierung von Produktionszellen. Solche viralen Vektoren werden in der Regel mit VSV-G pseudotypisiert, um hohe Vektorkopienzahlen in Säugetierzellen zu erreichen. Dies erfordert jedoch ein Arbeiten unter S2 Bedingungen. Um dies zu umgehen, wurden virale Vektoren mit dem ecotropen Hüllprotein PVC211, einem molekularen Klon des Friend *murinen Leukämievirus* (MLV) ausgestattet, was die Transduktion von CHO-Zellen und hämatopoetischen Stammzellen der Maus ermöglicht, aber auch Experimente in S1 Laboren erlaubt. Ziel dieser Arbeit war es, die Effizienz des Gentransfers zu optimieren, indem von PVC211 abgeleitete Varianten des Hüllproteins Env erzeugt wurden, denen das R-Peptid fehlt und die somit auch ohne Reifung bereits fusogen sind. Keine der erzeugten Varianten gelang es MLV-Vektoren effizient zu pseudotypisieren jedoch pseudotypisierten zwei Varianten erfolgreich HIV-1-Partikel. Besonders die HIV-1-Vektoren, die mit der Hüllvariante eMLV-GaLVΔR pseudotypisiert wurden, zeigten eine sehr viel höhere Infektiosität als Wildtyp-Env. HIV(eMLV-GaLVΔR) Vektorpartikel dürften sich als ein nützliches Werkzeug für die Etablierung produktiver Produktionszellen erweisen.

Schlüsselbegriffe: *Sleeping Beauty* Transposon, mRNA, retroviraler Vektor, PVC211, pseudotyp-Vektoren

Publications included in this cumulative thesis

1. **N. Tschorn**, K. Berg, J. Stitz
   Transposon vector-mediated stable gene transfer for the accelerated establishment of recombinant mammalian cell pools allowing for high-yield production of biologics
   Biotechnology letters, **2020**, *42*, 1103-1112
   (doi: 10.1007/s10529-020-02889-y.)

2. **N. Tschorn**, C. Söhngen, K. Berg, J. Stitz
   Ecotropic HIV-1 vectors pseudotyped with R-peptide-deleted envelope protein variants reveal improved gene transfer efficiencies
   Virology, **2022,** *577*, 124-130
   (doi: 10.1016/j.virol.2022.09.008.)

3. **N. Tschorn**, Y. van Heuvel, J. Stitz
   Transgene expression and transposition efficiency of two-component Sleeping Beauty transposon vector systems utilizing plasmid or mRNA encoding the transposase
   Molecular Biotechnology, **2022**
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Abbreviations

APOBEC3G	apolipoprotein B mRNA editing enzyme, catalytic subunit 3G
BSL	biosafety level
BET	bromodomain and extraterminal
CO_2	carbon dioxide
CHO	hinese hamster ovary
CD4	cluster of differentiation 4
cDNA	complementary DNA
CMV	*cytomegalovirus*
C-tail	cytoplasmic tail
DNA	deoxyribonucleic acid
Env	envelope protein
GOI	gene of interest
GAPDH	glycerylaldehyde 3-phosphate-dehydrogenase
GaLV	*gibbon ape leukemia virus*
GLP-1-Fc	glucagon-like peptide-1 Fc fusion protein
VSV-G	G-protein of the *vesicular stomatitis virus*
GFP	green fluorescence protein
Gag	group-specific antigen
HSCs	hematopoietic stem cells
HEK	human embryo kidney
hFVIII	human blood coagulation factor 5
HIV-1	*human immunodeficiency virus* 1
IgG	immunoglobulin G
ITRs	inverted terminal repeats
LV	lentiviral vector
LTRs	long terminal repeats
LDL	low-density lipoprotein
mRNA	messenger RNA
MOI	multiplicity of infection
mCAT-1	murine cationic amino acid transporter-1
MLV	*murine leukemia virus*
MLV_{ampho}	amphotropic MLV
MLV_{eco}	ecotropic MLV
Nef	negative factor
NPC	nuclear pore complex
OPI	overproduction inhibition

PBS	phosphate buffered saline
PB	*piggyBac*
PEI	polyethylenimine
Pol	polymerase
PTMs	post-translational modifications
PIC	pre-integration complex
PTBP2	polypyrimidine tract binding protein 2
RCRs	replication-competent retroviruses
RRE	rev response element
RNA	ribonucleic acid
SIN	self-inactivating
SV	*simian virus*
SB	*Sleeping Beauty*
Pit	sodium-dependent Pi transporter
SNV	*spleen necrosis virus*
SU	surface unit
TAR	transactivation response
TSS	transcriptional start site
TM	transmembrane region
VCN	vector copy number
Vif	viral infectivity factor
Vpr	viral protein R
Vpu	viral protein U
WPRE	*woodchuck hepatitis virus* posttranscriptional regulatory element

1 Introduction

Biopharmaceuticals are drugs produced in biological systems. The vast majority of the approved biopharmaceuticals are produced in mammalian cells as these products require appropriate post-translational modifications (PTMs) [1]. To accommodate the demand for high quality products at elevated quantity, the mammalian cell line used for production has to exhibit high productivity. Productivity is characterized by the rate of protein expression per cell within a defined time frame. The productivity of a recombinant mammalian cell line depends on a number of parameters. Aside from optimizing cell cultivation conditions such as composition of the growth media and the cultural environment, a high vector copy number (VCN) of the gene of interest (GOI) encoding for the therapeutic protein integrated in the host cell genome is indispensable for high and sustained productivity [2]. Besides other strategies, two systems are able to facilitate this – transposon vectors and retrovirus-derived vectors. Both efficiently mediate stable integration of the GOI at high VCN into genomic DNA of mammalian cells, and thus are powerful tools for the establishment of protein producer cells [3, 4].

1.1 Transposon vectors

One method to transfer foreign nucleic acids into mammalian cells at elevated VCN for biotechnological approaches is the employment of DNA transposon vectors. The most prominent DNA transposons are *piggyBac* (PB) isolated from the cabbage looper moth and *Sleeping Beauty* (SB) reconstructed from three fish species, namely white cloud minnow, atlantic salmon and rainbow trout. The first reconstructed SB transposon vectors exhibited low activity [5]. Recombination of generated transposase gene mutants led to a hyperactive transposase enzyme called *SB100x* referring to the factor of improvement as compared to the first reconstructed enzyme [6, 7]. DNA Transposons are composed of a transposase gene flanked by inverted terminal repeats (ITRs). After expression of the transposase gene, the enzyme recognizes short sequences in the ITRs and cuts out the transposable element for subsequent re-integration into a new gene locus. This event is called transposition. Transposons differ in the target sequence they re-locate the transposable element. *PiggyBac* preferably integrates into the four-nucleotide TTAA while *Sleeping Beauty* favors the dinucleotide TA as target sequence [8, 5]. These relatively short sequences allow for a close-to-random integration in the genome [9, 10]. SB exhibits only a slight bias towards transcription units and their flanking regions [11, 12]. However, PB transposon vectors are predominantly enriched in highly expressed genes with an integration preference for transcriptional start sites (TSSs), DNase I hypersensitive sites, DNA-binding sites and transcription factors, and thus bear resemblance to the *murine leukemia virus* (MLV)-mediated insertion profile. An explanation for this is the physical interaction of PB transposases with the bromodomain and

extraterminal (BET) proteins also tethering to MLV integrase [13, 14], and thus poses a risk to applications *in vivo*.

1.2 Two-component vector system

Based on the natural transposon structure, two-component vector systems were developed for optimized gene transfer. The transposon vector, also called donor vector, encompasses the GOI and is flanked by the ITRs (Figure 1).

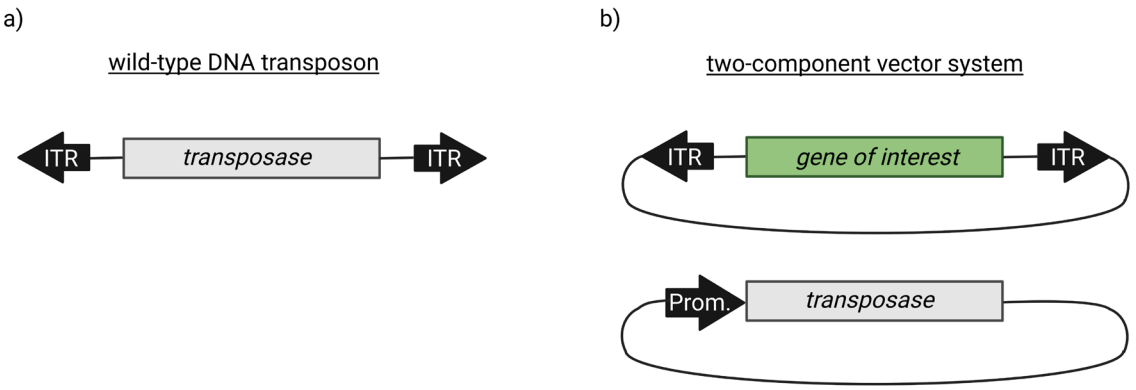

Figure 1: Organization of a wild-type DNA transposon and a two-component transposon vector system. a) The wild-type DNA transposon consists of the transposase gene and is flanked by the inverted terminal repeats (ITRs). (b) The two-component vector system comprises the donor vector carrying the gene of interest surrounded by the ITRs and the helper vector coding for the transposase enzyme. Illustration was created with Biorender.com

The transposase gene is located on a separate plasmid facilitating the titration of donor vector and transposase plasmid for efficient transposition since higher amounts of the transposase enzyme leads to decreased transposition events, a phenomenon called overproduction inhibition (OPI) [15]. Transposition occurs in a variety of cell types and the integrated genetic sequences remain stable [16]. Transposon vectors mediate gene transfer at high efficiencies and multiple copy numbers. This is in contrast to classic stable plasmid transfection mostly resulting in significantly lower copy numbers [17].

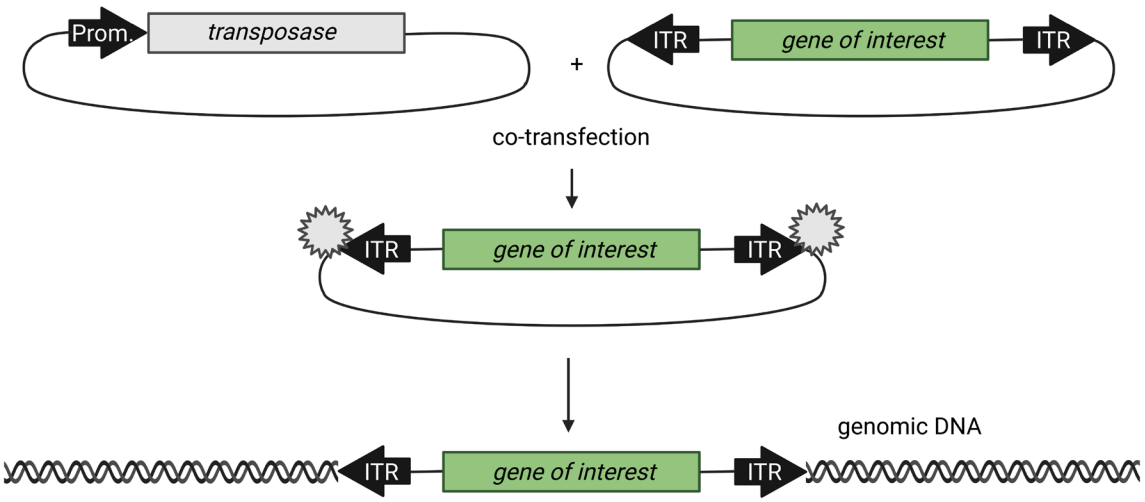

Figure 2: Schematic illustration of the transposition mechanism. Upon co-transfection of the helper vector encoding the transposase and the donor vector containing the gene of interest framed by the ITRs, the transposase gene is expressed, binds to specific sequences in the ITRs, cuts out the gene of interest harboring from the plasmid backbone and integrates it into the genomic DNA of the target cell. Illustration was created with Biorender.com

If plasmids are used for transfection, the bacteria-derived sequences in the plasmid backbone are transferred into the host cell genome and can potentially lead to gene silencing of the GOI. Utilizing transposon vectors for the establishment of stable producer cell lines instead, circumvents the mentioned drawback as they only integrate the transposable element within the ITRs, and thus minimizes the risk of gene silencing [18]. However, transposon vector systems have one disadvantage as the vector expressing the transposase enzyme is plasmid-based and could stably integrate into the genomic DNA of the host cell. The undesired integration can lead to sustained expression of the transposase gene and ongoing transposition events of already integrated GOI leading to instability of the cell line, and thus potential loss of productivity [19]. One solution to this problem is to introduce the transposase as transcripts or as protein into the target cell [20, 21]. Due to the size and complexity of proteins, the cumbersome production, purification and delivery into the cell, replacing plasmid-based transposase with transposase protein is concomitant with high costs and time expenditure. Therefore, mRNA-based transposase expression can be a useful alternative for transposition experiments as the mRNA synthesis can be performed *in vitro* in small reaction tubes and transfection is conducted utilizing cationic lipid based transfection reagents such as polyethylenimine (PEI) as shown by Bire and colleagues in 2013 for *piggyBac*-derived vectors [22]. A detailed introduction and discussion of the advantages of transposon vector systems for the establishment of stable producer cell lines in relation to retroviral gene transduction is provided in the review article included in this thesis [23].

1.3 Retroviral vectors for gene transfer

Retroviral vectors are utilized to transfer nucleic acids into mammalian cells for gene therapeutic purposes but can also be instrumental for the establishment of protein producer cell lines [24–28, 4]. Derived from γ-retroviruses such as MLV, these replication-incompetent vectors stably integrate their genetic cargo into the target cell genome upon transduction, and thus enable sustained expression of the gene of interest. Retroviral transfer vector mRNAs are structured similarly to the mRNAs of their parental viruses except coding for a gene of interest instead of viral genes necessary for replication. In the retroviral genome the 5´ long terminal repeat (LTR) entails a promotor/enhancer and drives transcription. The 3´LTR is responsible for transcription termination and polyadenylation of the transcripts. The *gag* gene encodes for the viral core proteins matrix, capsid and nucleocapsid and the *pol* gene codes for the viral enzymes, namely protease, reverse transcriptase and integrase. The *env* gene encodes for the envelope proteins (Env) mediating attachment to target cell receptors for viral entry [29]. To avoid the formation of replication-competent retroviruses (RCRs) during MLV vector particle production, the envelope gene *env* and the structural genes *gag* and *pol* are embedded in separate plasmids lacking the packaging signal Psi (ψ) [30] as illustrated in Figure 3. The transfer vector plasmid carrying the GOI exclusively entails the ψ and the LTRs.

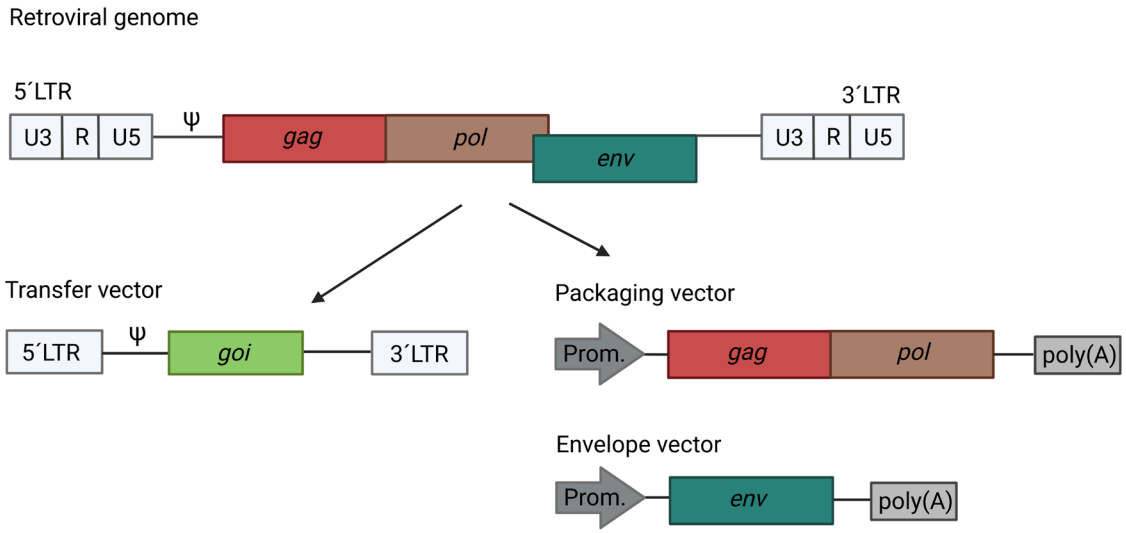

Figure 3: Schematic illustration of the γ-retroviral genome and the retroviral vector components. The simple retroviral genome consists of the *gag* gene encoding the core proteins such as matrix, capsid and nucleocapsid, the *pol* gene coding for the viral enzymes reverse transcriptase, protease and integrase and the envelope gene *env* facilitating the expression of the Env proteins mediating receptor recognition and cell entry via membrane fusion. To ensure safety and to avoid packaging of viral structural genes, the viral components are inserted into separate expression constructs. The transfer vector contains the packaging signal Psi (ψ) mediating the packaging of the gene of interest (*goi*) mRNA flanked by the LTRs while the packaging vector provides the genes for the expression of Gag and Pol. The envelope vector codes for the Env proteins. Illustration was created with Biorender.com

To produce retroviral vector particles, the three aforementioned plasmids can be transiently or stably co-transfected into suitable cell lines such as human embryonic kidney 293 (HEK293) cells and its related cell lines, human fibro-sarcoma HT-1080 cells or Chinese hamster ovary (CHO) cells, and thus generating transient or stable packaging cell lines [31–34]. The produced retroviral particles are released into the supernatant of the producer cell line and can be directly applied to transduce susceptible target cells. Depending on the stability of the utilized viral vector and envelope proteins, vector particles can be harvested and concentrated employing ultrafiltration or ultracentrifugation to maximize vector particle density for subsequent titration experiments without the loss of infectivity [35–37]. For the production of glucagon-like peptide-1 Fc fusion protein (GLP-1-Fc), a drug for the treatment of type 2 diabetes, MLV vectors pseudotyped with the envelope protein of *vesicular stomatitis virus* (VSV-G) were utilized to transduce suspension CHO- S cells yielding a productivity of 3.15 g/L [27]. The commercial production of Pritumumab, a human immunoglobulin G (IgG) monoclonal antibody for the treatment of patients with glioma, is conducted in CHO cells transduced with the GPEx® system composed of MLV vectors pseudotyped with VSV- G produced in HEK293 cells [38].

1.4 Lentiviral vectors for gene transfer

Lentiviral vectors (LVs) are most frequently derived from *human immunodeficiency virus* 1 (HIV-1) and belong to the *Retroviridae* family. The parental lentiviruses exhibit, in contrast to the simple γ- retroviruses, a complex mRNA splice pattern having multiple splice donors and acceptors facilitating the expression of six additional gene products. In addition to the genes *gag*, *pol* and *env,* the lentiviral genome entails the regulatory genes *tat* and *rev* and the accessory genes *nef*, *vif*, *vpr* and *vpu* necessary for viral replication and enhanced infectivity. The Tat protein binds to the transactivation response (TAR) element encoded by the HIV-1 LTRs to elongate transcription [39, 40]. The Rev protein interacts with the Rev response element (RRE), a *cis*-acting RNA localized in the *env* gene and is responsible for the nuclear export of HIV-1 transcripts into the cytoplasm [41, 42]. Vif, Vpr, Vpu and Nef enhance pathogenicity *in vivo*. In particular, Nef regulates CD4 receptor presentation on infected cells to prevent HIV-1-mediated superinfection in order to inhibit depletion by the immune system [43]. Vif binds and eliminates APOBEC3G, a cytosine deaminase responsible for inhibiting reverse transcription [44]. Vpu increases particle release antagonizing tetherin, which inhibits virion release from infected cells [45]. Vpr plays a crucial role in mediating nuclear import of the pre-integration complex (PIC) [46].

As illustrated in Figure 4, the first-generation lentiviral vectors were based analogous to γ- retroviral vectors on three plasmids: the packaging plasmid harboring the majority of the HIV-1 genome except for the packaging signal ψ and the LTRs that were replaced by a

promotor and poly (A) signal, a transfer vector entailing the GOI, the ψ signal, the RRE and an envelope expression plasmid. As the natural tropism of HIV-1 is limited to CD4-expressing cells, the envelope plasmid encodes for the envelope proteins of other viruses such as *vesicular stomatitis virus* (VSV) allowing for the transduction of a broad range of cell types. In the development process of second-generation lentiviral vectors, it was aimed to improve safety by deleting the coding sequences of the accessory genes *vpu*, *vpr*, *nef* and *vif* from the packaging plasmid as they are not essential for vector production [47]. Further improvements towards safety of lentiviral vectors led to the development of a third-generation vector system enabling a Tat-independent transcription by deleting the U3 region from the 3´LTR in the transfer vector. During reverse transcription of these so called self-inactivating (SIN) vectors, the deletion in the 3´LTR is transferred to the 5´LTR and results in transcriptional inactivation in the target cell genome, and thus reduces the risk of formation of RCRs and the transcriptional activation of aberrantly located genes [48]. The U3 region in the 5´LTR was replaced with a constitutive promotor derived from *Rous sarcoma virus* (RSV) enabling transcription of viral RNA [49]. The packaging construct finally only encodes for Gag/Pol and the regulatory gene *rev* is provided *in trans* on a separate plasmid.

Lentiviral vector particles are commonly produced upon transient transfection since the production is hampered by the cytotoxicity of the HIV-1 protease and the VSV-G envelope proteins [50, 51]. For stable production, inducible packaging systems were developed including promotors only activated during the production period to prevent premature cell death. The most prominent inducible expression systems are Tet-On and Tet-Off systems allowing for controlled gene expression in the presence of tetracycline (Tc) [52, 53]. The utilization of such systems involves an additional purification step removing the Tc and consequently alternative methods were developed to circumvent this. Constitutive packaging systems with non-cytotoxic envelope proteins were established such as the STAR packaging cell line. This cell line is based on second-generation HIV-1 vectors equipped with amphotropic MLV 4070A envelope proteins harboring a truncated cytoplasmic tail (C-tail) derived from *gibbon ape leukemia virus* (GaLV) reaching titers of 1×10^7 TU/mL [54]. In 2018, Tomás and his colleagues established the LentiPro26 packaging cell line using a mutated HIV-1 protease revealing lower activity but enabling stable and constitutive production of lentiviral vector particles achieving titers of 1×10^6 TU/mL [55].

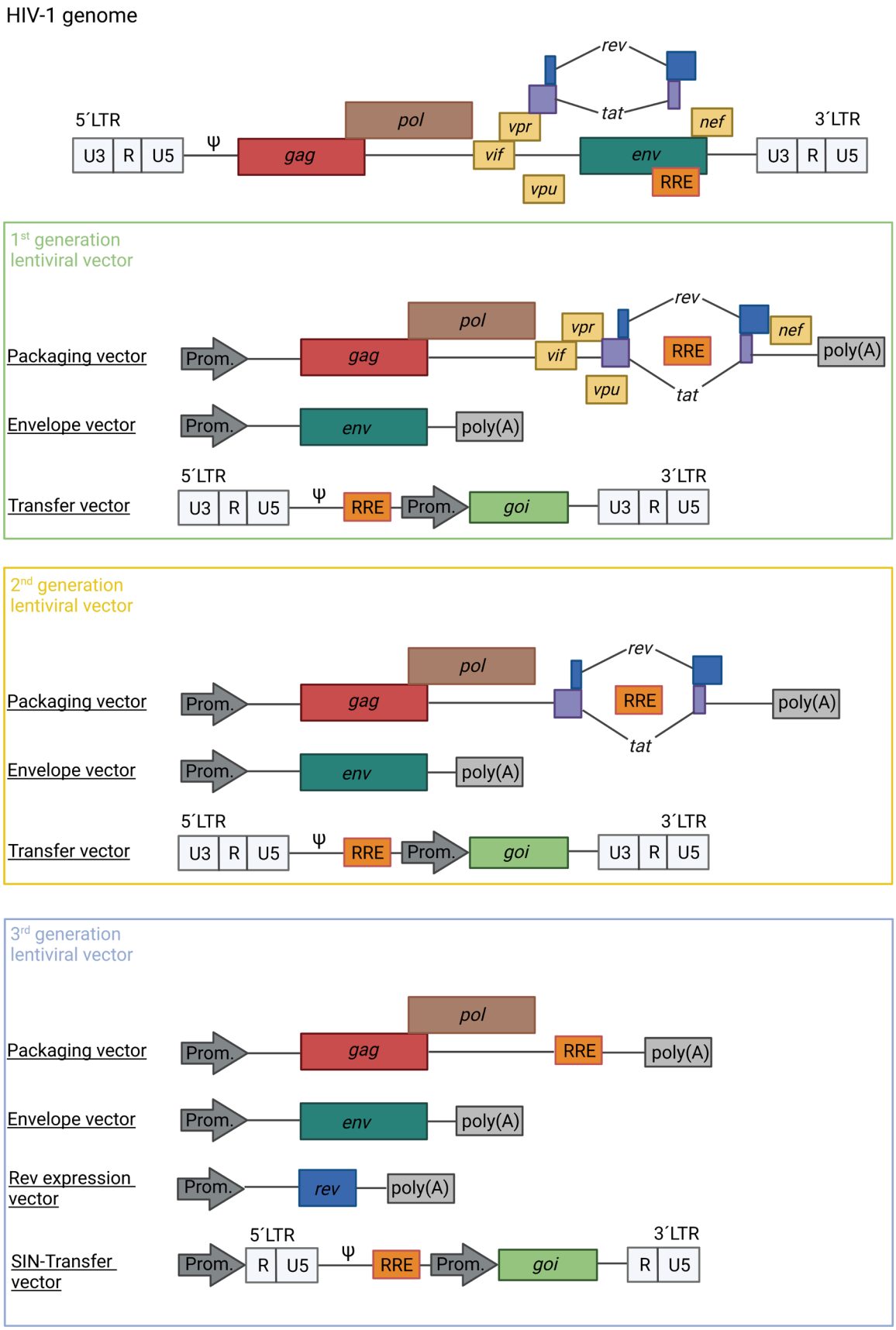

Figure 4: Schematic illustration of the HIV-1 genome and the developed lentiviral vector generations. Genomic RNA is flanked by long terminal repeats (LTRs) necessary for transcription initiation and termination. The *gag* gene encodes for the core proteins forming the viral core. The *pol* gene codes for the viral enzymes protease, reverse transcriptase and integrase necessary for viral

replication. *Env* encodes the envelope protein mediating attachment to the target cell facilitating viral entry. *Vif*, *vpr*, *vpu*, and *nef* code for the accessory proteins. *Tat* and *rev* encode for the regulatory proteins. The first-generation lentiviral vector system contained a packaging plasmid harboring the major part of the HIV-1 genome. The *env* gene was excluded and embedded into a separate envelope vector. The transfer vector encompassed the gene of interest (*goi*) and was driven by an internal promotor derived from a heterologous virus. The packaging signal Psi (ψ) exclusively included in the transfer vector, ensured encapsulation of the transfer mRNA and the Rev response element (RRE) mediated nuclear export of viral transcripts. In the second-generation of LVs the accessory genes were removed except for the *tat* and *rev* gene. In the third-generation of LVs transcription occurred independently from Tat and the *rev* gene was transferred to a separate expression plasmid. For safety improvement, the U3 region in the 3′ LTR was deleted in order to generate a self-inactivating (SIN) transfer vector. A constitutive promotor from other viruses was fused to the 5´LTR to ensure transcription of viral RNA. Illustration was adapted from Rintz et al., 2022 [56] and was created with Biorender.com

1.5 Optimization of retroviral vectors

Over the last decades, γ-retroviral and lentiviral vectors were optimized obtaining elevated titers to enhance transduction efficiencies. The expression levels of the viral packaging, transfer and envelope genes in the packaging cells are important for improved retroviral particle production. Strong promotors derived from heterologous viruses such as *cytomegalovirus* (CMV) or *simian virus 40* (SV 40) were commonly used to ensure higher expression levels of the viral genes and the transfer gene [57]. To further enhance expression levels, a *woodchuck hepatitis virus* post-transcriptional regulatory element (WPRE) can be inserted downstream of the GOI [58]. WPRE enhances transcript termination resulting in elevated mRNA levels presumably by supporting mRNA processing but the exact mechanism remains unknown [59, 60]. Depending on the origin of the viral vector components used for particle production, codon optimization of the viral genetic sequences can improve protein expression in the producer cells by changing synonymous codons preferably used by the translation machinery of the desired host cell for protein expression [61, 62].

Besides enhancements on protein expression levels, envelope protein engineering is a promising strategy to elevate titers as the Env proteins mediate gene transfer upon binding to the cognate target receptor and is thus crucial for efficient transduction. Retroviral envelope proteins interact with specific host cell receptors to initiate cell entry, which limits the range to susceptible target cells. To avoid restriction to specific cell types, retroviral vectors were generated using envelopes from heterologous viruses. This so-called pseudotyping enables modifying vector tropisms for specific applications. Retroviral particles naturally occur with different tropisms. The ecotropic γ-retrovirus MLV (MLV_{eco}) Env proteins bind to the murine cationic amino acid transporter-1 (mCAT-1), a receptor exclusively expressed on murine and rat cells, while amphotropic MLV (MLV_{ampho}) particles recruits the sodium-dependent P_i transporter-2 (PiT-2) in mouse cells and the human homologous receptor for entering human cells, including hematopoietic stem cells (HSCs), and were thus favorably used in gene therapy

[63, 64]. To further enhance transduction efficiencies and to broaden the tropism, retroviral vectors are commonly pseudotyped with VSV-G recruiting the low-density lipoprotein (LDL) receptor ubiquitously presented on various cell types of different donor organisms [65, 66]. Experimental work utilizing such VSV-G pseudotype vectors has to be conducted under biosafety level 2 but they are frequently used to efficiently transfer GOIs into CHO cells, the gold standard cell line for industrial protein production [67–69]. The ecotropic envelope of $PVC211_{mc}$, a molecular clone of Friend MLV, is an exception as it mediates gene transfer into CHO cells recruiting the hamster homologue of mCAT-1 [70]. It was also recently shown that retroviral vectors pseudotyped with PVC211 Env facilitate gene transfer into murine HSCs allowing for utilization in preclinical mouse models for gene therapy research [71]. One limitation of MLV-mediated gene transduction is its restriction to dividing target cells as the PIC enters the cell during mitosis when the nuclear envelope is fragmented and host chromosomes are accessible for binding the PIC to the chromosomes [72, 73].

In contrast, viral vectors derived from the HIV-1 enable gene transfer independent of the host cell cycle. The viral capsid containing the genetic cargo enters the nucleus through the nuclear pore complex (NPC) by interacting with the proteins Nup62 and Nup358 [74, 75]. This allows for efficient transduction of cells with low mitotic activity [76]. HIV-1-derived vectors can also be pseudotyped with numerous heterologous envelope proteins to broaden or limit their tropism to specific target cell types as their natural tropism using HIV-1 Env is restricted to CD4 receptor-expressing cells [77]. HIV-1-based vectors are commonly pseudotyped with VSV- G enabling transduction of a variety of cell types such as stem cells. However, the VSV-G protein is cytotoxic in higher concentrations, and thus sustained expression in stable packaging cells is not feasible [78, 79]. To circumvent this limitation, HIV-1 vectors were equipped with envelope proteins from the *Retroviridae* family. Envelope proteins of MLV_{ampho} and MLV_{eco} were shown to be successfully incorporated into HIV-1 particles [80, 81]. Pseudotyping with the Env proteins of GaLV, recruiting the PiT-1 receptor on human cells, revealed no infectivity in contrast to MLV Env pseudotyped lentiviral vectors. This is due to the differences in amino acid sequences of the C-tail harboring the recognition site for cleavage by the viral protease necessary to render the Env proteins fusogenic. To circumvent this, envelope glycoproteins were modified by exchanging C-tails from different retroviruses or introducing truncations in the cytoplasmic region [82, 83].

1.6 Modifications of envelope proteins

Retroviral envelope proteins are composed of a surface unit (SU) binding to a cognate host cell receptor and a transmembrane protein (TM) enabling fusion between the viral and host cell membrane to facilitate viral entry (Figure 5). In MLV and GaLV Env proteins fusogenicity is activated by proteolytic cleavage of a short peptide in the C-tail of the TM, the so called R-

peptide. Cleavage of the R-peptide is mediated by the viral protease and occurs after the release of the virus particle from the infected cell during maturation.

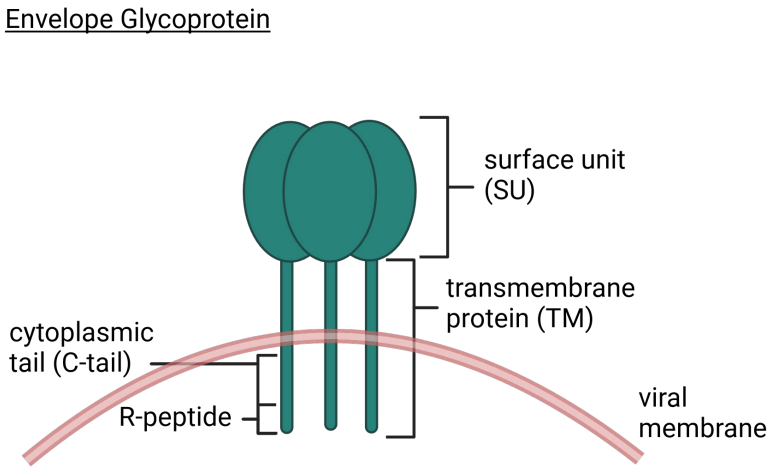

Figure 5: Structure of the retroviral envelope glycoprotein. The envelope proteins are incorporated as trimers into the viral membrane and consist of a surface unit (SU) connected to the transmembrane protein (TM) by a disulfide bond. The cytoplasmic tail (C-tail) of TM entails a short peptide sequence called R-peptide which is cleaved off by the viral protease then mediating fusion with the cell membrane after binding to the cognate receptor. Illustration was created with Biorender.com

After proteolytic cleavage, conformational changes in the envelope proteins occur and facilitate membrane fusion upon receptor binding, and thus viral entry [84, 85]. Deletion of the R-peptide by inserting a stop codon in the *env* gene coding sequence and following expression of ΔR Env in target receptor-positive cells results in cell-cell-fusion and formation of clusters of multinucleated cells called syncytia [86, 87]. Several studies investigated if R-peptide-deleted retroviral envelope protein variants decorating MLV or HIV-1 vector particles would enhance gene transfer assuming that the enhanced fusion activity correlates with elevated vector titers. The deletion of the R-peptide of MLV_{ampho} Env resulted in reduced titers when decorated on MLV and in slightly reduced titers when pseudotyping HIV-1 [88, 89]. It was assumed that R-peptide deletion alters surface unit conformation and has a negative effect on MLV particle formation [89, 88]. GaLV envelope proteins were shown to only mediate infectivity lacking the R-peptide when pseudotyped on HIV-1 particles in contrast to MLV particles efficiently incorporating full length GaLV envelope proteins [83, 90]. To circumvent the inability to form infectious HIV-1 vector particles, the GaLV-derived transmembrane or cytoplasmic domains were exchanged with those of MLV_{ampho} obtaining titers comparable to wild-type MLV_{ampho} envelope pseudotype HIV-1-derived particles [83]. Based on these findings, further chimeric GaLV/MLV_{ampho} envelope variants were constructed and assessed for their ability to mediate elevated gene transfer efficiencies [89, 62]. Tomás and his colleagues fused the transmembrane region of MLV_{ampho} 407A to the GaLV surface unit and compared titers achieved with the same chimeric envelope variant suffering from R-peptide deletion. Both

chimeric envelope variants efficiently pseudotyped HIV-1 particles but titers were increased using the R-peptide-lacking envelope variant hypothesizing inefficient R-peptide cleavage by the HIV-1-protease and thus reduced infectivity [89].

Another study investigated if MLV and HIV- 1 vectors pseudotyped with a chimeric Env variant composed of the avian spleen necrosis virus (SNV) N-terminal sequence fused to the GaLV C-tail facilitates the transduction of SNV-susceptible D17 cells. In addition, envelope variants harboring R-peptide deletions and an amino acid exchange corresponding to the MLV R-peptide cleavage site were generated. All chimeric Env protein variants formed infectious MLV pseudotype particles. As expected, the chimeric variant encompassing the full length GaLV C-tail coding region failed to mediate infectivity when decorated on HIV-1 particles. However, deletion of the R-peptide and a single amino acid exchange restored formation of infectious HIV-1 pseudotype vectors [91]. These findings showed that envelope engineering is a promising strategy to overcome envelope incorporation limitations and to elevate gene transfer efficiencies.

2 Research aims

The aim of this thesis was to develop and optimize transposon vectors and retroviral vectors enabling efficient gene transfer, and thus the rapid establishment of high-yield producer cells. The vectors should mediate stable integration of genes of interest at high vector copy numbers and the resulting recombinant cells should exhibit constant productivity. The transposon vector system used in this thesis was derived from the *Sleeping Beauty* transposon and consists of two components entailing a donor vector coding for the gene of interest and a transposase expression vector. However, the plasmid-based transposase vector could stably integrate into the target cell genome and cause sustained expression of the transposase gene leading to genetically unstable producer cell pool and thus potential loss of productivity. To investigate this, stable recombinant cell pools were examined for transposase gene integration and expression. To provide an alternative for transposase-encoding plasmids, the transposase was transcribed *in vitro* into mRNA and co-transfected with the donor vector DNA for subsequent examination of vector copy numbers and expression levels of the GOI. The results of this study were published in *Molecular Biotechnology* [92].

Retroviral vectors are commonly pseudotyped with VSV-G to mediate efficient gene transfer. However, sustained expression of the glycoprotein is cytotoxic for the vector producing cells limiting its use to transient and inducible packaging cells. In addition, experiments with these pantropic vectors have to be conducted under BSL-2. Thus, it was intended to develop viral vectors facilitating efficient gene transfer under BSL-1 conditions. Retroviral vectors equipped with the ecotropic envelope $PVC211_{mc}$, a molecular clone of Friend MLV, fulfill this requirement and enable transduction into CHO cells, the gold standard cell line for protein production. To further elevate gene transfer efficiencies, the ecotropic envelopes were genetically modified by introducing R-peptide deletions and further modifications of the C-tail of PVC211 Env proteins subsequently used to pseudotype MLV and HIV-1 vectors. It was assumed that the enhanced fusogenicity induced by R-peptide deletion could elevate gene transfer efficiencies. Consequently, Env protein variants were assessed for their fusogenicity, incorporation into γ-retroviral and lentiviral vector particles and their potential to elevate pseudotype vector titers as compared to wild-type PVC211 Env using mCAT-1 receptor-negative packaging cells not allowing for receptor interference and syncytia formation. The findings of this study were published in *Virology* [93].

3 Publications

3.1 Publication 1

Transposon vector-mediated stable gene transfer for the accelerated establishment of recombinant mammalian cell pools allowing for high-yield production of biologics

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REVIEW

Transposon vector-mediated stable gene transfer for the accelerated establishment of recombinant mammalian cell pools allowing for high-yield production of biologics

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Abstract Stable recombinant mammalian cells are of growing importance in pharmaceutical biotechnology production scenarios for biologics such as monoclonal antibodies, growth and blood factors, cytokines and subunit vaccines. However, the establishment of recombinant producer cells using classical stable transfection of plasmid DNA is hampered by low stable gene transfer efficiencies. Consequently, subsequent selection of transgenic cells and the screening of clonal cell populations are time- and thus cost-intensive. To overcome these limitations, expression cassettes were embedded into transposon-derived donor vectors. Upon the co-transfection with transposase-encoding constructs, elevated vector copy numbers stably integrated into the genomes of the host cells are readily achieved facilitating under stringent selection pressure the establishment of cell pools characterized by sustained and high-yield recombinant protein production. Here, we discuss some aspects of transposon vector technologies, which render these vectors promising candidates for their further utilization in the production of biologics.

Keywords Mammalian cells · *piggyBac* · Protein production · *Sleeping Beauty* · Transposon vector

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Introduction

With the growing demand for biotherapeutics at highest quality standards, mammalian cells are increasingly used for production. In 2018, 84% of the marketed biotechnologically produced drugs were generated using mammalian cells. The vast majority of these biologics are therapeutic monoclonal antibodies produced in Chinese hamster ovary (CHO) cells (Walsh 2018).

Recombinant mammalian protein producer cell lines are not only required for the final industrial scale production of proteins of interest (POIs). They are already needed in early phases of drug discovery and development. POIs are produced at research laboratory-scale to facilitate their biochemical and biophysical characterization followed by preclinical trials in small animal models in vivo. The classical approach to establish recombinant cell lines uses circular plasmid

DNA for stable transfection (Manceur et al. 2017). Although a wide range of transfection methods and reagents are available, some host cells show low transfectability. Even if the transient transfection efficiency—i.e. the epichromosomal cellular uptake of plasmids—is sufficient, the stable integration of linearized plasmid DNA into the host cell genome is a rare event. Depending on the host cell type, the donor organism and the transfection protocol used, most stably transfected cells only harbor low vector copy numbers (VCN) ranging from single to about five plasmid molecules randomly integrated into the host cell genome (Chusainow et al. 2009; Kolacsek et al. 2014). The integrated plasmid sequences do not only contain the expression cassette entailing a promoter/enhancer required to drive the expression of the gene of interest (GOI) encoding the POI and a polyadenylation signal (p(A)), but also long bacteria-derived sequences. At least an origin of replication (*ori*) and an antibiotic-resistance gene allowing for the selection and subsequent amplification of the plasmid in transformed bacteria are required. These bacterial sequences are recognized as foreign and targeted by the methylation machinery in mammalian cells. This results in silencing of proximate eukaryotic or viral promoter/enhancer driving the expression of the GOI (Riu et al. 2007) and can consequently prevent sustained high-level POI production. Typically, polyclonal or pooled cell populations do not produce satisfying POI yields. Thus, the screening of hundreds or even thousands of cell clones to identify a protein producer cell demonstrating efficient, stable and sustained expression levels of POI is indispensable (Wang et al. 2018). In summary, using stable transfection, all aforementioned limitations contribute to the time-consuming and cost-intensive processes to establish protein producer cells. Novel vector technologies are required to accelerate the generation of transgenic cells at early drug development stages. Vector systems derived from transposons were developed and further improved in the past 15 years allowing the rapid establishment of stable recombinant mammalian cell pools for the high-yield production of biologics.

DNA transposon biology and host cell interaction

DNA transposons are fossil mobile DNA elements. A wide range of individual DNA transposons from different families and donor organisms were characterized in detail over the last three decades e.g. *Tol2* originating from medeka fish, *Sleeping Beauty* - synthetic sequences derived from transposons found in the white cloud minnow, atlantic salmon and rainbow trout—and *piggyBac* isolated from the cabbage looper moth (Fraser et al. 1996; Ivics et al. 1997; Kawakami et al. 1998). All DNA transposons are composed of a transposase gene and flanking inverted terminal repeats (ITRs; Muñoz-López and García-Pérez 2010). The enzyme transposase recognizes specific short target sequences, called directed repeats (DRs) located in the ITRs. Upon binding, the transposase cuts out the transposon sequence from the surrounding genomic DNA of the host cell. The formed complex consisting of the mobilized transposon DNA fragment and the still bound transposases is now able to change its position to a new location in the cell genome. The transposases open the genomic DNA backbone at the new *locus* and insert the transposon fragment. The ligation of the open DNA ends is mediated by cellular key factors of the non-homologous end joining pathway (NHEJ) within the double strand break (DSB) repair system (Mátés et al. 2007). Thus, this so called transposition uses a cut-and-paste mechanism.

The examination of the sequences targeted by the respective transposases for re-integration into the genomic DNA of the host cell revealed differences between various transposons. While *Tol2* of the *hAT* family could not be shown to prefer a specific sequence, members of the *Tc1/mariner* family like *Sleeping Beauty* (SB), *Frog Prince* and *Minos* as well as *piggyBac* (PB; superfamily PB) clearly favor defined insertion motifs. With the dinucleotide TA for *Tc1/mariner* transposons and the four-nucleotide motif TTAA for PB, these target sequences are very short, and thus would allow close- to-random integration over the entire host cell genome (Grabundzija et al. 2010). This assumption was further supported by the findings that *Tol2*, SB and PB did not show any preference for specific host cell chromosomes. *Tc1/mariner* transposons including SB were demonstrated to perform close-to-random integration. Although not very pronounced, there seems to be a weak bias in mammalian cells towards the insertion into transcribed regions and their regulatory sequences located upstream (Yant et al. 2005; Huang et al. 2010; Gogol-Döring et al. 2016). In contrast, *Tol2* and PB

favor certain specific genomic regions. Both, *Tol2* and PB, insert mostly upstream and in close proximity to transcriptional start sites (TSSs), CpG-islands and DNase I hypersensitive sites (Huang et al. 2010). For PB it was recently shown (Gogol-Döring et al. 2016) that the cellular BET proteins interact with the transposase and guide the accumulation of insertions to TSSs. In this regard, PB shows a high similarity to the γ-retrovirus murine leukemia virus (MLV; Wu et al. 2003; de Jong et al. 2014; Gogol-Döring et al. 2016).

Only a few cellular proteins interacting with the transposase have been described to date. In a yeast two-hybrid screen the transcription factor Myc-interacting protein zinc finger 1 (Miz1) was identified to interact with SB transposase (Walisko et al. 2006). As a result the expression of cyclin D is down-regulated in transgenic human cells leading to a temporary arrest in cell cycle phase G_1. Integration into the host cell genome appears to be more efficient during a prolonged G_1 phase. The DNA-bending high mobility group protein 1 (HMGB1) was shown to be crucial to facilitate efficient transposition. While transposition was largely limited in HMGB1-deficient murine cells, this restriction was abrogated by transient recombinant over-expression of HMGB1 and partially overcome by HMGB2. It is assumed, that at least HGMB1 serves as a co-factor for binding of the transposase to the target DR sequences in the ITRs, and thus supporting the formation of the synaptic transposase-DNA complex during transposition (Zayed et al. 2003). In contrast, transposition of PB appears to be largely cell factor independent as it can be experimentally reconstituted in vitro using purified PB transposase and DNA elements (Burnight et al. 2012). Like retroviruses, SB as well as PB seem to exploit the cellular barrier to autointegration factor (BAF) to promote transposon integration into the host genome at high efficiencies by preventing autointegration (Wang et al. 2014).

DNA transposon vectors

As illustrated in Fig. 1, in a two-component DNA transposon-derived vector system the transposase gene and the ITRs are separated onto two different plasmids. The transposase construct minimally entails a suitable promoter active in the desired host cell, the sequence encoding the transposase and a 3'-located p(A). The donor or transposon vector encompasses an expression cassette with the GOI flanked by the ITRs. Upon co-transfection of target cells with both plasmids, the transposase is expressed and cuts out the GOI expression cassette framed by the ITRs from the plasmid backbone. The subsequent stable insertion of the transposon vector into the genome concludes the transposition process (Ivics et al. 1997). In contrast to transgenic cells established by classical stable transfection using plasmids, the integrated transposon vectors do not contain any bacterial sequences originating from the plasmid vector backbone. Consequently, silencing of the promoter driving GOI expression should presumably occur at much lower frequency (Riu et al. 2007). Amongst other features, this renders transposon vectors attractive tools for the establishment of recombinant protein producer cells.

Vector systems derived from SB and PB are most frequently used for biotechnological applications. However, the first generation of transposon vectors derived from SB was characterized by low transposition efficiencies (Ivics et al. 1997). The stepwise optimization of the SB transposase by the introduction of accumulating mutations led in a pioneering study to the evolved enzyme SB100X revealing a 100-fold improved activity as compared to the wild-type protein (Mátés et al. 2009; Voigt et al. 2016). The transposase of PB, namely mPB, was first adapted to mediate 20-fold enhanced transposition efficiency in mammalian cells employing codon optimization (Cadiñanos and Bradley 2007; Liang et al. 2009). Following the example of improving transposase activity by means of molecular evolution (Mátés et al. 2009), the mPB enzyme was further advanced. The resultant hyPB was demonstrated to mediate 10-fold enhanced transposition activity in mammalian cells as compared to mPB (Yusa et al. 2011). Cui and co-workers (Cui et al. 2002) described the optimization of the ITR sequences of SB transposon vectors leading to a four-fold increase of transposition events in human HeLa cells. Later attempts to improve transposition efficiencies of SB vectors by the introduction of additional point mutations within the ITRs did unfortunately not lead to further improvements (Scheuermann et al. 2019).

The achieved transgenic rate—i.e. the percentage of stably genetically modified cells as a result of stable integration of the donor vector into the host cell genome—using SB and PB two-component vector

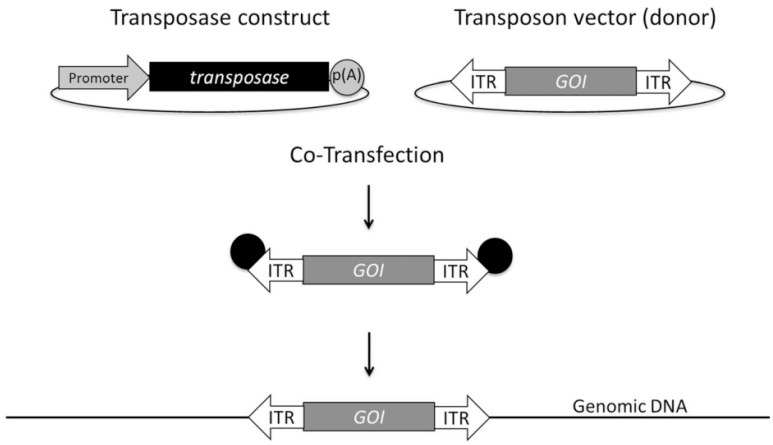

Fig. 1 Two-component transposon vector systems consist of a transposase construct minimally encompassing a promoter/enhancer upstream of the transposase coding region and a polyadenylation signal (p(A)). The transposon or donor vector contains the gene of interest (GOI) flanked by the inverted terminal repeats (ITRs). Upon co-transfection into suitable host cells, the transposase expressed in trans binds to the ITRs and cuts out the transposon vector fragment from the bacterial plasmid backbone and mediates the integration into the cell genome using a cut-and-paste mechanism

systems obviously depends on the transfectability of the desired target cell and the transfection protocol employed, which may require individual optimization. The efficiency of transposition can be divided into three different subsequent events: (i) the excision of the transposon vector from the plasmid backbone, (ii) the overall stable transgenic rate and (iii) the average VCN per cell (Kolacsek et al. 2014). The transposition efficiencies observed in different cell types are highly variable (Izsvák et al. 2000; Troyanovsky et al. 2016). Besides the amount of plasmid DNA transfected per target cell count (high versus low dose), optimization of the transposition rate and VCN per cell can be improved by determining the optimal ratio of transposase construct to transposon vector (Grabundzija et al. 2010). For the utilization of SB-derived vector systems, it has to be emphasized that an excess of transposase hampers efficient transposition—a phenomenon termed overexpression inhibition (OPI). It was hypothesized that a high concentration of transposase saturates the target DR sequences in the ITRs slowing synapsis formation of transposon ends (Liu and Chalmers 2014). Consequently, the optimal SB transposase construct to donor ratio, depending on the genetic design of the expression cassettes, ranges in most cell types from 1/5 to 1/30. PB transposase does not suffer from OPI (Wilson et al. 2007). Nevertheless, also for PB systems the optimal balance of vector components has to be examined for every target cell aiming to achieve the highest transgenic rate.

Higher transgenic rates are generally achieved using larger amounts of transposon vector molecules in high dose transfection protocols (e.g. >500 ng DNA per 1×10^5 cells). Depending on the host cell line, its transfectability and its assistance in transposition, about 10 to 30% of the transfected cells are stably modified. The DNA dosage also influences the integrated copy numbers per cell. Using low dose approaches (15–50 ng per 1×10^5 cells) the vast majority of transgenic cells show single vector integrations. Here, usually 1 to 10% of the transfected cells reveal stable vector integration. In contrast and upon high dose transfection, PB transposon vectors reach single to single-digit number of vector insertions per cell, respectively. However, SB100X facilitates up to 40 integrations under these conditions (Grabundzija et al. 2010). The high vector copy numbers per cell make SB vectors attractive for the establishment of stable producer cell lines. However, PB vectors seem to have the advantage of targeting favorable sites close to TSSs resulting in higher levels of transgene expression. Besides the high transposition rates achieved with both transposon vector systems, SB and PB qualify for a wide range of biotechnological

applications due to their considerable high cargo capacity. SB vectors were reported to be instrumental in gene transduction carrying payloads of up to 6 kb. Larger transgene cassettes lead to declining transposition frequencies (Izsvák et al. 2000; Rostovskaya et al. 2012). However, SB and PB vectors were demonstrated to mediate stable transgene transfer of even larger DNA molecules such as artificial bacterial chromosomes (BACs) with sizes of up to impressive 200 kb outperforming retroviral vectors with maximum cargo capacities of about 6 kb (Li et al. 2011; Rostovskaya et al. 2012).

Transposon vectors for the accelerated establishment of mammalian protein producer cell pools

Due to their ability to stably transduce genetic cargo into a variety of mammalian cell types, transposon vectors, particularly derived from SB and PB, provide a toolbox for many applications. These include the generation of transgenic animals, the development of gene knock-out screens in gene function discovery, the establishment of inducible pluripotent stem cells (iPSCs) and the utilization in somatic gene therapy (Di Matteo et al. 2012; Yusa 2015; Narayanavari et al. 2017; Tipanee et al. 2017). In these fields, transposon vectors are increasingly used as alternatives to retroviral vector-mediated gene transfer (Vargas et al. 2016).

The generation of polyclonal pools of genetically modified mammalian cells for the purpose of recombinant protein production was first demonstrated using MLV and HIV-1 vectors mediating gene transduction (Oberbek et al. 2011; Stitz 2011; Elegheert et al. 2018). These approaches capitalized on the stable integration of multiple vector copies per cell and the sustained expression of the transgenes as a result of the favored insertion sites located in the proximity of transcriptional start sites and active cellular transcription units (Craigie and Bushman 2014; Gogol-Döring et al. 2016). The productivity of the recombinant cell pools and cell clones established upon viral vector-mediated gene transduction was remarkable. However, the utilization of viral vector technology requires (i) the establishment of stable or transient viral packaging cells, upon co-expression of at least three different constructs, namely, the transfer vector harboring the transgene of choice, a packaging construct minimally encompassing the structural genes *gag* and *pol* encoding for the viral core proteins and enzymes and an envelope construct enabling the expression of the Env proteins facilitating vector particle cell entry. (ii) The subsequent titration of produced vector particle preparations in suitable susceptible target cells has to be conducted. (iii) In case vector particles with a tropism for human cells or pantropic vectors pseudotyped with the G-protein of vesicular stomatitis virus (VSV-G) are used, a biosafety level 2 laboratory (BSL-2) is indispensable. In summary, the complexity of viral vector production and characterization reduces the attractiveness of retrovirus technology for the establishment of transgenic cell pools for protein production.

The utilization of transposon vectors is comparably convenient and straightforward as illustrated in Fig. 2. The required optimization of transfection protocols and the assessment of the optimal transposase construct to transposon vector ratio for the host cell of choice are standard practices easily established in any laboratory and are quickly performed by experienced staff. Usually, the generated recombinant mammalian cells are classified as genetically modified organisms of BSL-1, provided that the mobilized payload is not hazardous itself e.g. oncogenes. PB and SB vector systems are both instrumental in the rapid establishment of transgenic cells in vitro. Multiple stable insertions of vectors in the host cell genome are readily achieved. With a possible payload of far more than 6 kb, both transposon vector systems exceed the capacity requirements for the purpose of protein production. Even two protein encoding expression cassettes *in cis* are easily mobilized, e.g. for the co-production of heavy and light chains of monoclonal antibodies (mAbs; Ahmadi et al. 2017).

Matasci et al. (2011) were the first to use PB vectors to establish pools of CHO cells producing tumor necrosis factor receptor (TNFR) fused to the Fc-fragment of human immunoglobulin G1 (IgG1). The bicistronic donor vector entailed a cytomegalovirus promoter/enhancer (P_{CMV}) driving the expression of the fusion protein and a herpes simplex virus thymidine kinase promoter ($P_{HSV\text{-}TK}$) coupled to a *pac* gene mediating resistance against puromycin. Using a mPB transposase expression construct to donor vector ratio of 1:9, transgenic cells were established upon co-transfection. One day post transfection, cells were subjected to selection by expansion

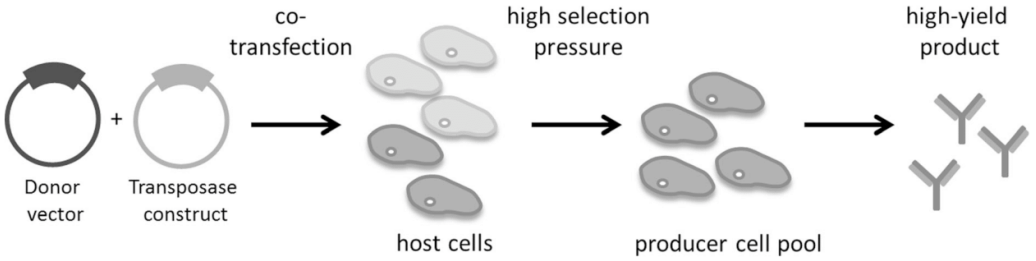

Fig. 2 Rapid establishment of protein producer cells using a two-component transposon vector system by co-transfection of the donor vector and the transposase construct into host cells. One or two days post transfection, selection pressure can be applied to generate a high-yield producer cell pool

in the presence of puromycin at a concentration of 10 and 50 µg/ml. Both resultant stable cell pools—PB(10) and PB(50), indicating the selection pressure applied—facilitated considerable product yields of 42 and 50 mg/l TNFR:Fc, respectively. A number of cell clones were isolated from both pools and analyzed for their productivity. While more than 34% of the PB(50)-derived clones revealed a productivity reaching 50 to 100 mg/l, only 28.6% of the clones originating from the parental PB(10) pool were as productive. This difference in cell productivity was even more visible for yields between 25 and 50 mg/l with 55.8% of all PB(50)-derived clones and only again 28.6% of PB(10) cell clones. Only less than 10% of the PB(50) cell clones produced less than 25 mg/l. In contrast, 40% of PB(10) clones were significantly less productive than the parental pool. As expected, enhanced productivity correlated with higher VCN per cell ranging from an average of three copies for the least productive clones up to six copies for the best performing ones. In conclusion, this demonstrated the rapid establishment of cell pools with advanced productivity employing PB vectors and very stringent selection. Noteworthy, cell productivity of pools and clones was stable over a period of three months in the absence of selection (Matasci et al. 2011).

In 2016, the research team led by Dr. Wurm used the above described expression cassettes in transposon vectors derived from *Tol2*, SB and again PB to compare the three vector systems for their potential to establish CHO producer cell pools and clones (Balasubramanian et al. 2016a). The optimal donor to transposase construct ratio for each vector system using mTol2, mPB and SB100X transposases was assessed. *Tol2* vectors showed dramatically lower transposition efficiencies as compared to SB and PB. In fact, even with the best performing donor to transposase construct ratio employed, at least over 60% of the inserted *Tol2* vectors were demonstrated to be a result of stable transfection rather than transposition. Consequently, and in the absence of selection pressure, cell pools established with PB and SB vectors generated significantly higher transgenic rates of about 20% stably modified cell populations as compared to less than 5% using the *Tol2*-derived system. In addition, SB and PB vectors reached more than three-fold higher transgene expression levels than *Tol2* vectors. However, and upon application of a low selection pressure (10 µg puromycin/ml) for ten days, pools of cells were established reaching productivities ranging from 80 mg/l of TNFR:Fc using *Tol2* vectors to 95 mg/l for PB and SB vectors. On average, ninefold higher yields were obtained using transposase-mediated gene transfer as compared to conventional stable transfection of plasmids. In 14-day fed-batch cultures of pools, TNFR:Fc levels of up to 900 mg/l were achieved. Over a period of three months in the absence of selection pressure, productivity decreased to approximately 50% of the initial yields for all pools generated with the three vector systems. This underscored the necessity of stringent selection to establish sustained high-yield expression.

PB vectors were also utilized to establish CHO cell pools for the production of monoclonal antibodies. The expression of heavy and light chain was driven by a P_{CMV} whereas a SV40 promoter mediated the expression of glutamine synthetase (GS). These expression cassettes present on one donor vector were flanked by insulator sequences to minimize post-transpositional silencing. Stable recombinant cell

pools cultivated in shaker flasks were established producing four different mAbs. Yields of 2.3 to up to remarkable 7.6 g/l were reported (Rajendra et al. 2016). Unfortunately, the identity of the produced antibodies was not disclosed. The high productivities of the cell pools were shown to originate from up to seven VCN per cell leading to high expression levels of mRNA. Most importantly, the cell pools revealed a very high homogeneity resulting from highly productive clones composing the vast majority of the population. The scale-up from shaker flasks to a 36 l bioreactor delivered yields of 4.7 g/l for one of the monoclonal antibodies (Rajendra et al. 2017a). CHO pools established using PB vector-mediated gene transduction produced mAbs of the same quality as compared to mAbs harvested from clonal producer cell lines generated by stable transfection. This finding was also confirmed in a second study conducting capillary electrophoresis-sodium dodecyl sulfate (CE-SDS), glycan analysis, analytical size-exclusion chromatography (aSEC) and peptide mapping analysis using liquid chromatography and mass spectrometry (LCMS; Rajendra et al. 2017b).

High yields of multiple products can also be achieved by the generation of cell pools using simultaneous co-expression from multiple individual transposon vectors upon co-transfection with a transposase construct. The team around Balasubramanian (Balasubramanian et al. 2016b) demonstrated the establishment of CHO cell pools producing three model proteins, namely, enhanced green fluorescent protein (EGFP), secreted alkaline phosphatase (SEAP) and a monoclonal antibody. Not surprisingly, the productivity of the pools was superior when each individual transgene encompassing transposon vector carried a different selectable marker allowing for the application of three separate selection pressures at the same time. Accordingly, we recently reported on the rapid establishment of highly efficient viral packaging cell lines (VPCs) using SB-derived transposon vectors in human HT-1080 fibrosarcoma cells (Berg et al. 2019). All three vector components required—the packaging and *env*-construct and the transfer vector as well—were co-transfected with the transposase construct and allowed for subsequent triple-selection. Within only three weeks, VPCs were established reaching vector particle yields of over 1.0×10^6 transducing units per ml (TU/ml). In contrast, VPCs that were generated by conventional stable plasmid transfection achieved 20-fold lower titers and required an extended establishment time of three months.

The previously mentioned findings demonstrate the utility of transposon vectors for the future generation of cell pools to rapidly produce second generation biologics composed of multiple components. Examples for these complex biologics are viral vector particles for somatic gene therapy and enveloped virus-like particle (VLP) vaccines displaying single or multiple target antigens at high density on their surface. There is a demand in the pharmaceutical industry for accelerated producer cell line development without lowering product yields or compromising product quality to maintain the provision of biologics at economically affordable prices to the growing market.

Conclusions and outlook

Amongst others, transposon vector systems derived from *Sleeping Beauty* and *piggyBac* are most frequently used for biotechnological applications. Transposases were optimized and donor vectors further developed enabling the rapid establishment of highly productive recombinant cell pools. Upon a brief optimization of the co-transfection method and the donor vector to transposase construct ratio, a wide range of host cells is susceptible to these relatively new vector systems. The technology capitalizes on the efficient stable integration of multiple transgene expression cassettes per cell. Using the industry gold standard CHO as host cells, transgenic rates of 20% are readily achieved. Antibiotic resistance marker genes coupled to the transgene of choice facilitate the stringent selection required to establish highly productive pools of cell clones in a short time. Transposon vectors were shown to overcome the limitations of conventional stable plasmid transfection and will thus prove their utility and value for the future development of novel biologics and their industrial scale production.

However, the development of these vectors is still in an early pioneering stage. Most importantly, the vector design can certainly be further improved. Some advancement was already achieved in enhancing expression of transgenes by insertion of matrix attachment regions (MARs; Ley et al. 2013). To minimize gene silencing, the utilization of flanking

cHS4 DNA insulators was instrumental (Sharma et al. 2012). Transposon vectors encompassing inducible transgene expression cassettes for the production of cytotoxic proteins were generated (Kowarz et al. 2015; Michael and Nagy 2018). To avoid the use of antibiotics during transgenic cell selection and product manufacturing, vectors were designed for the establishment of cell pools upon chemically induced dimerization of growth factor receptors (Kacherovsky et al. 2012). Novel panels of transposon vectors will have to be tailored combining multiple genetic elements and vector components to further enhance expression levels and to meet specific requirements of future industrial production scenarios.

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3.2 Publication 2

Ecotropic HIV-1 vectors pseudotyped with R-peptide-deleted envelope protein variants reveal improved gene transfer efficiencies

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Brief Communication

Ecotropic HIV-1 vectors pseudotyped with R-peptide-deleted envelope protein variants reveal improved gene transfer efficiencies

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ABSTRACT

Viral vectors derived from human immunodeficiency virus type 1 (HIV-1) mediate efficient stable gene transduction. Consequently, these vectors are utilized in gene therapeutic approaches. We here aimed for improving HIV-1 pseudotype vector formation using envelope proteins (Env) of ecotropic murine leukemia virus (MLV) suffering deletions of the R-peptide and further amino acid substitutions in their cytoplasmatic domains. All examined Env variants revealed cell-surface expression and showed elevated fusogenicity as compared to wildtype (eMLV-wt) Env but failed to efficiently pseudotype MLV particles. However, two variants generated ecotropic HIV-1 pseudotype vectors with superior infectivity. Most importantly, pseudotyping with the variant eMLV-GaLVΔR encompassing the R-peptide-deleted cytoplasmic domain of the gibbon ape leukemia virus Env yielded titers three-fold higher than HIV(eMLV-wt) vectors. We anticipate that superior ecotropic HIV(eMLV-GaLVΔR) pseudotype vectors will be of utility in preclinical gene therapy studies aiming at the genetic modification of primary murine cells.

1. Introduction

Viral vectors derived from the retrovirus family are frequently used in somatic gene therapy. Particularly, vectors based on murine leukemia virus (MLV) and the human immunodeficiency virus type 1 (HIV-1) mediate gene transduction at high efficiencies and subsequent stable integration of the gene of interest into the target cell genome. These characteristics allow for example the delivery of therapeutic genes *ex vivo* e.g., into hematopoietic stem (HSCs) and early progenitor cells in approaches to treat diseases like adenosine deaminase-deficient severe combined immunodeficiency (ADA-SCID; Aiuti et al.,2009) or Wiskott-Aldrich syndrome (WAS; Ferrua et al., 2019). To elevate gene transduction, retroviral vectors are commonly pseudotyped with the glycoproteins of vesicular stomatitis virus (VSV-G). However, the expression of VSV-G is cytotoxic, and thus does not facilitate the establishment of stable packaging cell lines constitutively producing vector particles (Yee et al., 1994). In addition, the resulting pantropic pseudotype vectors require experimental work being conducted under biosafety level 2 (BSL-2) conditions as the recruited low density lipoprotein (LDL)-receptor is expressed ubiquitously on different cell types from different donor organisms (Finkelshtein et al., 2013; Koch et al., 2006). Prior to clinical gene therapy trials, preclinical mouse models are frequently employed to evaluate and to optimize gene transfer efficiencies. Here, retroviral vectors pseudotyped with the envelope proteins (Env) of ecotropic MLV are often used with a host cell tropism restricted to rodent cells recruiting the receptor murine cationic amino acid transporter (mCAT), and thus enabling production under biosafety level 1 (BSL-1) conditions (Albritton et al., 1989). We recently demonstrated the efficient gene transfer into murine HSCs and progenitor cells mediated by ecotropic MLV vectors pseudotyped with Env of Friend MLV (FrMLV) molecular clone PVC-211mc (van Heuvel et al., 2021).

To improve gene transduction efficiencies, retroviral Env proteins can be modified by introducing mutations in den transmembrane region (TMR) and truncation of the C-terminus of the transmembrane Env protein (TM), respectively (Russell and Cosset, 1999). The C-terminal 16 amino acids of the MLV TM form the so-called R-peptide, which is cleaved off by the viral protease during particle maturation. Cleavage of the R-peptide induces conformational changes in the Env proteins resulting in enhanced fusogenicity and mediating particle-cell-entry upon receptor recognition (Bobkova et al., 2002). We here report the generation of FrMLV Env variants lacking the R-peptide and further amino acid substitutions in their cytoplasmatic domains anticipating

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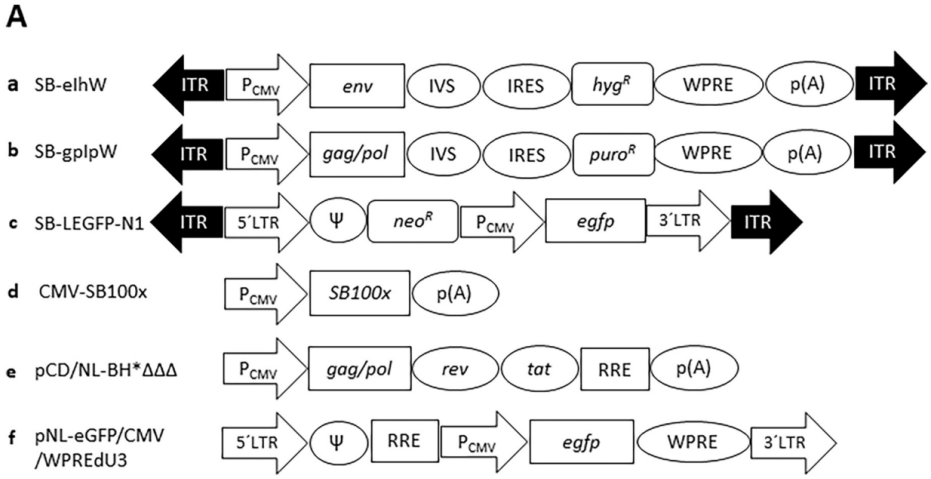
that the expected elevated fusogenicity would improve vector particle titers. We consequently generated expression vectors encoding for wildtype (eMLV-wt) PVC-211mc Env as well as the R-peptide-deleted variant eMLV-ΔR.

Previous studies demonstrated pseudotyping of MLV and HIV-1 vector particles with chimeric avian spleen necrosis virus/gibbon ape leukemia virus (SNV/GaLV) Env variants. The variant CGaΔR encompassing the entire SNV Env ectodomain and TMR fused to the GaLV-originating cytoplasmic tail (CT) lacking the R-peptide was most efficiently incorporated and mediated the highest vector titers of MLV and



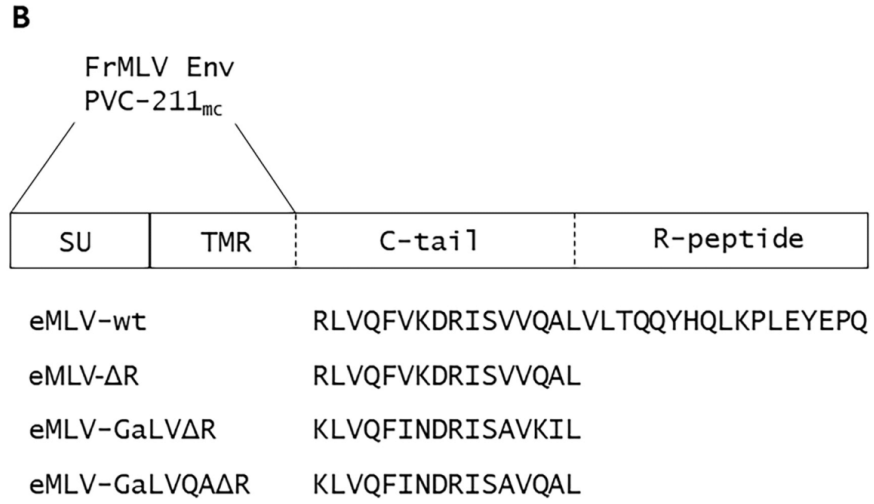


Fig. 1. Schematic illustration of the constructs used in this study (A). In the envelope construct (a) SB-eIhW, a cytomegalovirus (CMV) promoter/enhancer element (P_{CMV}) drives the expression of ecotropic envelope gene *env* derived from the Friend MLV molecular clone PVC- 211 and its derivates, followed by a synthetic intron (IVS), an internal ribosome entry signal (IRES) and a hygromycin-resistance gene (hyg^R). The Woodchuck hepatitis virus post transcriptional regulatory element (WPRE) and the polyadenylation signal (p(A)) of the bovine growth hormone gene are located downstream. The inverted terminal repeats (ITRs) flank the expression cassette at the 5′- and 3′-end. The packaging construct (b) SB-gpIpW harbors the MLV *gag/pol* genes and the puromycin-resistance gene ($puro^R$). The transfer vector construct (d) 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*. The transposase construct (d) CMV-SB100X harbors the human codon-optimized sequence coding for the hyperactive *Sleeping Beauty* transposase variant *SB100x*. (B) Amino acid sequences of the envelope eMLV-wt and the generated envelope variants. All Env variants, suffer deletions of the R-peptide. Variant eMLV-ΔR is the matured form of eMLV-wt Env. The remaining C-tails of eMLV-GaLVΔR and eMLV-GaLVQAΔR originate from gibbon ape leukemia virus (GaLV). In variant eMLV-GaLVQAΔR two amino acids were exchanged with the corresponding amino acids of eMLV-wt Env.

HIV-1 pseudotype vectors even exceeding the utilization of wt SNV Env (Stitz et al., 2006). Based on these findings, we generated a chimeric Env variant eMLV-GaLVΔR Env. Yet in another report, GaLV Env proteins entailing alterations within their CTs were selected for mediating efficient formation of transduction-competent HIV-1-derived vectors. Besides the R-peptide-deleted Env, variant GaLV-RTM revealed the highest potency upon R-peptide cleavage. The resultant matured Env protein CT showed only two amino acid exchanges as compared to wt GaLV - QA for KI (Merten et al., 2005). We therefore employed this CT to construct the chimeric Env variant eMLV-GaLVQAΔR. All three Env variants and eMLV-wt Env were examined for cell-surface expression, fusogenicity and for their ability to mediate efficient gene transduction of pseudotyped MLV and HIV-1 vectors upon incorporation into particles.

2. Results

2.1. All R-peptide-deleted Env variants mediate syncytium-formation

Env variant genes derived from PVC-211 were inserted into *Sleeping Beauty* donor expression vector SB-eIhW as illustrated in Fig. 1 A (Berg et al., 2019). The Env variants all encompass the entire PVC-211 Env ectodomain and transmembrane spanning region. While the eMLV-wt Env protein harbors the complete cytoplasmic domain (C-tail), all three variants lack the R-peptide as depicted in Fig. 1 B. The N-terminal part of C-tail of variant eMLV-ΔR is identical to that of eMLV-wt Env but devoid of the R-peptide. In contrast, the Env variant eMLV-GaLVΔR entails the R-peptide-deleted C-tail of GaLV Env. Variant eMLV-GaLVQAΔR shows two amino acid substitutions as compared to eMLV-GaLVΔR, i.e. KI were exchanged by QA.

The flow cytometric analysis of HT-1080 cells transiently transfected with the respective envelope constructs using gp70-SU-specific antibody and anti-rabbit polyclonal antibody conjugated with Alexa Fluor 488 demonstrated surface expression for eMLV-wt Env and all three Env variants (Fig. 2A). To examine the fully functional expression of Env variants, a syncytium-formation assay was conducted. Naïve HT-1080 cells were transiently co-transfected with respective envelope constructs and an EGFP expression construct and subsequently co-cultured with HT-1080/mCAT cells recombinantly expressing the ecotropic receptor (Berg et al., 2019). HT-1080 cells only transfected with the EGFP expression construct served as negative controls. Co-cultures were subjected to fluorescence microscopic analysis and monitored over a time period up to three days post transfection. As expected and depicted in Fig. 2B showing representative pictures taken at day one post transfection, no syncytia were observed in the eMLV-wt Env-expressing cell population. In contrast, all three Env variants mediated syncytium-formation. This demonstrated the functional expression of all Env variants, namely, cell surface expression, receptor-binding and fusogenicity. However, notably more syncytia with more nuclei were observed in cultures expressing eMLV-ΔR and eMLV-GaLVΔR as compared to eMLV-GaLVQAΔR indicating a reduction of fusogenicity mediated by only two amino acid residue exchanges.

2.2. Truncated Env variants fail to efficiently pseudotype MLV vector particles

We first established stable MLV-based packaging cell pools by co-transfection and subsequent selection of HT-1080 cells using the *Sleeping Beauty* vector system components CMV-SB100x (transposase construct), SB-gpIpW (packaging construct), SB-LEGFP-N1 (transfer vector) - all illustrated in Fig. 1 A - and the respective envelope variant expression construct as previously described in details (Berg et al., 2019). Cell-free supernatants of packaging cell pools were used to transduce NIH/3T3 murine target cells in three independent experiments. Three days post transduction, FACS analysis revealed efficient transduction using MLV(eMLV-wt) vectors reaching a mean titer of 3.0 $\times$ 10^5 TU/mL with a standard deviation (SD) of 1.5 $\times$ 10^5. MLV (eMLV-GaLVΔR) and MLV(eMLV-ΔR) vectors yielded titers one and even two magnitudes lower titers, namely, 1.1 $\times$ 10^4 (SD 8.5 $\times$ 10^3) and 9.9 $\times$ 10^2 TU/mL (SD 6.0 $\times$ 10^2), respectively. The titers obtained in all individual transduction experiments are shown inTable 1 in the

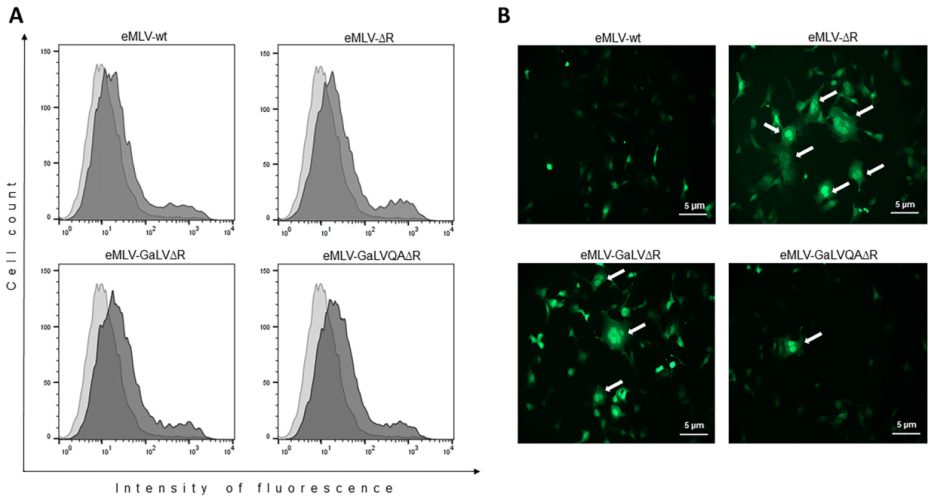


Fig. 2. Surface expression of envelope proteins on HT-1080 cells (A). Cells were transiently transfected with the respective envelope expression constructs. Three days post transfection, recombinant cells were stained with primary rabbit antibodies recognizing the surface unit gp70 of Friend MLV and secondary Alexa Fluor 488-coupled anti-rabbit antibodies and were analyzed for surface expression utilizing FACS (dark grey areas). Stained naive HT1080 cells served as negative controls (light grey areas). (B) Syncytium-formation induced by the respective envelope variants. HT-1080 cells were co-transfected with expression constructs encoding for the envelope proteins and the fluorescence protein EGFP. After 6 h of incubation, cells were co-cultured with HT-1080 cells recombinantly expressing mCAT. Pictures were taken after 24 h of co-culture at 200-fold magnification. Scale bars represent 5 µm and white arrows indicate syncytia. Representative images from three independent experiments are shown.

Table 1
HIV1 pseudotype vector titers obtained upon titration in HT-1080/mCAT target cells in three independent experiments. Titers are reported as transducing units per mL (TU/mL). Mean values from titrations are shown with the respective standard deviations (SD); n.d. (not detectable, titers $\leq 10^3$ TU/mL).

Envelope	Titer [TU/mL]			Mean ± SD
	Exp.1	Exp.2	Exp.3	
eMLV-wt	5.2×10^5	5.5×10^5	5.4×10^5	$5.3 \times 10^5 \pm 1.6 \times 10^4$
eMLV-ΔR	5.3×10^5	5.7×10^5	1.2×10^6	$7.5 \times 10^5 \pm 3.5 \times 10^5$
eMLV-GaLVΔR	1.9×10^6	1.4×10^6	2.3×10^6	$1.9 \times 10^6 \pm 4.4 \times 10^5$
eMLV-GaLVQAΔR	n.d.	n.d.	n.d.	n.d.

supplementary data section.

Vector particle pellets were analyzed for Env variant incorporation using antibodies specific for gp70-SU and MLV p30-CA capsid proteins and secondary antibodies coupled to horse radish peroxidase (HRP) performing Western blot analysis. Equal volumes of cell-free supernatants of the respective confluent packaging cell lines were harvested from T75 flasks and subjected to ultracentrifugation. Samples from naïve HT1080 and Env-negative vector particles (no Env) served as negative controls. Detection of the p30-CA revealed comparable amounts of vector particles produced in all packaging cell pools. The gp70-SU proteins of eMLV-wt Env were also readily detected. However, variants eMLV-ΔR, eMLV-GaLVΔR, and eMLV-GaLVQAΔR incorporation remained undetectable (supplementary data section, Fig. 1).

2.3. The Env variants eMLV-wt, eMLV-ΔR and eMLV-GaLVΔR are incorporated into lentiviral vectors

Western blot analysis of the HIV-1 pseudotype vector particle pellets resulting from equal volumes of cell-free supernatants was performed employing antibodies directed against gp70-SU. Samples of naïve HEK293T and Env-negative vector particles (no Env) served as negative controls. As shown in Fig. 3A, the gp70-SU proteins of the Env variants eMLV-wt, eMLV-ΔR and eMLV-GaLVΔR were readily detected. However, variant eMLV-ΔR was observed at much smaller quantities as compared to eMLV-wt and eMLV-GaLVΔR indicating its lower incorporation efficiency. Incorporation of eMLV-GaLVQAΔR remained undetectable. Parallel Gag quantitation using p24 ELISA revealed comparable amounts of Env displaying vector particles as illustrated in Fig. 3B.

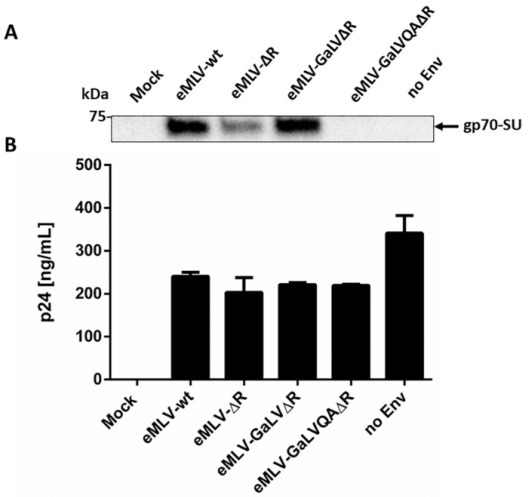


Fig. 3. Incorporation of Env proteins in HIV-1 vector particles (A). Cell-free supernatants harvested from lentiviral packaging cells and naïve HEK293T (Mock) cells serving as negative controls, were pelleted using ultracentrifugation. Western blot analysis of vector particle pellets was performed using polyclonal antibodies directed against FrMLV gp70-SU. The position of the molecular weight marker is presented on the left. (B) To ensure that comparable amounts of vector particles were produced, cell-free supernatants were analyzed using ELISA to detect capsid p24 and precursor Gag proteins. The assay was performed in duplicate. Error bars indicate standard deviation.

2.4. HIV-1(eMLV-GaLVΔR) vectors reach titers superior to HIV-1(eMLV-wt) vectors

To assess the potential of truncated Env variants to mediate efficient lentiviral pseudotype gene transduction, HEK293T cells were transiently co-transfected with a packaging construct, a transfer vector encoding the reporter gene *egfp*, both derived from HIV-1, and the respective ecotropic envelope expression vectors. Two days post transfection, cell-free vector particle preparations were harvested and subjected to transduction experiments using HT-1080/mCAT target cells. Target cells exposed to Env-negative vector particles served as negative controls. FACS analysis of target cells for EGFP expression performed three days post transduction revealed high gene transfer efficiencies mediated by Env variants eMLV-wt, eMLV-ΔR and eMLV-GaLVΔR. As shown in Table 1, detected mean vector titers ranged from 5.3×10^5 for eMLV-wt Env, to 7.5×10^5 and 1.9×10^6 TU/mL for eMLV-ΔR Env and eMLV-GaLVΔR Env, respectively. Gene transduction using variant eMLV-GaLVQAΔR was not detectable (n.d.; titers $\leq 10^3$ TU/mL). Notably, utilization of eMLV-GaLVΔR Env advanced titers reached with HIV-1 (eMLV-wt) by more than three-fold.

3. Discussion

New gene therapeutic strategies and approaches using lentiviral vectors are most frequently pre-clinically tested in primary murine cells and mouse models (Gopinath et al., 2015). In contrast to the utilization of envelope proteins mediating an extented host range including non-rodent target cells, pseudotyping of such vectors with envelope proteins derived from ecotropic MLV allow for the generation of vector particles and subsequent gene transduction under biosafety level 1 conditions (NIH, 2019). The Env proteins of FrMLV PVC-211mc are here of particular interest as they mediate efficient gene transduction not only into murine cell lines and primary hematopoietic stem and progenitor cells but also hamster cells (Berg et al., 2019; Stitz, 2011; van Heuvel et al., 2021).

With the aim to further enhance the transduction competence and therefore gene transfer efficiency of ecotropic retroviral and lentiviral vectors, we generated Env variants derived from PVC-211mc lacking the R-peptide and with truncated cytoplasmic tails originating from gibbon ape leukemia virus. We anticipated that the truncated Env variants would reveal enhanced fusogenicity, and thus could potentially elevate vector particle infectivity (Bobkova et al., 2002; Merten et al., 2005; Stitz et al., 2006).

Employing FACS analysis, CT-truncated and eMLV-wt Env proteins were demonstrated to reveal comparable cell-surface expression levels on HT-1080 cells transiently transfected with the respective envelope constructs. This underscored the previous report of Kubo and co-workers (Kubo et al., 2019) that the absence of the R-peptide does not alter surface expression. As expected and utilizing a syncytium-formation assay, all R-peptide-deficient Env proteins were shown to exhibit superior fusogenicity as compared to eMLV-wt Env, also revealing their ability to bind to the mCAT receptor (Bobkova et al., 2002).

However, when vector particles were harvested from established stable MLV-derived packaging cell lines and subjected to transduction experiments using NIH/3T3 target cells, Env variants eMLV-GaLVΔR and eMLV-ΔR, respectively, mediated gene transfer efficiencies of at

least one and two magnitudes lower as compared to eMLV-wt Env. No detectable gene transduction was achieved utilizing eMLV-GaLVQAΔR proteins. Western blot analysis of vector particle pellets was conducted using antibodies directed against the surface unit gp70 and secondary antibodies coupled with HRP. In contrast to the truncated Env variants, eMLV-wt Env proteins were readily detected. Our findings are partially in accordance with previous reports by Januszeski and Aguilar examining Moloney MLV(MoMLV) Env variants (Aguilar et al., 2003; Januszeski et al., 1997). They reported a dramatic decrease of infectivity upon R-peptide-deletion. However and in their studies, gp70-SU was still detectable in particles. In conclusion, this indicated that the R-peptide play a pivotal role in mediating efficient active incorporation of Env into MLV particles by interacting with Gag proteins recruiting Env during particle assembly (Rein et al., 1994; Schneider et al., 2011).

Since the generated Env variants could not efficiently incorporate into MLV particles, we decided to examine pseudotyping of HIV-1 vector particles with the ecotropic Env variants. To assess the incorporation of Env variants into HIV-1-derived vector particles, Western blot analysis employing antibody directed against the eMLV Env protein gp70-SU was conducted. In parallel, supernatants of packaging cells (VPCs) were examined for Gag concentration employing a p24 ELISA. All Env-positive VPCs showed comparable amounts of p24, and thus quantities of particles. Env variants eMLV-wt, eMLV-ΔR and eMLV-GaLVΔR were readily detected in lentiviral vector particle pellets. However, eMLV-wt and eMLV-GaLVΔR were much more efficiently incorporated than eMLV-ΔR. Variant eMLV-GaLVQAΔR Env incorporation was not detectable, despite only two amino acid substitutions as compared to variant eMLV-GaLVΔR at the very C-terminus of the proteins, namely, QA and KI, respectively. This could indicate that the CT of eMLV-GaLVQAΔR mediates exclusion of the Env proteins from lipid rafts, the areas HIV-1 assembly and budding, and thus prohibits Env incorporation into particles (Beer et al., 2005; Nguyen and Hildreth, 2000). In contrast, Env variants eMLV-wt, eMLV-ΔR and eMLV-GaLVΔR would necessarily need to co-locate with Gag during particle assembly. However and due to the very limited homology of C-type retroviruses such as MLV and GaLV and HIV, it appears unlikely that the CT of these variants would mediate direct Gag-Env-interaction. It is rather feasible to assume that the Env variants decorate lentiviral particles upon passive incorporation (Checkley et al., 2012).

Cell-free supernatants were also subjected to transduction experiments using HT-1080/mCAT cells. These cells recombinantly express the ecotropic receptor and are highly susceptible to transduction mediated by MLV- and HIV-1-derived pseudotype vectors facilitating gene transfer efficiencies two-to three-fold superior as compared to murine NIH/3T3 fibroblast target cells. Pseudotyping with variants eMLV- ΔR and eMLV-GaLVΔR reached titers superior to HIV(eMLV-wt) vectors. This was most prominent for HIV(eMLV-GaLVΔR) vectors showing a more than three-fold elevated mean vector titer of 1.9×10^6 TU/mL as compared to 5.3×10^5 TU/mL using eMLV-wt Env. This finding is in alignment with the study of Song and co-workers (Song et al., 2019) reporting that the exchange of the ΔR-CT of wildtype MLV with that of GaLV Env also facilitated the formation of infectious pseudotype particles. However, a comparison of wt Env and the R-peptide-deleted variant encompassing CT of GaLV Env was not conducted in this previous study. Notably, the CT of the Env variant GaLVΔR was previously demonstrated to mediate highly efficient incorporation into HIV-1-derived vector particles and elevating vector titers upon exchange of the CT of heterologous spleen necrosis virus (SNV) Env proteins (Stitz et al., 2006). Env variant eMLV-GaLVQAΔR failed to mediate efficient infectivity. Its CT domain, yet with a C-terminal R-peptide and fused to the GaLV transmembrane region and ectodomain, was previously reported to be efficiently incorporated into particles and processed by the HIV-1 protease facilitating high lentiviral pseudotype vector infectivity (Merten et al., 2005).

In conclusion, our study demonstrated that efficient pseudotyping of HIV-1-derived vectors was observed using eMLV-wt Env and the R-peptide-deleted variants eMLV-ΔR and eMLV-GaLVΔR, while eMLV-GaLVQAΔR failed to incorporate at detectable amounts. The Env variant eMLV-GaLVΔR mediated the highest vector titers exceeding the yields of HIV(eMLV-wt) by more than three-fold. We therefore anticipate that HIV(eMLV-GaLVΔR) vector particles will be of utility to enhance gene transfer efficiencies into murine primary cells and preclinical mouse models in the development of future gene therapeutic strategies.

4. Materials and methods

4.1. Cells

Human fibro-sarcoma HT-1080 cells and its recombinant derivates (ATCC CCL-121), murine fibroblast NIH/3T3 cells (ATCC CRL-1658) and human embryonic kidney HEK293T cells (ATCC CRL-3216) were grown in high glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-Glutamin and 10% FBS (Gibco BRL, Germany). Cells were cultured at 37 °C in a humidified atmosphere at 5% CO_2. For passaging, cells were detached using 1 mM EDTA in PBS.

4.2. Construction of envelope expression vectors

The envelope vector (SB-eIhW) the packaging construct SB-gpIpW, the transfer vector LEGFP-N1 and the transposase vector CMV-SB100x used in this study were previously described in detail (Berg et al., 2019). Human codon optimized (GenScript, USA) *env* gene of the Friend MLV clone PVC-211mc (GenBank accession no. AAA46478) served as a template for amplification of *env* gene variants. R-peptide-deleted eMLV-ΔR *envelope* gene was amplified using the oligonucleotides 5′-ATCACCATGGCATGTTCCACACTGTCCAAG AGCCC-3′ and 5′TTTAA ACCAGCACACTGGTTACAGAGCCTGGACCACTGAGATCCTGT CTTTAACAAATTGGACCAATCGATTCAGAATGCAAGGCCCGAACAG-3′. The cytoplasmic domain encoding region of the GaLV *env* (GenBank accession no. AF055060.1) was used to design the primers 5′-ATCAC CATGGCATGTTCCACACTGTCCAAGAGCCC-3′ and 5′-TTTAAACCAGCA CACTGGTTACAGAATTTTAACTGCACTTATCCTATCATTGATGAATTG-AACTAACTTATTCAGAATGCAAGGCCCGAACAG-3′ employed to introduce R-peptide-deleted cytoplasmic domain of GaLV p15-TM in the Env variant eMLV-GaLVΔR. The envelope variant eMLV-GaLVQAΔR was based on the amino acid sequences published by Merten and colleagues in 2005 (Merten et al., 2005). For amplification, the primers 5′-ATCAC CATGGCATGTTCCACACTGTCCAAGAGCCC-3′ and 5′-TTTAAACCAGCA CACTGGTTACAGTGCCTGAACTGCACTTATCCTATCATTGATGAATTG-AACTAACTTATTCAGAATGCAAGGCCCGAACAG-3′ were used. The generated *env* genes flanked by the restriction site for *EcoRV* and *BstXI* were digested accordingly and inserted into pIhW (Berg et al., 2019) opened with the same restriction enzymes to obtain the respective envelope constructs.

4.3. Flow cytometric analysis of cell-surface expression

HT-1080 cells were transiently transfected with the Env variant-encoding constructs. Three days post transfection, 1×10^6 cells were detached and incubated with 1 μg of polyclonal rabbit antibody directed against FrMLV gp70-SU (Genscript, NY) in 100 μL for 1 h on ice. Cells were washed with PBS and anti-rabbit Alexa-Fluor 488-conjugated secondary antibody (Abcam, USA) was used for detection of Env-expressing cells employing a S3e flow cytometer (Bio-Rad, USA).

4.4. Syncytium-formation assay

To determine the fusogenicity of the respective Env variants, 1×10^5 HT-1080 cells were co-transfected with 1.5 μg of the respective envelope constructs and 1 μg of an EGFP expression construct. After 6 h, cells were washed twice with PBS and overlaid with 1×10^5 HT-1080/mCAT cells overexpressing the receptor of ecotropic MLV (Berg et al., 2019). On the

following day, fixation of cells was performed using 4% Histofix solution (Carl Roth, Germany) for 10 min. Fixation solution was removed and cells were overlaid with PBS for subsequent fluorescence microscopy (Axio Vert a1, Zeiss, Germany) at a magnification of 200×.

4.5. Establishment of MLV-based packaging cell pools

To ensure comparability of different Env variants pseudotyping MLV-derived vector particles, a cell line lacking the envelope gene (no Env) was first generated. For the establishment of no Env, HT-1080 cells were co-transfected with 0.75 μg SB-gpIpW, 1.5 μg SB-LEGFP-N1 and 0.3 μg of the transposase construct CMV-SB100x using TransIT®-LT1 transfection reagent (Mirus, USA) according to the manufacturer's instructions. Two days post transfection, cells were transferred to 10 cm culture dishes and subjected to selection in the presence of neomycin and puromycin. Cell clones were generated using biological cloning. Four cell clones were expanded and examined for EGFP and Gag expression levels employing FACS and Western blot analysis, respectively (data not shown). The cell clone revealing the highest mean fluorescence intensity and Gag expression was used as a parental cell line for the generation of stable Env variant-positive vector producing cell lines (VPCs). To establish stable VPCs with the different envelope variants, cells of the described clone were transfected with 2.5 μg of respective envelope expression plasmid (eMLV-wt, eMLV-ΔR, eMLV-GaLVΔR, eMLV-GaLVQAΔR). Two days post transfection, cells were subjected to escalating selection pressure to reach a final concentration of 5 μg/mL of puromycin, 800 μg/mL of G418 and 300 μg/mL hygromycin.

4.6. Generation of lentiviral pseudotype vector particles

For the production of lentiviral particles pseudotyped with ecotropic Env variants, 1×10^7 HEK293T cells were seeded into T175 flasks to reach confluency of 80%. Cells were transiently transfected using 40 kDa polyethylenimine transfection reagent (PEI; Polysciences Inc., Germany) at a mass ratio of 1:3 (DNA:PEI) according to the manufacturer's instructions using 14 μg of packaging construct pCD/NL-BH*ΔΔΔ, 21 μg of transfer vector pNL-eGFP/CMV/WPREdU3 (both plasmids were kindly provided by the Jakob Reiser Lab: Addgene # 17,531 (Zhang et al., 2004) and # 17,579 (Ricks et al., 2008)) and 7 μg of the respective envelope variant encoding constructs. Transfection and subsequent analysis experiments were conducted independently in triplicate.

4.7. Western blot analysis

Viral vector-containing cell-free supernatants of VPCs were harvested as described and pelleted at 4 °C and 25,000 rpm for 1.5 h using a SW28 swing-out rotor and an Optima XE centrifuge (Beckman Coulter, USA). Vector particle pellets were re-suspended in 80 μL Laemmli-Buffer containing 50 mM Dithiothreitol (DTT) and lysed at 95 °C for 10 min. Equal volumes of lysed vector particle pellets were loaded on a 4–15% acrylamide gel for SDS-PAGE and subsequent blotting on a PVDF membrane (Carl Roth, Germany). TSB-buffer supplemented with 0.05% tween 20% and 2% milk powder was used for blocking. Envelope protein was detected using a rabbit polyclonal antibody specific for gp70-SU (GenScript, USA) at a dilution of 1:2000. Secondary antibody labeled with HRP against rabbit-IgG (1:10,000) was used for protein detection (Abcam, USA). The substrate ECLplus (Thermo Scientific, Bremen, Germany) and a ChemiDoc XRS + imager (Bio-Rad, USA) were employed for luminescence signal visualization.

4.8. Gag quantitation using ELISA

Cell-free supernatants of VPCs were analyzed for their Gag concentration using the HIV-1 p24 capsid ELISA kit (VPK-107; Cell biolabs, USA) to quantify vector particles. ELISA was conducted according to the manufacturer's instructions.

4.9. Viral vector titration

Supernatants of pseudotyped lentiviral VPCs were harvested two days post transfection from HEK293T cells. To obtain cell-free vector preparations, harvested supernatants were passaged through a 0.45 μm pore size PVDF filter (Carl Roth, Germany). To assess the viral vector titers, 8×10^4 HT-1080/mCAT target cells were seeded in 2 mL per well in six-well dishes one day prior to transduction (Nunc, Wiesbaden, Germany). Different dilutions of vector samples in total volumes of 1 mL were employed for transduction experiments. On the following day, 1 mL of fresh media was added to transduced cells. Three days post transduction cells were analyzed employing flow cytometry (S3e; Bio-Rad, USA) to determine the percentage of EGFP-positive cells. Vector titers were calculated as described previously by Salmon and Trono (2007).

CRediT authorship contribution statement

Natalie Tschorn: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, preparation, Visualization. **Christian Söhngen:** Methodology. **Karen Berg:** Methodology. **Jörn Stitz:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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3.3 Publication 3

Transgene expression and transposition efficiency of two-component Sleeping Beauty transposon vector systems utilizing plasmid or mRNA encoding the transposase

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ORIGINAL PAPER

Transgene Expression and Transposition Efficiency of Two-Component Sleeping Beauty Transposon Vector Systems Utilizing Plasmid or mRNA Encoding the Transposase

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Abstract
The use of two-component transposon plasmid vector systems, namely, a transposase construct and a donor vector carrying the gene of interest (GOI) can accelerate the development of recombinant cell lines. However, the undesired stable transfection of the transposase construct and the sustained expression of the enzyme can cause genetic instability due to the re-mobilization of the previously transposed donor vectors. Using a *Sleeping Beauty*-derived vector system, we established three recombinant cell pools and demonstrate stable integration of the transposase construct and sustained expression of the transposase over a period of 48 days. To provide an alternative approach, transcripts of the transposase gene were generated in vitro and co-transfected with donor vector plasmid at different ratios and mediating high GOI copy number integrations and expression levels. We anticipate that the use of transposase mRNA will foster further improvements in future cell line development processes.

Keywords Transposon vector · *Sleeping Beauty* · mRNA transfection · Transposition · Transgene expression · Producer cell line

Introduction

Transposon vector technology can be used to mediate highly efficient stable gene transfer allowing for the rapid establishment of transgenic cells and is thus utilized in gene therapeutic strategies as well as the development of cell lines for the production of biologics [1–4]. Two-component plasmid vector systems are most frequently used for non-therapeutic applications derived from *Sleeping Beauty* and *PiggyBac* encompassing a transposase encoding vector and a separate donor vector containing the expression cassette with the gene of interest (GOI) flanked by the inverted terminal repeats (ITRs) [5, 6]. The expressed transposase enzyme recognizes specific sequences in the ITRs and subsequently cuts out the ITR-flanked expression cassette from the donor vector plasmid and integrates the linear DNA fragment into the target cell genome. This event called transposition thus facilitates stable gene integration via a cut and paste-mechanism [7, 8]. Transposition mediated by *Sleeping Beauty*-derived transposon vectors can mediate the integration of GOIs at high copy numbers per genome and enable sustained expression [9]. When the expression of the GOI in a transposon vector is coupled to a selectable reporter gene such as antibiotic resistance genes, highly productive cell pools can be established in a couple of days and weeks. This approach circumvents the time-consuming establishment and screening of cell clones to reach high productivity [10–12].

The optimal ratio of donor vector and transposase vector is crucial for efficient transposition. A phenomenon called overproduction inhibition (OPI) caused by larger amounts of transposase enzyme appears to decrease transposition activity [13, 14]. Moreover, *Sleeping Beauty* vector-mediated transposition efficiencies vary in different cell lines and types from a range of donor species [10, 11]. This requires the optimization of transfection protocols and the careful titration of donor to transposase construct ratio to obtain high transgenic rates and GOI copy numbers per cell as a

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result of transposition [13, 15–17]. However, as plasmids are transfected, also stable transfection events occur, namely, the unintended integration of the donor vector including the bacteria-derived backbone and the transposase construct. Especially the undesirable integration of the transposase gene and sustained expression of the transposase enzyme can mediate the mobilization of transposed donor vector cassettes resulting in either the reintegration into new *loci* or the degradation of the mobilized DNA fragments [18]. This genetic instability in the target cell line can thus lead to a loss of productivity.

Consequently, we here established three recombinant cell pools using co-transfection of donor and transposase plasmids and subsequent selection pressure and examined transposase gene integration and expression over a period of 48 days. To circumvent the use of transposase encoding plasmids, the *SB100x* transposase gene of *Sleeping Beauty* was transcribed in vitro into mRNA as previously described by Bire et al. [19], who established this methodology for *PiggyBac*-derived vectors. Human HEK293 cells were either co-transfected with a constant amount of donor vector and the *SB100x* transcripts in different ratios or, alternatively, with an optimized quantity of transposase plasmid construct. Recombinant cell pools were established and analyzed for transgene expression levels and vector copy numbers resulting for transposition and stable transfection, respectively.

Materials and Methods

Cells

Adherent human embryonic kidney HEK293 cells (ATCC CRL-3216) were grown in high glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-Glutamine and 10% fetal bovine serum (FBS); Gibco, Dreieich, Germany). Cells were expanded at 37 °C in a humidified atmosphere at 5% CO_2. For passaging, cells were detached using 1 mM Ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS). Prior to seeding, cell count was determined using an automated cell counter (fluidlab R-300; Anvajo, Dresden, Germany).

Plasmids

The donor vector SB-EGFP-IpW and transposase expression plasmid CMV-SB100x were previously described by Berg et al. [11, 20]. Donor vector SB-VEGF-IpW was constructed by linearizing SB-EGFP-IpW upon restriction using the enzymes *EcoRV* and *XbaI* removing the enhanced green fluorescence protein (EGFP) encoding fragment and inserting instead an accordingly digested human codon-optimized sequence encoding human vascular endothelial growth factor (VEGF; Genscript, Piscataway Township, NJ, USA).

Transfection and Selection of HEK293 Cells

HEK293 cells were transfected using TransIT®-LT1 transfection reagent (Mirus, Madison, WI, USA) according to manufacturer´s instructions. On the day of transfection, 5×10^5 cells were seeded in 2 mL per well using six-well plates (Nunc, Wiesbaden, Germany). Cells were co-transfected in triplicate with 2.5 µg of SB-VEGF-IpW and 250 ng of transposase vector CMV-SB100x corresponding to a ratio of 1:1/10. Two days post transfection, cell pools were subjected to a selection pressure of 0.5 µg/mL of puromycin (InvivoGen, Toulouse, France), which was increased stepwise to a final concentration of 10 µg/mL over a period of 48 days.

Detection of Transposase Coding Sequence in Genomic DNA

Genomic DNA of recombinant cell pools was harvested using the EchoLUTION Cell Culture DNA Kit (BioEcho, Cologne, Germany). Equivalents of 10,000 genomes (60 ng) of genomic DNA originating from transfected HEK293 cells and naïve cells serving as a negative control were used to perform genomic PCR using the Q5® High-Fidelity DNA Polymerase following the manufacturer's instructions (NEB, Ipswich, MA, USA). 0.001 pg of the transposase expression plasmid (equivalent to 10^2 copies) served as a positive control. In parallel, serial dilutions of transposase expression plasmid mixed with 60 ng genomic DNA of naïve HEK293 cells were prepared to serve as a reference standard. Using the oligonucleotides gDNA_SB100x-for: 5′-CTAGCGTGC AGACAATCGTGAGGAAGTACAAG-3′ and gDNA_ SB100x-rev: 5′-TGGCCCTCGCAGACAGCGAATTAA TTGAATTC-3′ generated amplicons of a size of 436 bp. PCR was performed employing an initial denaturation step of 30 s at 98 °C, followed by 35 cycles of denaturation for 10 s at 98 °C, annealing for 15 s at 63 °C and elongation for 15 s at 72 °C, and a final extension step for 2 min at 72 °C.

Detection of SB100x Transcripts

RNA of transfected HEK293 cells was harvested using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Concentration of purified RNA was determined utilizing a NanoQuant Plate™ Reader (Tecan Trading AG, Männedorf, Switzerland). Purified RNA (1 µg) was subsequently reverse transcribed to cDNA using the LunaScript® RT SuperMix Kit (NEB, Ipswich, MA USA). For transposase gene detection, PCR was performed with 2 µL of cDNA solution serving as a template and

oligonucleotides SB100x-for: 5′-ACAGGACATTCTGGA GAAACGTGCTGTGG-3′ and SB100x-rev: 5′- TTCTTC AGCTCGGCCCACAGATTCTCG-3′. PCR was conducted with the aforementioned PCR program. As an internal reaction control, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers GAPDH cDNA for: 5′-CGGAGT CAACGGATTTGGTC-3′ and GAPDH cDNA rev: 5′-TGG GTGTCGCTGTTGAAGTC-3′ were used amplifying 800 bp long DNA fragments.

SB100x-mRNA Synthesis and Transfection into HEK293 cells

The *SB100x* transposase coding sequence [20] was inserted into IVTRup (a gift from Ángel Raya (Addgene plasmid #101362)). The vector was linearized by restriction using the enzyme *EcoRV*. The Gibson cloning primers: Gibson-SB100x-for: 5′-GGCTGGGCTGGTACCTGTGCCCGG TGTCAGGCCAGCTGTAGTTGCAGCGGTCAGCTGCCG CTCTCTGGCCCTGTGCGAACTGCTGTGCCAGGTGCA CCCTGGGGGACCAGGCTGCCTGGGCTTCCTGGA ACTGGTGAAGCTGCCGCCACTTCCTCTATGCTCTCT CCAGCAGGCAATTCTGGGTAAACGATCTTCATTTGC CTATAAAGCTGCACAGCTCACAGGCCTTGGACCGTT TCTGCCCCAGCCCCAGCATTGGCCCTTTGGACAGAC TCTGAAACCGTGCGCAGAACGCACCCTGTCATTACA AATGACTCCTGGAGGCAGTCCCCGGGGGCCTGG CAGGAGCACCTGTGTTTCTGTGGGGTCTGAAAA TGACAGACCAATCGCTTGGGCCCGGGAGGCGGA GGTTGCAGTGGGCCGAGATCGAGACATTGCCCTCCA GCC-3′ and Gibson-SB100x rev: 5′-AGGGCATGGCCA GAAGGCAAGCCCCGCAGAAGGCAGCGATATCTT AGTATTTGGTAGCGTTTCCTTTGAACTGCTTCACC-3′ were used to amplify the *SB100x* coding sequence followed by the insertion into the linearized vector to generate the plasmid IVTRup-SB100x. The plasmid was used as a template for subsequent PCR amplification employing the oligonucleotides mRNA T7 Promoter for: 5′-GAATTGTAATAC GACTCACTATAGGGCGAATTGG-3′ and mRNA Poly(T)-3′UTR rev: 5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTCTAGACCCTCA C-3′. The resulting cDNA was used for in vitro transcription (IVT) of SB100x-mRNA employing HiScribe™ T7 ARCA mRNA Kit (NEB, Ipswich, MA, USA) according to the manufacturer's instructions. To remove template DNA, 2 units of DNase I were added to each reaction tube containing the mRNA for 15 min at 37 °C. After DNase I treatment, mRNA was purified using the Monarch® RNA Cleanup Kit (NEB, Ipswich, MA, USA). RNA concentration and purity were determined using a NanoQuant Plate™ Reader (Tecan Trading AG, Männedorf, Switzerland). Aliquots were stored at − 80 °C [21].

Prior to transfection, HEK293 cells were detached using 1 mM PBS/EDTA and seeded at 8×10^5 cells per well into six-well plates. Different amounts of SB100x-mRNA were co-transfected with a constant amount of 1.5 µg of donor plasmid SB-EGFP-IpW. Cells were transfected with 40 kDa linear polyethylenimine transfection reagent (PEI MAX®; Polysciences Inc., Warrington, PA,USA) at a mass ratio of 1:4 (DNA:PEI) in duplicate. Reaction tubes containing the donor vector or SB100x-mRNA, respectively, were prepared separately. In addition, co-transfection of the donor vector and the transposase encoding plasmid CMV-SB100x at a ratio of 1:1/10 was conducted. Cells transfected only with the donor vector served as controls. To ensure comparable transfection efficiencies, fill-up plasmid DNA of pUC18 was added to the reaction tubes to ensure constant total amounts of nucleic acids. Two days post transfection, 1×10^5 cells were transferred to 10 cm dishes and 2 µg/mL of puromycin was added to the cultivation media. Cells were cultured as described in the materials and methods section for 16 days at constant selection pressure.

Flow Cytometric Analysis of EGFP Expression

Recombinant HEK293 cell pools and naïve HEK293 cells serving as negative controls were detached and washed twice with PBS. Cells were resuspended in 1 mL Fluorescence Activated Cell Sorting (FACS)-buffer containing 1% fetal calf serum (FCS) in PBS/1 mM EDTA for subsequent flow cytometric analysis (S3e; BioRad, Hercules, CA, USA). The mean fluorescence intensity (MFI) mediated by EGFP expression (EGFP-MFI) was determined using the flow cytometry analysis software FlowJo (BD Biosciences; Franklin Lakes, NJ, USA).

Determination of Vector Copy Numbers

Genomic DNA of recombinant cell pools was isolated using the EchoLUTION Cell Culture DNA Kit (BioEcho, Cologne, Germany) according to the manufacturer's instructions. Primer/probes for the detection of Woodchuck Hepatitis virus Posttranscriptional Regulatory Element (WPRE) (WPRE for: 5′-GAGGAGTTGTGGCCCGTTGT-3′; WPRE rev: 5′-TGACAGGTGGTGGCAATGCC-3′; WPRE probe: FAM-CTGTGTTTGCTGACGCAAC-BHQ1), Ampicillin (Amp for: 5′-CAGCAATAAACCAGCCAGCC-3′; Amp rev: 5′-ACTTACTCTAGCTTCCCGGC-3′; Amp probe: FAM-GCAACTTTATCCGCCTCCATC-BHQ1) and Polypyrimidine Tract Binding Protein 2 gene (PTBP2) as an internal reference (PTBP2 for: 5′-TCTCCATTCCCTATGTTC ATGC-3′; PTBP2 rev: 5′-GTTCCCGCAGAATGGTGA GGTG-3′; PTBP2 probe: JOE-ATGTTCCTCGGACCA ACTTG- BHQ1) [22] were utilized for Taqman® quantitative PCR employing the StepOne™ Real-Time PCR System

(Thermo Scientific, Waltham, MA, USA). DNA-free samples and genomic DNA of naïve HEK293 cells served as negative controls. Serial dilutions of the reference VCN-standard plasmid containing WPRE, ampicillin resistance gene (Amp^R), and PTBP2 sequences were used for standardization. Details of the cloning strategy are available upon reasonable request. Statistical significance was determined using the GraphPad Prism software (GraphPad software; San Diego; CA; USA) utilizing the 1-way-ANOVA test.

Results

Detection of the SB100x Gene in Genomic DNA of Transgenic HEK293 Cells

Recombinant cell pools were established upon co-transfection with the donor vector SB-VEGF-IpW and transposase vector CMV-SB100x illustrated in Fig. 1 and expanded for 48 days under escalating selection pressure reaching a final puromycin concentration of 10 µg/mL at day 38. To monitor integration of the *SB100x* gene into cell genomes, genomic DNA of recombinant cell pools was isolated at different time points of cultivation. Genomic DNA of naïve HEK293 cells and a template-free sample (H_2O) served as negative controls. PCR was conducted using *SB100x*-specific primers generating amplicons with a size of 436 bp. In parallel, serial dilutions of the transposase expression plasmid were mixed with 60 ng of genomic DNA of naïve HEK293 cells equivalent to 10,000 genomes to assess PCR sensitivity. As shown in Fig. 2 and with a sensitivity of 10 copies in 1000 genomes, *SB100x* genes were detectable throughout the entire cultivation and selection period of 48 days post transfection. Signal intensities, and thus the *SB100x* gene frequency, decreased from seven to 48 days post transfection indicating yet *SB100x* gene copy numbers of $> 10^2$ and $< 10^3$ integrated in 10,000 genomes.

Detection of SB100x Transcripts in Recombinant HEK293 Cells

To assess whether integrated transposase genes also mediated the expression of transcripts, RNA of recombinant cell pools was isolated 17 and 48 days post transfection. RNA was subsequently transcribed into cDNA and PCR was conducted employing *SB100x*-specific oligonucleotides. As an internal reference, primers specific for the amplification of GAPDH were used to ensure that similar amounts of RNA templates were applied. As shown in Fig. 3, transposase transcription was readily detected 17 and 48 days post transfection. Amplicon signal intensity decreased from day 17 to day 48, while GAPDH amplification remained constant. This demonstrated the sustained expression of transposase resulting from the unintended stable integration of the respective expression construct.

Detection of SB100x Transcripts in Recombinant HEK293 Cells Using mRNA for Transposition

To prevent undesired integration of the transposase gene into the target genome, cDNA of *SB100x* was transcribed in vitro into SB100x-mRNA for subsequent co-transfection with the donor vector SB-EGFP-IpW (Fig. 1). Different ratios of SB100x-mRNA to donor vector were co-transfected into HEK293 cells to determine the optimal condition facilitating efficient transposition. Two and 16 days post transfection, genomic DNA was isolated and subjected to PCR using *SB100x*-specific oligonucleotides. As shown in Fig. 4A and two days post transfection, the transposase gene was detectable in all cell pools co-transfected with SB100x-mRNA or plasmid SB100x. Detected signal intensities decreased with higher dilution of transfected SB100x-mRNA. This indicated the contamination of SB100x-mRNA with the IVTRup-SB100x-derived amplicon DNA. However, after 16 days of cultivation

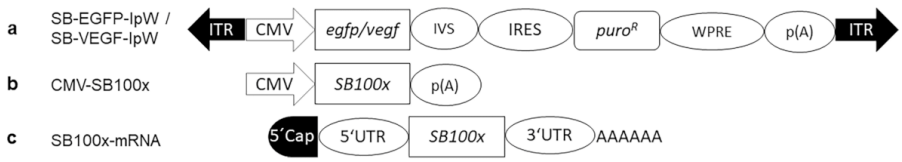

Fig. 1 Schematic illustration of the genetic elements of transposon and transposase vector constructs. (a) A CMV promoter/enhancer element (P_{CMV}) drives expression of the donor vectors and (b) the plasmid-based helper vector construct. The expression cassettes of the donor plasmids encompass the reporter gene enhanced green fluorescent protein (e*gfp)* or vascular endothelial growth factor (*vegf*) followed by an synthetic intron (IVS) and an internal ribosome entry side (IRES). Thus, the puromycin resistance gene ($puro^R$) allows also for the selection of cells expressing of the other reporter gene and is followed by the woodchuck hepatitis posttranscriptional regulatory element (WPRE) and a polyadenylation signal (p(A)) of the bovine growth hormone (bGH). The expression cassettes of the the donor vectors are flanked by inverted terminal repeats (ITRs) of *Sleeping Beauty* (SB). The transposase construct CMV-SB100x encodes the human codon-optimized transposase variant *SB100x*. (c) The SB100x-mRNA comprises the transposase coding sequence and is flanked by untranslated regions (UTRs). The 5′Cap stabilizes the mRNA and protects it from degradation

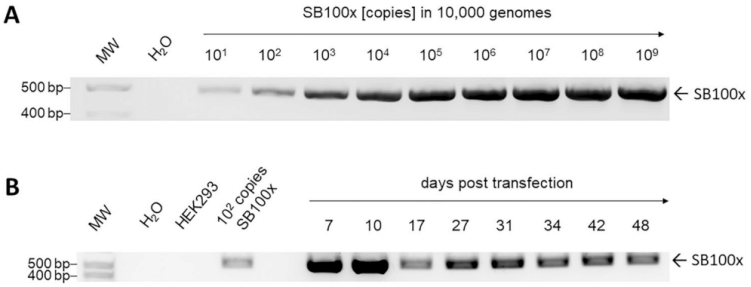

Fig. 2 Detection of *SB100x* coding sequences in genomic DNA. **A** Different amounts of plasmid CMV-SB100x were mixed with 60 ng genomic DNA of naïve HEK293 cells equivalent to 10,000 genomes to determine the sensitivity of detection. A PCR reaction mixture without plasmid DNA template served as a negative control (H_2O). **B** Exemplarily, one representative data set of the detection of *SB100x* gene in genomic DNA of recombinant HEK293 cell pools performed in triplicate is shown. Genomic DNA was isolated from selected cell pools upon co-transfection of donor vector SB-VEGF-IpW and transposase expression construct CMV-SB100x at different days post transfection to investigate transposase gene integration. 0.001 pg of the transposase vector mixed with 60 ng genomic DNA of naïve HEK293 cells equivalent to 10,000 genomes served as a positive control (10^2 copies). Genomic DNA of naïve HEK293 cells and template-free samples (H_2O) served as negative controls

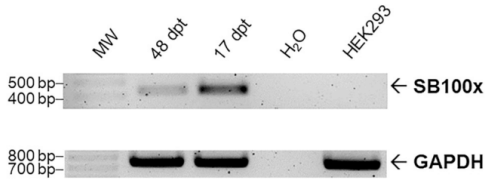

Fig. 3 Detection of *SB100x* transcripts in HEK293 cell pools. RNA of selected cell pools was isolated 17 and 48 days post transfection (dpt) and served as templates for cDNA synthesis. Equivalent volumes of synthesized cDNA were used for the amplification of DNA fragments of the *SB100x* coding sequences. GADPH amplification served as an internal and positive control. RNA isolated from naïve HEK293 cells and template-free samples (H_2O) served as negative controls. Data shown are representative results of experiments conducted in triplicate

under constant selection pressure, the transposase gene was exclusively still detectable in genomic DNA of cells co-transfected with plasmid SB100x. Cell pools transfected with SB100x-mRNA showed no longer a contamination with SB100x DNA at a sensitivity of detection of 10^2 copies per 10,000 genomes. It could therefore be assumed, that the IVTRup-SB100x plasmid was detected at 2 days post transfection in its epichromosomal form and did not stably integrate into the cell genomes at high frequency.

In parallel and to examine transposase expression, RNA was isolated and transcribed in vitro into cDNA for subsequent PCR after 16 days of cultivation. Transposase gene expression was readily detected only in samples of cells co-transfected with plasmid SB100x. In contrast and underscoring the findings employing genomic PCR, no transposase transcripts were detected in cells transfected with SB100x-mRNA.

FACS Analysis 16 Days Post Transfection Using SB100x-mRNA

To investigate the optimal ratio of donor vector to SB100x-mRNA for elevated transposition efficiencies resulting in high reporter gene expression levels, cells were subjected to flow cytometric analysis to determine EGFP mean fluorescence intensity (MFI) 16 days post transfection under constant selection pressure. As shown in Fig. 5A, cells transfected exclusively with the donor construct revealed the lowest MFI (987 ± 112) followed by the cell pools transfected with SB100x-mRNA at a ratio of 1:1/10 (1592 ± 79) and 1:1/20 (1175 ± 32). The highest MFI showed the cells transfected with plasmid-based transposase construct (3595 ± 104). Cells transfected with mRNA at a ratio of 1:1; 1:1/2 and 1:1/5 exhibited comparable and second highest mean fluorescence intensities (2583 ± 74; 2490 ± 0; 2367 ± 49).

Assessment of Vector Copy Number

Vector copy numbers (VCNs) stably integrated in host cell genomes were examined 16 days post transfection employing TaqMan® qPCR. VCNs were determined using WPRE-specific primers probes binding within the donor vector expression cassette. PTBP2 primers served as internal reference. As shown in Fig. 5B, all cell pools transfected with SB100x-mRNA and cells transfected with the donor construct only reached a copy number of three copies or more of the donor vector. Co-transfection of the donor vector and

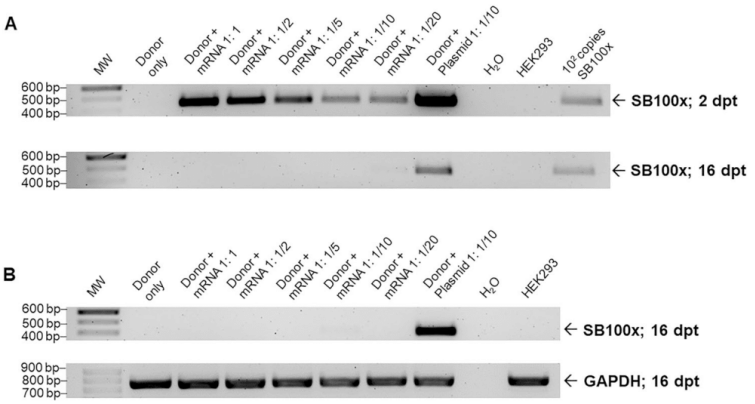

Fig. 4 Detection of the transposase coding region in genomic DNA and RNA preparations of HEK293 cells after co-transfection. **A** Different ratios of donor vector SB-EGFP-IpW to SB100x-mRNA were transfected into HEK293 cells in duplicate and genomic DNA was isolated 2 and 16 days post transfection (dpt) for subsequent sensitive PCR analysis. In addition, co-transfections of the donor vector and the transposase plasmid CMV-SB100x and transfection with the donor vector exclusively were conducted. Transposase plasmid mixed with 60 ng genomic DNA of naïve HEK293 cells equivalent to 10,000 genomes served as positive control (10^2 copies). Genomic DNA of naïve HEK293 cells and template-free samples (H_2O) served as negative controls. **B** Detection of *SB100x* transcripts in HEK293 cells. RNA of cell pools was isolated 16 dpt. Equivalent volumes of synthesized cDNA were used for the amplification of DNA fragments of the *SB100x* coding sequence. Amplification of GAPDH served as an internal positive control. Naïve HEK293 cells and template-free samples (H_2O) served as negative controls. Data shown are representative results of experiments conducted in duplicate

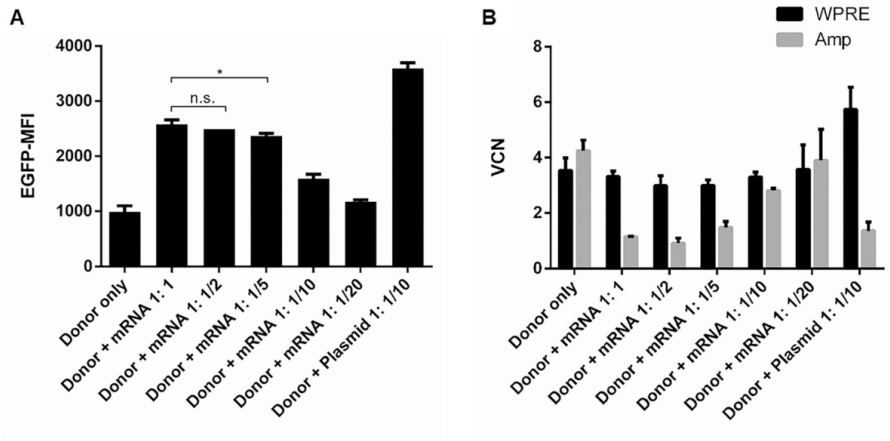

Fig. 5 Flow cytometric analysis of HEK293 cells and determination of vector copy number (VCN) per genome from different cell pools 16 days post transfection after constant selection performed in duplicate. **A** HEK293 cells were co-transfected with different ratios of donor vector SB-EGFP-IpW to SB100x-mRNA and were analyzed for mean fluorescence intensity of EGFP expression (EGFP-MFI) employing flow cytometry. In addition, cells transfected with the donor vector only and co-transfected with the vector CMV-SB100x were analyzed. Mean values are indicated and error bars represent standard deviations. (*) A P value ≤ 0.05 is indicated for the ratios Donor + mRNA 1:1 and Donor + mRNA 1:1/5. The difference of mean EGFP-MFI values of ratios Donor + mRNA 1:1 and Donor + mRNA 1:1/2 is not statistically significant (n.s.; using the one-way ANOVA analysis). **B** Determination of VCN 16 days post transfection. Genomic DNA was isolated of HEK293 cell pools transfected with different amounts of SB100x-mRNA or transposase expression plasmid CMV-SB100x and subjected to Taqman®-qPCR using oligonucleotides allowing for the amplification of DNA fragments of WPRE in the donor vector and ampicillin (Amp^R) present in the backbones of all constructs used. An additional primer/probe set amplifying the *ptbp2* gene was used as an internal reference. Mean values are shown and error bars represent standard deviations resulting from two independent experiments

plasmid-based transposase resulted in a mean integration of six transposon vector copies.

To investigate whether integrated VCNs were a consequence of stable donor plasmid transfection or transposition, qPCR using ampicillin-specific primers was conducted allowing for the detection of plasmid backbones of donor vector, transposase construct, and in some cases pUC18 used as fill-up DNA. Transfection with SB100x-mRNA with a ratio of 1:1 and 1:1/2 to donor vector SB-EGFP-IpW revealed the lowest amount of stably integrated plasmid with a mean plasmid integration of around 1 per cells (1.1 ± 0.0; 0.9 ± 0.2). Cells transfected with SB100x-mRNA and donor vector at a ratio of 1:1/5 as well as co-transfection with the plasmid CMV-SB100x yielded about two plasmid copy numbers (1.5 ± 0.2; 1.4 ± 0.3), respectively. Transfection of mRNA in a ratio of 1:1/10 and 1:1/20 and transfection with the donor vector only led to the integration of around three to four copies of ampicillin resistance genes as a result of undesired stable transfection.

Discussion

Transposon vector technology has fostered the establishment of recombinant cell lines for the production of biologics, vaccine candidates, and viral vector particles [1]. However, when the transposase is expressed from plasmid DNA, the undesired integration as a result from stable transfection and sustained expression of the transposase gene may cause genetic instability of selected polyclonal recombinant cell lines due to ongoing transposition events.

We thus investigated the integration and expression of the transposase gene after co-transfection of a donor vector and a transposase construct followed by subsequent selection of three stable cell pools. HEK293 cells were co-transfected with the donor vector SB-VEGF-IpW and transposase expression vector CMV-SB100x in a ratio of 1:1/10. The ratio was previously optimized in our laboratory for efficient transposition in HEK293 cells resulting in high expression of the gene of interest. Two days post transfection, cells were subjected to expansion in the presence of increasing concentrations of puromycin and were cultivated until 48 days post transfection. Genomic DNA of recombinant cell pools was isolated at different time points and examined for transposase gene integration. Employing sensitive PCR, cell pools revealed *SB100x* gene integration over the entire period of 48 days with an approximate VCN of $> 10^2$ per 10,000 genomes. The much higher VCN at day 7 and 10 can be attributed to high amounts of unintegrated epichromosomal plasmid shortly after transfection.

To investigate whether the integrated *SB100x* genes also mediated the expression of the respective *SB100x* transcripts, RNA of recombinant cells was isolated 17 and 48 days post transfection and subjected to RT-PCR analysis. Sustained transposase gene expression was readily detected at both time points. Signal intensity decreased from day 17 to day 48 most likely indicating gene silencing due to DNA methylation attracted by the bacteria-derived sequences in the plasmid backbone [23, 24].

To circumvent the use of transposon encoding plasmid DNA, we transcribed the *SB100x* DNA sequence in vitro into mRNA and co-transfected different ratios of donor vector to SB100x-mRNA with the transposon vector plasmid SB-EGFP-IpW into HEK293 cells in duplicate. To compensate for reduced mRNA amounts, samples were supplemented with the fill-up plasmid pUC18. Control cells were transfected with the donor vector—plus fill-up pUC18—and together with the transposase construct CMV-SB100x, respectively. Transgenic cells were continuously selected in the presence of puromycin. Genomic DNA of selected cell pools were again analyzed for the presence of the *SB100x* gene using sensitive PCR. The transposase gene was readily detected in all samples taken 2 days post transfection including the mRNA co-transfected cells. It is likely to assume that this was caused by a contamination with the PCR amplicon generated from parental construct IVTRup-SB100x used for in vitro transcription of the mRNA. The protocol used here should therefore in future extend the time for DNase I treatment and elevate the enzyme concentration to eliminate future contaminations. However, the contaminating PCR amplicon must mostly have remained epichromosomal since all samples of mRNA co-transfected cells were tested negative at 16 days post transfection. In contrast and yet again, cells co-transfected with the plasmid CMV-SB100x integrated transposase genes were readily detected. Using RT-PCR analysis, only these cells were also shown to express the transposase genes.

To assess efficient reporter gene expression and donor vector transposition, cell pools were also examined for EGFP expression and vector copy numbers 16 days post transfection employing FACS analysis and quantitative Taqman® qPCR, respectively. Cell pools co-transfected with transposase vector CMV-SB100x revealed the highest EGFP-MFI followed by cell pools transfected with SB100x-mRNA in a ratio of 1:1, 1:1/2, and 1:1/5. Cell pools co-transfected mRNA with a ratio of 1:1/10 and 1:1/20 revealed comparable mean fluorescence intensities to donor only transfected cell pools. This showed that reporter gene expression could be increased by transfecting more transposase mRNA but reached a maximum using a ratio of 1:1/5.

To examine whether high EGFP expression levels were a result of efficient transposition, Taqman® qPCR was performed using primers amplifying the mRNA-stabilizing element WPRE with in the transgene expression cassette of the donor vector and Amp^R-specific primers able to amplify from the backbones of all plasmids used in this

study, namely, SB-EGFP-IpW, CMV-SB100x, and pUC18. As expected from the highest expression level of EGFP, cells co-transfected with the donor vector and the CMV-SB100x expression plasmid revealed also the highest VCN of about 6 for the transposon vector, while only less than 2 copies of the plasmid backbones were detected indicating efficient transposition. The cell pool co-transfected with plasmid-based transposase generated copy numbers of about 6 copies per cell and is in accordance with the results of Kolacsek et al. [25].

Cells co-transfected with the donor vector and fill-up plasmid revealed lower VCN of around 4 for the donor vector, but even slightly higher amounts of integrated Amp^R genes demonstrating that the integrations resulted from stable transfection and due to the adjacent bacterial sequence were prone to gene silencing [7, 8, 26]. Similar patterns were observed in cells co-transfected with transposase RNA at ratios of 1:1/10 and 1:1/20, respectively. However, with the high amount of fill-up plasmid used here to compensate decreasing vector component DNA concentrations, it is very likely that a large proportion of plasmid backbone detected originated from pUC18 integrations. This would in turn mean that most transposon vector copies resulted from transposition events and would explain higher EGFP expression levels as compared to the control cells. Cells transfected with RNA to plasmid ratios 1:1, 1:1/2, and 1:1/5 revealed much lower amounts of backbone sequences ranging from 1 to 2 but similar donor vector copies of about 3 per genome, and thus mostly integrated by transposition. This supports the findings of comparable high expression the EGFP transgene.

The elevated copy numbers using CMV-SB100x were presumably a result of the prolonged expression of the transposase gene over some of days as compared to the utilization of transposase mRNA, which only last for a couple of hours [19]. We assume this could be compensated conducting first co-transfections of donor vector and transposase mRNA followed by repeated serial transfections of only the mRNA e.g., at two or three successive days. During this time period, donor plasmid should still be available in sufficient quantities in the cells to enable efficient transposition. In summary, we anticipate that the avoidance of undesired transposase gene integration into the host cell genome, and thus sustained expression, using transposase IVT mRNA transcripts will be utilized in future cell line development processes.

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Declarations

Competing Interests The authors declare that they have no conflict of interest.

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4 Summary and Outlook

One part of this thesis was to develop transposon vectors for the fast establishment of mammalian producer cells characterized by elevated copy numbers and sustained productivity. Previous studies have already shown that the *Sleeping Beauty* two-component vector system mediated efficient gene transfer and stable integration of the GOI [9]. However, the plasmid-based transposase vector could undesirably integrate into the target genome resulting in the potential re-mobilization and depletion of the already stably integrated gene of interest. This study further supported this assumption as *SB100x* genes in the genomic DNA and its transcripts were readily detected in HEK293 cells 48 days post co-transfection of the plasmid transposase construct and the respective donor vector. To circumvent this, the transposase gene was transcribed *in vitro* into mRNA for subsequent transposition experiments analyzing the expression levels of the GOI and vector copy numbers 16 days post transfection. Presented data demonstrated that the achieved vector copy numbers using *SB100x*-mRNA for transposition experiments was lower compared to plasmid-based *SB100x*-mediated transposition. This was also mirrored in decreased GOI expression levels analyzed employing FACS analysis. However, when cells were examined 16 days post transfection for transposase expression using RT-PCR, *SB100x* transcripts were exclusively detected in cell pools transfected with plasmid-based transposase. This study demonstrated that the utilization of *SB100x*-mRNA is a reasonable alternative to plasmid-based *SB100x* transposase avoiding undesired integration and subsequent expression of the transposase gene.

A potential explanation for lower VCN using *SB100x*-mRNA for transposition is the short half-life of mRNA molecules compared to plasmids resulting in a shortened time frame for transposase gene expression, and thus less transposition events. To compensate for this, serial transfections of *SB100x*-mRNA could be performed two and three days post initial co-transfection assuming sufficient epichromosomal amounts of the donor vector for following transposition events enhancing VCN and therefore cellular productivity. Another possible way to elevate VCN in target cells is a simultaneous transfection of three donor vector plasmids coding for the same transgene but harboring different selection markers. Upon applying stringent triple selection pressure, an enrichment of high VCN in cells can be achieved. This concept of triple co-transfection and co-selection was previously successfully applied in the establishment of stable high titer retroviral vector packaging cell pools [31, 71]. A further approach to obtain elevated VCN could be performing a serial transfection of SB- and PB-derived vectors as their transposases favor different target sequences and should not re-mobilize already integrated transfer genes.

Future applications of developed SB vectors could include the establishment of monoclonal antibody-producing cell lines which has already been demonstrated using PB vectors yielding

high antibody titers of up to 7.6 g/L [94, 95]. SB vectors may also be instrumental for the generation of cell pools producing human blood coagulation factor VIII (hFVIII). The hFVIII-producing human-derived expi293F cell pool, generated employing PB vectors, prove to be highly productive (9.0 U/10^6 cells/24 h) without the need for screening and generation of cell clones [96]. It is likely to assume that SB vectors might even exceed productivities as SB-mediated gene transfer obtained higher VCN compared to PB vectors [17].

The second part of this thesis aimed at the optimization of gene transfer utilizing retroviral vectors derived from MLV and HIV-1. Such vectors facilitate stable integration of their genetic cargo at high copy numbers enabling the establishment of highly productive producer cells. Retroviral vectors are mostly pseudotyped with VSV-G mediating efficient gene transfer but requiring working under biosafety level 2. To circumvent this, vectors utilized in this thesis were equipped with ecotropic envelope proteins allowing experiments under biosafety level 1 conditions. Envelope variants originating from Friend MLV PVC-211_{mc} lacking the R-peptide and C-tails derived from MLV and GaLV were generated anticipating that their fusogenicity elevates gene transfer efficiency. Titration experiments showed that all generated envelope variants failed to efficiently pseudotype MLV particles. In contrast, two of four generated envelope variants successfully pseudotyped HIV-1 vector particles. Gene transfer was most enhanced by exchanging the C-tail of ecotropic MLV envelope proteins with the C-tail of GaLV Env lacking the R-peptide. This chimeric envelope variant named eMLV-GaLVΔR exceeded titers mediated by the wild-type Env PVC211 about threefold, and will thus prove valuable for preclinical gene therapeutic applications and the establishment of protein producer cells.

The generated Env protein variant eMLV-GaLVΔR is – in contrast to VSV-G – not cytotoxic in human cells, and thus stable HIV-1-derived vector packaging cells could be established. To generate a stably HIV(eMLV-GaLVΔR)-producing cell pool, SB transposon-derived vectors could be used for the stable integration of all viral expression cassettes at high copy numbers into the genomes of host cells upon transposition. This approach was already shown to be beneficial for the establishment of stable ecotropic MLV-derived packaging cells outperforming vector particle-producing cells generated by conventional stable transfection using plasmids [31]. With a stable, continuously HIV(eMLV-GaLVΔR)-producing vector packaging cell, a constant particle harvest could be performed. A stable LV packaging cell line is preferable as consistent vector quality is achieved, which is a key factor in the industrial production of viral vectors. In contrast, current lenti-and retroviral vector particle production, building on the transient transfection of cognate viral vector constructs into HEK293 derivate host cells, suffers from considerable batch-to-batch variations with altering vector titers [97].

The research group around Humbert *et al.* developed a stable packaging cell line producing LVs pseudotyped with the Env proteins of *cocal virus* generating constant titers of about

1.0 x 10^6 TU/mL over a cultivation time of four month [98]. In another study, a stable LV packaging cell line was constructed using the non-toxic Env proteins derived from *feline endogenous retrovirus* RD114 enabling transduction of human cells. HIV(RD114) vector particle titers produced by this packaging cell line were relatively stable for over five months [99]. HIV(eMLV-GaLVΔR) vector particles harvested from future stable packaging cells could be used for repeated transductions of CHO or recombinant CHO-mCAT-1 suspension cells to establish high-yield protein producer cells. The approach to utilize lentiviral vector-mediated gene transduction into CHO cells was proven to be instrumental for the production of hemagglutinin H5 of *avian influenza virus* achieving an average production rate of 5.1 µg/mL [69]. In another example, also performing repeated transduction of CHO cells with LVs, production of hFVIII yielded productivities of 2.5 mg/L exceeding previously reported productivities [100]. Transduction can be performed at high multiplicities of infection (MOI) aiming for transgene enrichment in target cells, and thus elevating productivity as demonstrated for the production of erythropoietin (EPO) in CHO cells yielding 206 µg/mL [101]. However, the aforementioned studies were conducted in BSL-2 laboratories. The HIV(eMLV-GaLVΔR) vector particles developed in this thesis should facilitate producer cell line establishment conducted in a BSL- 1 environment, and thus further simplify the utilization of lentiviral vector-mediated gene transduction in the future establishment of highly efficient protein producer cell lines.

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List of publications

1. van Heuvel, Y., Schatz, S., Hein, M., Dogra, T., Kazenmaier, D., **Tschorn, N. (5 %);** Genzel, Y., Stitz, J.(2023) MuPACK.e, a novel suspension retroviral packaging cell line facilitating high vector yields and production in bioreactors. Frontiers in Bioengineering and Biotechnology. https://doi.org/10.3389/fbioe.2023.1076524

2. **Tschorn, N. (75 %)**, van Heuvel, Y., Stitz, J. (2022) Transgene Expression and Transposition Efficiency of Two-Component Sleeping Beauty Transposon Vector Systems Utilizing Plasmid or mRNA Encoding the Transposase. *Mol Biotechnol*. https://doi.org/10.1007/s12033-022-00642-6

3. **Tschorn, N. (75 %)**, Söhngen, C., Berg, K., Stitz, J. (2022) Ecotropic HIV-1 vectors pseudotyped with R-peptide-deleted envelope protein variants reveal improved gene transfer efficiencies. Virology 577:124–130. https://doi.org/10.1016/j.virol.2022.09.008

4. **Tschorn, N. (90 %)**, Berg, K., Stitz, J. (2020) Transposon vector-mediated stable gene transfer for the accelerated establishment of recombinant mammalian cell pools allowing for high-yield production of biologics. Biotechnol Lett 42:1103–1112. https://doi.org/10.1007/s10529-020-02889-y

5. Berg, K., Schäfer, VN.**, Tschorn, N. (5 %)**, Stitz, J. (2020) Advanced Establishment of Stable Recombinant Human Suspension Cell Lines Using Genotype-Phenotype Coupling Transposon Vectors. Methods Mol Biol 2070:351–361. https://doi.org/10.1007/978-1-4939-9853-1_20

6. Berg, K., Schäfer, VN., **Bartnicki, N. (5 %),** Eggenschwiler, R., Cantz, T., Stitz, J. (2019) Rapid establishment of stable retroviral packaging cells and recombinant susceptible target cell lines employing novel transposon vectors derived from Sleeping Beauty. Virology 531:40–47. https://doi.org/10.1016/j.virol.2019.02.014

Appendix

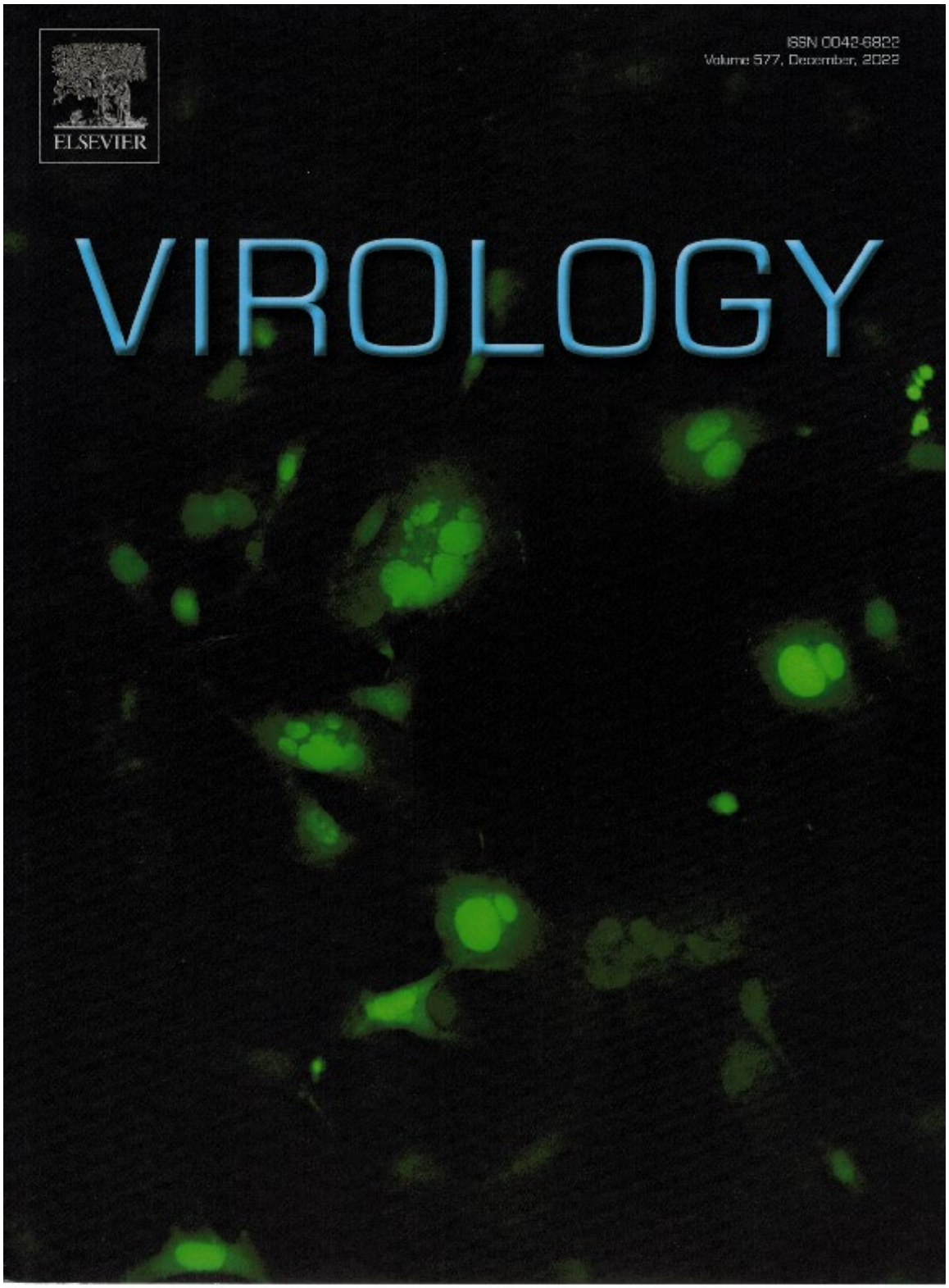

Figure 6: Fluorescence microscopic image of eMLV-ΔR Env and GFP-expressing HT-1080 cells forming syncytia with mCAT-1-positive cells. This picture was part of publication Nr. 2 included in this thesis [93] and was chosen as cover art for *Virology* Vol. 577, December 2022 (ISSN 0042-6822)