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Driving DNA transposition by lentiviral protein transduction

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Gene vectors derived from DNA transposable elements have become powerful molecular tools in biomedical research and are slowly moving into the clinic as carriers of therapeutic genes. Conventional uses of DNA transposon-based gene vehicles rely on the intracellular production of the transposase protein from transfected nucleic acids. The transposase mediates mobilization of the DNA transposon, which is typically provided in the context of plasmid DNA. In recent work, we established lentiviral protein transduction from Gag precursors as a new strategy for direct delivery of the transposase protein. Inspired by the natural properties of infecting viruses to carry their own enzymes, we loaded lentivirus-derived particles not only with vector genomes carrying the DNA transposon vector but also with hundreds of transposase subunits. Such particles were found to drive efficient transposition of the *piggyBac* transposable element in a range of different cell types, including primary cells, and offer a new transposase delivery approach that guarantees short-term activity and limits potential cytotoxicity. DNA transposon vectors, originally developed and launched as a non-viral alternative to viral integrating vectors, have truly become viral. Here, we briefly review our findings and speculate on the perspectives and potential advantages of transposase delivery by lentiviral protein transduction.

Keywords: DNA transposition, protein transduction, *piggyBac*, *Sleeping Beauty*, lentiviral vector, IDLV

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It is fascinating how ancient and extremely primitive DNA transposable elements are making an impact in a world of modern and advanced genetics that is constantly climbing to new technological heights. In spite of their genetic simplicity, parasitic mobile DNA elements have infiltrated and colonized the genomes of all living creatures, exploiting the capacity to relocate between genomic loci and multiply in numbers. Fossil remnants of these once actively jumping elements have lost the mobility due to accumulating mutations, and hide in genomes across the animal kingdom as inactive marks of a past genetic history. The *piggyBac* DNA transposon is one of the exceptions to the rule. This element was first identified when it actively jumped from its insect host, the cabbage looper moth *Trichoplusia ni*, into the genome of a baculovirus.[1] The active mobility of the *piggyBac* transposon led to its use in genetic analyses of insects,[2] but its capacity to insert genes in mammalian cells was not unveiled until recently.[3,4] Screening of a library of mutant transposases led to the identification of a hyperactive *piggyBac* transposase, hyPBase, with even higher activity in human systems. Another DNA transposon, the *Sleeping Beauty* element was the first transposable element shown to efficiently transpose in mammalian cells.[5] This element, a member of the *Tc1/mariner* transposon family, was reconstructed from salmonid transposable elements by consecutive steps of mutagenesis, facilitating its subsequent use in genetic engineering,[6,7] animal transgenesis,[8-10] forward genetic screens,[11,12] and therapeutic gene transfer.[13-15] Engineering of the hyperactive SB100X transposase variant, generated by a high-throughput PCR-based DNA shuffling approach,[16] and vectors with improved inverted

repeats[17,18] have added further efficacy to the system.

In their most typical composition, DNA transposons contain a single gene encoding the transposase protein. The gene is flanked by two inverted terminal repeats that serve as binding sites for transposase protein during cut-and-paste transposition. Interaction of transposase subunits binding to each of the terminal regions of the transposon facilitates the close association of the transposon ends allowing first excision from a donor and insertion into an acceptor site. Conventional DNA transposon-based vector systems are based on the co-delivery of (i) donor plasmids carrying the DNA transposon vector including the gene of interest and (ii) plasmids carrying the transposase expression cassette. Alternatively, in vitro-transcribed RNA molecules may serve as a source of the transposase.[19] Also, viral vectors, including lentiviral, adenoviral, and adeno-associated viral vectors, have been adapted as carriers of the transposase expression cassette.[20-23]

Common for all current DNA transposon systems is the need for intracellular production of the transposase. Ideally, DNA transposition is achieved within a short time-frame defined by a short-term boost of protein production and activity. It is challenging, however, to control the level and longevity of expression after plasmid transfection or viral vector transduction, and even transient expression strategies may cause sustained expression of the transposase, at least in slowly proliferating tissues and cell types. Also, in many cell types DNA and RNA transfection or nucleofection procedures tend to create massive nuclear accumulation of the transposase, which may on one hand be desired for optimal efficacy but on the other increase the risk of inserting numerable copies of the transposon or harm the cells otherwise. Adding to this, delivery of transposase-encoding plasmid DNA or viral vectors comes with the inherent risk of stably integrating the transposase expression cassette (often driven by a strong promoter) in the genome of the treated cells. It is known that transposases may interfere with normal cell cycle progression[24] and cause premitotic cell cycle arrest and even apoptosis through mechanisms involving activation of p53 and c-Jun.[25] By processes that are potentially linked to such toxicity, too high transient transposase expression levels lead to reduced efficacy of DNA transposition.[14,26] We have previously discussed these aspects in detail.[27] This type of regulation, normally referred to as overproduction inhibition (OPI), may possibly reflect natural regulatory mechanisms or, alternatively, that artificial overproduction of the transposase may harm the cells and even cause cell death.

In light of the uncertainties and potential safety precautions associated with intracellular production of effector proteins, like transposases and endonucleases, it is important to scrutinize other means of delivering such proteins to cells. Direct delivery of protein itself is an obvious alternative that, if successful, guarantees short-term activity and limits potential cytotoxicity. Fused to proteins of interest, cell-penetrating peptides (CPPs), also referred to as protein transduction domains (PTDs), can facilitate cellular protein uptake[28] and hold the potential to carry drugs into the interior of cells. A recent study unveiled intrinsic cell-penetrating capacities of zinc-finger nucleases (ZFNs), allowing direct delivery of recombinant ZFN protein to mammalian cells in vitro.[29] However, recombinant production of functional *Sleeping Beauty* and *piggyBac* transposases has turned out to be extremely challenging, and the few published attempts did not successfully demonstrate efficacy of DNA transposition catalyzed by recombinant transposases.[30,31] Low levels of activity may be explained by problems related to production and purification of active transposase as well as to reduced cellular uptake potentially caused by entrapment of protein in the endosomes.[32]

To establish effective DNA transposition without the need of transferring transposase-encoding nucleic acids, we recently set out to investigate an alternative route for delivering transposase proteins to cells.[33] Inspired by the capacity of viruses to carry their own enzymatic proteins, we sought to examine the ability of lentivirus-like particles to incorporate heterologous proteins like the *piggyBac* and *Sleeping Beauty* transposases. Here, we briefly review our findings, published earlier this year in *Nucleic Acids Research*,[33] and speculate on the perspectives and further applications of transposase delivery by lentiviral protein transduction.

Early studies demonstrated that the structure of gamma-retroviruses is sufficiently flexible to allow incorporation of foreign proteins fused to the Gag polypeptide.[34,35] More recently, this approach was successfully employed to deliver the Flp recombinase ferried within Gag precursors in murine leukemia virus (MLV) particles.[36] In accordance with these findings, HIV-1-derived lentiviral particles were found to tolerate the inclusion of heterologous proteins fused to Gag. Work by the Komano group demonstrated release of proteins like β-lactamase, GFP, and caspase 3 upon virion maturation[37,38] and established a basis for exploiting lentiviral Gag precursors as carriers of proteins and drugs. Using a related approach, Schenkwein and coworkers fused heterologous proteins to the integrase protein within the Pol region of the GagPol polypeptide.[39] Despite the fact that lentiviral particles contain considerably fewer GagPol than Gag polypeptides, the strategy effectively supported transfer of the mCherry reporter protein and p53, the latter of which was found to trigger apoptosis in virus-treated cells. Hitch-hiking of proteins in Gag and GagPol polypeptides offers attractive alternatives to previous approaches based on the viral incorporation of proteins fused to the HIV-1 accessory protein Vpr.[40,41] Vpr-based fusions have been successfully used to deliver reporter proteins, Cre recombinase, and I-SceI meganuclease to virus-treated cells,[40,42,43] but the relatively few copies of Vpr in the particles[44] and the potential toxicity of Vpr[45] may restrict the applicability of this approach. **Table 1** provides an overview of the strategies that have been successfully utilized to incorporate and transfer foreign proteins of interest (POIs) in lentiviral particles.

To convert lentiviral particles into transposase protein delivery vehicles, we introduced the transposase in the N-terminus of the Gag polypeptide at a position between the matrix protein and an artificially introduced myristoylation signal derived from the Lyn kinase.[37] Also, in the modified packaging construct, we

Table 1. Overview of strategies used to deliver proteins of interest (POIs) by lentiviral protein transduction

POI incorporation strategy	Transferred POI
Gag-POI (POI fused to p6)	GFP,[46] YFP,[47,48] CFP,[47,48] mCherry,[49] pHluorin[49]
POI-Gag-Pol (POI fused to MA)	GFP,[37] β-lactamase,[37] Caspase-3[38]
MA-POI-CA (POI inserted between MA and CA)	GFP[50]
Gag-Pol-POI (POI fused to IN)	mCherry,[39] p53,[39] λ repressor,[51] LexA,[52] I-PpoI,[53] Zif268,[54] E2C[55,56]
Vpr-POI (POI fused to Vpr)	GFP,[57,58] SN,[41] CAT,[41] IN,[59,60] RT,[60] PR,[61] Cre,[43] I-SceI,[42] Luc,[62] A3G,[63] HSV-TK,[64] Iinamarase[64]
POI-WXXF (POI fused to Vpr-binding WXXF-motif)	CAT,[65] IN,[66] scAb[67]
Nef-POI (POI fused to Nef)	GFP,[75,76] HSV-TK[76,77]

introduced the D64V mutation in the integrase gene,[68] certifying that the normal lentiviral integration machinery of such particles was not active. Assisted by HIV-1 protease cleavage sites flanking the transposase protein, the transposase was effectively liberated from Gag and GagPol polypeptides upon virion maturation. Western analyses demonstrated potent incorporation of both SB100X and hyPBase transposasases in virions. Our first indications of the efficacy of virally delivered transposases came from observations of high levels of *piggyBac* DNA transposition. In HeLa cells, treatment with hyPBase-loaded virus particles triggered higher levels of transposition than we normally observe with a standardized plasmid co-transfection protocol. A schematic overview of DNA transposition driven by a conventional plasmid-based system and by lentiviral protein transduction is provided in **Figure 1**.

We have previously shown that DNA transposons are effectively mobilized from lentiviral DNA intermediates generated by reverse transcription of integrase-defective lentiviral vectors (IDLVs).[22,23] This led to the idea that lentiviral particles could potentially accommodate both the transposase and the transposon, essentially mimicking conventional lentiviral vectors carrying both integrase protein and the recognition sites for active gene insertion. During the analysis of transposase-loaded IDLVs, we encountered the obstacle that transfer of the vector was markedly restricted by the load of Gag-fused transposase molecules. However, this problem was solved by generating particles composed of both wildtype and transposase-containing Gag and GagPol (still carrying the D64V mutation). Such chimeric particles supported quite significant levels of *piggyBac* DNA transposition in a panel of cell types including human primary keratinocytes and normal human dermal fibroblasts. Notably, such transposition was not evident when the particles were loaded with a mutated, inactive variant of the hyPBase and when the particles were not able to get access to the cells due to lack of VSV-G pseudotyping.

With experimental evidence for this new gene delivery concept, we went on to characterize the transduced cells in more detail using confocal microscopy. This analysis showed, perhaps surprisingly, quite low cellular levels of the transposase, which was visible only in few concentrated foci within transduced cells. In comparison, cells transfected with hyPBase-encoding plasmid displayed massive nuclear accumulation of the transposase. Still, the level of transposition, as measured by colony formation after mobilization of a transposon containing the puromycin resistance gene, was higher using the viral approach. We believe that this observation reflects that more or less all the cells were transduced by the viral vector, whereas a lower percentage of cells were transfected with plasmid DNA. Following this reasoning, a high transduction rate may compensate for the potential limitations of DNA transposition from virally delivered substrates. In line with this notion, we made an interesting observation when genomic vector copy numbers were determined after DNA transposition. Notably, all analyzed puromycin-resistant clones generated after virus-mediated transposition contained a single copy of the transposon, whereas transposition after plasmid transfer resulted in clones with variable copy numbers, half of them with 5 or more (and up to 12) copies of the transposon. The background for such key differences between plasmid- and virus-based transposase delivery is schematically depicted in **Figure 2**.

Our data confirmed that DNA transposition after plasmid transfection may result in multiple insertions. This returning observation in the transposon field is the expected result of both strong and prolonged expression of the transposase as well as concomitant high levels of transposon donor plasmid in cells that are fairly easy to transfect. In clones developing from a transfected cell, DNA transposition is likely to keep going for several cell divisions until the plasmids are lost. This means that DNA transposons that are initially inserted in chromosomal DNA can be re-excised from the genome or, likely more frequent, that additional DNA transposons in a developing clone may be transferred from plasmid DNA to locations within the chromosomes. As a result, such 'clones' may be composed of subclones containing a variable number of insertions and, hence, may not be truly clonal. This will lead to clonal heterogeneity in terms of transgene expression. Much like diluted preps of conventional lentiviral vectors, transposase-loaded IDLVs balance the level of gene insertion in each transduced cell leading only to a single insertion. Thus, this technique can support applications where only one insertion of the transposon is desired. As a direct consequence, clones resulting from the short-term action of the transposase during protein transduction are more likely to display homogenous expression of the transgene.

Our data show that hyPBase delivered in lentiviral particles can get access to DNA transposons localized either in co-transfected plasmid DNA or in co-transduced IDLVs. This argues that transposase

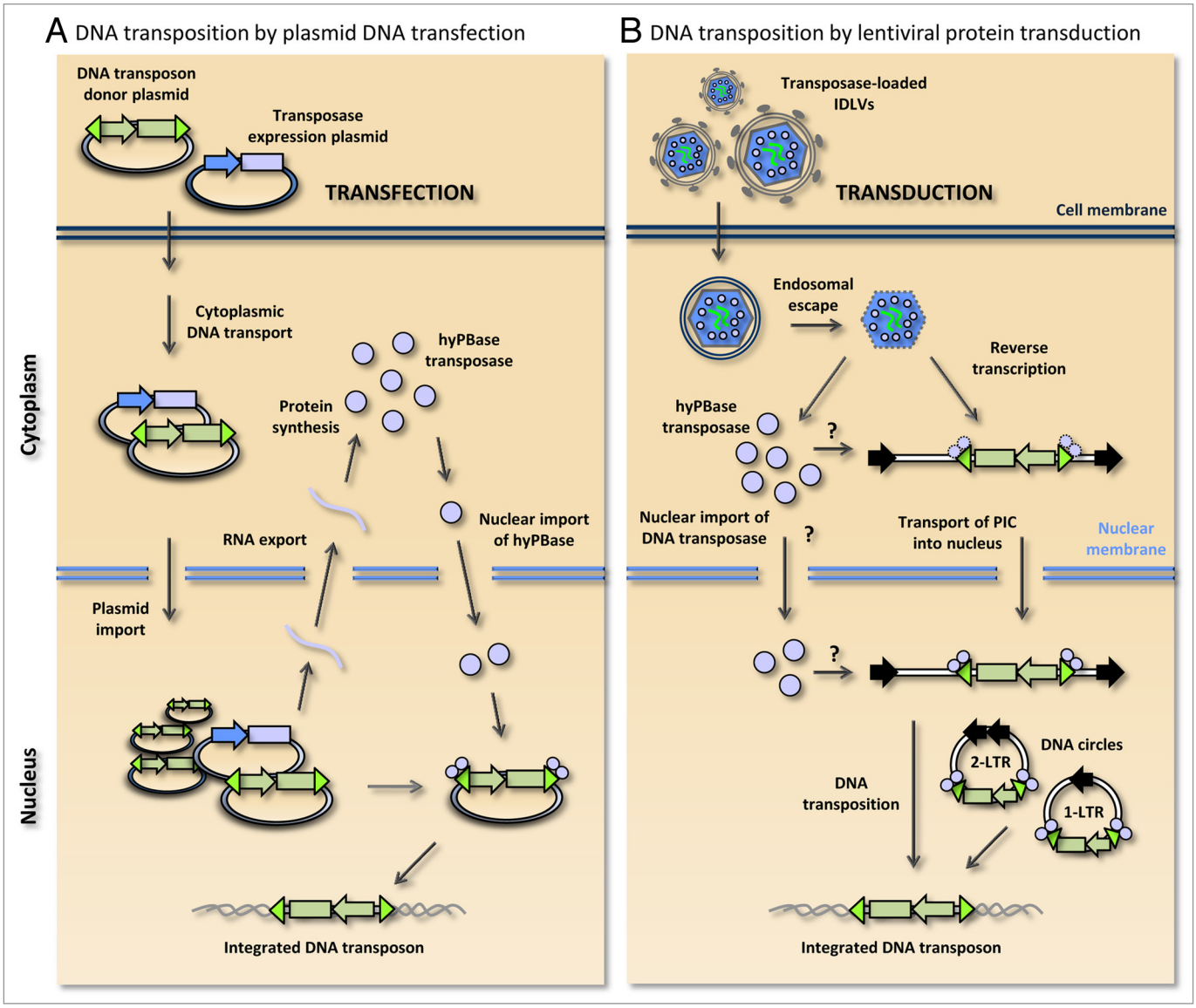

Figure 1. Schematic comparison of *piggyBac* DNA transposition by plasmid DNA transfection and lentiviral protein transduction. (**A**) DNA transposition by co-transfection of the DNA transposon donor plasmid and transposase-encoding plasmid. Transport through the cytoplasm and nuclear uptake lead to production of hyPBase transposase, which is subsequently imported into the nucleus. Within the nucleus the transposon-based gene vector (indicated in green) is excised from the donor plasmid and inserted into a genomic locus. (**B**) DNA transposition by lentiviral protein transduction in integrase-defective lentiviral vectors (IDLVs). Engineered lentiviral particles carry both the hyPBase protein (indicated by small light-purple circles) and the diploid RNA vector genome (indicated by green lines). Cell entry mediated by the VSV-G surface protein occurs through endocytosis and subsequent endosomal escape. Reverse-transcribed double-stranded DNA intermediates serve as transposon donors. Along with linear DNA substrates, 1-LTR and 2-LTR circles generated by homologous recombination and non-homologous end joining, respectively, may serve as transposon donors. Question marks indicate that it is not currently known whether transposase subunits are associated with the transposon in the cytoplasm or are imported into the nucleus prior to association with the transposon terminal repeats. It is currently unclear whether the transposase remains part of the pre-integration complex (PIC) during nuclear entry or is released from the PIC during cytoplasmic transport.

subunits at a certain stage during transduction are liberated from the invading pre-integration complex (PIC), allowing formation of transposition complexes on substrates delivered in trans. Still, the efficiency of the approach seems to be further improved by incorporating transposases in transposon-carrying IDLVs. In analogy with conventional lentiviral vectors carrying both the integrase and the substrate for the integration process, we believe that the proximity between transposases and the reverse-transcribed substrate within the PIC is supporting the overall efficiency of the process. In this way, the PIC is potentially offering an intracellular environment with high, local concentration of the transposase and, hence, compensating for the overall low levels of transposase in virus-treated cells.

As part of our endeavor to establish DNA transposition by protein delivery in human cells, we repeatedly tried to incorporate the hyperactive SB100X

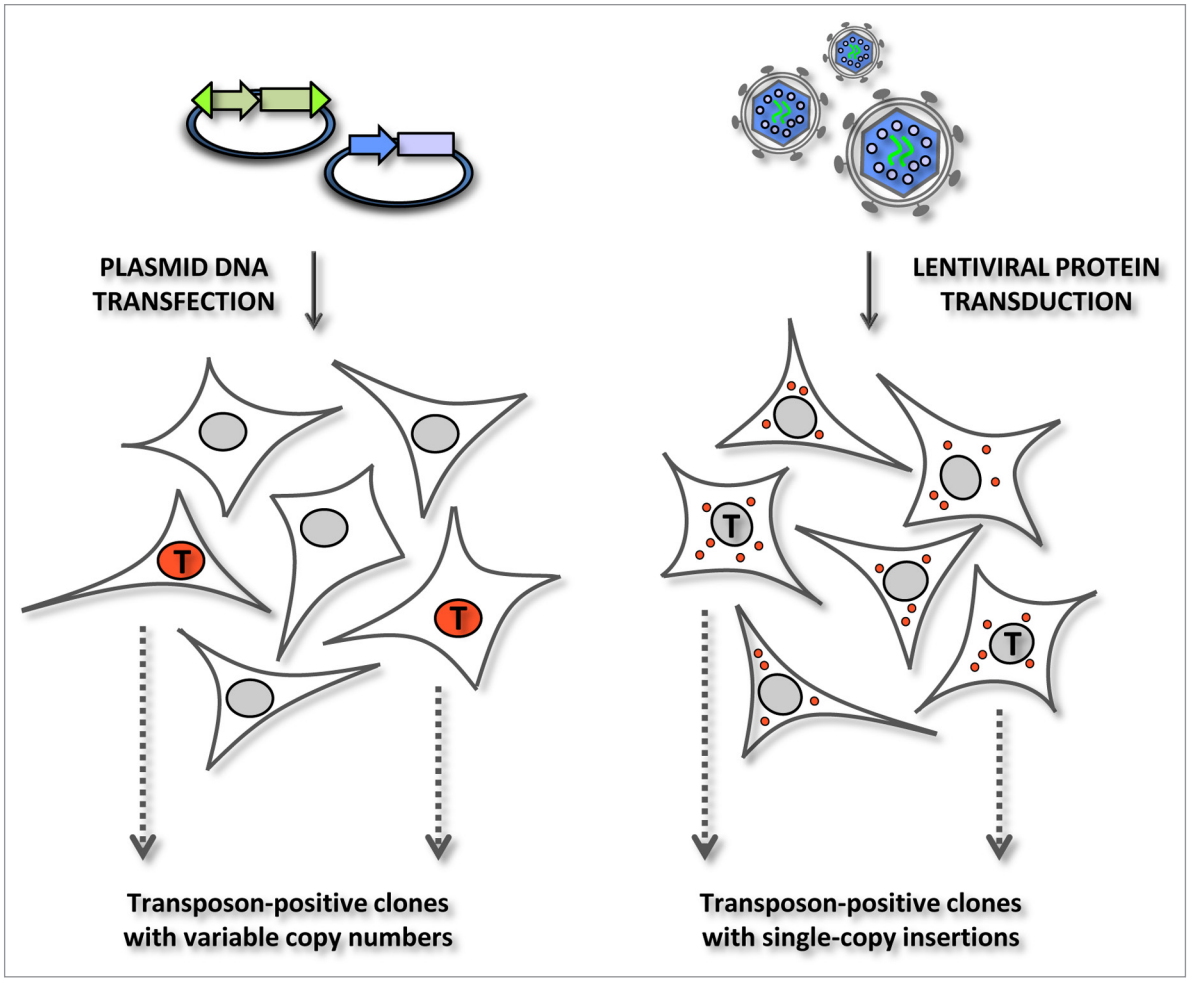

Figure 2. Comparative models of DNA transposition observed after plasmid DNA transfection and lentiviral protein transduction. Red marking indicates schematically the patterns of immunostaining that were observed by confocal microscopy of cells stained with an antibody specific for HA-tagged hyPBase transposase.[33] Plasmid DNA transfection leads to dramatic overexpression of the transposase in successfully transfected cells, whereas lentiviral protein transduction results in much lower overall levels of the transposase in virus-treated cells. Small red dots in transduced cells indicate that the transposase is present in all cells, but only observed in concentrated foci primarily within the cytoplasm. 'T' indicates cells in which successful transposition is achieved. Effective DNA transposition is a likely result of robust nuclear levels of transposase, potentially leading to several transposon insertions in a single cell/clone, whereas DNA transposition supposedly is less efficient and does not occur in all transposase-positive cells after protein transduction. As a result, however, all resulting clones contain only a single integrated copy of the transposon. See text for further details.

transposase[16] in lentiviral particles. We have seen that Gag-fused SB100X is indeed effectively packaged into virions and also liberated from Gag during virion maturation, but so far we have not been able to demonstrate efficacy of such virally delivered SB100X proteins. The lack of function is at least partially explained by the negative effect of the C-terminal 4-amino acid tag originating from the protease cleavage site between SB100X and Gag. The cleavage site is required to ensure proper release of the SB100X during virion maturation, but the N-terminal part of the cleavage site remains an integrated part of the protein after cleavage. In separate analyses of different plasmid-encoded SB100X variants with such small peptide tags fused to the C-terminus of the protein, we have seen that the function is completely abolished. This confirms the general notion that the C-terminus of SB transposases needs to be left untouched for full activity of the protein[69] and that *piggyBac* transposases are more flexible and less vulnerable to such changes.[70] To facilitate lentiviral SB100X protein transduction, it would be an alternative option to fuse SB100X to the C-terminal end of GagPol, allowing release of a protein with an additional tag only in the N-terminus.

DNA transposons are by definition non-viral transposable elements, and derived gene vectors were originally developed and launched as a non-viral alternative to viral integrating vectors. As such, they are already powerful tools in biomedicine and now moving into the clinic.[71] Why then bother adapting virus particles as carriers of transposases? By showing proof-of-concept that genomic engineering tools can be directly delivered by lentiviral particles, we are addressing one of the major potential challenges related to current systems of genomic engineering and editing. Is it appropriate and sufficiently safe to overexpress enzymes like transposases

and site-targeted nucleases within treated cells? Lentiviral protein transduction is the first approach that allows efficacious DNA transposition in human cells after direct protein delivery. Previous attempts to produce and deliver recombinant transposases have had limited success,[30,31] and lentiviral particles furthermore offer the opportunity of delivering transposases and transposon substrates simultaneously in a single vehicle. As an additional bonus, such hybrid particles generally seem to generate only a single genomic insertion of the DNA transposon, although this phenomenon needs to be further scrutinized in additional cell types. Also, the option of targeting DNA transposition to certain cell types by pseudotyping of the lentiviral particles deserves more attention in the future.

Early studies showed the applicability of plasmid-based DNA transposition system in vivo with initial focus on the mouse liver.[14,26] Many other tissues are significantly less accessible to plasmid DNA transfection, and low transfection rates are challenging in terms of wider in vivo use of both *Sleeping Beauty*- and *piggyBac*-based systems. It is tempting to speculate that transposase-loaded IDLVs could be paving the way for several new in vivo applications of *piggyBac* DNA transposition. One such application could be gene insertion by DNA transposition in skin. We have previously demonstrated efficient gene delivery and prolonged transgene expression in human skin intradermally injected with lentiviral vectors.[72,73] Future studies will show whether injection of IDLVs carrying the PB transposase can facilitate in vivo DNA transposition in skin progenitor cells. As we explore new ways of exploiting this transposase delivery approach, we are hard at work stretching the boundaries of lentiviral particles as transporters of foreign cargo. Most recently, we demonstrated targeted genome editing by lentiviral protein transduction of programmable nucleases co-delivered in virus particles with substrates for homology-directed recombination.[74]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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