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REVIEW

Transposon-based gene delivery vectors for gene therapy

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ABSTRACT The first gene therapy clinical trials were initiated more than two decades ago thanks to the previous development of viral vectors that allow high efficiency gene transfer into mammalian cells. Since then the application of viral gene transfer has been a successful treatment option for a variety of diseases. Hematopoietic stem cells (HSCs) represent the most frequently targeted cell population for the treatment of severe monogenic diseases as their gene therapeutic correction is a valid alternative to conventional HSC transplantation when a compatible donor is not available. Indeed, viral gene transfer was successfully applied in HSC-based *ex vivo* gene therapy of the blood and immune systems, albeit several studies have exposed serious adverse effects that were caused by the therapeutic vector induced inappropriate activation of proto-oncogenes. After these failures, researchers have developed new types of randomly integrating vectors that have proven safer in preclinical studies, which is consistent with interim reports of clinical trials also foreshadowing that they potentially have an improved safety profile. This review focuses on new and clinically relevant DNA transposon-based gene delivery vectors, and compares their properties with those of the old and new generation viral vectors.

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Introduction

Molecular genetic technologies have recently enabled a variety of novel therapeutic approaches to treat diseases by genetically modifying the cells of the patients, which are collectively referred to as gene therapy procedures. The major motivation for gene therapy has been the need to develop novel treatments for diseases for which there is no effective conventional treatment available. Gene therapy involves approaches like adding a functional copy of a gene to cells that have only non-functional copies or activating cells of the immune system so as to aid killing of diseased cells, just to mention the most common ones. The first human gene therapy clinical trial was performed in 1989 by Rosenberg and co-workers (1990) in the USA. These investigators used a γ-retrovirus vector, an early derivative of Moloney murine leukemia virus (MLV), to introduce the neomycin resistance gene into human tumor-infiltrating lymphocytes. They then reinfused the modified lymphocytes into patients with metastatic melanoma. This study demonstrated that γ-retroviral gene transduction for human gene therapy was feasible. Following the pioneering study of Rosenberg and co-workers (1990) the number of approved gene therapy clinical trials increased each year consistently over the next decade, reaching an annual global value around 100 by 1999 that was roughly maintained as an average annual number of approved trials during the last 15 years. By June 2014, 2076 gene therapy clinical trials had been completed, were ongoing or had been approved in 36 countries worldwide, according to the database of the Journal of Gene Medicine (http://www.wiley.com//legacy/wileychi/genmed/clinical/). During the first decade of the 2000s, the so far dynamic growth in the number of annually approved clinical trials has stopped due to that insertional oncogenesis having emerged as a major limitation in gene therapy protocols treating for primary immunodeficiencies (Farinelli et al. 2014). The long term outcome of the clinical trial started in Paris at the Necker-Enfants Malades Hospital in 1999 for treating X-linked Severe Combined Immunodeficiency (SCID-X1) well demonstrated that the integrating γ-retroviral gene delivery vectors frequently mediated activation of proto-oncogenes like LIM (Lin11, Isl-1, Mec-3) domain only 2 (LMO2) (Hacein-Bey-Abina et al. 2003). Gene therapy was initially successful at correcting immune dysfunction in 8 of the 9 SCID-X1 patients treated between March 1999 and April 2002, however 31-68 months after gene therapy acute lymphoblastic leukemia (ALL) developed in 4 of these 9 patients (Hacein-Bey-Abina et al. 2008; Hacein-Bey-Abina et al. 2010).

This serious issue raised new demands on the gene therapy

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vectors on which the scientific world is currently trying to meet.

A promising new approach potentially avoiding insertional oncogenesis is the application of site-directed nucleases instead of integrating gene delivery vectors. Site-directed nucleases are exploited to induce double-strand breaks (DSBs) and the following action of the cell intrinsic DNA repair systems harnessing the administered repair partner DNA may introduce the desired changes to the targeted chromosomal locus. Artificial nucleases, such as zincfinger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), and the RNA-guided endonuclease system (the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) system) are those site-directed nucleases currently tested mainly in preclinical studies. Very recently a report of a clinical study demonstrated the safety and feasibility of autologous transplantation of CD4-enriched T cells undergone a genetic modification at the *CCR5* gene locus by ZFN, into Human Immunodeficiency Virus (HIV) patients (Tebas et al. 2014). The purpose of this procedure was to induce acquired genetic resistance to HIV infection in an attempt to mimic the known inherited resistance displayed by persons with the *CCR5*-delta32 mutation (Liu et al. 1996).

Still the overall efficiency of genetic modifications mediated by site-directed nucleases is significantly lower as compared to randomly integrating gene delivery vectors (Radecke et al. 2010). Therefore, making the more effective randomly integrating vectors safer is necessary for a number of gene therapy protocols. One important direction of improvements in randomly integrating vector design was to develop lentivirus based vectors. Lentiviruses like the HIV are complex retroviruses and in contrast to γ-retroviruses they are able to infect nondividing and terminally differentiated cells. This is their significant advantage compared to γ-retroviral vectors. In addition to that, the development of self-inactivating (SIN) lentiviral vectors (Miyoshi et al. 1998) also increased their safety profile.

Transposon-based gene delivery vectors represent a promising new branch of randomly integrating vector development for gene therapy. They have some intrinsic advantages over viral vectors, and recently we have also been able to elevate their efficiency into the viral range. Here we will discuss the major drawbacks of integrating viral vectors and give an overview of the characteristics of those transposon systems that are already used or qualifying for use in gene therapy.

Insertional oncogenesis

The successful introduction of therapeutic genes into target cells is a prerequisite for gene therapy. Furthermore, for achieving long-term gene expression and clinical benefit, integration of the therapeutic genes to the chromosomal DNA of the target cells is also required.

From the early stages of gene therapy viral vectors represent the greater part of vehicles for transferring genes to human cells. This is due to their efficient infection rate and the ability to stably integrate therapeutic genes into the host genome. Most of the integrating viral vectors used for gene therapy originate from -retroviruses and more recently from lentiviruses (Ginn et al. 2013). They are characterized by an RNA genome. Their proviral complementary DNA is inserted to the host genome by the virus-derived reverse transcriptase and integrase (Kootstra and Verma, 2003). γ-retroviruses can only replicate in dividing cells as they cannot transmit their genome through nuclear pores. First generation γ-retroviral vectors were based on MLV containing intact long terminal repeats (LTRs) comprising strong promoter/enhancer elements driving the expression of therapeutic genes (Deichmann and Schmidt, 2013). Lentiviruses are complex retroviruses and most of the lentiviral vectors are HIV-1-based. Lentiviruses can also transduce non-dividing cells due to the nuclear import of their viral preintegration complex (Bukrinsky et al. 1993). These gene delivery vectors of viral origin inherit many intrinsic features from their parental viruses. In the early era of gene therapy, a nearly random integration pattern of viral vectors was expected (Stocking et al. 1993). In contrast, by now it is clear that viral integration is far from being random. MLV tends to integrate into regulatory regions of actively transcribed genes (eg. transcriptional start sites or CpG islands) (Cattoglio et al. 2010), whereas HIV-1 appears to be biased toward regions of active transcription by a mechanism driving its integrations to the outer shell of the nucleus in close proximity to the nuclear pores (Marini et al. 2015; Schroder et al. 2002). Gene delivery vectors derived from MLV and HIV-1 also exhibit the same integration patterns as their parental viruses (Fig. 1).

Insertional mutagenesis is a natural consequence of such integrating vector insertions. In addition to the potential disruption of host genetic elements, an even more dangerous phenomenon, the transcriptional deregulation of genes near the sites of vector integration is also associated to insertional mutagenesis. This phenomenon is caused by the genetic elements present on the vector or on the cargo DNA. Virus specific transcriptional enhancers within the viral LTRs have been identified as one of the major determinants of nearby host gene activation independent of retrovirus subtypes, and the MLV LTR enhancer has been shown to exhibit the strongest activity in this respect (Montini et al. 2009). To decrease the strength of host gene activation and increase vector safety, SIN retroviral vectors were designed. In these vectors, enhancer/promoter sequences of the LTRs are removed, thereby greatly reducing their potential of activating neighboring genes (Yu et al. 1986). Importantly, insertional host gene activation is also determined by the characteristics

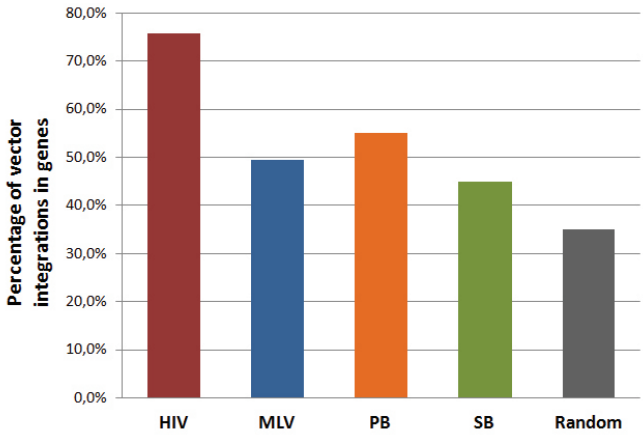

Figure 1. Insertional site preference of integrating vectors in HSCs and HSC-derived cells. Due to the similar experimental design of the studies of Huang et al. (Huang et al. 2010) and Cattoglio et al. (Cattoglio et al. 2010) analyzing transposon and viral vector integration profiles respectively, the observed frequencies of different vector integrations in RefSeq genes can be compared. Considering the known bias of γ-retroviral vector integrations towards 5′ regulatory regions, in addition to the integrations in RefSeq genes those viral integrations within ±50 kb from a known transcription start site (TSS) were as well determined as gene hits (Cattoglio et al. 2010). The random integration bar essentially indicates the gene content of the genome.

of transcriptional regulatory elements carried in the cargo. However, the internal cellular promoters, generally used in the cargo to drive the expression of therapeutic genes, induce deregulation of host gene expression less frequently, at a shorter range and to a lower extent as compared to intact viral LTRs (Maruggi et al. 2009).

Collectively, the biased viral integration patterns and the potential of the viral vectors to transcriptionally deregulate nearby genes raise the possibility of insertional oncogenesis driven by the most hazardous subset of insertional mutagenesis events. Indeed, insertional oncogenesis is currently the major limitation in classical gene therapy protocols using randomly integrating gene delivery vectors. Clinical experience has shown that viral vectors typically activate the expression of nearby tissue specific proto-oncogenes, resulting in the clonal outgrowth of those transformed cells. The scientific community first realized this serious drawback in relation to gene therapy treatment of primary immunodeficiencies.

Initial gene therapy efforts with the use of integrating vectors targeted primary immunodeficiency disorders. These are rare, inherited disorders caused by developmental defects of various cells of the immune system. The resulting impaired immune responses predispose patients to autoimmunity, infections and even tumorigenesis (Notarangelo, 2010). If the adverse consequences of an inherited genetic aberration are manifested in one of the early steps of hematopoietic lineage commitment, it leads to the complete lack or functional impairment of blood cell subsets. This is the case with SCIDs. One of the most common types of SCID is SCID-X1 which is caused by mutations in the X chromosomal gene coding for the interleukin-2 (IL-2) receptor common gamma chain (*IL2RG*). IL2RG is an important signaling component of many interleukin receptors directing the development and growth of lymphocytes. This monogenic disease is an excellent target of *ex vivo* gene therapy as delivery of a single corrected copy of *IL2RG* to autologous hematopoietic stem cells (HSCs) ensures successful reconstitution of the hematopoietic system following the transplantation of those genetically modified HSCs. High risk of γ-retroviral vector-based gene therapy became clear when in clinical trials (led in France and the UK) 5 of 20 patients treated for SCID-X1 developed T cell acute lymphoblastic leukemia (ALL) after infusion of autologous CD34+ T cells modified with the therapeutic MLV-based retroviral vector (Howe et al. 2008; Hacein-Bey-Abina et al. 2010). The causative relationship between the therapeutic vector and leukemia was confirmed as viral integrations were found near the promoter of the LMO2 lymphoid proto-oncogene leading to its increased expression (Fig. 2). Insertion site analyses revealed viral integrations also near to Cyclin D2 (CCND2) and MECOM (MDS1and EVI1 complex locus). Moreover, several integration sites were found in close proximity to other cancer-associated genes like the HoxB cluster, High-mobility group AT-hook 2 (HMGA2), jumonji, AT rich interactive domain 2 (JARID2) and Myeloid Cell Leukemia 1 (MCL1) (Hacein-Bey-Abina et al. 2003, 2010; Howe et al. 2008). This distribution of integration sites together with additional secondary genetic events led to the outgrowth of specific clones and finally leukemogenesis. Similar outcomes were observed when chronic granulomatous disease (CGD) and Wiscott-Aldrich syndrome (WAS) were treated with γ-retrovirus-based vectors (Ott et al. 2006; Braun et al. 2014). The comprehensive analysis of γ-retroviral integration sites early after gene therapy generally revealed a polyclonal pattern of haematopoiesis. However, „clonally skewed" vector insertion profiles, where clones with insertions near proto-oncogenes are enriched, were becoming evident during the follow-up studies, finally leading to the development of leukemias (Hacein-Bey-Abina et al. 2010; Braun et al. 2014).

Lately the initial SCID-X1 clinical trial was repeated using a SIN-γ-retroviral vector (Hacein-Bey-Abina et al. 2014). This new SIN-γ-retroviral vector was also based on MLV but the U3 enhancer was removed from the viral LTR and the expression of *IL2RG* gene was driven by the human elongation factor 1 short promoter (Zychlinski et al. 2008; Hacein-Bey-Abina et al. 2014) (Fig. 2). In its other properties the new vector was similar to the one used in the previous trial. Genome analyses revealed that the distribution of vector

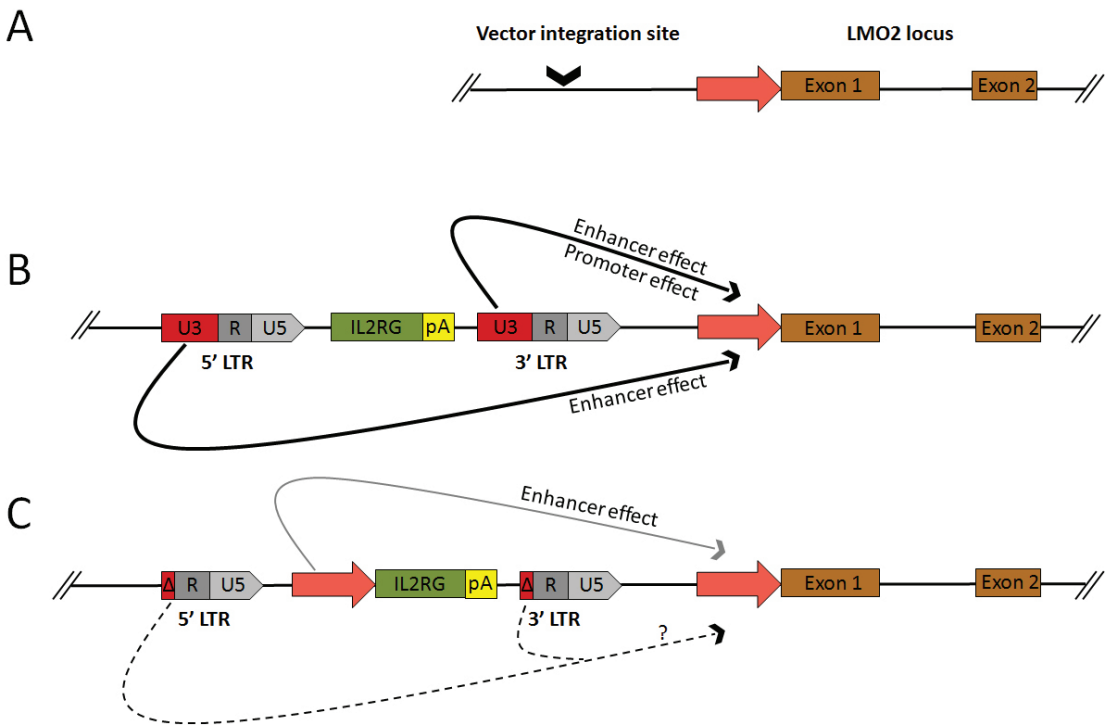

Figure 2. The mechanism of inducing insertional oncogenesis by MLV-derived γ-retroviral vectors. (A) Near the LMO2 locus a typical integration site for the MLV-derived vectors is shown. (B) The MLV-derived γ-retroviral vector and its potential impact on the adjacent LMO2 gene. (C) The MLV-derived SIN γ-retroviral vector and its potential impact on the adjacent LMO2 gene. Both vectors carry the IL2RG therapeutic gene. LTR, long terminal repeat; pA, polyadenylation signal; light red arrows, cellular promoters (EF1alpha promoter in the case of the therapeutic construct); U3, R and U5, are transcriptional control elements of the viral LTRs, the U3 element represents strong promoter/enhancer activity.

integration sites was largely similar using the SIN-γ-retroviral vector to patients' from the previous trial treated with the conventional γ-retroviral vector (Hacein-Bey-Abina et al. 2014). Elevated frequency of insertions has been detected near transcription start sites, gene-dense regions, and epigenetic marks associated with active transcription units. Thus, the alterations to the SIN-γ-retroviral vector did not have major effects on global associations of integration sites with these genomic features. However, "clonal skewing" has been less evident in the new clinical trial within the current observation period. Most importantly in the new SIN-γ-retroviral vector -based clinical trial, to date -with a median follow-up of 33 months- no serious adverse event occurred in the treated patients (Hacein-Bey-Abina et al. 2014). This suggests that removal of the viral enhancer reduced the potential of the γ-retroviral vector to transcriptionally activate nearby genes (Fig. 2).

Still the γ-retroviral and lentiviral genomic integration patterns may include a potential risk for carcinogenesis (Fig. 1). Therefore, other gene delivery vectors with significantly safer genomic integration patterns and lower potential to transcriptionally deregulate nearby genes are attractive alternatives of retroviral gene delivery.

General considerations supporting application of DNA transposons for gene therapy

In parallel with the development of safer viral vectors, alternative vector systems for gene therapy purposes are also under investigation. Among these, here we will focus on transposon-based vector systems.

Transposons are mobile genetic elements found in all eukaryotic genomes. Through their mobilization they are largely contributing to the diversity and evolution of host genomes (Prak and Kazazian 2000). Transposable elements can be classified into two major groups, such as DNA transposons and retrotransposons. Retrotransposons utilize "copy and paste" mechanism to spread in the host genome. The RNA intermediate of the element is reverse transcribed and inserted in a new genomic location. They have large evolutionary impact, and some of the currently active mammalian retrotransposons are also associated with human diseases (Belancio et al. 2008; Cordaux and Batzer 2009). Because of this and due to their

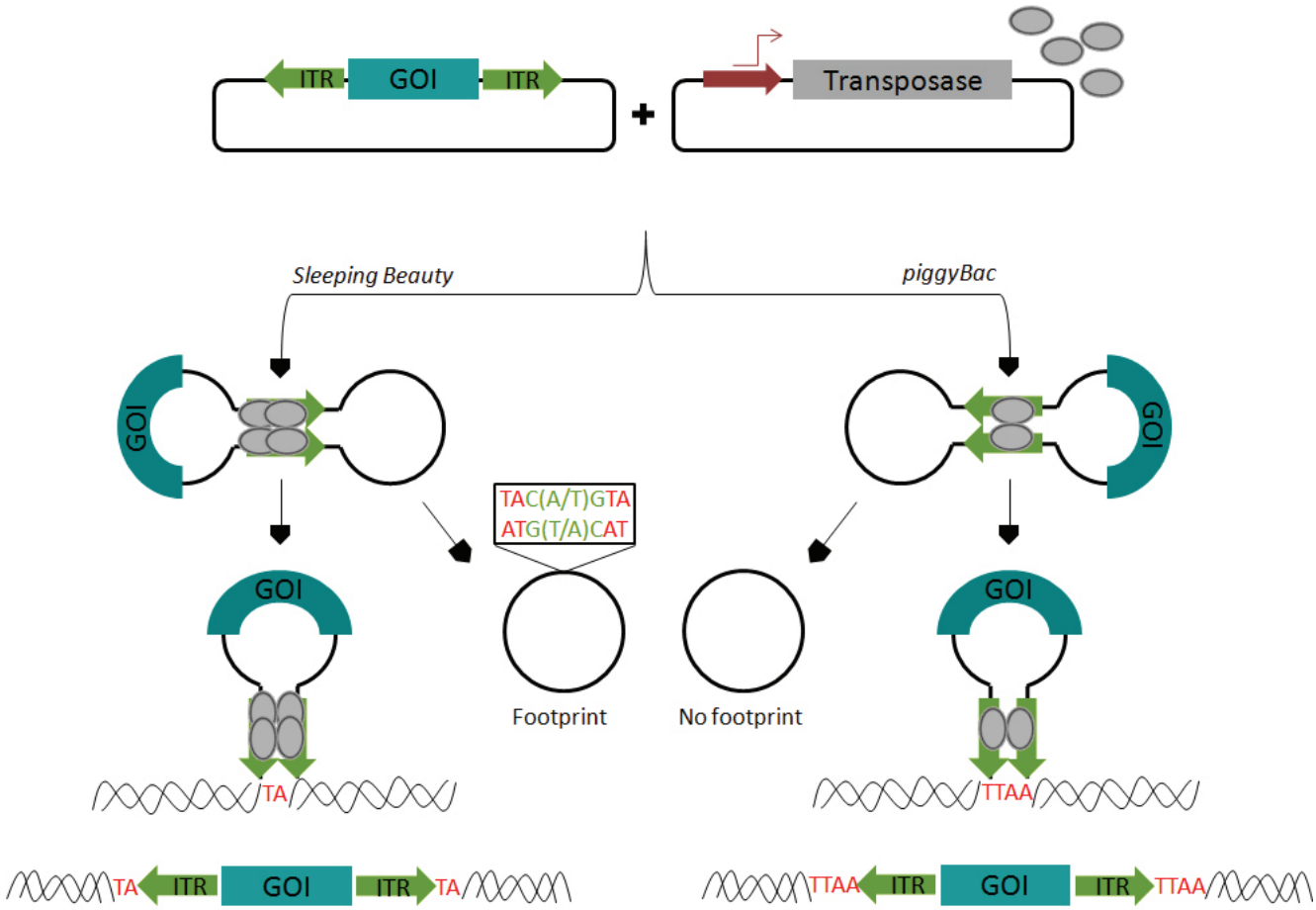

Figure 3. Schematic representation of the two-component DNA transposon-based gene delivery system and the mechanism of the 'cut and paste' transposition of *Sleeping Beauty* and *piggyBac* transposons. GOI, gene of interest; ITR, inverted terminal repeat; red arrow, promoter.

uncontrollable nature, retrotransposons are not good candidates as gene delivery vehicles for therapeutic purposes. Here, we rather focus on DNA transposons which mostly move in the host genome through a "cut and paste" mechanism (Fig. 3). Typically these elements are used for gene delivery in animal models and human gene therapy applications (Ivics et al. 2009). Their broad range of applicability is due to their simple genetic composition and easy handling as plasmid-based gene delivery tools. Native DNA transposons typically contain a single gene coding for the transposase protein. This gene is flanked by two inverted terminal repeats (ITRs) which contain binding sites for the transposase. During the "cut and paste" mechanism of their transposition the transposase binds to the ITRs and catalyzes excision and subsequent integration of the element. An appealing feature of this system is that the transposase protein can act on any sequence flanked by the ITRs. In conventional DNA transposon-based vector systems the transposase source is separated from the gene of interest placed between the ITRs. This way any gene of interest can be introduced efficiently into the host genome and the system's gene transfer activity is controlled and ensured by the trans-supplemented transposase (Fig. 3).

Therefore, gene therapy community has turned toward DNA transposons that share attractive features making them favorable tools for such applications. Some of their remarkable features designating them as a potentially better alternative than viral vectors for therapeutic gene delivery are mentioned below.

Cargo capacity

During design of viral vectors the packaging limit of the used virus should be taken into account. Size of the therapeutic constructs is limited by the packaging capacity of the virus capsid. Both the previously mentioned γ-retroviral and lentiviral vectors have the cargo capacity of ~8 kb (Thomas et al. 2003). In general, transposon-based vector systems can move larger genetic payload enabling the integration of complex or

multiple transgenes with a clinically relevant efficiency (Li et al. 2011; Turchiano et al. 2014)

Immunogenicity

One of the aims while constructing gene delivery vectors is to prevent the triggering of the immune system. Throughout the years of their gene therapy usage, viral vectors have been extensively engineered to reduce their immunogenicity. Nearly all coding regions of the viral genome is deleted, only those have been left intact which play role in packaging and integration. In practice, those remaining ones are provided *in trans* for the production of recombinant viral particles (Blesch 2004). Still, these systems need several viral proteins to function properly. These proteins have the potential to induce harmful immune-mediated and inflammatory responses.

In this regard, transposons serve as good alternatives. They are circular plasmid DNA, they do not contain viral proteins, in fact, the only protein needed for their function is the transposase. Therefore, they are significantly less immunogenic as compared to viral vectors. Although cytotoxicity can occur when applying transposon-based vectors due to the short-time transposase protein overexpression, this causes significantly lower immune response than the presence of viral helper proteins. Mechanisms of host response to non-viral vectors have not been completely elucidated. Toll-like receptor-9 (TLR-9) and DNA-dependent activator of interferon regulatory factors (DAI) are known to recognize unmethylated foreign DNA. These lead to sometimes overlapping signaling pathways to induce immune response (Baccala et al. 2009; Sharma and Fitzgerald 2011). Knowing of these mechanisms in detail will further help in the construction of immunologically safer transposon-based vectors.

Integration site preference

As already mentioned in the first section, different viral vectors prefer different genomic loci to integrate. γ-retroviral and lentiviral vectors show pronounced preference for integrating near to transcriptional start sites (Cattoglio et al. 2010) and into active transcriptional units (Schroder et al. 2002; Trono 2003), respectively. Accumulating data available related to the molecular mechanism of integration site selection of viral vectors. Probably the best known viral integrase (IN)-cellular cofactor interaction is the lentiviral IN-lens epithelium-derived growth factor (LEDGF) interaction which target lentiviral integration into active transcription units (Cherepanov et al. 2003) to the outer shell of the nucleus (Marini et al. 2015). Recent studies identified a host cellular cofactor of MLV, bromodomain containing protein 2 (BRD2), a member of the bromodomain and extraterminal domain (BET) family of chromatin binding proteins (Gupta et al. 2013). The authors also showed that with small-molecule BET inhibitors MLV integration can be reduced *in vivo*, and integration site preference can be shifted away from transcription start sites, thereby utilizing such an approach a safer MLV integration pattern may be achieved for gene therapeutic applications.

As opposed to viral vectors some DNA transposons, such as *Sleeping Beauty*, show significantly less preference for active genes or 5' regulatory regions in the genome (Fig. 1) (Yant et al. 2005; Wang et al. 2008), (see details in the next section). Although they exhibit a weak bias in integration site selection, the molecular determinants behind that are largely unknown.

Gene transfer efficiency

Among DNA transposons, predominantly members of the *Tc1/mariner* (*Sleeping Beauty*, Ivics et al. 1997) *piggyBac* (*piggyBack*, Ding et al. 2005) and *hAT* (Tol2, Urasaki et al. 2006; TcBuster, Woodard et al. 2012) families are used for transgene integration purposes in mammalian cells. These systems are all useful tools for experimental gene delivery in mammalian cells or in embryos. However, the initial gene transfer efficiencies exhibited by them in clinically relevant primary cell types were not comparable to that of the viral systems.

In the last decade hyperactive versions of the DNA transposons *Sleeping Beauty* and *piggyBac* have been developed exhibiting highly efficient gene transfer capabilities already reaching the viral efficiency range. Because of these and their numerous other useful features -detailed in the next sections- *Sleeping Beauty* and *piggyBac* are the most promising tools for gene therapy applications.

As they originate from different phylogenetic background, their biological properties are as well different. Knowing these properties in detail provides a basis for choosing the best-suited transposon vector system for the given therapeutic application (Table 1).

The Sleeping Beauty transposon system

Sleeping Beauty (SB) is a *Tc1/mariner* element, that was reconstructed almost two decades ago from inactive salmonid fish transposon sequences (Ivics et al. 1997). Since then it has shown to be active in several vertebrate species. One of its favorable features for using in human gene therapy is that it has no close relatives in the human genome, therefore the trans-mobilization of related host elements can be ruled out (Prak and Kazazian 2000).

The minimal sequence requirement for SB integration is a TA dinucleotide (Vigdal et al. 2002). This is duplicated upon transposition. Excision of an integrated SB element results in 3 bp 3´-overhangs. Repair of this overhang, together with the TA target site duplication, creates a characteristic transposition footprint (Fig. 3) (Liu et al. 2004). It was also shown that

Table 1. Main features of the DNA transposons *Sleeping Beauty* and *PiggyBac*.

Main characteristics	*Sleeping Beauty*	*PiggyBac*
Target sequence	TA (Vigdal et al. 2002)	TTAA (Fraser et al. 1996)
Integration site preference	Fairly random (31-39% into genes) (Yant et al. 2005)	Transcriptional units (47-67% into genes) (Ding et al. 2005; Burnight et al. 2012)
Footprint	C(A/T)GTA (Ivics et al. 1997)	None (Elick et al. 1996)
Cargo size	~10 kb (Izsvak et al. 2000)	~100 kb (Li et al. 2011)
Hyperactive transposase	SB100x (Mates et al. 2009)	hyPBase (Yusa et al. 2011)
Overproduction inhibition	Yes (Grabundzija et al. 2010)	Less affected (Wilson et al. 2007; Grabundzija et al. 2010)
Transposase tagging	Apparent efficiency drop (Wilson et al. 2005; Ivics et al. 2007)	No apparent efficiency drop (Owens et al. 2012)

target site selection is rather determined by DNA structure as SB insertion sites are associated with highly deformable sequences surrounding the TA dinucleotides (Geurts et al. 2006).

Extensive studies on SB integration site preference revealed that it shows no or weak preference for transcription units, it also does not prefer 5' regulatory regions and most of its gene hits are localized in introns (Yant et al. 2005). As gene therapy applications require vectors with the least preference for target genes to avoid insertional mutagenesis, SB is a promising system for such applications. However, despite the fairly random integration pattern, intragenic integrations can still occur holding the possible risk of genotoxicity. As it was already discussed in the case of viral vectors this is largely due to the strong promoter/enhancer activity of viral LTRs present in the therapeutic vectors. In contrast to the LTR of viral vectors, the ITRs of SB have very low intrinsic promoter /enhancer activity (Moldt et al. 2007). To further reduce the risk of insertional oncogenesis, generation of transposons capable of targeted integration into specific loci is desirable (Voigt et al. 2008). The main strategy for this is the fusion of sequence-specific DNA-binding domains to the transposase protein. Unfortunately, the activity of SB is largely compromised upon such fusions both C- and N-terminally (Wilson et al. 2005; Ivics et al. 2007).

In order to achieve optimal transposition with SB, the amounts of the components of the transposon system should be carefully optimized. High concentration of the transposase protein leads to a phenomenon called "overproduction inhibition", of which the exact molecular mechanism is not known. It is suggested that transposase proteins in excess are assembled in higher-order oligomers, or sequestrated in the cytoplasm or in nucleoli, thereby transposition activity is decreased (Lohe and Hartl 1996; Bire et al. 2013). In most comparison studies SB revealed the most prone to overproduction inhibition among the tested transposases (Grabundzija et al. 2010; Bire et al. 2013). The proper amount of the transposase expressing construct depends on the cell type and transfection conditions, while the amount of the transposon vector is mainly dependent on the desired transgene copy number. These are needed to be optimized for every particular application.

As the transposition activity of the original SB transposase is not robust enough to make it suitable for gene therapy applications, large efforts were made to increase its efficiency. As a result SB100x has been generated by a high-throughput, *in vitro* molecular evolution (Mates et al. 2009). This hyperactive transposase is 100-fold more potent than the original SB in HeLa cells. Since its creation it has been successfully applied for germline transgenesis purposes in rodents, (Katter et al. 2013; Ivics et al. 2014c) rabbits (Ivics et al. 2014b) and pigs (Ivics et al. 2014a) and for stable gene transfer in clinically relevant cells (Mates et al. 2009; Jin et al. 2011). It is also widely used in human gene therapy applications (Singh et al. 2014) (see the next section).

One limitation of the SB transposon system is the progressive loss of its transposition efficiency with increasing cargo size (Karsi et al. 2001). Although recent studies demonstrate that the SB system is able to carry large transgenes, even bacterial artificial chromosomes (BACs) (Rostovskaya et al. 2012), under such conditions the reduced transposition efficiencies are not compatible with gene therapeutic use. Therefore, when large therapeutic constructs (>10 kb) are needed to apply, another transposon system with larger cargo capacity would be a better choice.

The *piggyBac* transposon system

The *piggyBac* (PB) element is identified in the Cabbage Looper moth genome (Fraser et al. 1985). As it was found to be highly active in a wide range of insect species it has become the generally used vector for the germline modification of insects belonging to various orders (Handler 2002).

PB transposase targets TTAA tetranucleotide sequences without any structural requirement (Li et al. 2005) and excision of an integrated PB element frequently leaves behind

no footprint (Elick et al. 1996) (Fig. 3). PB on the genomic scale exhibits bias toward transcriptional start sites, CpG islands and actively transcribed loci (Ding et al. 2005). In case of the PB system, fusion of DNA-binding domains to the transposase may give the possibility of targeted insertion, as PB retains its transposition activity upon addition of such domains on both the 5' and 3' ends. Although the number of off-target integrations remained high, this may be a powerful approach to direct PB insertion to safe genomic loci (Owens et al. 2012). In most comparison studies with other transposases, PB exhibits the lowest sensitivity for overproduction inhibition even at high transposase concentrations (Wilson et al. 2007; Grabundzija et al. 2010). In contrast, a more recent study of Kolacsek et al. (2014) found PB more prone to overproduction inhibition then any of the SB variants tested.

The PB transposase has been modified in order to have optimal codon usage in mammalian cells and the new codon-optimized transposase *mPBase* has been effectively used for transposition in mouse cells (Cadinanos and Bradley 2007). So far, the most hyperactive PB transposase is *hyPB* identified in a screen of a large mutant library in *S. cerevisiae* (Yusa et al. 2011). One of the favorable features of both *mPBase* and *hyPB* is that they can move large genetic cargos up to 100 kb (Li et al. 2011), making the PB system a good candidate for integration of large therapeutic constructs.

PB has been successfully used for the reprogramming of mouse and human fibroblast cells to induce pluripotent stem cells (iPS) (Woltjen et al. 2009). Based on its characteristic that it does not leave footprints upon excision (Ding et al. 2005), the reprogramming factors were even removed from iPS cells by re-expressing the transposase protein (Yusa et al. 2009; Woltjen et al. 2011).

What may be of concern in terms of potential insertional oncogenesis is that the PB ITRs have significant enhancer/promoter activity. It was previously shown that the 5' ITR of PB acts as a promoter and the 3' ITR displays enhancer activity (Cadinanos and Bradley 2007; Shi et al. 2007; Wilson et al. 2007). Nevertheless, a recent study demonstrated that this promoter activity is directional and both activities can be isolated by using insulators (Saha et al. 2015). However, random integration of an insulator-containing vector can be detrimental if it blocks the interaction between an endogenous promoter and its critical enhancer. Therefore, application of insulator elements as safety precaution should be carefully considered.

Another safety concern for clinical application of the PB system is that numerous PB ITR-like sequence containing DNA elements and domesticated PB transposase-like genes are present in the human genome. The presence of ~2000 PB-like DNA elements in the genome raises the question of whether there is a risk of genomic mobilization or rearrangement upon expression of the exogenously delivered PB transposase (Feschotte 2006). Lately it has been demonstrated that, it seems unlikely that expression of the PB transposase can induce endogenous element mobilization or genomic rearrangements, even though only a few of possible PB ITR-like elements were directly investigated (Saha et al. 2015). Transposase domestication occurs when the host recruits the transposase or some of its protein domains encoded by a mobile element in order to perform cellular functions (Feschotte 2008). In the human genome 5 domesticated PB transposase-like genes, piggyBac transposable element derived 1-5 (PGBD1-5) are present (Sarkar et al. 2003). Among these, PGBD3 and 5 are under thorough investigation. PGBD3 has been resulted from the landing of a PB element to intron 5 of the human Cockayne syndrome Group B (CSB) gene, leading to the generation of a CSB-PiggyBac fusion protein expressed in Cockayne syndrome (Newman et al. 2008). Whereas PGBD5 has found to be neural-specific and it may have played role in neural evolution (Pavelitz et al. 2013). Presence of these domesticated elements may raise the possibility of protein-protein interaction with administered transposase proteins resulting in functional interference, a phenomenon whose existence has not yet been proved.

Current stage of transposon applications for gene therapy

The SB and PB DNA transposon systems have been studied extensively to treat both genetic and acquired diseases. They proved their efficiency both *in vitro* and *in vivo* gene transfer in clinically relevant cell types and preclinical animal models.

Successful preclinical studies with the SB system include: expression of human blood coagulation factor IX in factor IX-deficient mice (Yant et al. 2000); correction of murine tyrosinemia type I by delivering the functional human fumaryl acetoacetate hydrolase gene (FAH) (Montini et al. 2002); correction of junctional epidermolysis bullosa in human patient-derived cells (Ortiz-Urda et al. 2003); stable expression of insulin in the livers of diabetic mice (He et al. 2004); expression of blood coagulation factor VIII and factor IX and phenotypic correction in mouse hemophilia models (Ohlfest et al. 2005b; Hausl et al. 2010); correction of mucopolysaccharidosis type I and VII in a mouse model (Aronovich et al. 2007; Aronovich et al. 2009); correction of Fanconi anemia type C in human patient-derived cells (Hyland et al. 2011); long-term reduction of jaundice in Gunn rats (Wang et al. 2009) and correction of lung allograft fibrosis in a rat model (Liu et al. 2006). Anti-cancer therapy with SB was also done in mice having intracranial human glioblastoma (Ohlfest et al. 2005a); gastrointestinal cancer (Bao et al. 2012) and osteosarcoma (Huang et al. 2012).

PB was also successfully used in preclinical cancer cell

gene therapy approaches. It was applied for a gene-directed enzyme prodrug therapy to treat mouse ovarian cancer and human ovarian adenocarcinoma cells (Kang et al. 2009a, 2009b), for treatment of B-lineage malignancies (Manuri et al. 2010) and for the generation of Epstein-Barr Virus (EBV)-specific cytotoxic T-cells targeting Human Epidermal growth factor Receptor 2 (HER2) positive cancer cells (Nakazawa et al. 2011). It was also used for modeling treatment of α1-antitrypsin deficiency (Burnight et al. 2012) and hemophilia A in mice (Matsui et al. 2014).

Among DNA transposons for the time being only the SB system is involved in clinical trials. The first clinical trial using the SB transposon system was launched in 2008 in the United States. The strategy involves targeting modified T-cells to tumor-associated antigens (TAAs). TAAs are present on the surface of tumor cells and not on healthy cells, thereby specific recognition and destruction of the tumor cells is possible. Both autologous and allogeneic T-cells are used for *ex vivo* modification, during which a transposon vector carrying a chimeric antigen receptor (CAR) gene specific for a TAA is stably introduced into their genome, to produce the CAR T-cells. Currently, there are eight ongoing phase-I clinical trials in the United States involving CAR T-cells generated with the SB system to treat different malignancies (http://www.wiley.com//legacy/wileychi/genmed/clinical/). Most of the ongoing clinical trials are aiming to treat B-lymphoid malignancies by CAR T-cells targeting the B-cell antigen CD19. On the basis of initial clinical data next generation clinical trials can be planned. Recent studies attempt to increase transpositional efficiency by using the hyperactive SB100X transposase and to further decrease possible genotoxicity caused by the continuous transposase expression by using in vitro-transcribed mRNA as the transposase source. Furthermore, another approach is under development with which T-cells from a healthy donor can be pre-prepared to express CARs which can largely broaden the human application of CAR T-cells (Singh et al. 2014).

A European project led by the University of Geneva is also moving towards clinical trials targeting age-related macular degeneration (AMD). Scientist within the TargetAMD research network (http://www.targetamd.eu/) are aiming to develop an SB transposon-based gene therapy for the treatment of the exudative form of AMD. This disease causes vision loss due to abnormal vascularization of the retina. This process is stimulated by increased levels of vascular endothelial growth factor (VEGF) and can be inhibited by the induction of pigment epithelium-derived factor (PEDF) which is an anti-angiogenic protein (Holekamp et al. 2002). Overexpression of the latter protein in modified cells is expected to restore VEGF/PEDF balance, thereby suppressing neovascularization of the retina and providing a long-term cure for exudative AMD. The therapeutic approach comprises the subretinal transplantation of autologous retinal pigment epithelial cells modified with an SB transposon vector carrying the recombinant PEDF gene. Therapeutic gene delivery is achieved using the SB100X hyperactive transposase as it has been previously shown to mediate effective and stable transgene delivery into ARPE19 cells, as well as into primary retinal and iris pigment epithelial cells (Johnen et al. 2012). Very recently, by applying this SB-mediated PEDF transgene delivery into retinal pigment epithelial cells before their transplantation, the corneal neovascularization was significantly reduced in a rabbit model of AMD (Kuerten et al. 2015).

Concluding remarks

Development of the field of gene therapy brought up numerous efficient and promising treatment opportunities giving hope to cure diseases that have proven to be incurable by conventional treatments. The majority of gene therapy protocols require efficient delivery of therapeutic genes into relevant target cells, followed by their robust chromosomal integration for long-term expression and clinical benefit. Clinical trials treating primary immunodeficiencies with retroviral vectors demonstrated efficient gene transfer and correction of the immunodeficiency (Farinelli et al. 2014). After the initial success, however, their long-term clinical outcome highlighted the risk of adverse effects associated to therapeutic gene delivery (Farinelli et al. 2014). It became clear that retroviral vectors typically activated the expression of nearby tissue specific proto-oncogenes, resulting in insertional oncogenesis. This called for the development of nonviral gene delivery vectors with significantly safer integration patterns and lower potential to transcriptionally deregulate nearby genes. Recently, SB and PB transposon-based vectors, efficient enough in mammalian cells, represent a promising new toolkit of randomly integrating vectors for gene therapy (Mates et al. 2009; Yusa et al. 2011). It is not yet known whether their attractive features will indeed be translated to an improved safety profile of gene therapy protocols. However, it should be emphasized that so far neither of the SB and PB vectors exploited for preclinical applications has provided any evidence of triggering insertional oncogenesis (Montini et al. 2002; Hausl et al. 2010; Matsui et al. 2014).

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