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Nonviral Gene Delivery with the *Sleeping Beauty* Transposon System

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Abstract

Effective gene therapy requires robust delivery of therapeutic genes into relevant target cells, long-term gene expression, and minimal risks of secondary effects. Nonviral gene transfer approaches typically result in only short-lived transgene expression in primary cells, because of the lack of nuclear maintenance of the vector over several rounds of cell division. The development of efficient and safe nonviral vectors armed with an integrating feature would thus greatly facilitate clinical gene therapy studies. The latest generation transposon technology based on the *Sleeping Beauty* (*SB*) transposon may potentially overcome some of these limitations. *SB* was shown to provide efficient stable gene transfer and sustained transgene expression in primary cell types, including human hematopoietic progenitors, mesenchymal stem cells, muscle stem/progenitor cells (myoblasts), induced pluripotent stem cells, and T cells. These cells are relevant targets for stem cell biology, regenerative medicine, and gene- and cell-based therapies of complex genetic diseases. Moreover, the first-in-human clinical trial has been launched to use redirected T cells engineered with *SB* for gene therapy of B cell lymphoma. We discuss aspects of cellular delivery of the *SB* transposon system, transgene expression provided by integrated transposon vectors, target site selection of the transposon vectors, and potential risks associated with random genomic insertion.

Introduction

The ability to efficiently deliver foreign genes into cells provides the basis of using gene therapy to cure genetic diseases. The vast majority of gene delivery systems currently tested in clinical trials are based on viral vectors. Because viruses are highly specialized at crossing through cellular membranes by infection, they are efficient at delivering nucleic acids to target cell populations. However, some viral vectors, including those derived from adenoviruses or adeno-associated viruses, remain largely episomal, requiring readministration in order to maintain a desired level of transgene expression over time. However, repeated delivery can provoke immune responses against vector-encoded proteins (reviewed in Hartman *et al.*, 2008). In contrast, retroviruses integrate their therapeutic cargo into the genome, resulting in long-term transgene expression. A concern with using retroviral vectors is genotoxicity associated with mutagenic effects elicited by insertion of the vector into or near genes (Hacein-Bey-Abina *et al.*, 2003, 2008; Baum *et al.*, 2004). Such risk is especially pronounced with gammaretroviral vectors based on the murine leukemia virus (MLV) that preferentially integrate into transcription start sites (Wu *et al.*, 2003). Furthermore, HIV-derived lentiviral vectors are potential mutagens due to their biased insertion into transcription units (Schroder *et al.*, 2002; Bushman, 2003). Indeed, adverse events provoked by insertional mutagenesis of MLV-based vectors and resulting aberrant T cell proliferation have been observed in clinical trials for gene therapy of X-linked severe combined immunodeficiency (SCID-X1) (Hacein-Bey-Abina *et al.*, 2003, 2008; Thrasher *et al.*, 2006). Additional concerns include the possibility that transcriptional silencing may compromise expression of the integrated transgene (reviewed in Ellis, 2005), and that large transgenes may inhibit viral reverse transcription and packaging, thereby setting limitations to vector design for clinical use of retroviral vectors. Even though the second-generation, self-inactivating (SIN) recombinant retroviral vectors that lack strong enhancer elements in their long terminal repeats (LTRs) (Schambach *et al.*, 2006, 2007; Modlich *et al.*, 2009) may be able to address some of the inadvertent side effects such as insertional oncogenesis, the high costs associated with the manufacture of

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clinical-grade retroviral vectors as well as regulatory issues limit their widespread clinical translation. As a result, significant efforts and developments have been made toward crafting gene transfer vector systems that are as efficient but safer than viruses in clinical gene transfer.

Nonviral vector systems generally suffer from inefficient cellular delivery and limited duration of transgene expression due to the lack of genomic insertion and resulting degradation and/or dilution of the vector in transfected cell populations. Developments of nonviral delivery techniques, including liposomal formulations, nanoparticles, advanced electroporation methods such as nucleofection, and cell-penetrating peptides can significantly enhance the transfer of nucleic acids into therapeutically relevant cell types, even *in vivo.* In postmitotic tissues, nonviral vectors may provide long-lasting transgene expression; however, in the absence of long-term nuclear maintenance in dividing cell types such as stem cells, even efficient introduction of nucleic acids into cells does not guarantee long-term transgene expression. One class of nonviral vector systems that could potentially offer long-term expression in dividing cell types is based on scaffold/matrix attachment region (S/MAR)-containing episomal vectors that can promote replication and maintenance in mammalian cells (Hagedorn *et al.*, 2011). Yet another type of therapeutic vector would ideally unite the advantages of integrating viral vectors (i.e., long-lasting transgene expression) with those of nonviral delivery systems (i.e., lower immunogenicity, enhanced safety profile and reduced costs of manufacture). Transposable elements (transposons) could potentially offer such an alternative.

The *Sleeping Beauty* Transposon as a Nonviral Vector for Gene Therapy

DNA transposons are discrete pieces of DNA with the ability to change their positions within the genome via a cut-and-paste mechanism called *transposition*. In nature, these elements exist as single units containing the transposase gene flanked by terminal inverted repeats (TIRs) that carry transposase-binding sites (Fig. 1A). However, under laboratory conditions, it is possible to use transposons as bicomponent systems, in which virtually any DNA sequence of interest can be placed between the transposon TIRs and mobilized by *trans*-supplementing the transposase in the form of an expression plasmid (Fig. 1B) or mRNA synthesized *in vitro*. In the transposition process, the transposase enzyme mediates the excision of the element from its donor plasmid, followed by reintegration of the transposon into a chromosomal locus (Fig. 1C). This feature makes transposons natural and easily controllable DNA delivery vehicles that can be used as tools for versatile applications, including gene therapy.

On the basis of transposon fossils that are presumed to have been active >10 million years ago in fish genomes, an ancient transposon was resurrected, and named *Sleeping Beauty* (*SB*) after the famous fairy tale collected by the Brothers Grimm, because it was literally awakened after a long evolutionary "sleep" (Ivics *et al.*, 1997). *SB* was the first transposon ever shown capable of efficient transposition in vertebrate cells, thereby enabling new avenues for genetic engineering in animal model species (reviewed in Ivics *et al.*, 2009) as well as for human gene therapy (Yant *et al.*, 2000; Ivics and Izsvak, 2006). As a nonviral alternative to viral vectors, the potential of the *SB* system has been thoroughly probed (Izsvák and Ivics, 2004; Fernando and Fletcher, 2006; Ivics and Izsvak, 2006; VandenDriessche *et al.*, 2009; Hackett *et al.*, 2010; Izsvák *et al.*, 2010).

The advantage of *SB* transposon-based gene delivery is that it combines the favorable features of viral vectors with those of naked DNA molecules. Namely, owing to permanent genomic insertion of transgene constructs (Fig. 1C), transposition-mediated gene delivery can lead to sustained and efficient transgene expression in preclinical animal models (Hackett *et al.*, 2010). However, in contrast to viral

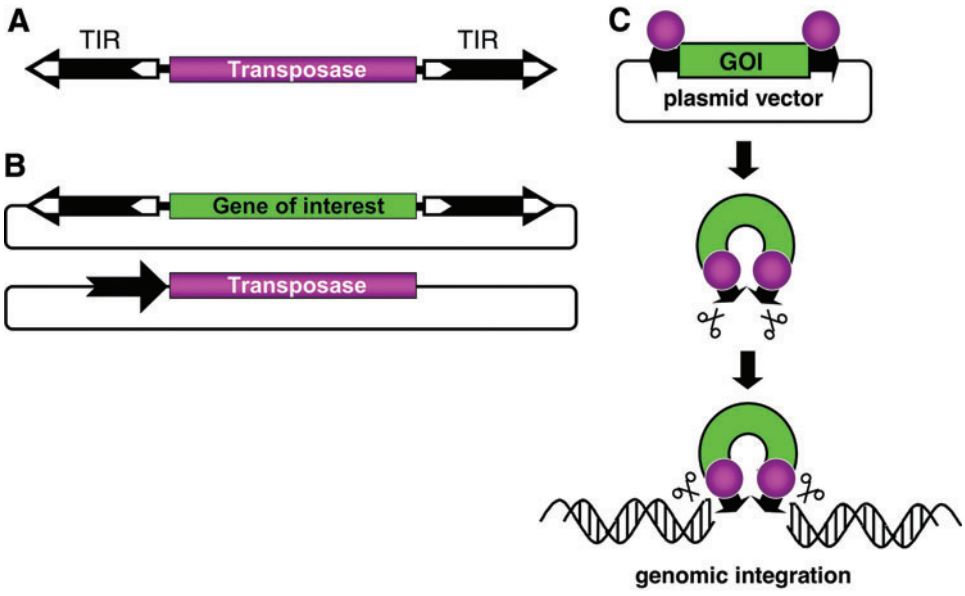

FIG. 1. General organization and use of transposable elements as gene vectors. **(A)** Autonomous transposable elements consist of terminal inverted repeats (TIRs; black arrows) that flank the transposase gene. **(B)** Bicomponent transposon vector system for delivering transgenes that are maintained in plasmids. One component contains a DNA of interest between the transposon TIRs carried by a plasmid vector, whereas the other component is a transposase expression plasmid, in which the black arrow represents the promoter driving expression of the transposase. **(C)** The transposon carrying a DNA of interest is excised from the donor plasmid and is integrated at a chromosomal site by the transposase. Color images available online at www.liebertonline.com/hum

vectors, transposon vectors can be maintained and propagated as plasmid DNA (Fig. 1B and C), which makes them simple and inexpensive to manufacture, an important consideration for the implementation of future clinical trials. Further advantages of the *SB* system include its reduced immunogenicity (Yant *et al.*, 2000), no strict limitation of the size of expression cassettes (Zayed *et al.*, 2004), and improved safety and toxicity profiles (Ivics *et al.*, 2007; Moldt *et al.*, 2007; Walisko *et al.*, 2008; VandenDriessche *et al.*, 2009). Because transposition proceeds through a cut-and-paste mechanism that involves only DNA, transposon vectors are not prone to incorporating mutations by reverse transcription (that are generated in retroviral stocks at reasonable frequencies), and can tolerate larger and more complex transgenes. Unlike the LTRs of retroviruses, the TIRs of *SB* vectors have low enhancer/promoter activity (Moldt *et al.*, 2007; Walisko *et al.*, 2008). The insertion of chromatin boundary elements (insulators) flanking the transposon-contained expression cassettes (Fig. 2A) to prevent accidental *trans*-activation of cellular promoters further improved the safety profile of the *SB* system (Walisko *et al.*, 2008). Furthermore, synthetically produced mRNA can serve as a source of the transposase, thereby limiting the duration of transposase expression and lowering the risk of "rehopping" of the already integrated transposon-based vector (Wilber *et al.*, 2006). Chromosomal integration of *SB* transposons is random, which is potentially mutagenic (see below), but no *SB*-associated adverse effects have been observed in preclinical animal studies (Fernando and Fletcher, 2006; Ivics and Izsvak, 2006; Hackett *et al.*, 2010; Izsvák *et al.*, 2010). Last, *SB* transposons can be harnessed to integrate plasmid-based small hairpin RNA (shRNA) expression cassettes into chromosomes to obtain stable knockdown cell lines by RNA interference (Fig. 2B) (Kaufman *et al.*, 2005). Such technologies have been evaluated as a potential approach to the therapy of acquired immunodeficiency syndrome by stable RNA interference with *SB* vectors knocking down the CCR5 and CXCR4 cell surface coreceptors that are required for viral entry as a first step to confer resistance to HIV (Tamhane and Akkina, 2008).

Delivery of the Sleeping Beauty *vector system*

In *ex vivo* gene delivery, the therapeutic gene vector is introduced into a selected cell population that had been isolated from the patient, and the treated cells are transplanted back into the same patient. Because transposons are not infectious, it is necessary to combine the plasmid-based transposon vectors with technologies capable of efficient delivery of these nonviral vectors into cells. Because the efficiency of transposition is dependent on the efficiency of uptake of the introduced plasmids into the cell nuclei, delivery is a rate-limiting factor in transposition, and is thus of paramount importance. In principle, any technology developed for transferring nucleic acids into cells can be combined with transposon vectors. Unfortunately, there is no generally applicable method, and procedures must be established for each cell type. In difficult-to-transfect cells, including primary stem cells, delivery of transposon-based vectors can be significantly facilitated by nucleofection, a procedure based on electroporation that transfers nucleic acids directly into the nucleus. Indeed, nucleofection facilitated transposition in various stem cells including $CD34^+$ hematopoietic progenitors (Hollis *et al.*, 2006; Izsvák *et al.*, 2009; Mates *et al.*, 2009; Sumiyoshi *et al.*, 2009; Xue *et al.*, 2009), primary T cells (Huang *et al.*, 2008, 2009; Singh *et al.*, 2008) and human embryonic stem cells (Wilber *et al.*, 2007; Orban *et al.*, 2009). Importantly, in the context of the hematopoietic system, this *ex vivo* gene delivery procedure apparently did not compromise the potential of transposon-marked $CD34^+$ cells to differentiate normally into the erythroid, megakaryocytic, granulocyte/monocyte/macrophage lineage (Mates *et al.*, 2009) as well as into the $CD4^+CD8^+$ T, $CD19^+$ B, $CD56^+$ $CD3^-$ natural killer (NK), and $CD33^+$ myeloid lineages (Xue *et al.*, 2009). The robustness and feasibility of this nonviral, transposon-based procedure may significantly facilitate clinical realization of *ex vivo* stem cell therapy for the treatment of hematopoietic disorders and cancer, which has led to its application in humans (Williams, 2008).

In *in vivo* gene delivery, the therapeutic gene vector is directly introduced into an organ, where expression of the therapeutic gene construct is desired. Complexing naked DNA with polyethylenimine (PEI) followed by intravenous injection is one of the most effective methods to deliver therapeutic genes to the lung (Belur *et al.*, 2007). This approach resulted in long-term expression of luciferase after delivery of *SB* transposon vectors to the lungs of mice (Belur *et al.*, 2003, 2007). Complexing of transposon vectors with PEI allowed expression of therapeutic levels (10% of normal) of the blood coagulation factor VIII (FVIII) as well as phenotypic correction of the bleeding disorder in a mouse model of hemophilia A (L. Liu *et al.*, 2006b). In a rat model of pulmonary hypertension, systemic administration of an *SB* vector harboring an endothelial nitric oxide synthase (eNOS) gene resulted in inhibition of induced pulmonary

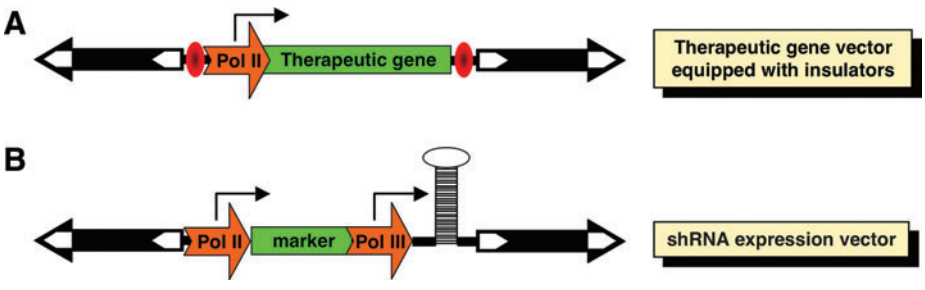

FIG. 2. Expression cassettes delivered by *Sleeping Beauty* transposon vectors. **(A)** A typical therapeutic expression cassette contains a ubiquitous or tissue-specific enhancer/PolII promoter that drives expression of a therapeutic gene. To enhance the safety of such a vector, the expression cassette might be flanked by insulator elements that will block *trans*-activation of endogenous promoters by the transposon insertion, and simultaneously protect the expression of the therapeutic gene from position effects. **(B)** Knockdown expression cassette including a PolII promoter that drives expression of a marker gene and a PolIII promoter that drives expression of a short hairpin RNA (shRNA). Color images available online at www.liebertonline.com/hum

hypertension (L. Liu *et al.*, 2006a). Furthermore, PEI-based systemic delivery of the *SB* transposon system encoding the human indoleamine-2,3-dioxygenase (hIDO) gene was evaluated in the context of lung transplantation-associated chronic complications, and found to elicit a remarkable therapeutic response, as evidenced by near normal pulmonary function in lung allografts (H. Liu *et al.*, 2006). Last, *SB*-mediated gene transfer significantly increased survival of mice bearing human glioblastoma xenografts by expressing antiangiogenic gene products (Ohlfest *et al.*, 2005a), and improved the efficacy of immunotherapy by facilitating sustained cytokine expression after local, intratumoral injections of vector–PEI complexes (Wu *et al.*, 2007).

As an alternative to complexing with a chemical compound, the use of physical delivery systems, such as hydrodynamic injection, can facilitate cellular uptake of naked DNA molecules (Liu *et al.*, 1999; Zhang *et al.*, 1999). This procedure involves rapid injection of a large volume of DNA solution through the tail vein. Delivery of therapeutic *SB* transposon constructs by hydrodynamic injection (Bell *et al.*, 2007) was first demonstrated to be applicable to mediate stable, long-term expression in mouse liver by Yant and colleagues, who reported sustained expression of α_1-antitrypsin in C57BL/6 mice (Yant *et al.*, 2000). This procedure has been successfully applied to confer a therapeutic benefit in several animal models of human diseases, including hemophilia B after delivery of human clotting factor IX (FIX) as a therapeutic gene product in FIX-deficient mice (Yant *et al.*, 2000) and tyrosinemia after hydrodynamic delivery of a fumarylacetoacetate hydrolase (FAH)-encoding *SB* transposon vector into the livers of FAH-deficient mice that showed selective outgrowth of genetically corrected hepatocytes (Montini *et al.*, 2002). Additional, successful preclinical testing of the *SB* system has been established in disease models for hemophilia A (Ohlfest *et al.*, 2005b) and mucopolysaccharidosis (Aronovich *et al.*, 2007, 2009).

Cell type-specific expression of the transgene is often desirable in order to exclude or limit ectopic transgene expression. One way to achieve this is to equip the transposon-contained expression cassette with tissue-specific promoters; this was shown to confer erythroid-specific expression of β-globin in a sickle cell disease model (Zhu *et al.*, 2007). Another strategy for targeted expression of therapeutic gene constructs is their targeted delivery into tissues of interest. Hyaluronan- and asialoorosomucoid-coated nanocapsules, were found to target *SB*-based vectors carrying an FVIII transgene to hepatocytes *in vivo* and to improve the phenotype of hemophilia A mice, after intravenous injection (Kren *et al.*, 2009). Similarly, packaging an *SB* vector expressing chUGT1A1 into proteoliposomes incorporating a fusogenic glycoprotein that promoted asialoglycoprotein receptor (ASGPR)-mediated endocytosis allowed successful *in vivo* delivery and sustainable, therapeutic gene expression in the hepatocytes of a rat model of Crigler-Najjar syndrome type 1 (Wang *et al.*, 2009). Importantly, in neither study was a significant host immune response toward the gene delivery vehicle or the transgene product observed (Kren *et al.*, 2009; Wang *et al.*, 2009). In summary, when combined with nonviral delivery approaches, the *SB* transposon system shows considerable efficacy in providing sustained levels of therapeutic gene expression both *ex vivo* and *in vivo*.

Target site selection of Sleeping Beauty *and its experimental manipulation*

The insertion pattern of most transposons is nonrandom, showing characteristic preferences for insertion sites at the primary DNA sequence level, and "hotspots" and "cold regions" on a genome-wide scale. *SB* displays considerable specificity in target site selection at the primary DNA sequence level in that TA dinucleotides are obligate target sites (Vigdal *et al.*, 2002). A palindromic AT-repeat consensus sequence with bendability and hydrogen-bonding potential was found to constitute the preferred target site (Vigdal *et al.*, 2002). It was later shown that a characteristic deformation of the DNA sequence may be a recognition signal for target selection (Liu *et al.*, 2005). This deformation, and the likelihood that a particular TA will be targeted by *SB*, can be computationally predicted (Liu *et al.*, 2005), which may allow a theoretical assessment of risks associated with transposon insertions in particular genomic regions (Geurts *et al.*, 2006). Nevertheless, a systematic assessment of potential genotoxic effects associated with genomic integration of transposon vectors will need to be performed either in cell-based assays and/or in animal models to provide clinically relevant data.

In contrast to the considerable specificity at the primary DNA sequence level, *SB* integration can be considered fairly random at the genomic level (Vigdal *et al.*, 2002; Yant *et al.*, 2005; Moldt *et al.*, 2011). Roughly one-third of *SB* insertions in mouse and human cells occur in transcribed regions, and because genes cover about one-third of the genome, such frequency suggests neither preference for nor disfavoring insertion into genes. The vast majority of those insertions that occur in genes are located in introns (Vigdal *et al.*, 2002), and, in contrast to most integrating viral vectors, the transcriptional status of targeted genes apparently does not influence the integration profile of *SB* (Yant *et al.*, 2005).

Target site selection properties suggest that *SB* might be safer for therapeutic gene delivery than the integrating viral vectors that are currently used in clinical trials. However, with any vector that integrates into chromosomes in a nearly random manner (theoretically, the *SB* transposon could insert into any of the $\sim 10^8$ TA sites in the human genome) comes the potential risk of insertional mutagenesis leading to transcriptional activation or inactivation of cellular genes (Baum *et al.*, 2004). Integration of the vector into a gene or its regulatory elements can knock out the gene, overexpress the gene, or alter its spatio/temporal expression pattern. Such genotoxic effects can have devastating consequences for the cell and the whole organism, including the development of cancer, as discussed previously (Baum *et al.*, 2004). As a possible strategy, introducing an imposed bias into the insertion profile, ideally, targeted integration of the therapeutic gene into a "safe" site in the human genome, could lower or eliminate possible hazards to the host cell. For targeted transposon insertion at least one component of the transposon system, either the transposon vector DNA or the transposase (or factors interacting with either of these components), needs to be engineered to be physically linked or interact with a heterologous DNA-binding domain (DBD), which is to tether the transposase/transposon complex to defined sites in the human genome, and to facilitate integration of the transposon into adjacent DNA (Voigt *et al.*, 2008). Fusions of the *SB* transposase with the GAL4 DBD

showed an enrichment of transposon insertions in a ~400-bp window around GAL4-binding sites in plasmid targets in human cells (Yant *et al.*, 2007). Target-selected *SB* insertion was also assessed by employing a molecular strategy based on engineering a *LexA* operator sequence into an *SB* transposon vector. Targeted transposition events into chromosomal S/MAR sequences as well as a chromosomally integrated tetracycline response element were recovered by coexpressing targeting fusion proteins containing LexA and either the SAF box, a protein domain that binds to S/MARs, or the tetracycline repressor (TetR) (Ivics *et al.*, 2007). Presumably, these targeting fusion proteins "sandwiched" the transposon and target DNA sites, allowing local insertion events. Last, targeted *SB* transposition within a 2.5-kb window around a chromosomally located tetracycline response element was observed at a frequency of >10% (Ivics *et al.*, 2007) by coexpressing the *SB* transposase with a targeting fusion protein consisting of TetR and a subdomain of the *SB* transposase spanning the N-terminal helix–turn–helix domain (N57) that mediates protein–protein interactions between transposase subunits (Izsvák *et al.*, 2002). Ongoing work in the authors' laboratory is dedicated to determining whether *SB* transposition can potentially be directed to physiologically relevant, endogenous sites in the human genome, by using both naturally occurring as well as synthetic DBDs.

Transgene expression

Any transgene vector system should ideally provide long-term expression of transgenes. By using classical, plasmid-based, nonviral delivery approaches, expression from the extrachromosomal plasmid rapidly declines after delivery. Transgenes delivered by nonviral approaches often form long, repeated arrays (concatemers) that are targets for transcriptional silencing by heterochromatin formation. In addition, long-term expression of transgenes delivered by retroviruses has been shown to be compromised by transcriptional silencing (Jahner *et al.*, 1982). It was shown that the zinc finger protein ZFP809 bridges the integrated proviral DNA of the murine leukemia virus and the tripartite motif-containing 28 transcriptional corepressor in embryonic stem cells (Wolf and Goff, 2009). Thus, sequence elements in the vector itself can predispose the cargo for silencing. The cut-and-paste mechanism of DNA transposition results in a single copy of the transgene per insertion locus, and thus concatemer-induced gene silencing is unlikely to be an issue with transposition-mediated gene transfer. Indeed, Grabundzija and colleagues found that transposon insertions delivered by the *SB* system only rarely (<2% of all insertions) undergo silencing in HeLa cells (Grabundzija *et al.*, 2010). Furthermore, stable transgene expression observed in ~300 independent insertions in this study suggests that *SB* rarely targets heterochromatic chromosomal regions for insertion, and that it is unlikely that certain sequence motifs in the transposon vector are recognized by mediators of silencing in the cell. An additional factor that may provoke transgene silencing is the cargo DNA, particularly the type of promoter used to drive expression of the gene of interest. Indeed, it was previously shown that transgene constructs delivered into mouse cells by *SB* transposition can be subject to epigenetic regulation by CpG methylation and that a determinant of epigenetic modifications of the integrating transposon vector is the cargo transgene construct, with the promoter playing a major role (Garrison *et al.*, 2007). However, with careful promoter choice, several studies have established that *SB*-mediated transposition provides long-term expression *in vivo*, as discussed previously. Notably, stable transgene expression from *SB* vectors was seen in mice after gene delivery in the liver (Yant *et al.*, 2000; Ohlfest *et al.*, 2005b; Aronovich *et al.*, 2009; Kren *et al.*, 2009), lung (Belur *et al.*, 2003; L. Liu *et al.*, 2006b), brain (Ohlfest *et al.*, 2005a), and blood after hematopoietic reconstitution *in vivo* (Mates *et al.*, 2009; Xue *et al.*, 2009). Thus, although our understanding of all the factors that will ultimately determine the expressional fate of an integrated transposon is still rudimentary, it appears that transposon vectors have the capacity to provide long-term expression of transgenes both *in vitro* and *in vivo*.

Gene transfer into stem cells by the SB100X *hyperactive transposase*

The hyperactive variant of the *SB* transposase, *SB100X*, yields efficient stable gene transfer after nonviral gene delivery into therapeutically relevant primary cell types, including stem or progenitor cells. For example, the use of the *SB100X* system yielded robust gene transfer efficiencies into human hematopoietic progenitors (Mates *et al.*, 2009; Xue *et al.*, 2009), mesenchymal stem cells, muscle stem/progenitor cells (myoblasts), induced pluripotent stem cells (iPSCs) (Belay *et al.*, 2010), and T cells (Jin *et al.*, 2011). These cells are relevant targets for stem cell biology and for regenerative medicine and gene- and cell-based therapies of complex genetic diseases. Importantly, expression of the *SB100X* hyperactive transposase did not adversely influence the differentiation or function of these adult stem/progenitor cells, nor was there any evidence of any cytogenetic abnormalities (Belay *et al.*, 2010). In the context of iPSC technology, the ability to coax the differentiation of pluripotent stem cells into clinically relevant, transplantable cell types is a key step toward their ultimate use in clinical applications, especially because undifferentiated iPSCs pose an intrinsic tumorigenic risk (Yamanaka, 2009). It was demonstrated that *SB* transposon-mediated delivery of the myogenic PAX3 transcription factor into iPSCs coaxed their differentiation into $MyoD^+$ myogenic progenitors and multinucleated myofibers (Belay *et al.*, 2010), suggesting that PAX3 may serve as a myogenic "molecular switch" in iPSCs, a finding that has implications for cell therapy of congenital degenerative muscle diseases, including Duchenne muscular dystrophy. Thus, the *SB100X* hyperactive transposase holds great promise for *ex vivo* and *in vivo* gene therapies.

Future Considerations/Outlook

There has been steadily growing interest in applying the *SB* system for gene therapy (Izsvák and Ivics, 2004; Essner *et al.*, 2005; Hackett *et al.*, 2005, 2010; Ivics and Izsvak, 2006; Izsvák *et al.*, 2010) in the context of several conditions including hemophilia A and B (Yant *et al.*, 2000; Ohlfest *et al.*, 2005b; L. Liu *et al.*, 2006b; Kren *et al.*, 2009; Hausl *et al.*, 2010), junctional epidermolysis bullosa (Ortiz-Urda *et al.*, 2002), tyrosinemia I (Montini *et al.*, 2002), Huntington disease (Chen *et al.*, 2005), sickle cell disease (Zhu *et al.*, 2007),

mucopolysaccharidosis (Aronovich *et al.*, 2007, 2009), cancer (Ohlfest *et al.*, 2005a; Peng *et al.*, 2009; Jin *et al.*, 2011), and type 1 diabetes (He *et al.*, 2004). In addition, important steps have been made toward *SB*-mediated gene transfer in the lung for potential therapy of α_1-antitrypsin deficiency, cystic fibrosis, and a variety of cardiovascular diseases (Belur *et al.*, 2003; Liu *et al.*, 2004).

The first clinical application of the *SB* system is currently ongoing, using autologous T cells gene-modified with *SB* vectors (Williams, 2008) carrying a chimeric antigen receptor (CAR) to render the T cells cytotoxic specifically toward CD19-positive lymphoid tumors (Huang *et al.*, 2008; Singh *et al.*, 2008). Lymphocytes represent a suitable initial platform for testing new gene transfer systems, as T cells can be genetically modified by viral and nonviral approaches without apparent resulting genotoxicity (Bonini *et al.*, 2003). Advantages of using the *SB* system for genetic modification of T cells include the ease and reduced cost associated with the manufacturing of clinical-grade, plasmid-based vectors compared with recombinant viral vectors.

Sleeping Beauty faces a number of challenges before widespread clinical translation. For *in vivo* delivery into the liver, the hydrodynamic technology needs to be calibrated first to large-animal models and then eventually to humans. This is not a simple task to solve, but important steps have been made toward this goal by developing computer-assisted protocols (Suda *et al.*, 2008). Another complicating issue with DNA delivery to the liver is the resulting tissue damage and the associated (at least transiently) elevated values of liver enzymes that may compromise clinical application of these procedures. A major hurdle in *ex vivo* delivery of the transposon components into relevant primary cell types is the toxicity of the transfection/electroporation protocols that is typically observed. In situations in which target cells are scarce and/or culturing and expansion of the transfected cells are impossible or cannot be solved without compromising cell identity and grafting potential, cytotoxicity of the transfection procedures is a serious issue that may undermine clinical applications. Development of nonviral chemical reagents promoting plasmid DNA delivery with reduced toxicity may provide a solution. Alternatively, the development of hybrid vector systems combining the natural ability of viruses to traverse cell membranes with efficient genomic insertion mediated by the *SB* system is a promising strategy. Indeed, components of the *SB* transposon have been incorporated into integrase-defective lentiviral particles that showed efficient gene transfer in a range of human cell types and an insertion profile favorable to conventional lentiviral vectors (Staunstrup *et al.*, 2009; Vink *et al.*, 2009; Moldt *et al.*, 2011). Hybrid adenovirus–*SB* vectors (Yant *et al.*, 2002) have been used to efficiently deliver *SB* transposon vectors expressing FIX into the liver in a hemophilic dog model (Hausl *et al.*, 2010). Retroviral vectors disabled in generating a cDNA copy of the retroviral vector have been shown to deliver the *SB* transposase mRNA into target cells with impressive efficiency (Galla *et al.*, 2011). Last, herpes simplex virus vectors with a tropism toward infecting neural progenitor cells have been used to target *SB* insertions in the central nervous system in an *in utero* gene delivery system in the mouse (Bowers *et al.*, 2006; de Silva *et al.*, 2010). Furthermore, not only the procedures that are used to deliver the transposon vector components into cells, but those components themselves, may elicit unwanted effects in the cell. For example, it was found that overexpression of the *SB* transposase can have cytotoxic effects (Galla *et al.*, 2011). The molecular mechanism of transposase cytotoxicity is currently not understood, but it is unlikely to be due to uncontrolled cleavage of genomic DNA by the transposase (Galla *et al.*, 2011). At any rate, careful dosing of the transposase as well as the transposon donor plasmids in gene delivery experiments appears to be of fundamental importance; luckily, with plasmid-based vectors such as the *SB* transposon system, this can easily be achieved. Last, potential genotoxic effects elicited by transcriptional upregulation of proto-oncogenes and other signaling genes on random transposon insertion is a relatively unexplored area of research. Investigations into these questions will be required to document the safety of the *SB* system for prospective clinical trials. The next phase of research will undoubtedly focus on these issues to introduce the *SB* transposon system as a nonviral gene delivery tool for gene therapy.

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