**EXPERT INSIGHT**

*Sleeping Beauty* transposon vectors for therapeutic applications: advances and challenges

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Transposable elements are natural, non-viral gene delivery vehicles capable of mediating stable genomic integration. The *Sleeping Beauty* (SB) transposon has the ability to cut-and-paste the ‘gene of interest’ into the genome, providing the basis for long-term, permanent transgene expression in transgenic cells and organisms. The SB transposon system is relatively well characterized, and has been extensively engineered for efficient gene delivery and gene discovery purposes in a wide range of vertebrates, including humans. The SB system is a safe and simple-to-use vector that enables cost-effective, rapid preparation of therapeutic doses of cell products. Recently, there has been a growing interest in using the SB system for therapy as evidenced by the large number of pre-clinical studies. SB moved swiftly from pre-clinical to clinical trials in almost a decade. In this article, we highlight the advancements and challenges associated with the SB system in various therapeutic applications. We also provide an overview that has been exploited by spin-off companies based on the SB system.

Submitted for review: Feb 21 2017  »  Published: Mar 30 2017

The high expectation on gene therapy continues to grow, as it holds the potential to provide a cure for myriad genetic diseases by substituting a corrupted gene with the respective functional one to achieve a therapeutic effect. The success of gene therapy is mainly dependent on the safety, efficacy, simplicity, cost-effectiveness and scalability of the vector system used for delivering and expressing the therapeutic gene of interest (GOI) into the cells. The numerous gene delivery approaches can be broadly classified as viral and non-viral mediated (Figure 1). Viral vectors have been exploited for clinical use based on their inherent gene delivery capabilities in multiple cell types. In fact, the majority of
Currently, there are around 600 and 800 gene therapy clinical trials involving retro/lentiviral and AV/AAV vectors, respectively [1]; however, despite their superb delivery capacity, viral vectors do have certain limitations. Whilst AV and AAV vectors are excellent delivery/expression vectors in non-dividing cells, they are diluted out by cell division. To achieve long-term therapeutic effect in dividing cells, multiple doses of administration are required, which induces adverse immune responses [2,3]. AAVs are also limited by their relatively low packaging capacity (<5 kb). By contrast, both retroviral and lentiviral vectors have the
capacity to mediate stable integration of the GOI, resulting in stable expression of the therapeutic gene. While lentiviruses have the capacity to infect both non-dividing and dividing cells, retroviruses such as the Moloney murine leukemia virus (MoLV) are restricted to dividing cells [4]. MoLV prefers to integrate into transcription start sites, whereas lentiviral vectors based on HIV exhibit bias towards integration into active genes [5]. Due to their biased integration pattern, retro- and lentiviral vectors are associated with an elevated risk of both insertional mutagenesis and trans-activation of oncogenes around the integration site [6,7]. Finally, viral production methods are complex, and generally involve high costs, partially associated with regulatory issues.

In contrast to viral vectors, non-viral vectors have been considered for their simplicity, safety and ease of production. Non-viral approaches include physical or chemical delivery methods such as transfection, electroporation/nucleofection, gene gun and nanodelivery. The bottleneck problems of classical non-viral delivery are its low efficacy and transient nature. However, there are currently over 400 non-viral gene therapy clinical trials [1].

Mobile genetic elements (transposons) are natural gene delivery vehicles capable of genomic insertion. DNA transposons have the ability to transpose within the genome by a cut-and-paste process; however, this process can be restricted to a single excision event from a transfected plasmid to the genome. Stable genomic integration provides the basis for permanent transgene expression in transgenic cells and organisms, thereby addressing the bottleneck problem of classical non-viral delivery. Sleeping Beauty (SB) is a resurrected, synthetic transposon, belonging to the Tc1/mariner family of transposons that is active in a wide variety of vertebrates, including human cells [8,9]. The potential of the SB-based non-viral integrating system as an alternative to viral vectors has been thoroughly investigated in the last two decades. The studies demonstrated its ability to mediate long-term gene expression in various human cell types, and revealed several advantageous safety features. Currently, the SB transposon vector is the most widely used alternative gene carrier to integrating viral vectors.

THE SB TRANSPOSON SYSTEM

Generally a transposon system includes a transposon and a transposase. The transposon acts as a carrier, which carries the gene to be inserted into the genome. The transposase is the workhorse catalyzing the process of transposition. Naturally, the transposase is located between the inverted terminal repeats (ITRs) of the transposon. Importantly, however, the transposase gene can be replaced with any GOI, and the transposase can govern transposition events when encoded by a separate plasmid in trans. Physical separation of the transposon from the transposase enabled optimization of transposon versus transposase ratio, and also provided the freedom of supplying the transposase in the form of mRNA, instead of DNA [10]. Both components of the SB
system, the transposon and the transposase, have been extensively engineered to improve transpositional activity.

**Engineering the SB transposon**

SB represents the first functionally active DNA transposon in vertebrates [8]. SB was engineered from ancient Tcl/mariner transposon fossils found within the Salmonid genomes by *in vitro* evolution [8]. The ITRs (230 bp) contains imperfect direct repeats (DRs) of 32 bp in length that serve as recognition signals for the transposase. Binding affinity and spacing between the DR elements within ITR has been crucial for efficient transpositional activities, suggesting that a constrained geometry is required during the pre-integration complex assembly [11,12]. Optimizing nucleotide residues (including mutations, deletions and additions) within the ITRs of the original SB transposon (pT) resulted in improved transposon versions, such as pT2, pT3, pT2B and pT4 (Table 1). For convenience of use, a whole series of transposon vectors with different reporter and selection markers are available [13].

**Engineer the SB transposase**

The SB transposase is a 39 kDa protein that possess DNA binding domains, a nuclear localization signal (NLS) and the catalytic domain, featured by a conserved amino acid motif (DDE). Various screens mutagenizing the primary amino acid sequence of the SB transposase resulted in hyperactive transposase versions (Table 2). SB100X is 100-fold hyperactive compared to the originally resurrected transposase (SB10) in certain cell types [13,14].

**Mechanism of transposition**

Transposition is a relatively well-characterized process, divided into excision and integration steps (Figure 2). First, the transposase recognizes the transposon, and binds the ITRs. During synaptic complex formation, the transposon ends are brought together by transposase monomers (presumably forming a tetramer) [15]. The transposase generates a DNA double-strand break upon excision [16], while single-stranded gaps at the integration site. The pre-integration complex containing the transposon bound transposase performs the integration into the host genome. SB transposition is a highly coordinated reaction that efficiently filters out abnormal, toxic transposition intermediates [12,17]. Excision leaves a footprint (3 bp) at the donor site. Integration occurs into TA dinucleotides of the genome, and results in target site duplications, generated by the host repair machinery [16,18,19]. Overall, SB appears to possess a nearly unbiased, close-to-random integration profile [20]. Transposon integration can be artificially targeted (~10%) to a predetermined genomic locus [20–24].

Several host factors of SB transposition, including HMGXB4, HMGB1, BANF1, KU70 and MIZ-1 have been identified [16,17,25–27]. These factors physically interact with the SB transposase, and assist in different steps of the transposition reaction. In addition to host encoded cellular factors, certain conditions (e.g., serum starvation, DNA methylation) are reported to affect SB transposition [27,28].
SALIENT FEATURES OF SB TRANPOSON TECHNOLOGY

Since its establishment, the SB transposon technology has been exploited for various applications, including gene delivery and gene discovery in diverse species. Recently, it has been intensively exploited for human therapeutic applications. The SB technology exhibits several advantageous features:

- Non-viral: the GOI can be easily cloned between the ITRs of the transposon, which can be simply co-delivered with the transposase in the form of plasmids (or plasmid/mRNA). Such procedures can be performed in biosafety level 1 (BSL-1) laboratory, not requiring any complex biohazard containment facilities;
- Well-characterized: the transposition mechanism of the SB system and its interaction with the host is relatively well characterized;
- Economical: in comparison to the production cost of viral vectors, Good Manufacturing Practice (GMP) grade plasmid production is relatively cheap, fast and less labor intense;
- Efficient and stable transgene expression: the hyperactive SB system has been demonstrated to support efficient and stable gene expression in various cell types. While the SB vector is not resistant to silencing (primarily dependent on the cargo), the expressed integration loci would faithfully produce the transgenic gene product long-term;
- Wide range of cell types: SB is capable of transposing in a wide variety of cell types, including therapeutically relevant primary cells [9];
- Not restricted to cycling cells: SB is able to transpose in non-dividing primary cells [29];
- Transgene integration is not restricted to efficient homologous recombination (HR): the transposase is capable of performing efficient transgene integration in cells, where the homologous recombination pathway of the host repair machinery is barely active;
- Maintains intact transgene structure: the SB vector is suitable to faithfully express complex transgenes;
- Cargo capacity: Although SB transposition is most optimal up to ~7.5 kb [9] of the transposon, the sandwich version (SA) has been shown to efficiently deliver cargos of >10 kb, thereby extending the cloning capacity of SB-based vectors. When combined with bacterial artificial chromosome (BACs), SB can deliver transgenes up to 100 kb [30].

TABLE 2

List of currently available SB transposases.

<table>
<thead>
<tr>
<th>Transposase</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB10</td>
<td>[8]</td>
</tr>
<tr>
<td>SB11 (3-fold higher than SB10)</td>
<td>[109]</td>
</tr>
<tr>
<td>SB12 (4-fold higher than SB10)</td>
<td>[66]</td>
</tr>
<tr>
<td>HSB1–HSB5 (up to 10-fold higher than SB10)</td>
<td>[108]</td>
</tr>
<tr>
<td>HSB13–HSB17 (HSB17 is 17-fold higher than SB10)</td>
<td>[111]</td>
</tr>
<tr>
<td>SB100X (100-fold higher than SB10)</td>
<td>[14]</td>
</tr>
<tr>
<td>SB150X (130-fold higher than SB10)</td>
<td>[24]</td>
</tr>
</tbody>
</table>

FIGURE 2

Sleeping Beauty transposition.

During SB transposition, the transposon unit with the inverted terminal repeats (ITRs) carrying the gene of interest is excised from the transposon vector by the transposase protein (red pie labeled as SB). The excised transposon is then integrated in the genome by the bound transposase protein.
Unbiased, close-to-random integration profile: the close-to-random integration profile of SB was confirmed by multiple studies, and was reported from various organisms and cell types [20,23,24,31–35].

Benign promoter/enhancer activity: the SB ITRs have negligible intrinsic promoter activity (less than ~100-fold vs MoMLV);

Low immunogenicity: as the transposase is provided separately from the integrating transposon vector, it is present only temporarily in the cells. Thus, the non-viral transposon system generally does not trigger adverse immune responses that are observed with certain viral vectors (AV, AAV);

No cross-mobilization in the human genome. None of the human genes are reported to recognize and mobilize the SB transposon (in contrast to the piggyBac [36,37]).

CURRENT APPLICATIONS OF SB TRANSPOSON TECHNOLOGY
The SB transposon system has been used for diverse genetic applications in vertebrate species, which can be broadly classified as

FIGURE 3
Applications of Sleeping Beauty transposon technology.

The SB transposon system has been successfully used for gene delivery (gene of interest) into a variety of animal models and cell types, including stem cells and primary cells (both in vitro and in vivo). It has been extensively exploited as a mutagenic tool for gene discovery applications. The approach of a forward genetic screen for modeling different cancers in animal models led to the discovery of numerous novel genes associated with cancer (functional oncogenomics). It has been used also a valuable tool in germline transgenesis in various animal species (generating transgenic animals). The SB gene delivery technology has been thoroughly used in several pre-clinical studies to model wide range of metabolic disorders, degenerative diseases and cancers. In addition, the SB system has also been utilized for mapping chromatin landscape, epigenome and 3D genome organization. Currently it is being evaluated in clinical trials as a non-viral gene delivery vector for various gene therapy applications like cancer immunotherapy, Alzheimer’s disease and age-related macular degeneration (AMD), all of which involves ex vivo modification of patient cells. The chart represents the distribution of various therapeutic applications of SB system that are categorized based on the approximate number of original research articles published as of March, 2017.
gene delivery and discovery (Figure 3) [38]. Briefly, the SB system has been used to generate stable transgenic cell clones in tissue culture [39] (reviewed in [40]). SB has been also highly valuable in generating transgenic animals, including fish, frog, rat, mouse, rabbit, pig, cow and sea squirt (for a recent review see [41]). Importantly, the SB-based transgenic technology is able to address previous problems of transgenesis, such as low efficacy, mosaicism, unstable gene expression [42], and offers novel ways for genetic engineering of even large animals [43]. SB was successfully employed in reprogramming somatic cells into induced pluripotent stem cells (iPSCs) that can be expanded and differentiated into different cell types [44–47]. The SB-based protocol has also been used for the production of iPSCs in various animal models [48–50]. In combination with an LTR7-based reporter, the SB system is suitable for genetic and phenotypic tagging to enrich embryonic stem and iPSC cultures for naive-like human pluripotent stem cells [51,52]. Furthermore, SB-based genetic screens have been used for gene annotation in both germline and somatic cells [53–55]. In somatic cells, SB is primarily employed in functional oncogenomics to identify novel genetic drivers of cancers [41,56,57]. SB has been also exploited in dissecting the regulatory architecture of the genome [58,59]. Nevertheless, it is beyond the scope of this review to provide comprehensive overview of all the various applications of the SB system, and readers are referred to the recent review articles.

TAILORING THE SB TRANPOSON TECHNOLOGY FOR CLINICAL APPLICATIONS

Here we provide an update on the cell and gene therapy applications. In the last decade, SB-based delivery vectors have been extensively used in pre-clinical animal models (for reviews see [38,60–63]). The encouraging pre-clinical results fueled its promotion towards clinical trials, and currently the technology is being evaluated to treat human diseases including cancer (lymphoma) (Figure 4), Alzheimer’s disease (AD) (Figure 5) and age-related macular degeneration (AMD) (Figure 6). The preliminary results support further clinical development of SB-based gene therapy approach.

The hyperactive SB transposon system

The latest optimized version of the SB system comprises of the hyperactive SB100X transposase and the pT4 transposon [12,14]. The SB100X, generated by molecular evolutionary strategy performs with significantly higher efficiency of genomic integration that in certain cells is even comparable to viral performances [14]. Since SB100X can integrate the therapeutic gene more efficiently, relatively lower amounts of DNA are required to achieve similar results compared to the less hyperactive versions [64]. Importantly, protocols optimized for non-hyperactive transposase versions need to be re-optimized to avoid unnecessarily high number of integrated copies of the therapeutic gene. The hyperactive transposon version, pT4 has optimized substrate recognition [12].
Switch from DNA to mRNA as a source of transposase

Electroporation/nucleofection of plasmid DNA can be highly toxic to certain cells, including primary stem cells [64]. By contrast, nucleofection of RNA is not significantly more toxic than the nucleofection alone, indicating that the toxicity is not caused by nucleofecting nucleic acid per se. Thus, switching the transposase source from plasmid DNA to mRNA could mitigate the toxicity of the delivery. Furthermore, supplying

**FIGURE 4**

Clinical application of Sleeping Beauty transposon technology for cancer immunotherapy.

*Sleeping Beauty* system has been successfully used in the clinical trials for engineering T cells to express chimeric antigen receptor (CAR) for use against leukemias and lymphomas. SB-modified T cells were used for autologous or allogeneic hematopoietic stem-cell transplantation (HSCT). Shown here is a schematic depicting the autologous cancer immunotherapy involving engineering patient’s own cells to recognize antigens presented by cancer cells and destroy them. The illustration shows chimeric antigen receptor (CAR)-T-cell-based adoptive immunotherapy for hematological malignancy. Peripheral blood CD4+ T cells isolated from the patient’s blood that are genetically modified using the SB system. The engineered cells express relevant cell surface CAR that can recognize the surface antigens of malignant cells. To generate sufficient number of engineered cells for clinical application the CAR-modified T-cells are subjected to *ex vivo* expansion, and then infused back into the respective patient where tumor cells are recognized and killed by CAR+ T-cells.
the transposase as mRNA would ensure its transient expression, and decrease the risk of remobilization of the integrated therapeutic gene (safety concern) [10].

**Extending the cloning capacity of the SB-based vector: the SA transposon**

The transposition efficiency of SB is inversely correlated with the actual size of the transposon over 7.5 kb [9]. The ‘sandwich’ (SA) configuration of the transposon was aimed to improve the mobilization of larger cargos. The SA transposon consists of four ITRs in total, with two complete elements flanking the GOI in an inverted orientation (Figure 7). Such an arrangement of a Tc1-like transposon was observed to mobilize large (>10kb) pieces of genomic DNA in

**FIGURE 5**

Gene therapy approach utilizing Sleeping Beauty transposon technology for the development of encapsulated cell biodelivery (ECB) device for Alzheimer’s disease (AD).

Illustration of ECB device for AD: Human retinal pigment epithelial cells (ARPE-19) are genetically engineered using the Sleeping Beauty system to express nerve growth factor (NGF). Engineered cells are encapsulated into an implant (semi-permeable hollow fiber membrane that allows the influx of nutrients and the efflux of NGF) providing an internal cell-supportive scaffold matrix for cell adherence and survival. The implant was validated by placing them in sterile containers filled with serum-free medium at 37 °C for up to 4.5 weeks. The validated implants are surgically implanted into the basal forebrain of AD patients. The cells are protected from immune rejection by the semipermeable membrane, and thus no immunosuppression is required during the treatment. The implanted ECB device secretes NGF that can arrest and might reverse the degeneration of the basal forebrain cholinergic neurons (as shown in the adjoining inlet at the bottom).
Exudative AMD involves degeneration of retinal pigment epithelial (RPE) cells due to extensive neovascularization resulting from imbalanced concentration of intraocular proteins like vascular endothelial growth factor (VEGF) and Pigment Epithelium-Derived Factor (PEDF). Illustration of gene therapy approach for AMD: Patient's own RPE cells are surgically isolated from the biopsy and genetically engineered using the Sleeping Beauty system to express PEDF. Engineered cells are transplanted back into the same patient swiftly in a short span of time (approximately 1 hour). Secreted PEDF protein interacts with its receptor and triggers an anti-angiogenic cascade that suppresses the neovascularization and mitigates retinal damage (as shown in the adjoining inlet at the bottom). Overexpression of PEDF restores the balance between PEDF and VEGF protein concentrations.
Drosophila [65]. Indeed, translating this observation to SB technology yielded the SA transposon with a superior ability to transpose >10 kb transgenes [66,67].

Shielding the transposon delivered transgene cassettes with insulators

SB facilitates the transgene expression in a copy-number dependent manner in transgenic animals, suggesting that the SB vector does not particularly alert the silencing machinery of the host [42]. Thus, incorporating insulator sequences in the SB-based vector might not be necessary to protect the transgene expression from silencing. On the other side, use of insulators motifs was demonstrated to effectively shield the promoter activity of the transgene cassette at the integration locus [26,68]. However, while insulators could prevent the transactivation of oncogenes, inserting insulator motifs could also have undesired effect on genome structure. Thus, the potential risks and benefits of using insulator sequences need to be carefully evaluated.

Delivery of the SB transposon system

Several non-viral gene delivery strategies have been tested for delivering SB constructs in vitro. In hard-to-transfect cell types electroporation/ nucleofection appears to be the most effective. A current limitation of this strategy for clinical applications is its capacity to engineer low number of cells. In principle, a flow through electroporation strategy could be beneficial to increase the number of engineered cells.

Besides nucleofection, nanoparticle-like carriers proved to be efficient to deliver the SB system. Notably, these carriers are also suitable to be combined with various targeting molecules that allow cell type specific transfer. In one example, hard-to-transfect mesenchymal stem cells (MSCs) could be targeted with high efficacy (~52%) by using engineered lipid-based nanoparticles (LBNs) encapsulating the SB system. These LBNs are chemically modulated to present synthetically reiterated MSC-targeting peptides on their surface [69]. Nanoparticle-like protocells with SB encapsulated inside can deliver the therapeutic gene into cancer cells, when folic acid is incorporated as a cancer cell-targeting motif [70]. Furthermore, special hepatocyte-targeted carriers, such as proteoliposomes containing galactose-terminated glycoproteins (e.g., the F protein of the Sendai virus) were demonstrated to effectively deliver the SB cargo into hepatocytes [71]. In a different approach, cell type-specific gene targeting using hyaluronan- and asialoorosomucoid-coated...
nanocapsules harboring the SB system were successfully used in vivo to direct genes to liver sinusoidal endothelial cells and hepatocytes, respectively [72]. Collectively, these studies imply that with the targeting ligand modification, the nanoparticle-like carriers can be developed as efficient gene delivery and targeting gene vehicles, highlighting their therapeutic potential.

Apart from the non-viral strategies, various SB-based viral hybrid technologies have been developed that can advantageously merge the excellent delivery properties of the viral vectors and the superior safety properties of the SB (Table 3 & Figure 8) (also reviewed in [41,63]). Currently, one of the most promising strategies is the in vivo gene transduction system based on a hybrid transposon/adenovirus vector [73] and hyperactive SB transposase (SB100X) [74]. This in vivo strategy is effective and safe, and performs without the requirement of ex vivo expansion and transduction of hematopoietic stem cells (HSCs) [75].

Eliminating bacterial sequences from the transposon vector
Non-viral delivery of large plasmid DNA molecules via electroporation is highly toxic to certain cell types,

<table>
<thead>
<tr>
<th>Hybrid technology</th>
<th>Delivering vehicle</th>
<th>Integration machinery</th>
<th>Packaging capacity</th>
<th>Advantages</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adeno/SB</td>
<td>Recombinant adenovirus</td>
<td>SB10</td>
<td>&gt;35 kb</td>
<td>Transduce dividing and non-dividing cells, and are one of the most efficient vehicles for in vivo gene delivery</td>
<td>[98]</td>
</tr>
<tr>
<td>HCAdV/SB</td>
<td>HCAdV</td>
<td>HSB5</td>
<td>&gt;36 kb</td>
<td>Showed negligible toxicity in mice and canine model for hemophilia B</td>
<td>[110]</td>
</tr>
<tr>
<td>HCAdV/SB</td>
<td>HCAdV</td>
<td>SB100X</td>
<td>&gt;36 kb</td>
<td>Lowest immuno- and toxicity compared to early generation adenoviral vectors</td>
<td>[74]</td>
</tr>
<tr>
<td>AAV/SB</td>
<td>Recombinant AAV</td>
<td>SB100X</td>
<td>&gt;5 kb</td>
<td>Stabilized transgene expression in combination with the high transduction efficiencies of AAV</td>
<td>[99]</td>
</tr>
<tr>
<td>HSV-1 amplicon/ SB</td>
<td>HSV-1</td>
<td>SB10; HSB5; SB12</td>
<td>≤130 kb</td>
<td>Efficient at delivering large transgenes to neurons specifically and provides stable long term expression</td>
<td>[100–103]</td>
</tr>
<tr>
<td>Baculo/SB</td>
<td>Baculovirus</td>
<td>SB11 SB100X</td>
<td>38 kb</td>
<td>Stable long term expression</td>
<td>[104]</td>
</tr>
<tr>
<td>IDLV/SB</td>
<td>IDLV</td>
<td>SB10; SB11; HSB3; SB80X and SB100X</td>
<td>8 kb</td>
<td>Un-biased random integration profile</td>
<td>[105]</td>
</tr>
</tbody>
</table>

Adeno: Adenovirus; Baculo: Baculovirus; HCAdV: High-capacity adenoviral vector; AAV: Adeno associated virus; IDLV: Integrase defective lenti virus; HSV-1: herpes simplex virus 1 amplicon.
The SB transposon system can be delivered (large rounded rectangle) into the cells by combining with any nucleic acid-delivery techniques like transfection (using the commercial transfection reagents) or nucleofection (electro-transfer of nucleic acids directly into the nucleus) or by complexing with liposomes (nucleic acids are packed directed into the liposomes) or by complexing with nanoparticles (liposome protamine/DNA lipoplex with targeting peptides) or by hybrid viral vectors (nucleic acids are packaged into virions). Once inside the cell, they can traverse the nuclear membrane (oval inside the cell) by a poorly understood process. Here delivery of the SB system via nucleofection is illustrated in the graphic. Transcription of the SB transposase gene results in an mRNA, which is translated into a protein in the cytoplasm. Note that the SB transposase can also be supplied in the form of an mRNA (as shown by the white dotted arrows) directly into the nucleus via nucleofection instead of plasmids. Using mRNA instead of DNA is beneficial in preventing genomic integration of the transposase gene and in reducing toxicity upon electroporation. The transposase protein then binds to the transposon ends (ITRs) resulting in excision and ultimately integrating into the chromosomal DNA of the genome. Stable genomic integration confers long-term expression of the gene of interest delivered by the transposon.
including primary T cells. Reducing the size of the DNA, and using supercoil DNA proved to be advantageous modifications in the delivery protocol [76,77]. Furthermore, the use of conventional plasmids as vectors that are propagated and isolated from bacteria raises a safety concern and a roadblock for broad clinical applications. In fact, the presence of bacterial backbone sequences on conventional plasmids such as antibiotic resistance gene and bacterial origin of replication has a number of negative consequences (Figure 9i). First, bacterial sequences are recognized and trigger gene silencing [78,79]. Second,
expression of antibiotic resistance genes can also induce undesired immune responses [79]. Furthermore, transmission of antibiotic resistance genes to the cells or the microbiota of the patient via horizontal gene transfer generates potential risks. For the above safety considerations regulatory agencies recommend avoiding the use of antibiotic resistance markers.

The need to eliminate redundant bacterial backbone sequences motivated researcher’s to consider new approaches that reduces the size and bacterial sequence content of the plasmids. These efforts resulted in the development of plasmid free of antibiotic resistance (pFAR; see below and Figure 9ii) and minicircle (MC) vectors (Figure 9iii).

pFAR vectors are produced under selection pressure in a genetically modified E. coli, which contains an amber mutation in the thymidylate synthase gene. Introduction of pFAR plasmids having the suppressor transfer RNA gene (Sup t-RNA) into the mutant E. coli can restore normal growth, providing a selection pressure for the maintenance of pFAR miniplasmids. The pFAR miniplasmids are much more efficient (in transfection as well as expression in vitro and in vivo) compared to the conventional plasmids (Figure 9ii) [80]. Importantly, the pFAR and SB technologies were successfully combined [Johnen S, In press], and would be used in the clinical trial to treat AMD.

Minicircle DNA vectors represent small and supercoiled molecules that are devoid of any bacterial sequences, and contain almost exclusively the GOI. They are produced by the inclusion of site-specific intramolecular recombination motifs between the GOI and bacterial backbone in the parental plasmid. SB transposon and transposase minicircle constructs have been examined and optimized for safety and efficacy in various cell types [77], including primary T cells [76]. A minicircles-based SB system has been also used for efficient germline transgenesis [81]. Minicircle vectors seem to improve the transfection efficiency and transgene expression, while decreasing the toxicity associated with DNA delivery (Figure 9iii).

Collectively, miniplasmid vectors could be optimized for a variety of cell types, might meet future regulatory requirements for gene therapy and vaccine products, and set a new standard in advanced cellular and gene therapy.

**Selection strategies for engineered cells expressing the gene of interest**

Certain clinical applications require a large number of engineered cells. Thus, high efficacy of delivery and the frequency of therapeutic gene integration are crucial. In addition, it might be necessary to further enrich engineered cell cultures by using selective culturing protocols. Ideally, the selection period should be short. The following selection strategies have been tested for selecting genetically modified cells using the SB system.

In a cancer immunotherapy application, T cells are genetically modified by nucleofecting the SB system to express chimeric antigen receptor (CAR) that redirects specificity towards tumors. For example, CD19-specific CAR+ T cells could be selectively expanded on K562-derived artificial presenting cells (aAPC) co-expressing human CD19 and in the presence of an...
array of co-stimulatory molecules. Co-expression of CD19 serves to specifically propagate the genetically modified T cells, leaving those cells that did not integrate the transposon to die from neglect. This method of expansion strategy can efficiently overcome the toxicity of nucleofection and yield sufficient numbers of CD19-specific CAR T cells for clinical applications [82,83].

In an alternative protocol, administration of irradiated PBMCs was used to overcome cell death following SB-mediated gene transfer of CAR-modified cytokine-induced killer cells (CIKs). This clinical-grade protocol enables both robust gene transfer and efficient T cell expansion [84].

In addition, the SB transposon system has also been used for multiplexed gene transfer in conjugation with methotrexate selection. The strategy allows stable expression of up to three different transgenes in human CD4+ T cells [85].

Alternatively to cytotoxic drugs, a chemically responsive amplification mechanism can be used for selecting the engineered cells. A nearly pure population of stably transduced cells can be generated by non-viral delivery of desired transgenes through a combination of SB transposon-mediated integration and selective amplification using a chemically induced dimerizer (CID) [86]. This positive selection strategy is responsive to a small molecule trigger. Using this fast and efficient selection strategy, engineered cell populations with >98% purity could be obtained within 1 week. Dimerizer-induced cell growth could provide cost and reproducibility advantages to natural ligand stimulation in ex vivo cell culture, and could be used to control engineered cell behavior in vivo.

A ‘traceless selection system’ can efficiently select for engineered cells, and can be also used to select against cells that retain expression of the transposase gene. In this approach, the transposase is expressed together with a tractable fluorescent reporter. The strategy is based on the observation that upon co-transfection of both the transposase and transposon constructs, the presence of the transposase also reports on successful transposition events with high frequency. This concept could be used to produce highly enriched, auxiliary gene-free, cell products [87] that meet important safety requirements.

Regulation of the transgene expression

Transcriptional regulation could control the timing and dose of the expression of the therapeutic gene. In principle, the SB vector, possessing negligible enhancer/promoter activity on its own [26,88] can be easily adopted for transcriptional regulation. For regulated transgene expression ‘all-in-one’ TET SB vectors display a relatively low signal-to-noise ratio, resulting in regulatory windows of around 25,000-fold [89]. For safety concerns it is desirable to secure even elimination of the therapeutic gene. In conjunction with the SB system, thymidine kinase (TK) can be used for conditional ablation of the therapeutic construct [90].

Supporting protocols to monitor the safety and efficacy

- Following SB-mediated integration determining the copy number of the therapeutic gene is usually performed by PCR-based assays [13,91]. At very limited amounts of DNA droplet
digital PCR can be successful to precisely determine the number of integrated vector copies;

- A high throughput integration site analysis belongs to the routinely performed safety studies. These analyses recover SB integration sites from the treated cells, and compare them to a computationally generated random genomic control [75]. SB exhibits a close-to-random integration profile with a small bias at repetitive elements [31]. The integration sites can be further investigated in various categories (e.g., library of safe harbor genomic sites, exons, regulatory regions, etc.) [35];

- A recently reported whole-body non-invasive imaging provides a method to examine long-term bio-distribution and persistence of the engineered cells. In this technology, bioluminescent imaging is used to monitor the signal emitted by firefly luciferase from the SB vector in vivo. The positron emission tomography (PET) is applied following injection of 2'-deoxy-2'-(18F)fluoro-5-ethyl-1-β-D-arabinofuranosyl-uracil ([18F]FEAU). Besides, monitoring safety, such a non-invasive imaging approach could be useful for assessing the efficacy of the therapeutic strategy [90].

Clinical evaluation of SB transposon system/technology

Assignees for SB patent applications are associated with two leading organizations (University of Minnesota and the Max Delbrück Center for Molecular Medicine (MDC)). As discussed above (Figures 4–6), SB transposon technology applications span the cell and gene therapy industry market that has experienced rapid growth in the last few years (Table 4 & Figure 10). The ‘simple’ search (with the term ‘sleeping beauty transposon’) using the patentscope search tool [92] of world intellectual property organization

<table>
<thead>
<tr>
<th>Company</th>
<th>Application</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discovery Genomics, Inc. (DGI)</td>
<td>Gene therapy</td>
<td>Diseases of blood</td>
</tr>
<tr>
<td>B-MoGen Biotechnologies Inc.</td>
<td>Gene delivery and gene editing</td>
<td>Providing tools and custom services</td>
</tr>
<tr>
<td>Intrexon Corp.</td>
<td>Gene therapy</td>
<td>Cancer immunotherapy</td>
</tr>
<tr>
<td>Ziopharm Oncology</td>
<td>Gene therapy</td>
<td>Cancer immunotherapy</td>
</tr>
<tr>
<td>Merck</td>
<td>Gene therapy</td>
<td>Cancer immunotherapy</td>
</tr>
<tr>
<td>Formula Pharmaceuticals, Inc.</td>
<td>Gene therapy</td>
<td>Cancer immunotherapy</td>
</tr>
<tr>
<td>Immusoft Corporation</td>
<td>Gene therapy</td>
<td>Rare diseases</td>
</tr>
<tr>
<td>NsGene A/S</td>
<td>Gene therapy</td>
<td>Neurological diseases (like Alzheimer’s disease, etc.)</td>
</tr>
<tr>
<td>Aldevron, LLC</td>
<td>Custom development and manufacturing services</td>
<td>Providing tools and custom services</td>
</tr>
<tr>
<td>Harbogen Biotechnologies, Inc.</td>
<td>DNA and related testing products development of precision medicine</td>
<td>Providing tools and custom services</td>
</tr>
<tr>
<td>Neuromics, Inc.</td>
<td>Bio-reagents company</td>
<td>Providing tools</td>
</tr>
<tr>
<td>Plasmid factory</td>
<td>Providing reagents and custom services</td>
<td>Providing reagents and custom services</td>
</tr>
<tr>
<td>Pharmead</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The data presented in the table is obtained either based on the available information or by searching various online sources as of March 2017. Note that companies that have confidentially licensed the SB transposon technology are not listed in the presented table.
Percentage share of the International Patent Classification (IPC). Majority of the patents involving the SB has been submitted in the category-C12N (inventions concerning microorganisms, enzymes; compositions thereof mutation or genetic engineering); A61K (inventions concerning pharmaceutical field-preparations for medical purposes); A01K (inventions concerning animal husbandry); A01N; C07H and G01N. Academic-industrial partnership can bridge the ‘gap’ between research done in academia and its translation into marketable products. Recently, the University of Minnesota’s patented SB-based gene delivery technology (SB11 and pT2) pooled with patents of cancer therapies practiced by the University of Texas’ MD Anderson Cancer Center. This bi-institutional technology sparked a landmark of $100 million licensing deal with biotech company Intrexon Corp. and pharmaceutical company Ziopharm Oncology [106]. 2 months later, the drugmaker Merck offered to pay Intrexon and Ziopharm nearly $1 billion, plus royalties for an upstart CAR T cancer drug development project [97]. Arrows indicate the license agreement(s) between the parties. Normal line indicates a mutual collaboration or industry-academic partnership. DGI: Discovery Genomics, Inc.; FORM: Formula Pharmaceuticals, Inc.; IMS: Immusoft Corporation; MDC: Max Delbrück Center for Molecular Medicine in the Helmholtz Association; NCI: National Cancer Institute; UoM: University of Minnesota; UoT MD A: University of Texas’ MD Anderson Cancer Center; XON: Intrexon Corporation; ZIOP: Ziopharm Oncology.
(WIPO) resulted in 19 patent applications or documents, involving SB. Majority of the patent applications are from the USA and Europe. Most of the SB patents are in the category C12N (inventions concerning microorganisms, enzymes; compositions thereof mutation or genetic engineering), followed by A61K (inventions concerning pharmaceutical field-preparations for medical purposes) and A01K (inventions concerning animal husbandry) (Figure 10A).

There are currently ten ongoing clinical trials in USA employing SB (Table 5). These trials use the SB11/pT2 version of the transposon system aiming to treat B-cell malignancies and metastatic breast cancer. In addition, the hyperactive SB100X-generated stable cell line in conjunction with Encapsulated Cell Biodelivery™ was trialed to treat AD patients (ClinicalTrials.gov identifier: NCT01163825) (Table 5). Another clinical trial is planned to launch in 2017, which would employ SB100X and pFAR technologies to treat AMD (TargetAMD [93]).

Because of the significant complexities associated with cell engineering and therapy, partnerships tend to link different players, including academic research institutions and the biotech/pharma industries (Figure 10B). In one example, University of Minnesota has combined its patented SB-based gene delivery technology (SB11 and pT2) with intellectual properties of cancer therapies practiced by the University of Texas’ MD Anderson Cancer Center. This joint deal was meant to create the first-of-its kind non-viral immunotherapy treatment to support the patient’s immune system to fight against cancer. In an attempt to develop and evaluate the potential of immunotherapy to treat solid tumors, Intrexon and ZioPharm announced a Cooperative Research and Development Agreement (CRADA) with the National Cancer Institute (NCI). This joint corporation will use T-cell receptors (TCRs) expressed by the non-viral SB system [94]. Recently, Immusoft acquired an exclusive access to use the SB transposon technology through acquisition of Discovery Genomics, Inc., which will be used for the development of autologous cell therapy products for treating a variety of diseases. Immusoft’s proprietary Immune System Programming (ISP™) technology would be used to program patients own B cells to generate miniature drug factories in the body. The Immusoft technology platform would use the SB transposon system for inserting genes encoding the correct human homolog of a missing or defective protein(s) to boost the patient’s own immune cells. Certain companies are providing reagents and custom services of SB transposon technology for research and clinical applications (Table 4).

In an attempt to replicate the success of SB in therapeutic applications, MDC has established an exclusive licensing agreement and strategic collaboration with Formula Pharmaceuticals, Inc. for the development of Cytokine Induced Killer (C.I.K.) cell-based Chimeric Antigen Receptor (CAR) immunotherapies. The collaboration platform is partially sponsored by the Helmholtz Association (MD-Cell, Innovation Lab), and would use MDC’s proprieties, the hyperactive SB100X transposase and pT4 transposon, the optimized components of the SB transposon-based gene transfer system [95].
<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Clinical trial ID</th>
<th>Disease</th>
<th>Gene</th>
<th>Gene type</th>
<th>Cell source</th>
<th>Target cells</th>
<th>Gene delivery</th>
<th>Administration route</th>
<th>Clinical phase</th>
<th>Status</th>
<th>Year approved/initiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>US-0922</td>
<td>CD 19+ B-lymphoid malignancies</td>
<td>CD19 antigen specific-zeta T-cell receptor</td>
<td>Receptor</td>
<td>Autologous</td>
<td>T lymphocytes</td>
<td>In vitro</td>
<td>Intravenous</td>
<td>I</td>
<td>Open</td>
<td>2008</td>
</tr>
<tr>
<td>2</td>
<td>US-1003</td>
<td>B-cell malignancies</td>
<td>CD19 antigen specific-zeta T-cell receptor</td>
<td>Receptor</td>
<td>Allogeneic</td>
<td>HLA matched T lymphocytes</td>
<td>In vitro</td>
<td>Intravenous</td>
<td>I</td>
<td>Open</td>
<td>2009</td>
</tr>
<tr>
<td>3</td>
<td>US-1022</td>
<td>B-cell malignancies</td>
<td>CD19 antigen specific-zeta T-cell receptor</td>
<td>Receptor</td>
<td>Allogeneic</td>
<td>Umbilical cord blood-derived lymphocytes</td>
<td>In vitro</td>
<td>Intravenous</td>
<td>I</td>
<td>Open</td>
<td>2010</td>
</tr>
<tr>
<td>4</td>
<td>US-1142</td>
<td>B-cell chronic lymphocytic leukemia</td>
<td>CD19 antigen specific-zeta T-cell receptor</td>
<td>Receptor</td>
<td>Autologous</td>
<td>CD4+ and CD8+ T lymphocytes</td>
<td>In vitro</td>
<td>Intravenous</td>
<td>I</td>
<td>Open</td>
<td>2012</td>
</tr>
<tr>
<td>5</td>
<td>US-1192</td>
<td>B-cell chronic lymphocytic leukemia</td>
<td>CD19 antigen specific-zeta T-cell receptor</td>
<td>Receptor</td>
<td>Autologous</td>
<td>CD4+ and CD8+ T lymphocytes</td>
<td>In vitro</td>
<td>Intravenous</td>
<td>I</td>
<td>Open</td>
<td>2012</td>
</tr>
<tr>
<td>6</td>
<td>US-1225</td>
<td>B-cell chronic lymphocytic leukemia</td>
<td>CD19 antigen specific-zeta T-cell receptor</td>
<td>Receptor</td>
<td>Autologous</td>
<td>CD4+ and CD8+ T lymphocytes</td>
<td>In vitro</td>
<td>Intravenous</td>
<td>I</td>
<td>Open</td>
<td>2013</td>
</tr>
<tr>
<td>7</td>
<td>US-1236</td>
<td>B-lineage malignancies</td>
<td>CD19 antigen specific-zeta T-cell receptor</td>
<td>Receptor</td>
<td>Allogeneic</td>
<td>Umbilical cord blood-derived lymphocytes</td>
<td>In vitro</td>
<td>Intravenous</td>
<td>I</td>
<td>Open</td>
<td>2013</td>
</tr>
</tbody>
</table>


**Clinical trials are anticipated to begin by the end of 2017.**
### TABLE 5

List of currently ongoing clinical trials using *Sleeping Beauty* transposon system.

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Clinical trial ID</th>
<th>Disease</th>
<th>Gene</th>
<th>Gene type</th>
<th>Cell source</th>
<th>Target cells</th>
<th>Gene delivery</th>
<th>Administration route</th>
<th>Clinical phase</th>
<th>Status</th>
<th>Year approved/initiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>US-1203</td>
<td>B-cell chronic lymphocytic leukemia</td>
<td>CD19 antigen specific chimeric antigen receptor (CAR) CD3 zeta and CD137 signaling</td>
<td>Receptor others</td>
<td>Autologous</td>
<td>CD4+ and CD8+ T lymphocytes</td>
<td><em>In vitro</em></td>
<td>Intravenous</td>
<td>I</td>
<td>Open</td>
<td>2013</td>
</tr>
<tr>
<td>9</td>
<td>US-1353</td>
<td>B-lineage malignancies</td>
<td>CD19 antigen specific-zeta T-cell receptor IL-15</td>
<td>Receptor cytokine</td>
<td>Autologous</td>
<td>Primary CD3+ lymphocytes</td>
<td><em>In vitro</em></td>
<td>Intravenous</td>
<td>I</td>
<td>Open</td>
<td>2014</td>
</tr>
<tr>
<td>Sweden</td>
<td>NCT 01163825</td>
<td>Alzheimer’s disease</td>
<td>NGF</td>
<td>Growth factor</td>
<td>Allogenic/ ATCC</td>
<td>ARPE-19</td>
<td><em>In vitro</em></td>
<td>Surgery</td>
<td>I</td>
<td>Unknown</td>
<td>2010</td>
</tr>
<tr>
<td>Switzerland</td>
<td>N.A</td>
<td>AMD</td>
<td>PEDF</td>
<td>Protein</td>
<td>Autologous</td>
<td>RPE</td>
<td><em>In vitro</em></td>
<td>Transplantation</td>
<td>lb/la</td>
<td>U.R*</td>
<td>2017**</td>
</tr>
</tbody>
</table>


**Clinical trials are anticipated to begin by the end of 2017.
TRANSLATIONAL INSIGHT
Despite periods of serious stagnation over the past few decades, the future of cell and gene therapy seems to be brighter. The turn-around began about a decade ago, and has been on an exponential trajectory by overcoming the early issues. Besides using traditional viral vectors, recent years have seen major breakthroughs in genome engineering systems, such as transposon-mediated gene delivery and CRISPR/Cas9-mediated genome editing tools. As discussed above, SB appears to be a relative low risk and efficient gene delivery vector, and represents a safer alternative to integrating viral vectors.

In parallel, rapid development occurred also in the CRISPR/Cas9 technology that can be developed for genome editing. These technologies became available in many species and have revolutionized genome engineering. These two approaches appear to have distinct features, and might occupy complementary niches in therapeutic applications. For example, due to its ability to specifically target sequences, the CRISPR/Cas9 system appears to be ideal for knocking out strategies. Currently it is tested in many pre-clinical studies, and could be on its way towards clinical applications in the near future. By contrast, although

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**FIGURE 11**
Milestones and developments in Sleeping Beauty transposon technology for various therapeutic applications as of March 2017.

- 1983: McClintock was awarded the Nobel Prize for the discovery of transposons
- 1997: Resurrection and establishment of SB transposon system
- 2002: Integration profile of SB was found to be fairly random
- 2007: First proof-of-concept and prototype for targeted transposition
- 2008: First clinical trial in USA using SB for B-cell malignancies
- 2009: Development of the hyperactive version of SB called SB100X
- 2010: SB-based gene therapy to treat Alzheimer’s disease
- 2017: SB-based gene therapy to treat AMD

12 clinical trials in two decades
proof-of-concept studies exist to demonstrate that SB can be targeted to predetermined genomic loci [23,24], the current SB system is not suitable for flexible and efficient sequence-specific genome targeting. Nevertheless, among integrating gene delivery vectors the SB system has the highest chance of landing in a genomic safe harbor [35]. On the other side, today’s CRISPR/Cas9 strategies to manipulate large genomic regions (knocking in) face clear limitations for clinical translation. The current knock-in protocols are tightly dependent on homologous recombination of the cellular DNA repair machinery that has low activity in many clinically relevant cell types. Thus, compared to CRISPR/Cas9, the SB system is more suitable for applications that require ‘gene insertion’ (especially large genes). Regarding safety, the SB-mediated transgene integration is highly regulated and precise, and does not generate unspecific double stranded breaks in the genome (off target).

In addition to ex vivo applications, an important milestone in the SB technology is in vivo delivery. This gene transduction system is based on a hybrid transposon/adenovirus vector and the hyperactive SB transposase (SB100X) [74]. This in vivo strategy is effective and safe in hematopoietic stem cells (HSCs), and performs without the requirement of ex vivo expansion and transduction [75,96]. This system may overcome some of the current difficulties associated with cell collection and manufacturing, and provide technical advances for gene therapy.

Collectively, the significant efforts invested in developing genome-engineering tools begin to pay dividends, as we witness an increasing interest in using them in various applications, including cell and gene therapy. The SB-mediated gene transfer is currently being evaluated in 12 clinical trials (Figure 11). In the coming years, the number of trials using genome-engineering systems is forecasted to increase, attracting further investment from the pharma as well as biotech companies.

ACKNOWLEDGEMENTS
We would like to acknowledge Dr Zoltan Ivics for his useful comments. ZI is supported by ERC Advanced Grant ERC-2011-AdG 294742-TRANSPOStress.

FINANCIAL & COMPETING INTERESTS DISCLOSURE
The authors have no relevant financial involvement with an organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. SN and ZI are employees of the MDC. ZI has several patent applications on Sleeping Beauty. No writing assistance was utilized in the production of this manuscript.

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Clinical trial database of National Institutes of Health: https://clinicaltrials.gov/


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