

Special Issue: Transposable Elements

## Review

Gene Therapy with the  
*Sleeping Beauty* Transposon  
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The widespread clinical implementation of gene therapy requires the ability to stably integrate genetic information through gene transfer vectors in a safe, effective, and economical manner. The latest generation of *Sleeping Beauty* (SB) transposon vectors fulfills these requirements, and may overcome limitations associated with viral gene transfer vectors and transient nonviral gene delivery approaches that are prevalent in ongoing clinical trials. The SB system enables high-level stable gene transfer and sustained transgene expression in multiple primary human somatic cell types, thereby representing a highly attractive gene transfer strategy for clinical use. Here, we review the most important aspects of using SB for gene therapy, including vectorization as well as genomic integration features. We also illustrate the path to successful clinical implementation by highlighting the application of chimeric antigen receptor (CAR)-modified T cells in cancer immunotherapy.

## Therapeutic Gene Delivery

The ability to deliver natural or synthetic genes into human somatic cell types provides the technological basis for gene therapy to treat inherited and acquired genetic diseases. Most (~70%) gene delivery systems used in ongoing clinical trials of gene therapy are based on viral vectors (Gene Therapy Clinical Trials Worldwide). Viral vector systems are attractive for gene delivery, because viruses have evolved the ability to deliver nucleic acids to target cells by infection. However, some viral vectors, including those derived from adenoviruses or adeno-associated viruses (AAV), are not equipped for chromosomal integration and, thus, remain largely **episomal** (see [Glossary](#)), leading to a gradual loss of the vector, especially in cycling cells. By contrast, retroviral vectors integrate their therapeutic cargo into the genome and, thus, have the potential to confer long-term transgene expression. Indeed, **hematopoietic stem cell** (HSC)-based gene therapy with integrating viral vectors has provided clear therapeutic benefit in primary immunodeficiencies (including SCID-X1 and ADA-SCID), thalassemia, and leukodystrophies [1–4]. A concern with gammaretroviral vectors developed from the mouse leukemia virus (MLV) is that mutagenic effects elicited by insertion of the vector into or near proto-oncogenes may result in **genotoxicity** [5–9]. In fact, **serious adverse events** associated with vector integration have been observed in clinical trials for SCID-X1 [5,7,10,11], X-CGD [12], and WAS [13]. To improve safety, self-inactivating (SIN) gammaretroviral vectors lacking a strong enhancer have been developed; these vectors have been shown to have reduced mutagenicity *in vitro* [14], and have been applied in multicenter clinical trials for SCID-X1, with promising efficacy and safety parameters [15]. Furthermore, although lentiviral vectors based on HIV appear to be safer than gammaretroviral vectors in gene therapy, recent studies indicate

## Trends

The mobility of transposons can be exploited for gene transfer in both experimental and therapeutic settings.

The SB transposon is a reconstructed element from fish genomes and likely the best-characterized transposon with activity in vertebrate cells.

The most active variants of SB support gene integration in human cells at a level approaching the efficiency of integrating viral vector systems.

SB has a close-to-random integration profile in the human genome, which contributes to the enhanced safety of SB in the context of therapeutic applications.

The nonviral SB transposon vector components can be combined with non-integrating viral particles to take advantage of their ability to deliver nucleic acids into cells.

Clinical trials with using SB as a vector are ongoing in the area of CAR-T cell engineering to treat patients with CD19<sup>+</sup> hematologic cancers.

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that some HIV integrations into genes associated with cancer or cell cycle regulation may confer a survival advantage on HIV-infected cells and thus a **clonal imbalance** of HIV integrations in patients with AIDS [16,17]. Finally, the high costs and significant delay (>6 months) associated with the manufacture of clinical-grade retroviral vector batches represent a significant hurdle in routine medical practice.

In contrast to viral vectors, nonviral vector systems, including nucleic acid vectors such as plasmid DNA, have been considered for their simplicity, safety, and ease of production. However, nonviral technologies generally suffer from inefficient cellular delivery, pronounced cellular toxicity, and limited duration of transgene expression due to the lack of genomic insertion and resulting degradation and/or dilution of the vector in transfected cell populations. Thus, just like non-integrating viral systems, nonviral vectors do not provide long-term nuclear maintenance and transgene expression in most therapeutically relevant somatic cell types. As a result, significant efforts have been made to craft novel gene transfer vectors that exceed the qualities of currently available vectors in: (i) efficacy (i.e., high-level stable gene transfer at low toxicity to the host cell); (ii) safety (i.e., low levels of genotoxicity and immunogenicity); and (iii) economic viability (i.e., acceptable costs on a per patient basis)

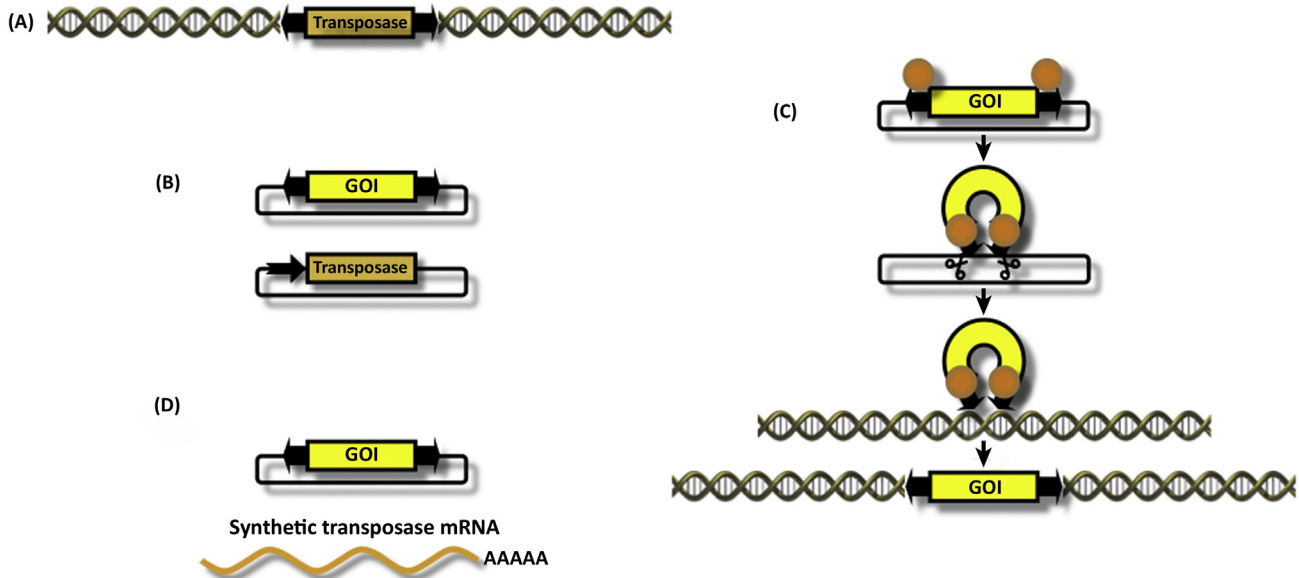
### Nonviral Gene Transfer Using the *Sleeping Beauty* Transposon

DNA transposons are genetic elements with the ability to change their positions within the genome [18]. The prevalent mode of transposition in the genome is via a cut-and-paste mechanism. In nature, these elements exist as mobile ('jumping') units of DNA containing a transposase gene flanked by terminal inverted repeats (TIRs) that carry transposase binding sites (Figure 1A). Importantly, it is possible to separate the two functional components of the transposon (the TIRs and the transposase) in the form of bi-component vector systems (reviewed in [19–21]). Transposon-based vectors enable incorporation of almost any DNA sequence of interest between the transposon TIRs and mobilization by *trans*-supplementing the transposase in the form of an expression plasmid (Figure 1B), similar to how nonautonomous viral vectors are produced in packaging cell lines. During the transposition process, the transposase enzyme mediates the excision of the element from its donor plasmid, followed by reintegration of the transposon into a chromosomal locus (Figure 1C). This feature makes transposons natural and easily controllable DNA delivery vehicles that can be used as tools for versatile applications in genetic engineering, including gene therapy (reviewed in [22]).

Based on ancient, inactive transposon sequences isolated from fish genomes, an active transposon was reconstructed, and named *Sleeping Beauty* (SB) after the famous fairy tale [23]. SB was the first transposon to be shown capable of efficient transposition in vertebrate cells, thereby enabling new avenues for genetic engineering, including gene therapy (reviewed in [21,22,24–30]). The advantage of SB transposon-based gene delivery is that it combines the favorable features of viral vectors with those of naked DNA molecules (Figure 2). Namely, owing to permanent genomic insertion of transgene constructs, transposition-mediated gene delivery can lead to sustained and efficient transgene expression in preclinical animal models [26]. However, in contrast to viral vectors, transposon vectors can be maintained and propagated as plasmid DNA, which makes them simple and inexpensive to manufacture, an important consideration for implementation and scale-up in clinical practice. Further advantages of SB as a gene-transfer system compared with viral vectors include its lower immunogenicity *in vivo* [31] [due to stable chromosomal integration, only a single administration of vector is required, in contrast to non-integrating viral vectors, whose repeated administration can provoke immune responses against vector-encoded proteins (reviewed in [32])], a greater capacity for genetic cargo [33] [SB has been shown to transpose bacterial artificial chromosomes (BACs) over 100 kb in length at reasonable efficiencies in human embryonic stem cells [34], whereas recombinant vectors based on AAV are not competent to package vector

### Glossary

- Chimeric antigen receptor (CAR):** an engineered fusion protein that contains an antigen-recognition domain derived from a single-chain variable fragment of a monoclonal antibody and a signaling domain typically derived from CD3- $\zeta$ .
- Clonal imbalance:** a reduction in polyclonality of cell populations as a result of a relative growth advantage of certain cell clones.
- Cytokine release syndrome:** a complication arising from cytokine release by activated T cells that generates a systemic inflammatory response.
- Engraftment:** a process through which transplanted cells establish themselves in the body of the recipient; this means that, in the context of stem cell transplantation, the stem cells begin to reproduce and give rise to differentiated cell types.
- Episomal:** extrachromosomal DNA.
- Genotoxicity:** a damaging effect on the genome.
- Hematopoietic stem cell:** stem cells located in the bone marrow that give rise to all cell types in the blood through hematopoietic differentiation.
- Leukemia:** blood cancer typically affecting lymphoid or myeloid white blood cells (leukocytes).
- Lymphoma:** a type of cancer that originates in the lymphatic system, including the bone marrow, spleen, thymus, lymph nodes, and lymphatic vessels.
- Neoantigen:** a patient-specific antigen that arises as a consequence of tumor-specific mutations.
- Nucleofection:** an electroporation-based transfection method for the delivery of nucleic acids into cells and their nuclei.
- Serious adverse event:** a life-threatening medical condition associated with the use of a drug in clinical trials.
- Stem cell memory T cell:** a subset of lymphocytes that has previously encountered an antigen, long-lived, self-renewing, and can expand and differentiate into effector cells upon re-exposure to the same antigen.



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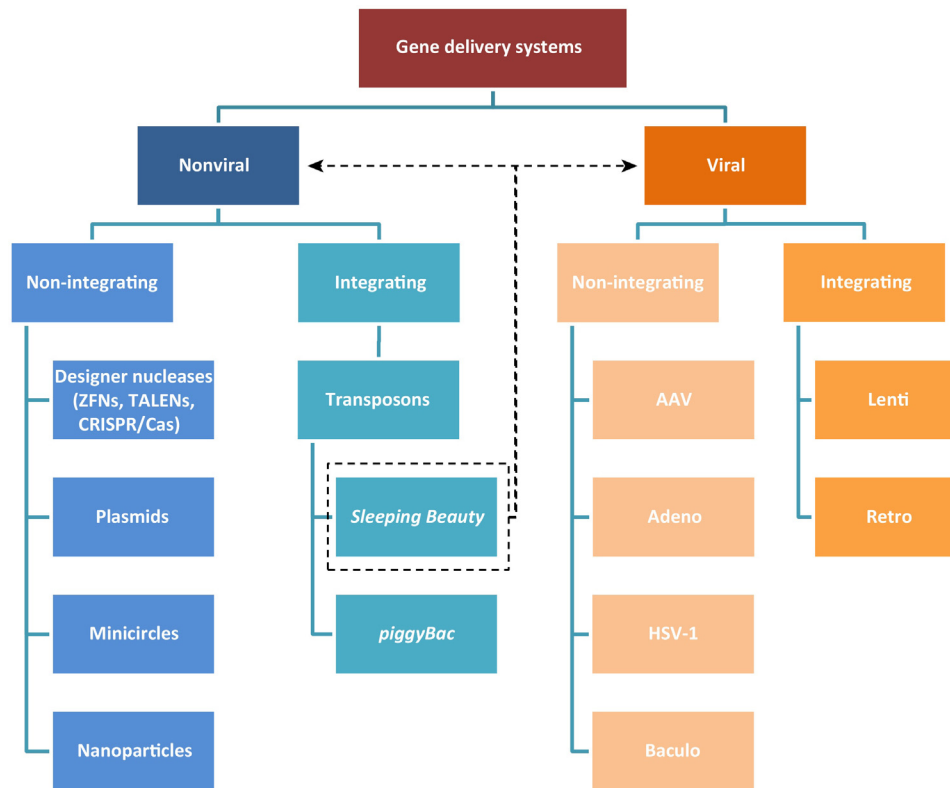
**Figure 1. The *Sleeping Beauty* (SB) Transposon System.** (A) Autonomous transposable elements comprise terminal inverted repeats (TIRs, black arrows) that flank the transposase gene (orange). (B) A bi-component, *trans*-arrangement transposon vector system for delivering transgenes that are maintained in plasmids. One component contains a gene of interest (GOI, yellow) between the transposon TIRs carried by a plasmid vector, whereas the other component is a transposase expression plasmid, in which the black arrow represents the promoter driving expression of the transposase. (C) The transposon carrying a GOI is excised from the donor plasmid and is integrated at a chromosomal site by the transposase. (D) Plasmid-based transposon cassettes can be mobilized by transposase supplied as *in vitro*-transcribed mRNA.

genomes >5 kb [35,36], and both retroviral and lentiviral vectors undergo a severe loss of titer beyond a vector size of ~8–10 kb [37,38], and its superior biosafety profile [29,39–42] (discussed below in ‘Safety aspects of *Sleeping Beauty* transposition’).

#### Optimized Vector Components for Enhanced *Sleeping Beauty*-Mediated Gene Delivery

Compared with the original version of the transposon (pT), attempts to improve the architecture of the transposon vector yielded several optimized variants (pT/2/3/4) [43–46]. The latest generation vector, pT4, was engineered to carry sequences optimized for transposase binding [46]. The efficiency of SB transposition is correlated inversely with the size of the transposon over 7.5 kb [47]. The ‘sandwich’ (SA) configuration of the SB transposon, comprising two complete transposon units flanking the cargo in an inverted orientation, enables superior transposition of larger transgenes (~three- and ~eightfold more efficient for a ~7-kb transposon with the first-generation SB transposase [33] and with the SB100X hyperactive transposase (Schorn and Ivics, unpublished data, 2010), respectively).

In an attempt to derive hyperactive transposase variants for advanced genetic engineering, amino acid substitutions spanning almost the entire SB transposase polypeptide have been screened for eliciting a change in catalytic activity. A second-generation SB transposase, called SB11 [48], is approximately threefold more active than the first-generation SB transposase, and has been primarily used in currently running clinical trials based on **CAR**-engineered T cells [49]. The most hyperactive SB transposase version currently available, SB100X, displays a ~100-fold hyperactivity compared with the originally resurrected transposase [50]. The SB100X transposase enables highly efficient germline transgenesis in relevant mammalian models, including mice, rats, rabbits, pigs, sheep, and cattle [51–56]. Moreover, the use of the SB100X system yielded robust gene transfer efficiencies into human HSCs [50,57], mesenchymal stem cells, muscle stem/progenitor cells (myoblasts), induced pluripotent stem cells (iPSCs) [58], and



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**Figure 2. Gene Delivery Vector Systems for Gene Therapy.** Vector systems are broadly classified as viral and nonviral vectors. Virus-derived vectors have the ability to infect and deliver DNA or RNA to a variety of cell types through transduction. Viral vectors can be further classified into integrating and non-integrating vectors on the basis of their ability to insert their genetic cargo into the genome of the target cell. Non-integrating viral vectors include vectors derived from adeno-associated virus (AAV), adenovirus (Adeno), herpes simplex virus 1 (HSV-1), and baculovirus (Baculo), whereas the most popular and widespread integrating viral vectors are derived from the HIV-1 lentivirus (Lenti) and the MLV gammaretrovirus (Retro). Non-integrating, nonviral gene delivery systems, such as designer nucleases, plasmids, and plasmid-like vectors, can be delivered either by transfection or nucleofection or can be complexed with liposomes and nanoparticles. However, natural gene delivery vehicles such as *Sleeping Beauty* (SB) and *piggyBac* transposons can overcome the problem of transient expression that is typically associated with non-integrating vector systems. Transposons have the inherent ability to stably integrate a gene of interest into the genome, thereby providing a long-term or possibly permanent therapeutic effect. The SB transposon system has been combined with both nonviral and viral techniques for cellular delivery (broken lines). Abbreviations: CRISPR/Cas, clustered regularly interspaced short palindromic repeat/CRISPR-associated; TALEN, transcription activator-like effector nuclease; ZFN, zinc finger nuclease.

T cells [59], which are relevant targets for regenerative medicine and gene- and cell-based therapies of complex genetic diseases.

The typical set-up for delivery of the SB transposon system into cells is supplying the two components of the vector system as conventional plasmids (Figure 1B). However, the transposase expression plasmid that is typically used to provide a transposase source in cultured cell lines can be replaced by mRNA synthesized in *in vitro* transcription reactions (Figure 1D). Co-delivery of SB transposase-encoding mRNA with an SB transposon plasmid to somatic cells was originally tested in a mammalian cell line *in vitro* and in the mouse liver *in vivo*, using the SB11 transposase [60,61]. By applying mRNA for intracellular delivery in therapeutically relevant cells *ex vivo*, some hurdles of gene transfer typical for DNA-based vectors can be avoided. For example, **nucleofection** of primary human cells, including HSCs and T cells, with mRNA was shown to cause significantly reduced cellular toxicity compared with nucleofection

with plasmid DNA [62,63]. Importantly, the implementation of an mRNA source for transient delivery of the SB transposase increases the biosafety of this approach, because mRNA does not bear the risk of chromosomal integration. Genomic integration of the SB transposase coding sequence into the genome would represent a finite risk in a gene therapy application, because such an event could lead to prolonged and uncontrollable transposase expression, resulting in continuous remobilization of the already integrated SB transposon.

A recent addition to the development of SB vectors with enhanced utility for clinical applications is the use of minicircle (MC) vectors as carriers of the SB transposon components. MC technology allows for a significant reduction of SB vector size by removing most of the backbone sequences from parental plasmids [64]. The first evident advantage of using MC vectors over plasmids is related to increased cell survival rates (up to ~threefold) following nucleofection of human T cells [63]. Along with decreased levels of cytotoxicity, nucleofection of T cells with SB transposon components supplied as MCs resulted in more efficient (up to ~fivefold) stable genome modification compared with conventional plasmid vectors [63]. The MC components are likely more efficient in transfection than plasmids because, due to their smaller size, they cross cellular membranes more efficiently than can plasmids [65,66]. In addition, the elevated levels of transposition observed with MC vectors are likely supported by the relatively short ~200-bp distance between the SB transposon ends in the MC-based transposon vector, owing to the depletion of the bacterial plasmid backbone. Indeed, SB transposition was shown to be more efficient when the length of DNA sequence outside the transposon unit was shortened, likely by aiding transposon and/or transposase complex formation [47]. In addition to efficacy, MC technology also offers biosafety advantages over conventional plasmid DNA vectors. Namely, the absence of bacterial plasmid backbone elements in therapeutic vectors is relevant in clinical applications, because antibiotic resistance genes included in a therapeutic cell product may raise safety concerns. A variant of the MC technology is represented by 'free of antibiotic resistance markers' (pFAR) miniplasmids [67] that, similar to MCs, lack antibiotic-resistance genes, thereby significantly enhancing the safety profile of nonviral gene delivery in clinical settings. Importantly, the pFAR and SB technologies were successfully combined [68], and are planned for use in a Phase I clinical trial to treat age-related macular degeneration [69].

Finally, in addition to the purely nonviral strategies, various SB-based viral hybrid technologies have been developed that can advantageously merge the excellent nucleic acid delivery properties of a non-integrating viral vector and the integrative properties of SB (reviewed in [24,30]). The SB system, including both the transposase and the transposon, can be packaged into various recombinant viruses for delivery (by transduction) into cells. In principle, these hybrid vectors could be used as alternatives to established viral vectors, and are suitable for cell type-specific gene engineering. Viral transposon hybrids have been established for integrase deficient lentivirus (IDLV) [70–72], adenovirus [73,74], AAV [75], herpes simplex virus [76,77], and baculovirus [78,79], where the SB transposon provides stable gene integration. Especially for *in vivo* approaches, hybrid vectors could be advantageous, because they: (i) bypass the need for repeated vector administration due to stable chromosomal transgene integration and expression; and (ii) may allow reduction of the applied viral dose, thereby alleviating vector-associated immune complications.

#### Safety Aspects of Sleeping Beauty Transposition

One of the most important risk factors associated with an integrating genetic element is genotoxicity, that is, mutational damage that can contribute to a pathologic shift to cellular homeostasis. The best-known examples of this are represented by clonal imbalance and tumorigenic transformation in some patients enrolled in clinical trials based on retroviral gene transfer into HSCs, as described above. Vector architecture, the enhancer and/or promoter

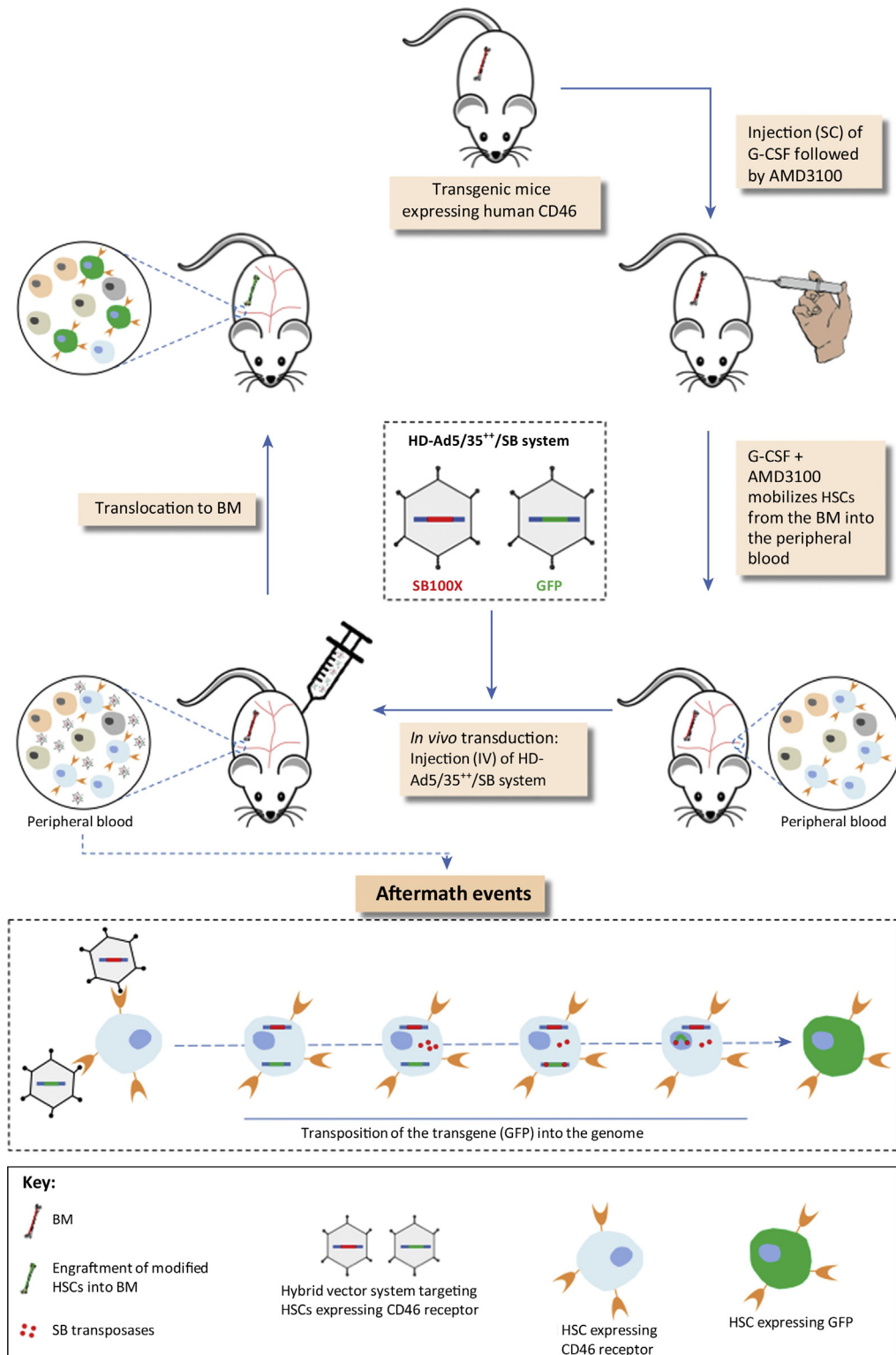
elements used to drive transgene transcription, copy numbers, the underlying disease, and insertion site distribution of the vectors can strongly influence the actual risk of insertional oncogenesis. In the context of the biochemistry of cut-and-paste transposition, at least two fundamental properties can contribute to genotoxicity of a transposon-based vector: (i) interaction of the transposase with endogenous human DNA sequences or human proteins with the transposon vector sequences; and ii) the genome-wide insertion profile of the vector. With respect to 'off-target' cleavage of the transposase, the use of the SB system appears to be safe in human cells. First, SB has been reconstructed from fish genomes [23], and the mammalian lineage does not contain transposons sufficiently similar to allow cleavage by the SB transposase. Sequences sufficiently similar to the SB TIRs could occur in the human genome by chance [39], and some of these sequences could bind the SB transposase. However, SB transposition is such a highly controlled process [46] that mobilization of these sequences is unlikely. Second, human cells do not express a protein with sufficient similarity to the SB transposase that could remobilize a genomically integrated SB vector. By contrast, the human PGBD5 transposase-derived protein can mobilize insect *piggyBac* (PB) transposon vectors in human cells [80]. Thus, despite the vast evolutionary distance between PGBD5 and insect PB transposons, there appears to be a cross-reaction between a catalytically active endogenous human transposase and transposon vector sequences that are exogenously delivered into human cells by gene transfer. The findings have potential implications for the genomic stability of PB vector insertions in human applications [81].

Characterization of the target site selection properties of different vector systems is useful for ranking the different vector types and designs according to their genotoxic potential [82]. A comparative study was conducted to address the target site selection properties of the SB and PB transposons as well as MLV-derived gammaretroviral and HIV-derived lentiviral systems in primary human CD4<sup>+</sup> T cells. The bioinformatic analyses included mapping against the T cell genome with respect to proximity to genes, transcriptional start sites (TSSs), CpG islands, DNaseI hypersensitive sites, chromatin marks, and transcriptional status of genes, and satisfying the criteria for genomic safe harbors (GSHs) [83,84]. The SB transposon displayed the least deviation from random with respect to its genome-wide distribution: no apparent bias was seen for either heterochromatin marks or euchromatin marks and only a weak correlation was detected with transcriptional status of targeted genes [85]. These analyses collectively established a favorable integration profile of the SB transposon, suggesting that SB is safer for therapeutic gene delivery than the integrating viral vectors that are currently used in clinical trials. Importantly, no SB-associated adverse effects have been observed in preclinical animal studies [26,28,86,87]. Finally, the future development of molecular strategies aiming at target-selected transgene integration with transposon-based vectors [88] may further enhance the safety profile of SB (see Outstanding Questions).

## Therapeutic Gene Delivery with the *Sleeping Beauty* Transposon System

### *In Vivo* Application of the *Sleeping Beauty* System in Preclinical Models

In an *in vivo* application, a gene vector system shuttling a therapeutic nucleic acid is delivered directly into the body, either systemically or, more typically, targeted to a given organ or cell type. Efficient *in vivo* delivery of transposon vectors is a challenge, because, unlike viruses, naked nucleic acids (DNA and mRNA) lack the capacity to pass through the cell membrane through infection. Thus, it is necessary to combine transposon vectors with technologies capable of efficient delivery of these nonviral vectors into cells (Figure 2). Currently, one of the most promising strategies is an *in vivo* gene transduction system based on a hybrid adenovirus/transposon vector [73] and the hyperactive SB100X transposase [74] (Figure 3). In a recent study, autologous HSCs were mobilized into peripheral blood, and HSCs were directly targeted using a hybrid adenovirus/transposon vector system *in vivo*, resulting in functional HSCs in a humanized animal model [89,90]. The procedure involved systemic, intravenous injection of an



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Figure 3. Illustration of *In Vivo* Transduction of Mouse Hematopoietic Stem Cells (HSCs) Using a Hybrid Adenovirus/Transposon Vector System. The ability to genetically modify HSCs without the need for myeloablative conditioning is relevant for the broader clinical application of gene therapy. (Top) Transgenic

(Figure legend continued on the bottom of the next page.)

integrating, helper-dependent hybrid adenovirus (HD-Ad5/35<sup>+</sup>)/SB vector system into the blood stream of transgenic mice expressing human CD46, a receptor that is uniformly expressed on HSCs, and allows for stable genetic engineering of HSCs *in vivo*. The potential advantage of the procedure is that it works without the need for the *ex vivo* expansion and transduction of HSCs. The potential disadvantage is that the efficiency of gene manipulation was not as high as those reported in clinical trials using lentiviral vectors and *ex vivo* cell processing; thus, this strategy needs further characterization and improvement. Nevertheless, this system has the potential to overcome existing technical and/or medical difficulties associated with cell collection and *ex vivo* manufacturing, and to provide real technical advances.

In addition, nanotechnology appears to open novel opportunities in efficient nonviral delivery, and can be combined with cell type-specific targeting [91,92]. Cell type-specific vector targeting using hyaluronan- and asialoorosomucoid-coated nanocapsules harboring the SB system were successfully used to direct gene vectors specifically to liver sinusoidal endothelial cells and hepatocytes, respectively [91]. These studies imply that nanoparticle-like carriers can be developed as efficient targeted gene delivery vehicles, highlighting their therapeutic potential.

#### *Ex Vivo* Application of the Sleeping Beauty System in Preclinical Models

In *ex vivo* gene delivery, the therapeutic gene vector is introduced into a selected cell population that was isolated from a donor, and the genetically engineered cells are transplanted into a patient (Figure 4, Key Figure). Depending on whether the donor is the patient themselves or another person, we differentiate between autologous or allogeneic cell products, respectively. Similar to *in vivo* applications, the efficiency of transposition is dependent on the efficiency of uptake of the introduced nucleic acids by the cells. In principle, any technology developed for transferring nucleic acids into cells can be combined with transposon vectors. In hard-to-transfect cells, including primary human cell types, delivery of transposon-based vectors can be significantly facilitated by nucleofection. Indeed, nucleofection facilitated transposition in CD34<sup>+</sup> HSCs [50,57,93–95], primary T cells [85,96–98], and human embryonic stem cells [61,99]. Importantly, in the context of the hematopoietic system, this *ex vivo* gene delivery procedure did not appear to compromise the **engraftment** and multilineage differentiation potential of CD34<sup>+</sup> cells [50,57].

SB transposition-based nonviral gene delivery has the outstanding potential to provide innovative and potentially curative treatments for an array of genetic disorders (reviewed in [24–28,30,69,86,100–102]). Prime examples for the use of SB in gene therapy include the treatment of hematologic disorders, lysosomal storage diseases, pulmonary disorders, dermatologic diseases, a variety of metabolic disorders, neurologic disorders, muscle disorders, and cancer (Table 1). This robust, nonviral, transposon-based procedure is currently being tested in human clinical trials [49], as discussed below.

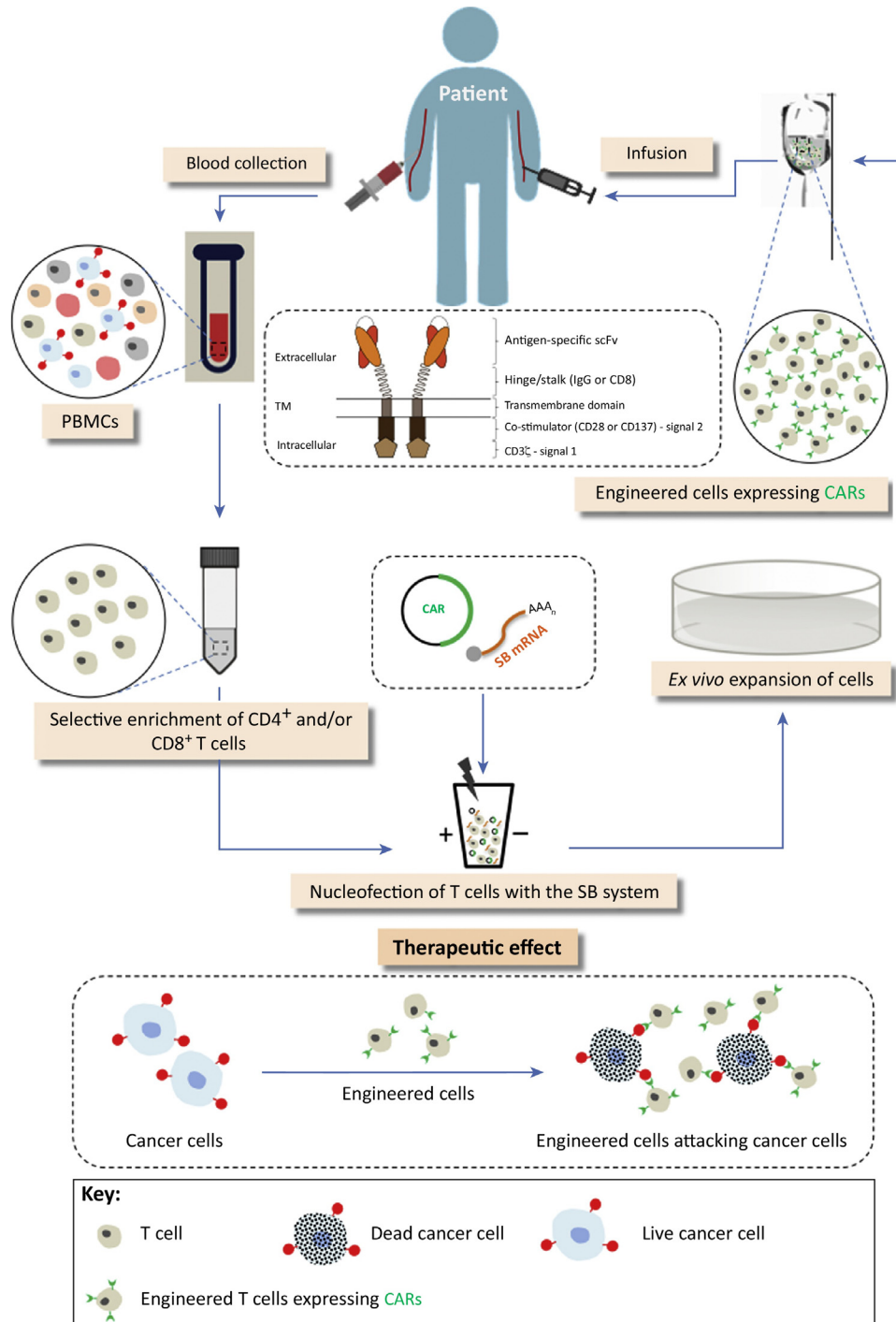
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mice expressing the human CD46 receptor are subcutaneously (SC) injected with granulocyte colony-stimulating factor (G-CSF) followed by AMD3100 to mobilize HSCs from the bone marrow (BM, in red) into the peripheral blood (PB) stream (red lines). For the *in vivo* transduction of the HSCs in the PB, an integrating hybrid vector system [HD-Ad5/35<sup>+</sup>/Sleeping Beauty (SB) vector system] is generated, wherein the SB transposon system components (SB100X transposase shown in red and an SB transposon carrying GFP as a transgene shown in green) are packaged into helper-dependent adenoviral (HD-Ad5/35<sup>+</sup>) vectors. Intravenous (IV) injection of the integrating hybrid vector system (middle) into the mice results in selective transduction of the HSCs expressing the human CD46 receptor, leading to transposition of the transgene (GFP) into the genome (bottom). The genetically modified HSCs (in green) expressing GFP are capable of engrafting back into the BM (in green), where they can undergo further expansion.



**Key Figure**

*Ex vivo* Application of the *Sleeping Beauty* (SB) System for Engineering T Cells to Express Chimeric Antigen Receptors (CAR) for Use against Leukemias and Lymphomas



### Genetic Modification of T Cells with *Sleeping Beauty* for CD19<sup>+</sup> Hematologic Cancers

The clinical application of the nonviral SB gene delivery system has been most extensively tested in patients with advanced B-lineage, CD19<sup>+</sup> acute **leukemia** and **lymphoma**. The SB system is used to genetically modify T cells with second-generation CAR targeting CD19, thereby redirecting T cells toward CD19-expressing tumors. Most previous and ongoing clinical trials have used viral transduction to modify T cells to express second-generation CARs comprising single-chain variable fragments (scFv) of monoclonal antibodies fused with either CD28 or 4-1BB (CD137) co-stimulatory domains in frame with chimeric CD3- $\zeta$  to provide 'signal 1 and 2' (i.e., signals initiated by CD3- $\zeta$  and CD28) (Figure 4). Administration of patient-derived, second-generation, CD19<sup>+</sup> CAR-T cells has shown activity in patients with chemotherapy-refractory B cell acute lymphoblastic leukemia (ALL) with response rates up to 90% [103–106], and overall response rates of up to 70% in patients with refractory B cell lymphomas [107–110]. Notably, toxicity, in the form of **cytokine release syndrome** (CRS) and/or neurotoxicity, has also been noted in patients, especially in those with significant response to therapy.

By contrast, a group at MD Anderson Cancer (MDACC) used the SB nonviral system to introduce a CAR into patient- and donor-derived T cells. Table 2 details the clinical CAR-T trials at MDACC utilizing the SB transposon/transposase system. Eighty patients have already been recruited to these clinical trials (Table 2), indicating the feasibility and robustness of the production of transposon-engineered CAR-T products. The first human experience was published describing the outcomes of 26 patients with relapsed (high risk) B cell ALL or lymphoma who received either patient-derived (autologous, ClinicalTrials.gov 00968760) or donor-derived (allogeneic, ClinicalTrials.gov 01497184) CAR-T cells administered in conjunction with autologous ( $n = 7$ ) or allogeneic ( $n = 19$ ) HSC transplantation (HSCT), respectively, to decrease the rate of disease relapse [49]. SB-mediated genetic transposition and stimulation resulted in 2200- to 2500-fold *ex vivo* expansion of genetically modified T cells, with 84% CAR expression, but without integration hotspots. Following autologous HSCT, the 30-month progression-free and overall survivals were 83% and 100%, respectively. After allogeneic HSCT, the respective 12-month rates were 53% and 63%. Importantly, the SB-modified T cells were administered when the transposase was no longer detectable by PCR. Thus, it is unlikely that continued expression of this fish-derived protein would be immunogenic in this setting. No acute or late toxicities, specifically no CRS or neurologic toxicity, were noted, and no exacerbation of graft-versus-host disease was observed. These survival data are approximately double that of historical controls based on autologous and allogeneic HSCT without the adoptive transfer of CAR-T cells. Despite a low antigen burden and unsupportive recipient cytokine environment, CAR-T cells persisted for an average of 201 days for autologous recipients and 51 days for allogeneic recipients [49]. The shorter

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**Figure 4.** The illustration shows CAR-T cell-based adoptive immunotherapy for hematologic malignancies. The CAR structure is depicted in the framed inset on the top. A homodimer of CARs on the cell surface is shown. CAR comprises an antigen-specific single chain variable fragment (scFv) derived from an antibody, which is held on the cell surface via a hinge/stalk region from IgG or CD8, and signals through CD3 $\zeta$  and (CD28 or CD137) co-stimulatory domains to mediate T cell activation. Depicted is autologous cancer immunotherapy that involves engineering the patient's own cells to recognize antigens presented by cancer cells and to destroy them. CD4<sup>+</sup> (or a mixture of CD4<sup>+</sup> and CD8<sup>+</sup> [63,69]) T cells are enriched from peripheral blood mononuclear cells (PBMCs) isolated from the patient's blood, and genetically modified using the SB system, typically by using nucleofection or another electroporation protocol. The framed inset in the middle depicts the use of *in vitro* transcribed mRNA as a source of the SB transposase and a transposon vector expressing a CAR. CAR-expressing engineered T cells are enriched and expanded *ex vivo* to generate sufficient numbers of cells for clinical application. CAR-engineered T cells are infused back into the respective patient, where tumor cells expressing the relevant tumor antigen are recognized and killed by the CAR-T cells. Infused T cells should be capable of a fully competent activation signal delivered through the CAR, enabling a panel of effector functions that includes: (i) sustained proliferation; (ii) serial killing; (iii) cytokine production; and (iv) resistance to activation-induced T cell death. One clinical sequela of competent CAR-based activity is the increase in the number of infused T cells, which, in the presence of a high tumor burden, results in synchronous activation leading to cytokine release syndrome.

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Table 1. Preclinical Studies with *Sleeping Beauty* Gene Transfer in Disease Models

Indication	Method of Delivery	Site of Delivery	Refs
Hematologic Disorders			
Hemophilia A and B	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	[31,120]
	Intravenous injection of DNA/polyethyleneimine (PEI) complexes	<i>In vivo</i> , mouse lungs	[138]
	Intravenous injection of nanocapsules	<i>In vivo</i> , mouse liver	[91]
	Intravenous injection of adenovirus/SB hybrid vector	<i>In vivo</i> , dog liver	[139]
Sickle cell disease	Transfection	<i>In vitro</i> , human cell lines	[140]
	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	[141]
	Transfection	<i>In vitro</i> , patient HSCs	[142]
Fanconi anemia	Transfection	<i>In vitro</i> , human cell lines	[143]
Congenital thrombotic thrombocytopenic purpura	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	[144]
Lysosomal Storage Diseases			
Mucopolysaccharidosis	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	[121,145,146]
Immunologic Diseases			
Severe combined immunodeficiency	Transfection	<i>In vitro</i> , human cell lines	[147]
Pulmonary Disorders			
Fibrosis	Intravenous injection of DNA/PEI complexes	<i>In vivo</i> , mouse lungs	[148]
Pulmonary hypertension	Intravenous injection of DNA/PEI complexes	<i>In vivo</i> , rat lungs	[149]
Dermatologic Disorders			
Junctional epidermolysis bullosa	Transfection	<i>Ex vivo</i> , patient epidermis	[150]
Dystrophic epidermolysis bullosa	Transfection	<i>Ex vivo</i> , human keratinocytes, followed by xenograft in mice	[151]
Metabolic Disorders			
Tyrosinemia type I	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	[61,152,153]
Type 1 diabetes mellitus	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	[154]
Hypercholesterolemia	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	[155]
Crigler–Najjar syndrome type I (hyperbilirubinemia)	Intravenous injection of proteoliposomes	<i>In vivo</i> , mouse liver	[92]
Neurologic Disorders			
Huntington disease	Transfection	<i>In vitro</i> , human cell lines	[156]
Alzheimer's disease	Transfection followed by encapsulated cell biodelivery	<i>In vitro</i> , human cell line, followed by graft in patient's brain	[157]
Muscular Dystrophy			
	Transfection	<i>In vitro</i> , mouse cell line, followed by transplantation into mice	[158]
	Transfection	<i>Ex vivo</i> , mouse myoblasts, followed by transplantation into mice	[159]
Cancer			
	Electroporation, TCR gene transfer	<i>Ex vivo</i> , human peripheral blood mononuclear cells, or T cells	[119,160]
	Electroporation, CAR gene transfer	<i>Ex vivo</i> , human peripheral blood mononuclear cells, or T cells	[59,96,97,161]
	Transfection	<i>In vitro</i> , hepatocellular carcinoma cell lines	[162]
	Intratumoral injection	<i>In vivo</i> , human glioblastoma xenografts in mice	[124]
	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	[163]

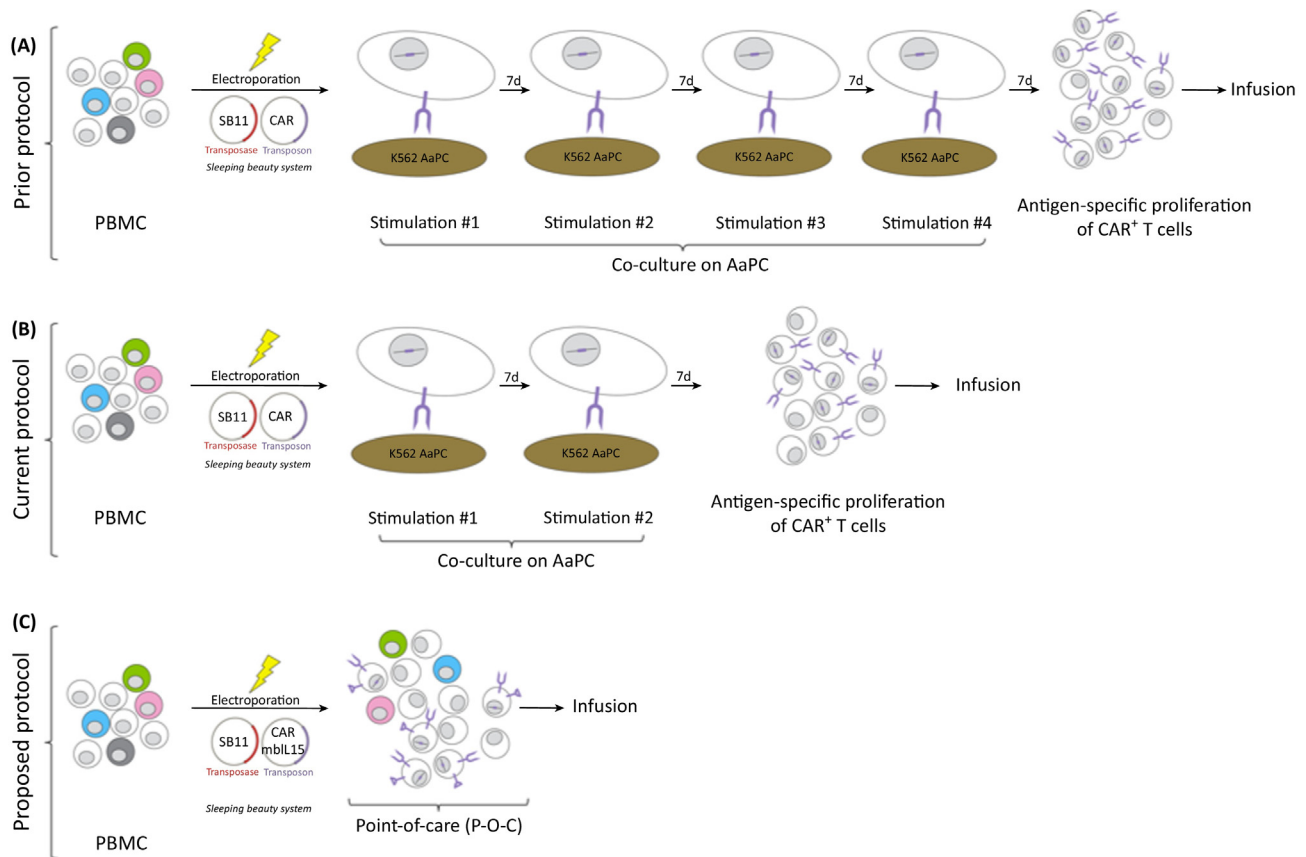
Table 2. Clinical Trials at MD Anderson Cancer Center Infusing *Sleeping Beauty*-Modified T Cells Expressing CD19-Specific CAR<sup>a</sup>

ClinicalTrials.gov	Protocol Schema	T Cell Dose	Enrolled
NCT00968760	CD19-specific T cells derived from patient; Arm 1: combined with auto-HCT as adjuvant therapy; Arm 2: infused for active disease after lymphodepletion	$5 \times 10^7/m^2$ – $5 \times 10^9/m^2$ (IL-2 last two cohorts)	23 (NHL, $n = 16$ ; ALL, $n = 6$ ; CLL, $n = 1$ )
NCT01497184	CD19-specific T cells derived from donor; Arm 1: Infused 6–12 wk after allo-HCT as adjuvant therapy; Arm 2: Infused for disease relapse after HCT following lymphodepletion	$10^6/m^2$ – $10^8/m^2$	36 (ALL, $n = 29$ ; NHL, $n = 6$ ; CLL, $n = 1$ )
NCT01362452	CD19-specific T cells derived from UCB donor, infused 6–12 wk after UCBT as adjuvant therapy	$10^6/m^2$ – $10^8/m^2$	4 (ALL, $n = 3$ ; NHL, $n = 1$ )
NCT01653717	CD19-specific T cells from patients with CLL after lymphodepleting chemotherapy	$10^7/m^2$ – $5 \times 10^{10}/m^2$	6
NCT02529813	CD19-specific T cells from patients after lymphodepleting chemotherapy	$10^5$ – $10^9/kg$	11 (ALL, $n = 5$ ; NHL, $n = 5$ ; CLL, $n = 1$ )

<sup>a</sup>Abbreviations: allo-HCT, allogeneic hematopoietic cell transplantation; auto-HCT, autologous hematopoietic cell transplantation; CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin's lymphoma; UCBT, umbilical cord blood therapy.

persistence of donor-derived T cells is likely due to the concomitant use of immunosuppressive medications as part of the standard of care to prevent graft-versus-host disease after HSCT. These data compare favorably to the persistence of CAR-T cells genetically engineered with viral vectors and, therefore, establish the proof-of-concept for the feasibility and safety of using SB-modified T cells. As a follow-up to this study, a clinical trial (ClinicalTrials.gov 02529813) is currently underway at MDACC infusing CAR-T cells in patients with refractory active ALL and lymphoma to evaluate refinements to the SB system.

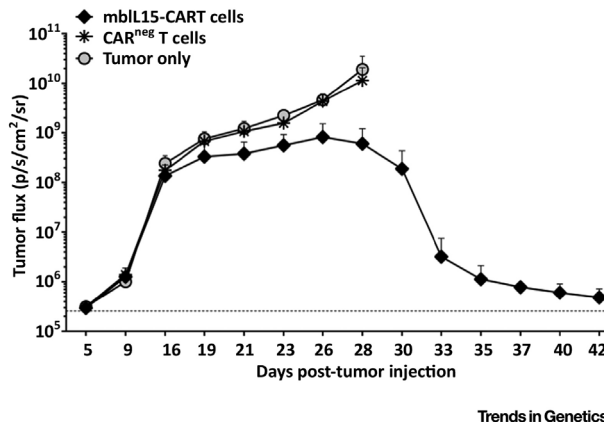
Nonviral gene transfer to T cells with the SB system offers advantages over other methods of gene transfer. For example, clinical-grade DNA plasmids from the SB system avoid the cost and complexity of manufacturing virus particles from retrovirus and lentivirus in compliance with good manufacturing practice (GMP) and provide a nimble approach to modifying the 'therapeutic cassette'. Additional improvements are underway based on reducing the time in tissue culture to produce SB-modified T cells for human applications (Figure 5). The first trials used SB-modified autologous and allogeneic T cells stably expressing a CD19-specific CAR based on approximately 4 weeks of co-culture on irradiated activating and propagating cells (AaPC) in the presence of IL-2 and IL-21 (Figure 5A). The AaPC were derived from the K-562 cell line and genetically modified to co-express the target antigen CD19 and co-stimulatory molecules [CD86, CD137L, and a membrane-bound (mb) version of IL-15 (mbIL15)] [111,112]. The manufacturing approach can be altered to shorten the time in culture to approximately 2 weeks for the following reasons: (i) GMP manufacturing is associated with high costs; (ii) progress of disease can render patients ineligible to receive an infusion and/or compromise their response; and (iii) 'younger' T cells have improved therapeutic potential [49]. It has also been demonstrated that improvements in the enzymatic activity of the SB transposase along with reduced size (MC vectors) of the DNA plasmid encoding the SB transposon can improve the efficiency of transposition [63]. The approximately fivefold improvement in rate of transposition with this technology is predicted to generate  $10^6$  CD19-specific CAR-T cells within 14 days of gene transfer (Figure 5B). This will be tested in an upcoming trial at the Universitätsklinikum Würzburg, Germany [69], based on a CAR design that has been validated in clinical trials using lentiviral transduction [106,113].



## Trends in Genetics

**Figure 5. Protocols for Genetic Modification of Human T Cells with the *Sleeping Beauty* (SB) System.** DNA plasmids encoding the SB transposon and transposase can be electroporated into T cells derived from autologous (or allogeneic) peripheral blood mononuclear cells (PBMC). The first-in-human application of SB was based on the selective propagation of transposed T cells stably expressing a CD19-specific chimeric antigen receptor (CAR) on  $\gamma$ -irradiated activating and propagating cells (AaPC) in the presence of soluble recombinant IL-2 and IL-21 in compliance with current good manufacturing practice (cGMP) for Phase I/II trials. Under these culture conditions, the CAR<sup>+</sup> T cells numerically expand and the T cells that are not genetically modified die by neglect. (A) The initial trial was based on four, approximately weekly additions of the AaPC. (B) The manufacturing process in an ongoing trial at MD Anderson Cancer Center was then shortened to approximately 14 days in compliance with cGMP based on two additions of AaPC. (C) An upcoming trial is based on further shortening the *ex vivo* culture time to under 2 days based on a technology referred to as point-of-care (P-O-C). This is based on the electrotransfer of DNA plasmids from the SB system encoding CAR and a membrane bound IL-15 (mbIL15). The coordinated signaling through CAR and mbIL15 provides a selective advantage for genetically modified T cells to sustain persistence after adoptive transfer. Thus, the time and expense associated with cell culture under cGMP can be avoided based on the proliferation of genetically modified T cells *in vivo* rather than *ex vivo*.

The SB system can also be adapted to express more than one transposon. In a technology dubbed ‘double transposition’, three DNA plasmids (two transposons and one transposase) have been electrotransferred into T cells to co-express CAR and mbIL15 [114]. The signaling through the second-generation CAR and the common  $\gamma$ -chain cytokine receptor results in a T cell that combines ‘signal 1 and 2’ with ‘signal 3’ [114]. This results in fully competent activation and the preservation of the key **stem cell memory T cell** ( $T_{SCM}$ ) subpopulation that is correlated with antitumor effects [115]. Indeed, the SB-modified CAR<sup>+</sup>mbIL15<sup>+</sup> T cells demonstrated superior persistence and killing of CD19<sup>+</sup> tumors in mice compared with current CAR<sup>+</sup> T cells. This was without apparent autonomous growth or transformation. The long-lived T cells were CD45RO<sup>neq</sup>CCR7<sup>+</sup>CD95<sup>+</sup>, phenotypically most similar to  $T_{SCM}$ , and furthermore had a memory-like transcriptional profile. Overall, these results demonstrate that CAR<sup>+</sup>mbIL15<sup>+</sup> T cells can sustain long-term persistence and effector function by signaling through mbIL15.



**Figure 6. T Cells Genetically Modified with the Sleeping Beauty (SB) System and Manufactured under ‘Point-of-Care’ Can Eliminate Cancer in a Preclinical Model.** The bioburden of disseminated CD19<sup>+</sup> tumors in mice was serially measured by biophotonic imaging (y axis). Background flux is shown by the broken line. Two populations of T cells were generated based on the electrotransfer of DNA plasmids from the SB system to express a CD19-specific chimeric antigen receptor (CAR) with mblL15 (diamond) or mock electroporated to generate CAR<sup>neg</sup> without mblL15 (star). The T cells were infused into the mice within 2 days of electroporation. As controls, mice received no immunotherapy (circle). These data reveal three phases of antitumor effects. Initially, the CAR<sup>+</sup>mblL15<sup>+</sup> T cells were ‘catching up’ with the tumor burden over the first 10 days (days 5–16), resulting in relative stability of tumor burden over the next 14 days (days 16–30), followed by resolution of the tumor mass (days 30–42).

This has implications for further improvements to manufacturing SB-modified T cells because the coordinated signaling via CAR and mblL15 results in a competitive survival advantage of these genetically engineered T cells (Figure 6). Thus, this protocol may further shorten the manufacturing time of a biologically active product (Figure 5C), because proliferation of genetically modified T cells occurs *in vivo* rather than *ex vivo*. Furthermore, the enforced expression of mblL15 may avoid the need for prior lymphodepleting chemotherapy to liberate endogenous IL-15, which is correlated with improved survival of CAR-T cells and antilymphoma effects [116].

Finally, human application of SB-modified CAR-T cells extends beyond targeting CD19. For example, clinical trials using viral-based gene transfer will test CARs specific for target antigens in other hematologic and solid tumors, including a SLAMF7-specific CAR for multiple myeloma and a ROR1-specific CAR for lymphoma, breast, and lung cancer [117,118]. The gene transfer technology enabled by SB to redirect T cell specificity can be extended beyond CAR. For example, TCRs targeting **neoantigens** can be stably expressed on SB-modified T cells, which provides the gateway to targeting metastatic epithelial cancers [119].

### Concluding Remarks and Future Perspectives

Several studies have established that SB-mediated transposition provides long-term expression *in vivo*. Notably, stable transgene expression from SB vectors was seen in mice after gene delivery to the liver [31,91,120,121], lung [122,123], brain [124], and blood after hematopoietic reconstitution *in vivo* [50,57]. Thus, it appears that SB transposon vectors have the capacity to provide long-term expression of transgenes both *ex vivo* and *in vivo*, suggesting that incorporating insulator sequences in SB vectors is not necessary to protect the transgene from silencing. The use of insulators was demonstrated to effectively shield the promoter activity of the transgene cassette at the integration locus [39,125]. However, although insulators could prevent transactivation of oncogenes, they could also have undesired effects on genome structure and function. Thus, the potential risks and benefits of using insulator sequences need to be carefully evaluated.

### Outstanding Questions

Efficient *in vivo* delivery of nonviral vector systems, including SB, is a challenge. Is it possible to devise cell type-specific reagents (either viral or nonviral) for efficient, targeted delivery of transposon vector components to target tissues *in vivo*?

*Ex vivo* delivery of SB transposon vector components to primary human cell types (including HSCs and T cells) currently depends on electroporation, which is toxic to the cells. Is it possible to develop transfection reagents for the nontoxic and efficient delivery of plasmid-based vectors into these cell types?

Close-to-random genomic integration is an advantage of SB when considering safety aspects of gene therapy by integrating genetic vectors. Could this be further improved by targeting transposon integration into genomic safe harbor sites?

Contemporary protocols to supply SB system components rely on delivering nucleic acids to cells. Can the transposase be supplied as purified recombinant protein?

In therapeutic applications, it is desirable to limit the numbers of integrated vector copies to a single copy per cell. Can strategies be devised to control copy number?

The first clinical applications of the SB system are currently ongoing using T cells gene-modified with SB vectors carrying a CAR to render the T cells cytotoxic specifically toward CD19<sup>+</sup> hematologic tumors [49,96,97]. Lymphocytes represent a suitable initial platform for testing new gene transfer systems, because T cells can be genetically modified using viral and nonviral approaches without apparent resulting genotoxicity. However, a major hurdle to the *ex vivo* delivery of the transposon components into relevant primary cell types is the toxicity of contemporary transfection and/or electroporation protocols. In situations where target cells are scarce and/or culturing and expansion of the transfected cells is impossible or cannot be solved without compromising cell identity and grafting potential, cytotoxicity of the transfection procedures is a serious issue that may undermine clinical applications. However, recent experimental data indicate that, by using transposon cassettes vectorized as MCs and by providing the transposase in the form of mRNA, cellular toxicity can be reduced [63] or overcome with *in vivo* selective proliferation based on cytokine signaling [114], thereby positioning clinical applications with SB well within reach, at least in the area of T cell engineering.

Alternative technologies for genetic engineering in clinically relevant cell types are rapidly advancing. Designer nucleases, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas nucleases are excellent tools for genome engineering, including gene editing and gene addition [126–129]. Designer nucleases are specialized in introducing a double-strand break (DSB) into the DNA, and, therefore, are highly efficient in mutagenizing a target site [130,131]. However, designer nucleases are *per se* non-integrating systems (Figure 2), because gene addition at the cut site is a process executed by the homology-directed repair (HDR) mechanism of the cells, the efficiency of which is considerably lower than introducing the DSB in the first place [132]. In other words, knocking out a gene by designer nucleases is more efficient than knocking in a gene into a specific site. In eukaryotic cells, DSBs can be repaired by at least two pathways: HDR and nonhomologous end joining (NHEJ). The two pathways act complementarily, but at different stages of the cell cycle: NHEJ is preferentially active during G1 and early S phase [133], whereas HDR is the preferentially used DSB repair pathway during late S and G2 phase, when homology templates are available [134]. Thus, HDR is strongly downregulated in most postmitotic cells [135] and, consequently, gene addition and gene repair require dividing target cells. In sharp contrast to designer nucleases, integrating viruses and transposable elements have evolved machineries for gene integration, which is a fundamental step of the life cycle of these genetic elements. This means that the efficiency of gene insertion by vector systems that are based on such genetic elements is robust, which is a key requirement for medically relevant applications. An additional benefit of integrating vectors over nuclease-based approaches is that some integrating vectors, particularly those based on transposons, can deliver their cargo into the genomes of nondividing cells [31,136].

The advantage of using the SB system for gene therapy includes: (i) the ease and reduced cost associated with manufacturing of clinical-grade, plasmid-based vectors compared with recombinant viral vectors; (ii) scalability: SB vectors can be manufactured in any quantity; (iii) easy quality control for clinical use; and (iii) indefinite storage with absolute fidelity. Since its resurrection, there has been a continuing interest in using the SB system for various applications, including gene therapy. Owing to its unique and salient features as a gene vector system, SB is currently being tested in 12 clinical trials worldwide and has also led to the foundation of multiple companies [137].

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## Resources

[www.abedia.com/wiley/vectors.php](http://www.abedia.com/wiley/vectors.php)

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