CELL & GENE THERAPY INSIGHTS

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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.

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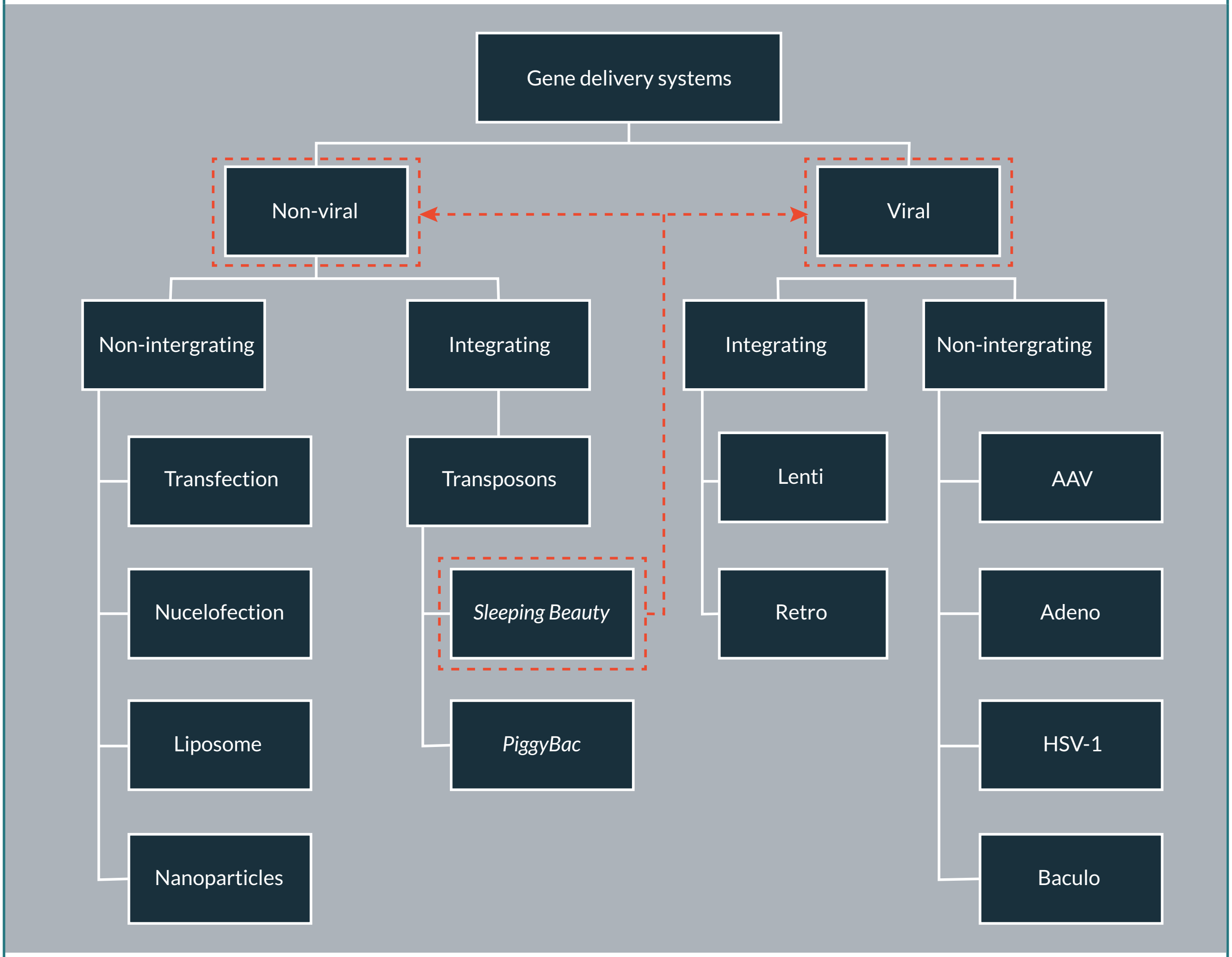
The high expectation on gene therapy continues to grow, as it holds the potential to provide a cure for myriad genetic diseases by substituting a corrupted gene with the respective functional one to achieve a therapeutic effect. The success of gene therapy is mainly dependent on the safety, efficacy, 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

FIGURE 1

Different gene delivery systems for gene therapy.



Gene therapy involves delivery of the gene of interest into therapeutically relevant cells. The delivery can be accomplished by several gene delivery systems as summarized in the graphic. Viruses possess the inherent ability to infect and deliver DNA into a wide variety of cell types. This phenomenon has been exploited for various gene therapy applications. Based on the integration ability viral vectors can be further classified into integrating and non-integrating vectors. Non-integrating viral vectors include vectors from adeno-associated virus (AAV), adenovirus (AV), herpes simplex virus 1 amplicon (HSV-1) and baculovirus (Baculo); while integrating viral vectors are derived from lentivirus and retrovirus. Because of the associated disadvantages with viral vectors, recently non-viral vectors have gained significance as an alternative delivery method. Naked DNA can be delivered either by transfection or nucleofection or can be complexed with liposomes and nanoparticles. However, these methods have a limitation of mediating the transient effect, due to the lack of stable genome integration. However, natural gene delivery vehicles like *Sleeping Beauty* and *PiggyBac* transposons can overcome the problem of transient expression. Transposons have the inherent ability to stably integrate the gene of interest into the genome, providing long term or possibly permanent therapeutic effect. *Sleeping Beauty* transposon system has been combined with both non-viral and viral delivery techniques (orange dotted line).

the gene therapy clinical trials currently use viral vectors. 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

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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 lenti-viral 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 *Tc1/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].

Engineering 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].

TABLE 1

List of currently available *SB* transposon vectors.

No.	Transposon	Ref.
1	*pT*	[8]
2	*pT2*	[11]
3	*pT3*	[108]
4	*pT2B*	[15]
5	*pT4*	[52]

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SALIENT FEATURES OF *SB* TRANSPOSON 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];

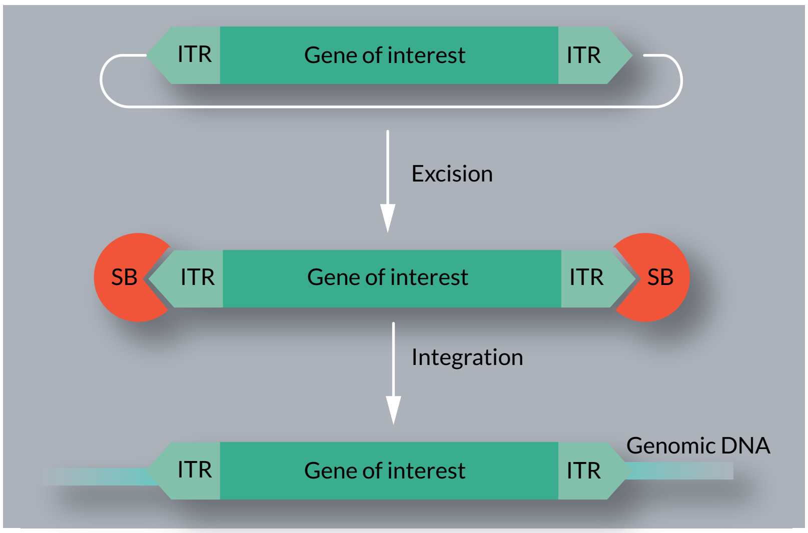
TABLE 2

List of currently available SB transposases.

Transposase	Ref.
SB10	[8]
SB11 (3-fold higher than *SB10*)	[109]
SB12 (4-fold higher than *SB10*)	[66]
HSB1–HSB5 (up to 10-fold higher than *SB10*)	[108]
HSB13–HSB17 (*HSB17* is 17-fold higher than *SB10*)	[111]
SB100X (100-fold higher than *SB10*)	[14]
SB150X (130-fold higher than *SB10*)	[24]

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.

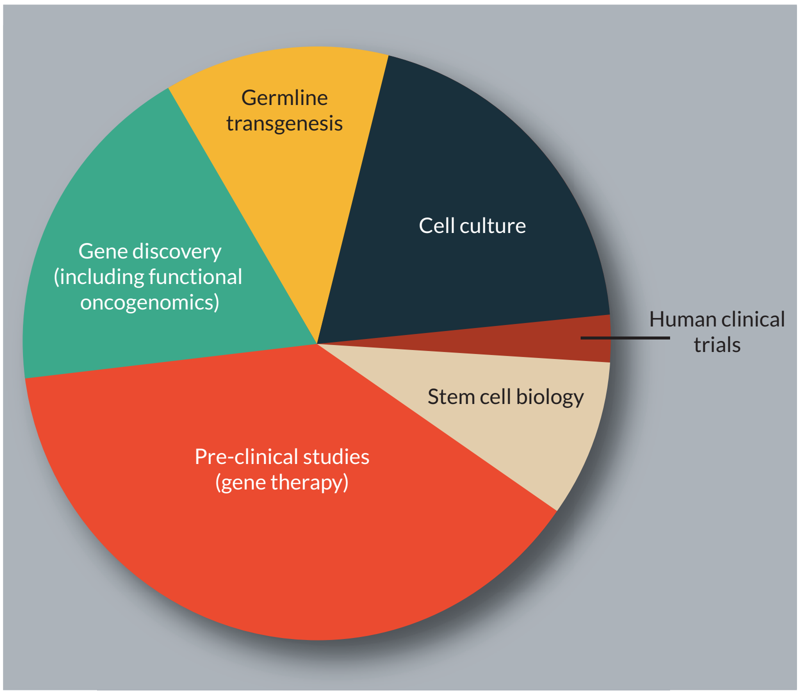
- **Unbiased, close-to-random integration profile:** the close-to-random integration profile of *SB* was confirmed by multiple studies, and was reported from various organisms and cell types [20,23,24,31–35];
- **Benign promoter/enhancer activity:** the *SB* ITRs have negligible intrinsic promoter activity (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.

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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* TRANSPOSON 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

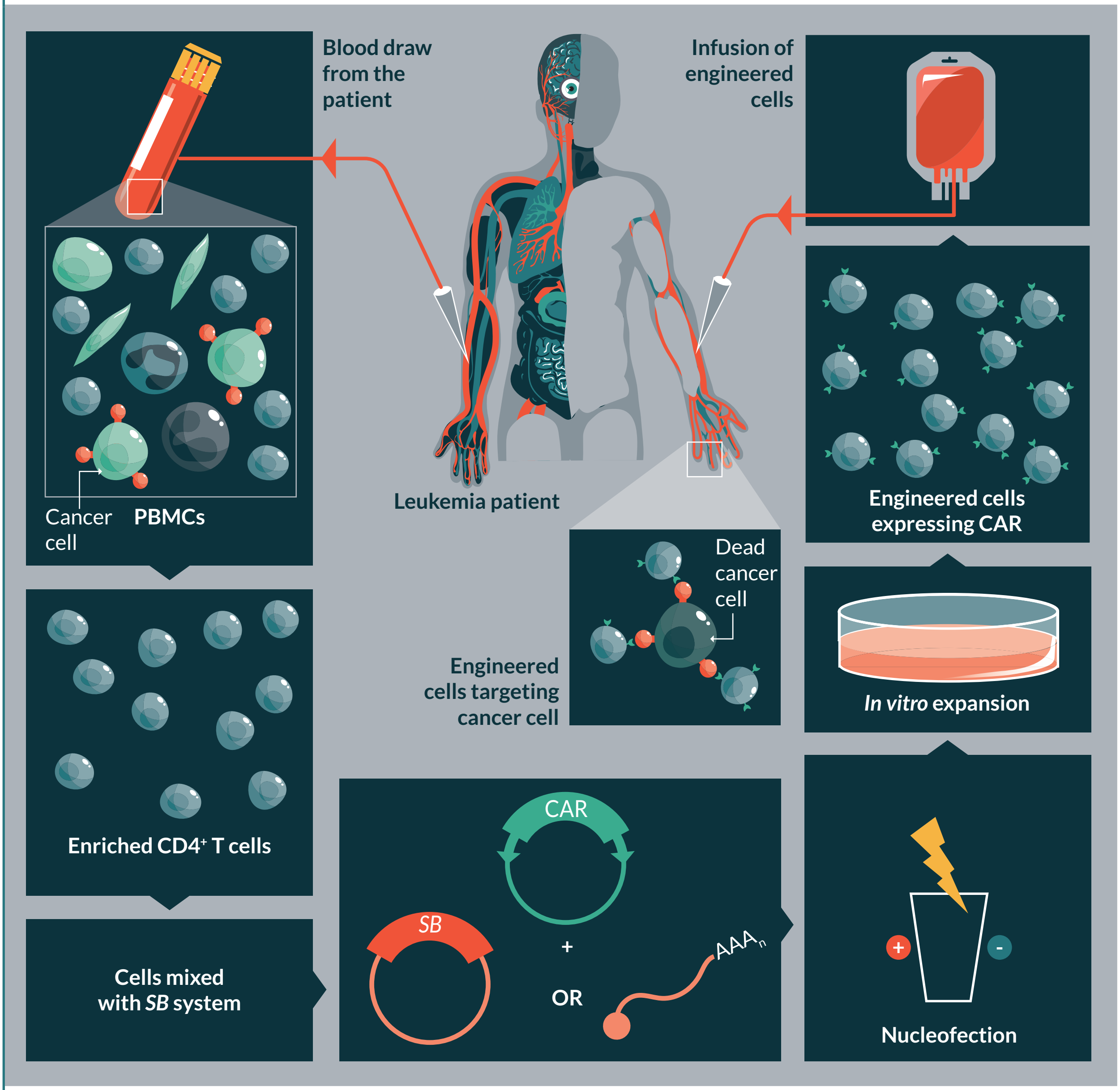
The latest optimized version of the *SB* system comprises of the hyperactive *SB100X* transposase and the *pT4* transposon [12,14]. The *SB100X*, generated by molecular evolutionary strategy performs with significantly higher efficiency of genomic integration that in certain cells is even comparable 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.

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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).

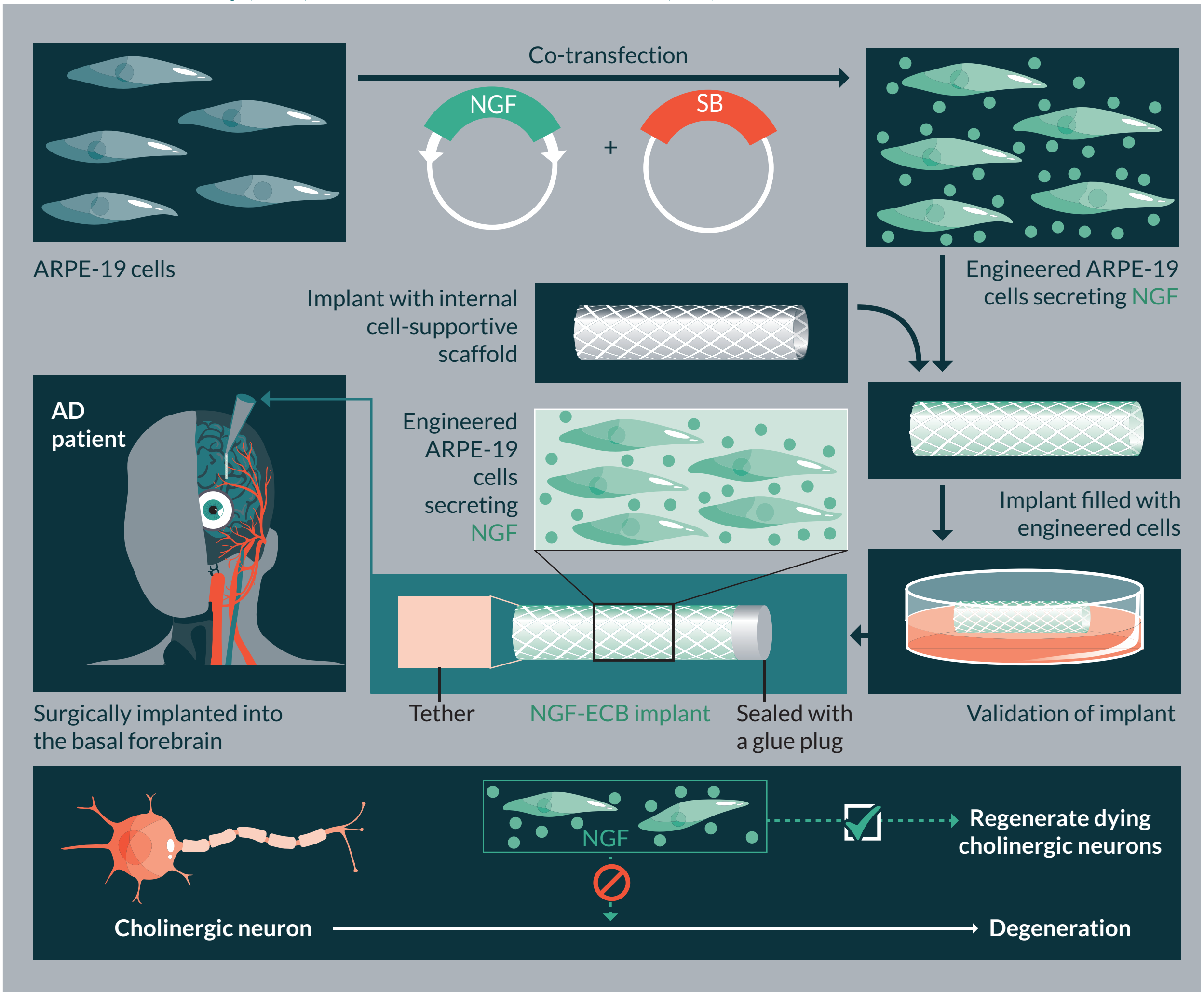
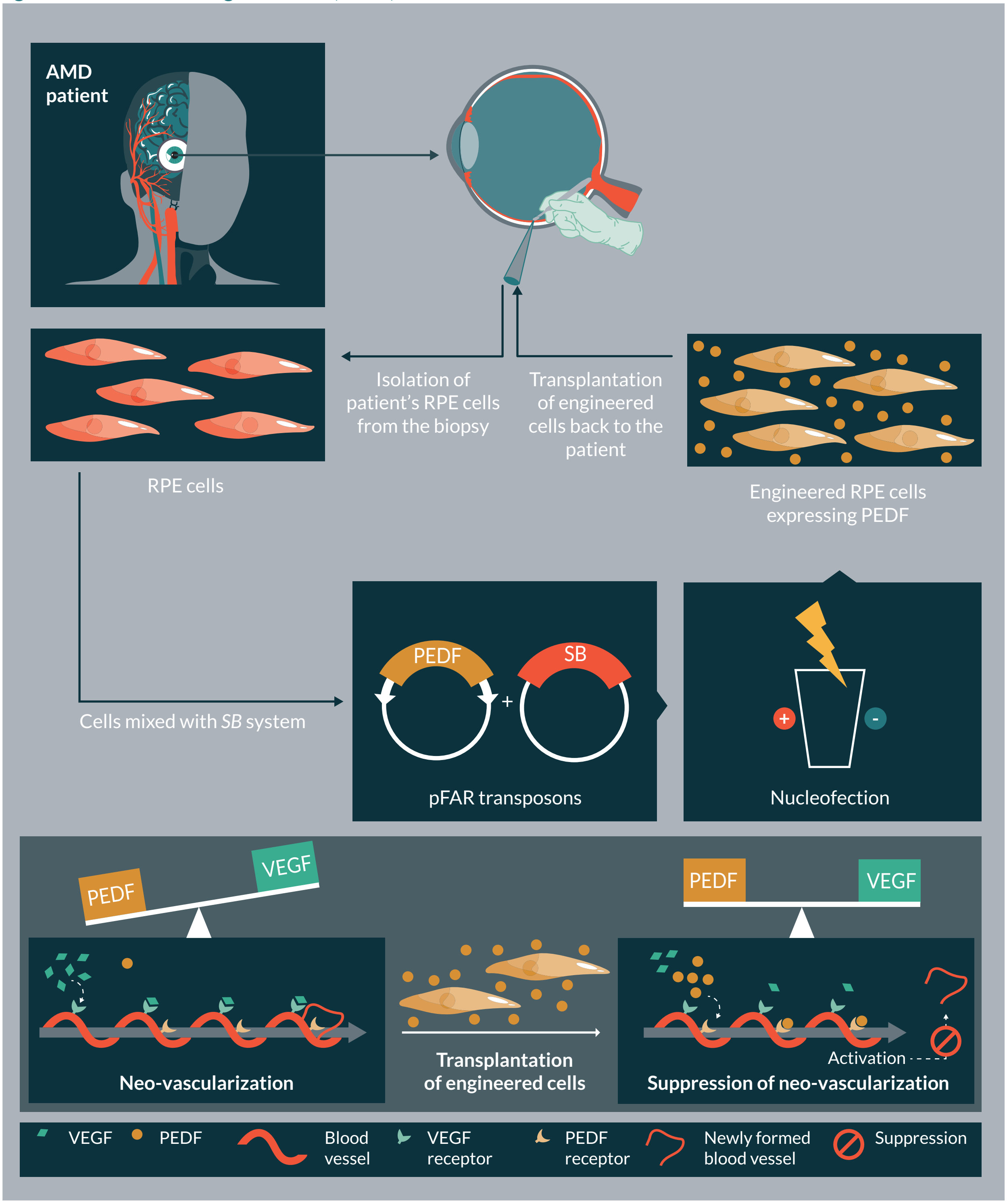


Illustration of ECB device for AD: Human retinal pigment epithelial cells (ARPE-19) are genetically engineered using the *Sleeping Beauty* system to express nerve growth factor (NGF). Engineered cells are encapsulated into an implant (semi-permeable hollow fiber membrane that allows the influx of nutrients 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).

FIGURE 6

Gene therapy approach utilizing Sleeping Beauty transposon technology for the treatment of exudative age-related macular degeneration (AMD).



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.

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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

FIGURE 7

Advancements in *Sleeping Beauty* transposon vectorization.

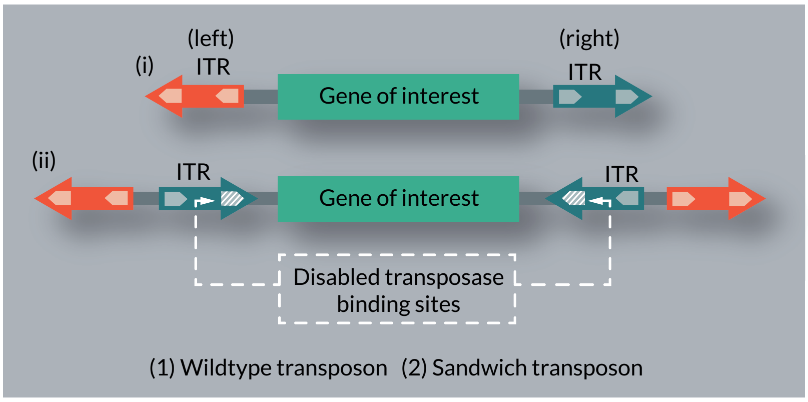

Illustration of the wild-type (i) and sandwich (ii) *SB* transposons. The sandwich transposon consists of two complete *SB* elements (ITRs) flanking the gene of interest in an inverted orientation. Because of the mutations (pentagon filled with wide downward diagonal lines) in the right ITRs (dark aqua blue arrow), only the full composite element can be mobilized. This arrangement of the sandwich *SB* transposon vector has enhanced capacity to efficiently transpose large cargos (>10kb).

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 3

List of various *Sleeping Beauty*–viral hybrid technologies.

<table>
<tr><th>Hybrid technology</th><th>Delivering vehicle</th><th>Integration machinery</th><th>Packaging capacity</th><th colspan="2">Advantages</th><th>Ref.</th></tr>
<tr><td>Adeno/SB</td><td>Recombinant adenovirus</td><td>SB10</td><td>>35 kb</td><td rowspan="3">Transduce dividing and non-dividing cells, and are one of the most efficient vehicles for in vivo gene delivery</td><td></td><td>[98]</td></tr>
<tr><td>HCAdV/SB</td><td>HCAdV</td><td>HSB5</td><td>>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>>36 kb</td><td>Lowest immunogenicity 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>>5 kb</td><td colspan="2">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 colspan="2">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 colspan="2">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 colspan="2">Un-biased random integration profile</td><td>[105]</td></tr>
</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.

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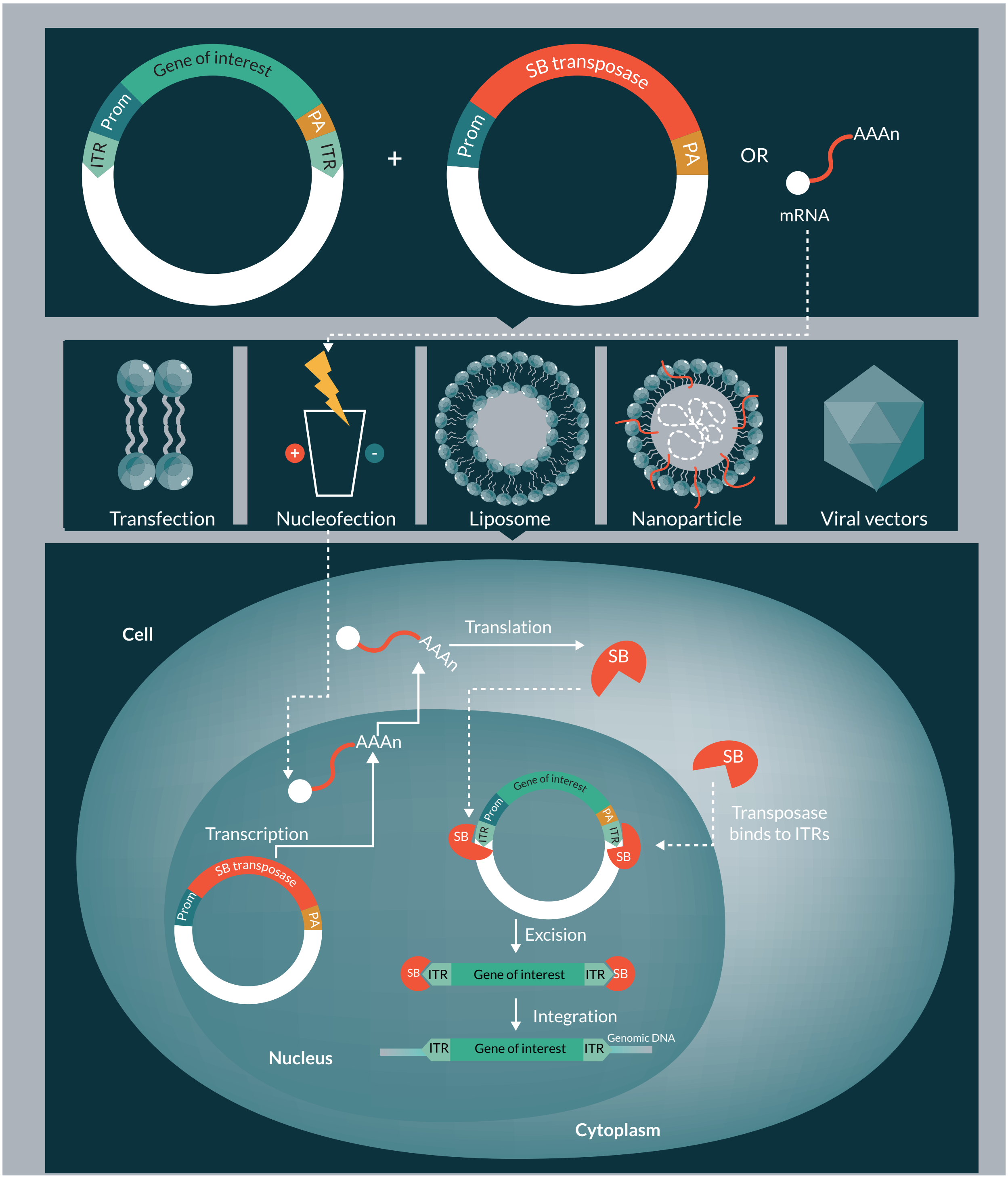
FIGURE 8

Delivery of the *Sleeping Beauty* transposon system into cells for cell & gene therapy applications.

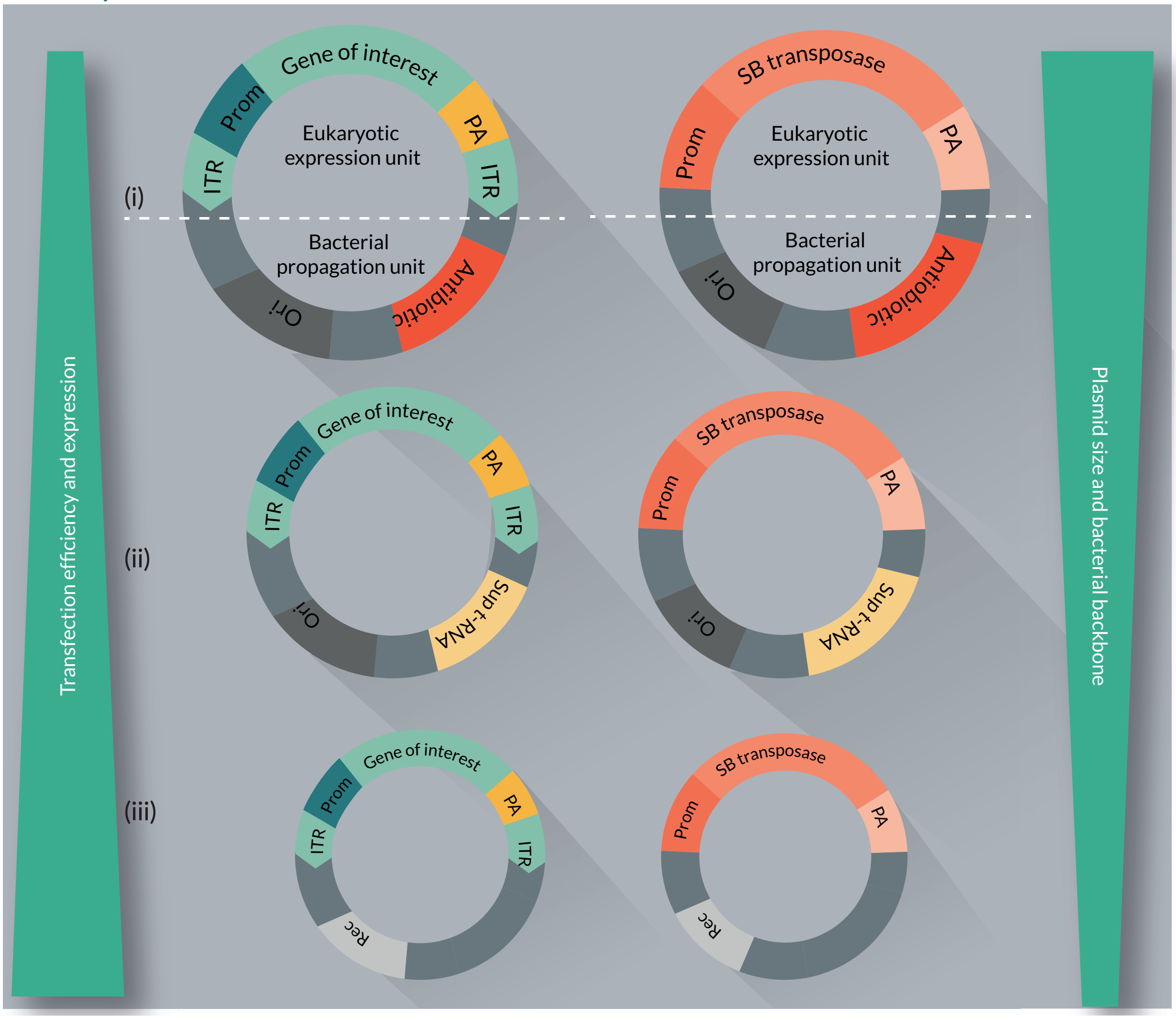


The *SB* transposon system can be delivered (large rounded rectangle) into the cells by combining with any nucleic acid-delivery techniques like transfection (using the commercial transfection reagents) or nucleofection (electro-transfer of nucleic acids directly into the nucleus) or by complexing with 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 electrotransfer. 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,

FIGURE 9

Schematic overview of alternative vector selection approaches according to the size and transfection efficiency.



Development of novel transposon vectors that are free of bacterial components for clinical applications. Structural components of conventional (i), pFAR (ii) and minicircle (MC; iii) plasmids are illustrated. Typical conventional plasmid (i) contains a bacterial propagation unit and an eukaryotic expression unit. Bacterial propagation components like origin of replication (*ori*) and antibiotic resistance gene are not desirable for clinical applications. Efforts in eliminating the bacterial components resulted in the development of pFAR and MC vectors. pFAR vectors are free of antibiotic resistance gene, replaced by a suppressor t-RNA gene (Sup t-RNA) for bacterial selection and propagation (see also text). pFAR vectors still have bacterial origin of replication (*ori*). MC represents vectors that contain no bacterial components. MCs are generated by an intramolecular recombination (Rec). Because of their reduced size and sequences of bacterial origin pFAR and MC miniplamids enable more efficient transfections and offer sustained expression compared to conventional plasmid vectors. Components of the conventional (i), pFAR (ii) and minicircle (MC; iii) plasmids are not proportional to the size and are not at scale. ITR-inverted terminal repeat; Prom-promoter; PA-polyadenylation.

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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

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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 4

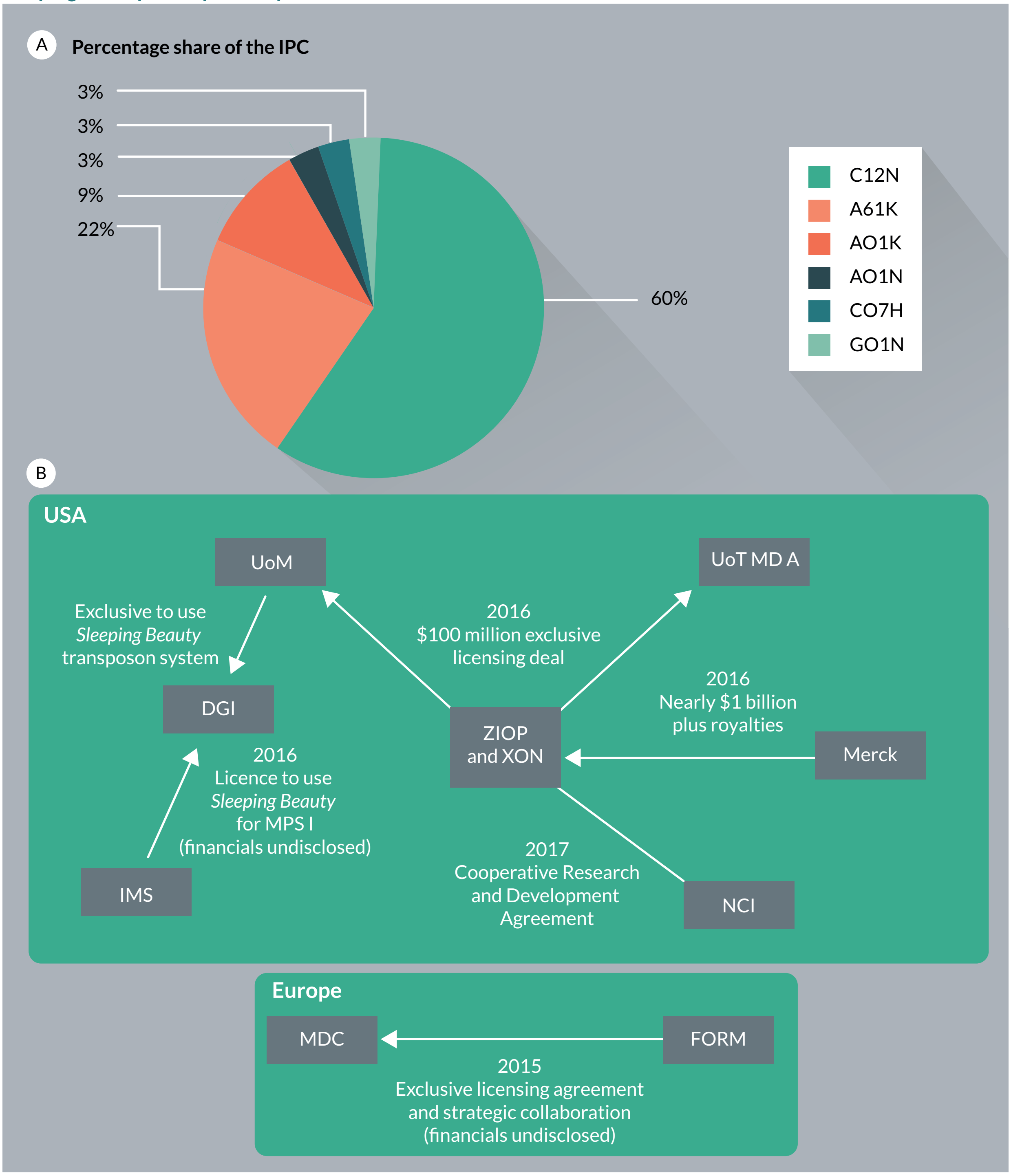
Industry interests in using *Sleeping Beauty* transposon technologies.

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

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.

FIGURE 10

Patent landscape and partnership relations of academic institutions and industrial partners involving the *Sleeping Beauty* transposon system as of March 2017.



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.

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(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 (*SB*11 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 join 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 proprietaries, the hyperactive *SB100X* transposase and *pT4* transposon, the optimized components of the *SB* transposon-based gene transfer system [95].

TABLE 5

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

Sl. No	Clinical trial ID	Disease	Gene	Gene type	Cell source	Target cells	Gene delivery	Administration route	Clinical phase	Status	Year approved/ initiated
USA											
1	US-0922	CD 19+ B-lymphoid malignancies	CD19 antigen specific-zeta T-cell receptor	Receptor	Autologous	T lymphocytes	*In vitro*	Intravenous	I	Open	2008
2	US-1003	B-cell malignancies	CD19 antigen specific-zeta T-cell receptor	Receptor	Allogeneic	HLA matched T lymphocytes	*In vitro*	Intravenous	I	Open	2009
3	US-1022	B-cell malignancies	CD19 antigen specific-zeta T-cell receptor	Receptor	Allogeneic	Umbilical cord blood-derived lymphocytes	*In vitro*	Intravenous	I	Open	2010
4	US-1142	B-cell chronic lymphocytic leukemia	CD19 antigen specific-zeta T-cell receptor	Receptor	Autologous	CD4+ and CD8+ T lymphocytes	*In vitro*	Intravenous	I	Open	2012
5	US-1192	B-cell chronic lymphocytic leukemia	CD19 antigen specific-zeta T-cell receptor	Receptor	Autologous	CD4+ and CD8+ T lymphocytes	*In vitro*	Intravenous	I	Open	2012
6	US-1225	B-cell chronic lymphocytic leukemia	CD19 antigen specific-zeta T-cell receptor	Receptor others	Autologous	CD4+ and CD8+ T lymphocytes	*In vitro*	Intravenous	I	Open	2013
7	US-1236	B-lineage malignancies	CD19 antigen specific-zeta T-cell receptor	Receptor	Allogeneic	Umbilical cord blood-derived lymphocytes	*In vitro*	Intravenous	I	Open	2013

The clinical trial data presented in the table is mined from the *Journal of Gene Medicine* database [1] and clinical trial database of National Institutes of Health [107] as of March 2017.
AMD: Age-related macular degeneration; ARPE-19: Human retinal pigment epithelial cell line; ATCC: American type culture collection; N.A: Not available; NGF: Nerve growth factor; PEDF: Pigment epithelium-derived factor; RPE: Retinal pigment epithelial cells; U.R*: Under review by regulatory authorities.
**Clinical trials are anticipated to begin by the end of 2017.

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TABLE 5

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

Sl. No	Clinical trial ID	Disease	Gene	Gene type	Cell source	Target cells	Gene delivery	Administration route	Clinical phase	Status	Year approved/ initiated
8	US-1203	B-cell chronic lymphocytic leukemia	CD19 antigen specific chimeric antigen receptor (CAR) CD3 zeta and CD137 signaling	Receptor others	Autologous	CD4+ and CD8+ T lymphocytes	*In vitro*	Intravenous	I	Open	2013
9	US-1353	B-lineage malignancies	CD19 antigen specific-zeta T-cell receptor IL-15	Receptor cytokine	Autologous	Primary CD3+ lymphocytes	*In vitro*	Intravenous	I	Open	2014
10	US-1360	Metastatic breast cancer	Murine MUC1 chimeric antigen receptor CD28/CD3 / OX40 caspase 9 IL-12	Receptor antigen suicide cytokine	Autologous	T lymphocytes	*In vitro*	Intravenous	I/II	Open	2014
Sweden											
11	NCT 01163825	Alzheimer's disease	NGF	Growth factor	Allogenic/ ATCC	ARPE-19	*In vitro*	Surgery	I	Unknown	2010
Switzerland											
12	N.A	AMD	PEDF	Protein	Autologous	RPE	*In vitro*	Transplantation	Ib/IIa	U.R*	2017**

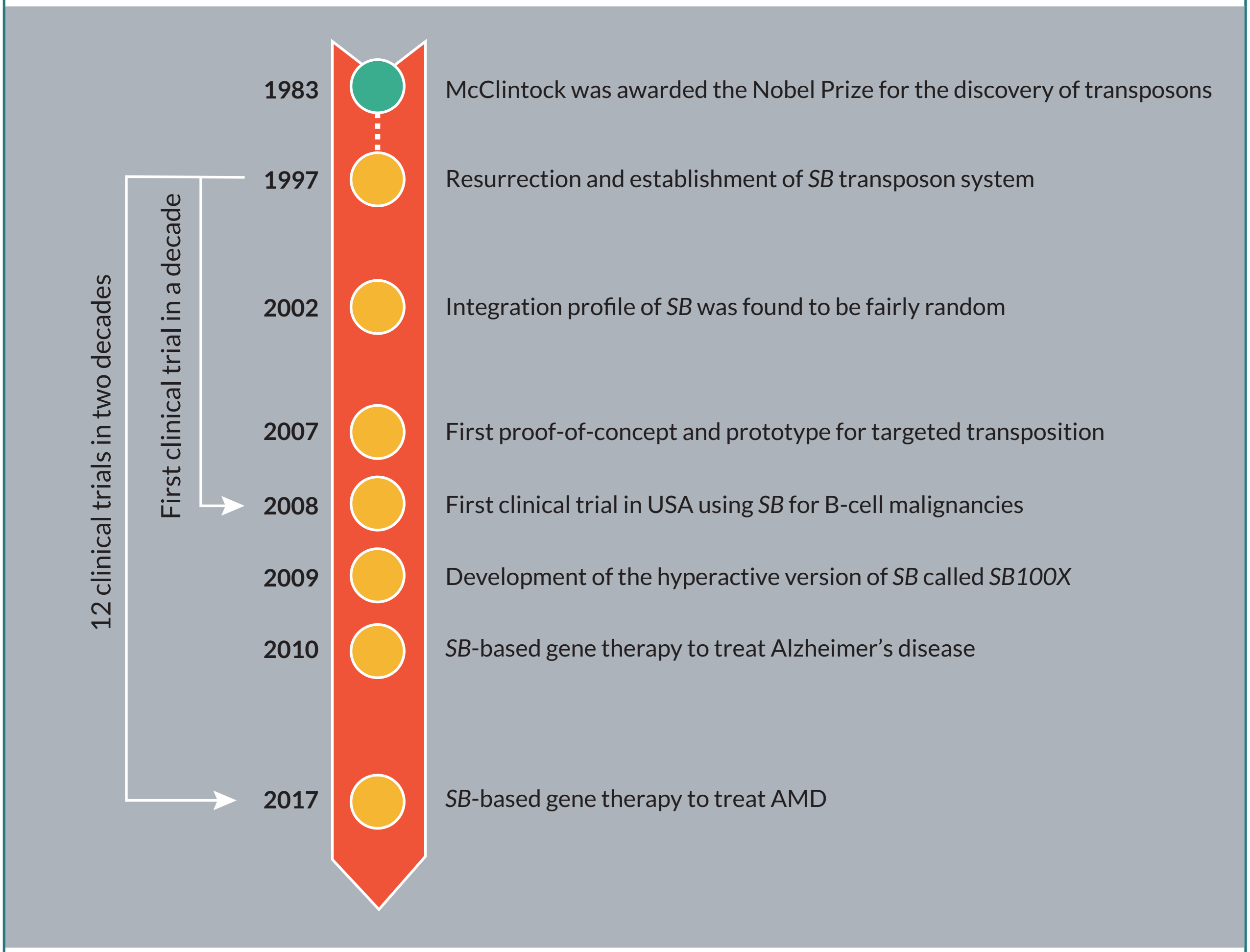
The clinical trial data presented in the table is mined from the *Journal of Gene Medicine* database [1] and clinical trial database of National Institutes of Health [107] as of March 2017.
AMD: Age-related macular degeneration; ARPE-19: Human retinal pigment epithelial cell line; ATCC: American type culture collection; N.A: Not available; NGF: Nerve growth factor; PEDF: Pigment epithelium-derived factor; RPE: Retinal pigment epithelial cells; U.R*: Under review by regulatory authorities.
**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 turnaround 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

FIGURE 11

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



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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.

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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.

REFERENCES

1. Vectors used in gene therapy clinical trials: http://www.abedia.com/wiley/vectors.php
2. Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. *Nat. Rev. Genet.* 2003; 4: 346–58.
3. Hartman ZC, Appledorn DM, Amalfitano A. Adenovirus vector induced innate immune responses: impact upon efficacy and toxicity in gene therapy and vaccine applications. *Virus Res.* 2008; 132: 1–14.
4. Roe T, Reynolds TC, Yu G, Brown PO. Integration of murine leukemia virus DNA depends on mitosis. *Embo J.* 1993; 12: 2099–108.
5. Lewinski MK, Yamashita M, Emerman M *et al.* Retroviral DNA integration: viral and cellular determinants of target-site selection. *PLoS Pathog.* 2006; 2: e60.
6. Hacein-Bey-Abina S, Von Kalle C, Schmidt M *et al.* LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 2003; 302: 415–9.
7. Baum C, von Kalle C, Staal FJ, Li Z *et al.* Chance or necessity? Insertional mutagenesis in gene therapy and its consequences. *Mol. Ther.* 2004; 9: 5–13.
8. Ivics Z, Hackett PB, Plasterk RH, Izsvak Z. Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. *Cell* 1997; 91: 501–10.
9. Izsvak Z, Ivics Z, Plasterk RH. Sleeping Beauty, a wide host-range transposon vector for genetic transformation in vertebrates. *J. Mol. Biol.* 2000; 302: 93-102.
10. Wilber A, Wangensteen KJ, Chen Y *et al.* Messenger RNA as a source of transposase for sleeping beauty transposon-mediated correction of hereditary tyrosinemia type I. *Mol. Ther.* 2007; 15: 1280–7.
11. Cui Z, Geurts AM, Liu G *et al.* Structure-function analysis of the inverted terminal repeats of the sleeping beauty transposon. *J. Mol. Biol.* 2002; 318: 1221–35.
12. Wang Y, Pryputniewicz-Dobrinska D, Nagy EE *et al.* Regulated complex assembly safeguards the fidelity of Sleeping Beauty transposition. *Nucleic Acids Res.* 2017 45: 311–326.
13. Kowarz E, Loscher D, Marschalek R. Optimized Sleeping Beauty transposons rapidly generate stable transgenic cell lines. *Biotechnol. J.* 2015; 10: 647–53.
14. Mates L, Chuah MK, Belay E *et al.* Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. *Nat. Genet.* 2009; 41: 753–61.
15. Izsvak Z, Khare D, Behlke J *et al.* Involvement of a bifunctional, paired-like DNA-binding domain and a transpositional enhancer in Sleeping Beauty transposition. *J. Biol. Chem.* 2002; 277: 34581–8.
16. Izsvak Z, Stuwe EE, Fiedler D *et al.* Healing the wounds inflicted by sleeping beauty transposition by double-strand break repair in mammalian somatic cells. *Mol Cell.* 2004; 13: 279–90.
17. Wang Y, Wang J, Devaraj A *et al.* Suicidal autointegration of sleeping beauty and piggyBac transposons in eukaryotic cells. *PLoS Genet.* 2014; 10: e1004103.
18. Luo G, Ivics Z, Izsvak Z, Bradley A. Chromosomal transposition of a Tc1/mariner-like element in mouse embryonic stem cells. *Proc. Natl Acad. Sci. USA* 1998; 95: 10769–73.
19. Yant SR, Kay MA. Nonhomologous-end-joining factors regulate DNA repair fidelity during Sleeping Beauty element transposition in mammalian cells. *Mol. Cell Biol.* 2003; 23: 8505–18.
20. Vigdal TJ, Kaufman CD, Izsvak Z *et al.* Common physical properties of DNA affecting target site selection of sleeping beauty and other Tc1/mariner transposable elements. *J. Mol. Biol.* 2002; 323: 441–52.
21. Ivics Z, Katzer A, Stuwe EE *et al.* Targeted Sleeping Beauty transposition in human cells. *Mol. Ther.* 2007; 15: 1137–44.
22. Yant SR, Huang Y, Akache B, Kay MA. Site-directed transposon integration in human cells. *Nucleic Acids Res.* 2007; 35: e50.
23. Ammar I, Gogol-Doring A, Miskey C *et al.* Retargeting transposon insertions by the adeno-associated virus Rep protein. *Nucleic Acids Res.* 2012; 40: 6693–712.
24. Voigt K, Gogol-Doring A, Miskey C *et al.* Retargeting sleeping beauty transposon insertions by engineered zinc finger DNA-binding domains. *Mol. Ther.* 2012; 20: 1852–62.
25. Zayed H, Izsvak Z, Khare D *et al.* The DNA-bending protein HMGB1 is a cellular cofactor of Sleeping Beauty transposition. *Nucleic Acids Res.* 2003; 31: 2313–22.
26. Walisko O, Schorn A, Rolfs F *et al.* Transcriptional activities of the Sleeping Beauty transposon and shielding its genetic cargo with insulators. *Mol. Ther.* 2008; 16: 359–69.
27. Walisko O, Izsvak Z, Szabo K *et al.* Sleeping Beauty transposase modulates cell-cycle progression through interaction with Miz-1. *Proc. Natl Acad. Sci. USA* 2006; 103: 4062–7.

 DOI: 10.18609/cgti.2017.014

28. Yusa K, Takeda J, Horie K. Enhancement of Sleeping Beauty transposition by CpG methylation: possible role of heterochromatin formation. *Mol. Cell Biol.* 2004; 24: 4004–18.
29. Huang X, Wilber AC, Bao L *et al.* Stable gene transfer and expression in human primary T cells by the Sleeping Beauty transposon system. *Blood* 2006; 107: 483–91.
30. Rostovskaya M, Fu J, Obst M *et al.* Transposon-mediated BAC transgenesis in human ES cells. *Nucleic Acids Res.* 2012; 40: e150.
31. Yant SR, Wu X, Huang Y *et al.* High-resolution genome-wide mapping of transposon integration in mammals. *Mol. Cell Biol.* 2005; 25: 2085–94.
32. Liu G, Geurts AM, Yae K *et al.* Target-site preferences of Sleeping Beauty transposons. *J. Mol. Biol.* 2005; 346: 161–73.
33. Geurts AM, Hackett CS, Bell JB *et al.* Structure-based prediction of insertion-site preferences of transposons into chromosomes. *Nucleic Acids Res.* 2006; 34: 2803–11.
34. Huang X, Guo H, Tammana S *et al.* Gene transfer efficiency and genome-wide integration profiling of Sleeping Beauty, Tol2, and piggyBac transposons in human primary T cells. *Mol. Ther.* 2010; 18: 1803–13.
35. Gogol-Döring A, Ammar I, Gupta S *et al.* Genome-wide Profiling Reveals Remarkable Parallels Between Insertion Site Selection Properties of the MLV Retrovirus and the piggyBac Transposon in Primary Human CD4(+) T Cells. *Mol. Ther.* 2016; 24: 592–606.
36. Henssen AG, Henaff E, Jiang E *et al.* Genomic DNA transposition induced by human PGBD5. *Elife* 2015; 4.
37. Ivics Z. Endogenous Transposase Source in Human Cells Mobilizes piggyBac Transposons. *Mol. Ther.* 2016; 24: 851–4.
38. Ivics Z, Izsvak Z. The expanding universe of transposon technologies for gene and cell engineering. *Mob. DNA* 2010; 1: 25.
39. Wachter K, Kowarz E, Marschalek R. Functional characterisation of different MLL fusion proteins by using inducible Sleeping Beauty vectors. *Cancer Lett.* 2014; 352: 196–202.
40. Ammar I, Izsvak Z, Ivics Z. The Sleeping Beauty transposon toolbox. *Methods Mol. Biol.* 2012; 859: 229–40.
41. Narayanavari SA, Chilkunda SS, Ivics Z. Sleeping Beauty transposition: from biology to applications. *Crit. Rev. Biochem. Mol. Biol.* 2016; 1–27.
42. Katter K, Geurts AM, Hoffmann O *et al.* Transposon-mediated transgenesis, transgenic rescue, and tissue-specific gene expression in rodents and rabbits. *FASEB J.* 2013; 27: 930–41.
43. Alessio AP, Fili AE, Garrels W *et al.* Establishment of cell-based transposon-mediated transgenesis in cattle. *Theriogenology* 2016; 85: 1297–1311 e2.
44. Davis RP, Nemes C, Varga E *et al.* Generation of induced pluripotent stem cells from human foetal fibroblasts using the Sleeping Beauty transposon gene delivery system. *Differentiation* 2013; 86: 30–7.
45. Grabundzija I, Wang J, Sebe A *et al.* Sleeping Beauty transposon-based system for cellular reprogramming and targeted gene insertion in induced pluripotent stem cells. *Nucleic Acids Res.* 2013; 41: 1829–1847.
46. Kaji K, Norrby K, Paca A *et al.* Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* 2009; 458: 771–5.
47. Fatima A, Ivanyuk D, Herms S *et al.* Generation of human induced pluripotent stem cell line from a patient with a long QT syndrome type 2. *Stem Cell Res.* 2016; 16: 304–7.
48. Kues WA, Herrmann D, Barg-Kues B *et al.* Derivation and characterization of sleeping beauty transposon-mediated porcine induced pluripotent stem cells. *Stem Cells Dev.* 2013; 22: 124–35.
49. Muenthaisong S, Ujhelly O, Polgar Z *et al.* Generation of mouse induced pluripotent stem cells from different genetic backgrounds using Sleeping beauty transposon mediated gene transfer. *Exp. Cell Res.* 2012; 318: 2482–9.
50. Talluri TR, Kumar D, Glage S *et al.* Derivation and characterization of bovine induced pluripotent stem cells by transposon-mediated reprogramming. *Cell Reprogram* 2015; 17: 131–40.
51. Wang J, Xie G, Singh M *et al.* Primate-specific endogenous retrovirus-driven transcription defines naive-like stem cells. *Nature* 2014; 516: 405–9.
52. Wang J, Singh M, Sun C *et al.* Isolation and cultivation of naive-like human pluripotent stem cells based on HERVH expression. *Nat. Protoc.* 2016; 11: 327–46.
53. Keng VW, Yae K, Hayakawa T *et al.* Region-specific saturation germline mutagenesis in mice using the Sleeping Beauty transposon system. *Nat. Methods* 2005; 2: 763–9.
54. Izsvak Z, Ivics Z. Sleeping Beauty hits them all: transposon-mediated saturation mutagenesis in the mouse germline. *Nat. Methods* 2005; 2: 735–6.
55. Kokubu C, Horie K, Abe K *et al.* A transposon-based chromosomal engineering method to survey a large cis-regulatory landscape in mice. *Nat. Genet.* 2009; 41: 946–52.
56. Copeland NG, Jenkins NA. Harnessing transposons for cancer gene

discovery. *Nat. Rev. Cancer* 2010; 10: 696–706.

57. Moriarity BS, Largaespada DA. Sleeping Beauty transposon insertional mutagenesis based mouse models for cancer gene discovery. *Curr. Opin. Genet. Dev.* 2015; 30: 66–72.

58. Ruf S, Symmons O, Uslu VV *et al.* Large-scale analysis of the regulatory architecture of the mouse genome with a transposon-associated sensor. *Nat. Genet.* 2011; 43: 379–86.

59. Pindyurin AV, de Jong J, Akhtar W. TRIP through the chromatin: a high throughput exploration of enhancer regulatory landscapes. *Genomics* 2015; 106: 171–7.

60. Izsvak Z, Hackett PB, Cooper LJ. Translating Sleeping Beauty transposition into cellular therapies: victories and challenges. *Bioessays* 2010; 32: 756–67.

61. Hackett PB, Largaespada DA, Switzer KC. Evaluating risks of insertional mutagenesis by DNA transposons in gene therapy. *Transl. Res.* 2013; 161: 265–83.

62. Swierczek M, Izsvak Z, Ivics Z. The Sleeping Beauty transposon system for clinical applications. *Expert Opin. Biol. Ther.* 2012; 12: 139–53.

63. Boehme P, Doerner J, Solanki M *et al.* The sleeping beauty transposon vector system for treatment of rare genetic diseases: an unrealized hope? *Curr. Gene Ther.* 2015; 15: 255–65.

64. Jin Z, Maiti S, Huls H, Singh H *et al.* The hyperactive Sleeping Beauty transposase SB100X improves the genetic modification of T cells to express a chimeric antigen receptor. *Gene Ther.* 2011; 18: 849–56.

65. Petrov DA, Schutzman JL, Hartl DL, Lozovskaya ER. Diverse transposable elements are mobilized in hybrid dysgenesis in Drosophila virilis. *Proc. Natl Acad. Sci. USA* 1995; 92: 8050–4.

66. Zayed H, Izsvak Z, Walisko O, Ivics Z. Development of hyperactive sleeping beauty transposon vectors by mutational analysis. *Mol. Ther.* 2004; 9: 292–304.

67. Turchiano G, Latella MC, Gogol-Doring A *et al.* Genomic analysis of Sleeping Beauty transposon integration in human somatic cells. *PLoS One* 9: 2014; e112712.

68. Sharma N, Hollensen AK, Bak RO *et al.* The impact of cHS4 insulators on DNA transposon vector mobilization and silencing in retinal pigment epithelium cells. *PLoS One* 7: e48421.

69. Wang DD, Yang M, Zhu Y, Mao C. Reiterated Targeting Peptides on the Nanoparticle Surface Significantly Promote Targeted Vascular Endothelial Growth Factor Gene Delivery to Stem Cells. *Biomacromolecules* 2015; 16: 3897–903.

70. Ma K, Fu D, Yu D *et al.* Targeted delivery of in situ PCR-amplified Sleeping Beauty transposon genes to cancer cells with lipid-based nanoparticle-like protocells. *Biomaterials* 2017; 121: 55–63.

71. Wang X, Mani P, Sarkar DP *et al.* *Ex vivo* gene transfer into hepatocytes. *Methods Mol. Biol.* 2009; 481: 117–40.

72. Kren BT, Unger GM, Sjeklocha L *et al.* Nanocapsule-delivered Sleeping Beauty mediates therapeutic Factor VIII expression in liver sinusoidal endothelial cells of hemophilia A mice. *J. Clin. Invest.* 2009; 119: 2086–99.

73. Yant SR, Ehrhardt A, Mikkelsen JG *et al.* Transposition from a gutless adeno-transposon vector stabilizes transgene expression *in vivo*. *Nat. Biotechnol.* 2002; 20: 999–1005.

74. Boehme P, Zhang W, Solanki M *et al.* A High-Capacity Adenoviral Hybrid Vector System Utilizing the Hyperactive Sleeping Beauty Transposase SB100X for Enhanced Integration. *Mol. Ther. Nucleic Acids* 2016; 5: e337.

75. Richter M, Saydaminova K, Yumul R *et al.* *In vivo* transduction of primitive hematopoietic stem cells after mobilization and intravenous injection of integrating adenovirus vectors. *Blood* 2016; 128: 2206–17.

76. Monjezi R, Miskey C, Gogishvili T *et al.* Enhanced CAR T-cell engineering using non-viral Sleeping Beauty transposition from minicircle vectors. *Leukemia* 2017; 31: 186–194.

77. Sharma N, Cai Y, Bak RO *et al.* Efficient sleeping beauty DNA transposition from DNA minicircles. *Mol. Ther. Nucleic Acids* 2013; 2: e74.

78. Simcikova M, Prather KL, Prazeres DM, Monteiro GA. Towards effective non-viral gene delivery vector. *Biotechnol. Genet. Eng. Rev.* 2015; 31: 82–107.

79. Tolmachov OE,. Building mosaics of therapeutic plasmid gene vectors. *Curr. Gene Ther.* 2011; 11: 466–78.

80. Marie C, Vandermeulen G, Quiviger M *et al.* pFARs, plasmids free of antibiotic resistance markers, display high-level transgene expression in muscle, skin and tumour cells. *J. Gene Med.* 2010; 12: 323–32.

81. Garrels W, Talluri TR, Ziegler M *et al.* Cytoplasmic injection of murine zygotes with Sleeping Beauty transposon plasmids and minicircles results in the efficient generation of germline transgenic mice. *Biotechnol. J.* 2016; 11: 178–84.

82. Singh H, Figliola MJ, Dawson MJ *et al.* Reprogramming CD19-specific T cells with IL-21 signaling can improve adoptive immunotherapy of B-lineage malignancies. *Cancer Res.* 2011; 71: 3516–27.

83. Singh H, Manuri PR, Olivares S *et al.* Redirecting specificity of T-cell populations for CD19 using the Sleeping

 DOI: 10.18609/cgti.2017.014

Beauty system. *Cancer Res.* 2008; 68: 2961–71.

84. Magnani CF, Turazzi N, Benedicenti F *et al.* Immunotherapy of acute leukemia by chimeric antigen receptor-modified lymphocytes using an improved Sleeping Beauty transposon platform. *Oncotarget* 2016; 7: 51581–97.

85. Kacherovsky N, Liu GW, Jensen MC, Pun SH. Multiplexed gene transfer to a human T-cell line by combining Sleeping Beauty transposon system with methotrexate selection. *Biotechnol. Bioeng.* 2015; 112: 1429–36.

86. Kacherovsky N, Harkey MA, Blau CA *et al.* Combination of Sleeping Beauty transposition and chemically induced dimerization selection for robust production of engineered cells. *Nucleic Acids Res.* 2012; 40: e85.

87. Mezzadra R, Hollenstein A, Gomez-Eerland R, and Schumacher TN. A Traceless Selection: Counter-selection System That Allows Efficient Generation of Transposon and CRISPR-modified T-cell Products. *Mol. Ther. Nucleic Acids* 2016; 5: e298.

88. Moldt B, Yant SR, Andersen PR, Kay MA *et al.* Cis-acting gene regulatory activities in the terminal regions of sleeping beauty DNA transposon-based vectors. *Hum. Gene Ther.* 2007; 18: 1193–204.

89. Heinz N, Schambach A, Galla M *et al.* Retroviral and transposon-based tet-regulated all-in-one vectors with reduced background expression and improved dynamic range. *Hum. Gene Ther.* 2011; 22: 166–76.

90. Najjar AM, Manuri PR, Olivares S *et al.* Imaging of Sleeping Beauty-Modified CD19-Specific T Cells Expressing HSV1-Thymidine Kinase by Positron Emission Tomography. *Mol. Imaging Biol.* 2016; 18: 838–848.

91. Kolacsek O, Pergel E, Varga N *et al.* Ct shift: A novel and accurate real-time PCR quantification model for direct comparison of different nucleic acid sequences and its application for transposon quantifications. *Gene* 2017; 598: 43–9.

92. PATENTSCOPE: https://patentscope.wipo.int/search/en/search.jsf

93. TargetAMD: http://www.targetamd.eu

94. Intrexon press release: http://investors.dna.com/2017-01-10-ZIOPHARM-and-Intrexon-Announce-Cooperative-Research-and-Development-Agreement-with-the-National-Cancer-Institute-Utilizing-Sleeping-Beauty-System-to-Generate-T-cells-Targeting-Neoantigens

95. Ascenion GmbH press release: https://www.mdc-berlin.de/44841773/en/news/archive/2015/20150803-formula_pharmaceuticals_and_the_max_delbr_

96. Ren J, Stroncek DF. Gene therapy simplified. *Blood* 2016; 128: 2194–2195.

97. Merck KGaA forges a $941M CAR-T development deal with Intrexon: http://www.fiercebiotech.com/biotech/updated-merck-kgaa-forges-a-941m-car-t-development-deal-in-trexon

98. Yant SR, Ehrhardt A, Mikkelsen JG, Meuse L, Pham T, Kay MATransposition from a gutless adeno-transposon vector stabilizes transgene expression *in vivo*. *Nat. Biotechnol.* 2002; 20, 999-1005.

99. Zhang W, Solanki M, Muther N *et al.* Hybrid adeno-associated viral vectors utilizing transposase-mediated somatic integration for stable transgene expression in human cells. *PLoS One* 2013; 8, e76771.

100. Bowers WJ, Mastrangelo MA, Howard DF, Southerland HA, Maguire-Zeiss KA, Federoff HJ. Neuronal precursor-restricted transduction via in utero CNS gene delivery of a novel bipartite HSV amplicon/transposase hybrid vector. *Mol. Ther.* 2006; 13, 580–8.

101. Peterson EB, Mastrangelo MA, Federoff HJ, Bowers WJ. Neuronal specificity of HSV/sleeping beauty amplicon transduction in utero is driven primarily by tropism and cell type composition. *Mol. Ther.* 2007; 15, 1848–55.

102. De Silva S, Mastrangelo MA, Lotta LT Jr, Burris CA, Federoff HJ, Bowers WJ. Extending the transposable payload limit of Sleeping Beauty (SB) using the Herpes Simplex Virus (HSV)/SB amplicon-vector platform. *Gene Ther.* 2010; 17, 424–31.

103. De Silva S, Mastrangelo MA, Lotta LT Jr *et al.* Herpes simplex virus/Sleeping Beauty vector-based embryonic gene transfer using the HSB5 mutant: loss of apparent transposition hyperactivity *in vivo*. *Hum. Gene Ther.* 2010; 21: 1603–13.

104. Luo WY, Shih YS, Hung CL *et al.* Development of the hybrid Sleeping Beauty-baculovirus vector for sustained gene expression and cancer therapy. *Gene Ther.* 2012; 19: 844–51.

105. Staunstrup NH, Moldt B, Mates L *et al.* Hybrid lentivirus-transposon vectors with a random integration profile in human cells. *Mol. Ther.* 2009; 17: 1205–14.

106. Intrexon, ZIOPHARM and MD Anderson in Exclusive CAR T Pact: http://investors.dna.com/2015-01-13-Intrexon-ZIOPHARM-and-MD-Anderson-in-Exclusive-CAR-T-Pact

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

108. Yant SR, Park J, Huang Y, Mikkelsen JG, Kay MA. Mutational Analysis of the N-Terminal DNA-Binding Domain of Sleeping Beauty Transposase: Critical Residues for DNA Binding and Hyperactivity in Mammalian Cells. *Mol. Cell Biol.* 2004; 24: 9239-47.

109. Geurts AM, Yang Y, Clark KJ *et al.* Gene transfer into genomes of human cells by the sleeping beauty transposon system. *Mol Ther*. 2003; 8:108-17.

110. Hausl MA, Zhang W, Müther N *et al.* Hyperactive Sleeping Beauty Transposase Enables Persistent Phenotypic Correction in Mice and a Canine Model for Hemophilia B. *Mol Ther.* 2010; 18:1896-1906.

111. Baus J, Liu L, Heggestad AD, Sanz S, Fletcher BS. Hyperactive transposase mutants of the Sleeping Beauty transposon. *Mol Ther.* 2005; 12:1148-56.

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