Wide awake and ready to move: 20 years of non-viral therapeutic genome engineering with the Sleeping Beauty transposon system

Hodge, R., Narayanavari, S., Izsvák, Z., Ivics, Z.

Human Gene Therapy
2017 OCT 01 ; 28(10): 842-855
doi: 10.1089/hum.2017.130

Publisher: Mary Ann Liebert, Inc. publishers

Wide Awake and Ready to Move: 20 Years of Non-Viral Therapeutic Genome Engineering with the *Sleeping Beauty* Transposon System

Russ Hodge[1], Suneel A. Narayanavari[1], Zsuzsanna Izsvák[1] and Zoltán Ivics[2,*]

[1] Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin, Germany
[2] Division of Medical Biotechnology, Paul Ehrlich Institute, Langen, Germany

*** Corresponding author**:
Zoltán Ivics
Paul Ehrlich Institute
Paul Ehrlich Str. 51-59
D-63225 Langen
Germany
Phone: +49 6103 77 6000
Fax: +49 6103 77 1280
Email: zoltan.ivics@pei.de

Key words: gene therapy vectors, chimeric antigen receptor, stable gene transfer, non-viral gene delivery, transposon/virus hybrid vectors, cell-specific targeting

Abstract

Gene therapies will only become a widespread tool in the clinical treatment of human diseases with the advent of gene transfer vectors that integrate genetic information stably, safely, effectively, and economically. Two decades after the discovery of the *Sleeping Beauty* (SB) transposon, it has been transformed into a vector system that is fulfilling these requirements. SB may well overcome some of the limitations associated with viral gene transfer vectors and transient non-viral gene delivery approaches that are being used in the majority of ongoing clinical trials. The SB system has achieved a high level of stable gene transfer and sustained transgene expression in multiple primary human somatic cell types, representing crucial steps that may permit its clinical use in the near future. Here we review the most important aspects of SB as a tool for gene therapy, including aspects of its vectorization and genomic integration. As an illustration we highlight clinical development of the SB system towards gene therapy of age-related macular degeneration and cancer immunotherapy.

Non-viral gene transfer using the *Sleeping Beauty* transposon

DNA transposons are genetic elements with the ability to change their positions within the genome. They mainly achieve this through a cut-and-paste mechanism. Natural transposons are mobile ("jumping") units of DNA encoding a gene for a transposase enzyme flanked by terminal inverted repeats (TIRs) that represent the sites where the transposase binds (**Fig. 1A**). A crucial point in turning transposons into vectors is the possibility of separating these two functions (the TIRs and the transposase) to establish a two-component system: one component supplying the transposase and the other component carrying a DNA sequence of interest between the TIRs (**Fig. 1B**). The transposase enzyme mediates the excision of the element from its donor plasmid, then reintegrates the transposon construct into a chromosomal locus (**Fig. 1C**). The result is an easily controllable DNA delivery vehicle that has a vast potential for diverse applications in genetic engineering, including gene therapies.

Although over the course of evolution transposons became dormant in vertebrates, it was possible to reconstruct an active sequence from ancient inactive transposon sequences isolated from fish genomes. This transposon was named *Sleeping Beauty* (SB) after the Grimm brothers' famous fairy tale[1]. SB was the first transposon ever shown to be capable of efficient transposition in vertebrate cells, and it opened entirely new avenues for genetic engineering. A vision developed of using SB for gene therapies (reviewed in [2-10]). A transposon-based gene delivery system would have the advantage of combining the favorable features of viral vectors with those of naked DNA molecules. First, permanent insertion of transgene constructs into the genome by the transpositional mechanism (in the case of SB this occurs at genomic TA dinucleotides[1]) leads to sustained and efficient transgene expression in preclinical animal models[6]. Second, in contrast to viral vectors, transposon vectors can be

maintained and propagated as plasmid DNA, which makes them simple and inexpensive to manufacture, another important consideration for implementations and a scale-up into real clinical practice. SB has further advantages as a gene-transfer system: its immunogenicity *in vivo* is much lower than that of viral vectors[11]; it can deliver larger genetic cargoes[12], and poses far fewer safety issues[9, 13-15]. Another transposon that has become a widely used, popular tool for a variety of applications including gene therapy[16-20] is the *piggyBac* (PB) transposon originally isolated from the cabbage looper moth[21].

Optimized vector components for enhanced Sleeping Beauty-mediated gene delivery

A number of improvements needed to be made to transform the original version of the SB transposon (pT) into an efficient tool for gene delivery in vertebrates. While originally the rate of transposition was low, steady improvements were made to optimize the vector architecture. Genetic engineering produced the variants pT/2/3/4, the most recent of which optimizes transposon binding[22-25]. In addition, the so-called “sandwich” vector architecture (containing two full-lengh copies of SB flanking a gene-of-interest) was developed to aid transposition of large transgenes[12].

Advanced genetic engineering also required variants of the transposase that were hyperactive compared to the original. Amino acid substitutions spanning almost the entire SB transposase polypeptide were screened to improve its catalytic activity. A second-generation SB transposase called SB11[26] proved to be about 3-fold more active than the first-generation SB transposase, and has been primarily employed in clinical trials based on chimeric antigen receptor (CAR)-engineered T cells that are currently underway[27]. Further improvements produced SB100X, the most hyperactive SB transposase version currently available, whose activity is ~100-fold that of the originally resurrected transposase[28]. SB100X transposase enables highly efficient germline

transgenesis in relevant mammalian models, including mice, rats, rabbits, pigs, sheep and cattle[29-34]. The system has also yielded robust gene transfer efficiencies into human hematopoietic stem cells (HSCs)[28, 35], mesenchymal stem cells, muscle stem/progenitor cells (myoblasts), iPSCs[36] and T cells[37], which are crucial targets for regenerative medicine and gene- and cell-based therapies aimed at complex genetic diseases. Importantly, recent insights into structure-function relationships in the SB transposase based on modeling[38] and crystallography[39] will likely be informative for structure-based design of next-generation SB transposases for therapeutic gene delivery.

Typically, the delivery of the SB transposon system into cells supplies the two components of the vector system as conventional plasmids (**Fig. 1B**). But the transposase expression plasmid typically used as the source of the transposase in cultured cell lines can be replaced by mRNA that is synthesized in *in vitro* transcription reactions (**Fig. 1D**), which was originally tested in a mammalian cell line *in vitro* and in the mouse liver *in vivo*, using the SB11 transposase[40, 41]. Although the nucleic acids carrying the SB vector components can only be partially be represented by RNA (the transposon is by definition DNA), the *ex vivo* application of mRNA for intracellular delivery of the transposase in therapeutically relevant cells avoids some of the hurdles typically encountered with DNA-based vectors. For example, nucleofecting primary human cells including HSCs and T cells with mRNA has a significantly lower toxicity than when plasmid DNA is used[42, 43]. Importantly, using mRNA source to transiently deliver the SB transposase increases biosafety, because mRNA does not run the risk of chromosomal integration. The risk of integrating the SB transposase coding sequence into the genome would represent a finite risk in gene therapy applications, because it could lead to the prolonged and uncontrollable expression of the transposase and could cause a continuous remobilization of the already integrated SB transposon.

Recently, a new generation of SB vectors have been produced, which will be even more useful for clinical applications by employing minicircle (MC) vectors as carriers of the SB transposon components. This technology permits a significant reduction in the size of SB vectors by removing most of the backbone sequences from parental plasmids[44]. The first evident advantage of MC vectors over plasmids has to do with the fact that they increase the surival rates of human T cells following nucleofection[43]. Alongside a lower cytotoxicity, stable genome modification with MCs was more efficient than with conventional plasmid vectors in T cells[43]. Transfection of MC components is more efficient than that of plasmids owing to their smaller size, which enables them to cross cellular membranes more efficiently than plasmids[45, 46]. Another reason for their higher levels of transposition is likely the relatively short distance between the ends of the transposons, ~200-bp in MC vectors, resulting from the removal of the backbone of the bacterial plasmid. Shortening the length of the DNA sequence lying outside the transposon unit leads to a much higher SB transposition, probably because it makes the formation of the transposon/transposase complex easier[47].

The biosafety advantages of MC technology also have to do with the absence of bacterial plasmid backbone elements in therapeutic vectors, a factor which is highly relevant in clinical applications. Otherwise the result may be an inclusion of antibiotic resistance genes in a therapeutic cell product. Variants of the MC technology are miniplasmids that are “free of antibiotic resistance markers” (pFAR)[48]; the lack of antibiotic-resistance genes significantly enhances the safety of methods of non-viral gene delivery in clinical settings. One of the newest innovations has been to combine the pFAR and SB technologies[49], and a Phase Ia/IIb clinical trial to treat age-related macular degeneration based on this approach is planned in the near future[50] (see further details on this approach below).

In addition to purely non-viral strategies, there has been a development of various SB-based viral hybrid technologies that merge the excellent nucleic acid delivery properties of a non-integrating viral vector and the integrative properties of SB in advantageous ways (reviewed in [3, 10]). One can pack the SB system, including both the transposase and the transposon, into various recombinant viruses for delivery (by transduction) into cells. In principle, these hybrid vectors could be used as alternatives to the viral vectors that have been established, and they are suitable for cell-type specific genome engineering. Such hybrids of viruses and transposons have been established for the integrase-deficient lentivirus (IDLV)[51-53], adenovirus[54, 55], AAV[56], herpes simplex virus[57, 58] and baculovirus[59, 60]. In these cases the SB transposon provides stable gene integration.

Safety aspects of Sleeping Beauty transposition

One of the most important risk factors associated with an integrating genetic element is genotoxicity: mutational damage that can shift cellular homeostasis toward some pathological path. This has happened in a number of recent clinical trials, in which retroviruses were used to transfer genes into HSCs, as described above: in some patients this has led to a clonal imbalance and tumorigenic transformation. Two fundamental properties of a transposon vector system potentially contribute to genotoxicity: i) if the transposase interacts with endogenous human DNA sequences or human proteins that are highly similar to the transposon vector sequences, or ii) if the vector is inserted into the genome at unsafe sites.

The SB system appears to be safe with respect to “off-target” cleavage of the transposase in human cells. Because the SB was reconstructed from fish genomes, the mammalian lineage does not contain transposons similar enough to it that they would be

cleaved by the SB transposase. It is always possible that chance might produce genomic sequences similar to the SB TIRs, some of which might bind the SB transposase. However, SB transposition is such a highly controlled process[25] that it is extremely unlikely that these sequences would mobilize. Secondly, human cells do not express a protein similar enough to the SB transposase to re-mobilize a genomically integrated SB vector. In sharp contrast, the human transposase-derived protein PGBD5 can mobilize insect PB transposon vectors in human cells[61]. Despite the vast evolutionary distance between human PGBD5 and insect PB transposons, there may be cross-reactions between an endogenous human transposase that is catalytically active and transposon vector sequences that are exogenously delivered into human cells by gene transfer. The findings suggest that there may be stability issues for applications involving PB vectors in human cells expressing PGBD5[62].

To estimate the genotoxic potential of different vector types and designs, it is important to characterize the properties, which influence their selection of target sites[63]. We previously carried out a comparative study of 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 $CD4^+$ T cells. Our bioinformatic analyses included mapping of integration sites generated by these four vector systems against the T cell genome with respect to their proximity to genes, transcriptional start sites (TSSs), CpG islands, DNaseI hypersensitive sites, chromatin marks, the transcriptional status of genes and criteria that qualify sequences as genomic safe harbors (GSHs)[64, 65]. Of the different systems, SB transposon targets displayed the least deviation from random in terms of genome-wide distribution. We found no apparent bias for either heterochromatin marks or euchromatin marks, and detected only a weak correlation with the transcriptional status of targeted genes[66]. Collectively, these analyses established that the SB transposon had a favorable integration profile compared to other vectors,

suggesting that it might be safer for therapeutic gene delivery than the viral vectors that are currently being used to integrate sequences in clinical trials. Importantly, no adverse effects have been associated with SB in preclinical animal studies[6, 8, 67, 68]. Finally, SB's safety profile can probably be further improved through molecular strategies that enhance target-selected transgene integration[69].

Therapeutic gene delivery with the *Sleeping Beauty* transposon system

In vivo application of the Sleeping Beauty system in pre-clinical models

In vivo applications use a gene vector system to shuttle a therapeutic nucleic acid delivered directly in the body; the delivery can be systemic, but more typically, it is targeted to a specific organ or cell type (**Fig. 2A**). The *in vivo* delivery of transposon vectors is challenging, because naked nucleic acids (DNA and mRNA) are unable to pass through the cell membrane through infection, unlike viruses. Thus, it is necessary to combine a transposon vector with a technology that can deliver a non-viral vector into cells. One of the most promising strategies is an *in vivo* gene transduction system based on a hybrid adenovirus/transposon vector[54] and the hyperactive SB100X transposase[55] (**Fig. 2A**). In a recent study, autologous HSCs were mobilized into peripheral blood, and directly targeted using such a hybrid adenovirus/transposon vector system *in vivo*, producing functional HSCs in a transgenic animal model[70, 71]. The procedure involves the systemic, intravenous injection of an integrating, helper-dependent hybrid adenovirus (HD-Ad5/35^{++})/SB vector system into the bloodstream. The hybrid vector targets human CD46, a receptor that is uniformly expressed on HSCs in these transgenic mice, and permits the stable genetic engineering of HSCs *in vivo*. This procedure has the potential advantage that it does not require the *ex vivo* expansion and transduction of HSCs. A potential disadvantage is that the efficiency of gene manipulation was not as high as that

reported from clinical trials using lentiviral vectors and *ex vivo* cell processing. This indicates that the strategy needs further characterization and improvements. Nevertheless, this system has the potential to overcome existing technical/medical difficulties associated with the collection and *ex vivo* manufacturing of cells, and thus represents a significant technical advance over current systems.

Immune complications following adenoviral vector delivery *in vivo* can lead to acute toxicity (reviewed in [72]) associated with activation of the innate inflammatory immune response. Importantly, the toxicity is dosage dependent, suggesting that it is possible to find a therapeutic window, in which the vector can be safely used. The other problem with adenoviral vectors that they cannot support long-term transgene expression due to the transient nature of the vector. Thus, especially for *in vivo* approaches, the use of hybrid adenovirus/transposon vectors could be advantageous, because i) due to stable chromosomal integration only a single administration of vector is required and, consequently ii) their use may allow reduction of the applied viral dose, thereby alleviating vector-associated immune complications. Indeed, delivery of adenovirus/transposon hybrid vectors was well tolerated in mice[71] and in dogs[73].

Ex vivo application of the Sleeping Beauty system in pre-clinical models

In *ex vivo* gene delivery, the therapeutic gene vector is introduced into a selected cell population that has been isolated from a donor, followed by the transplantation of the genetically engineered cells into a patient (**Fig. 2B**). We distinguish between autologous or allogeneic cell products depending on whether the donor is the same patient or another person. As for *in vivo* applications, the efficiency of transposition depends on the efficiency, at which the nucleic acids that are introduced are taken up by cells. In principle, any technology developed to transfer nucleic acids into cells can be combined

with transposon vectors. In cells that are hard to transfect, including primary human cell types, nucleofection can significantly facilitate the delivery of transposon-based vectors. This has been achieved in $CD34^+$ HSCs[28, 35, 74-76], primary T cells[66, 77-79] and human embryonic stem cells[41, 80]. Importantly, this *ex vivo* procedure did not appear to compromise the engraftment and multi-lineage differentiation potential of $CD34^+$ cells in the context of the hematopoietic system[28, 35].

SB transposition-based non-viral gene delivery has an outstanding potential to provide innovative treatments and potential cures for an array of genetic disorders (reviewed in [3, 5-8, 10, 50, 67, 81-83]). Prime examples for the use of SB in gene therapy include the treatment of haematologic disorders, lysosomal storage diseases, pulmonary disorders, dermatologic diseases, a variety of metabolic disorders, neurologic disorders, muscle disorders and cancer (**Table 1**). This robust, non-viral, transposon-based procedure is currently being tested in human clinical trials[27], discussed in the following section.

Sleeping Beauty **non-viral gene delivery for gene therapy of neovascular age-related macular degeneration**

Neovascular age-related macular degeneration (nvAMD) involves a degeneration of retinal pigment epithelial cells in the macula area and thus a loss of functions that are essential for central vision. Affecting over 13 million people world-wide, AMD is the fourth most common cause of blindness after cataract, retinopathy of prematurity, and glaucoma and is the leading cause of irreversible blindness in people over 50 years of age in developed countries[84]. The development of nvAMD has been traced to the development of subretinal neovascularization, caused by an overexpression of vascular

endothelial growth factor (VEGF)[84, 85]. Current treatments are based on monthly, life-long, intravitreal injections of inhibitors of VEGF, a strategy that is effective in 30-40% of patients. Anti-VEGFs have two major limitations: 60-70% of patients do not experience an improvement of vision, and treatments often produce adverse effects[86] including increased intraocular pressure, retinal detachment, endophthalmitis, photoreceptor cell death and a thinning of the inner neuronal layer of the retina[87, 88]. In addition, the logistical problems encountered by blind or low-vision patients who have to travel to a clinic on a monthly basis lead to a significant proportion of discontinued treatments, which has been reported at levels of 57% over 5 years[89] to as high as 71% within 24 months[90]. Obviously, new approaches are required for an efficient treatment of nvAMD.

The link between nvAMD and retinal pigment epithelial (RPE) cell degeneration has stimulated the idea that the condition could be treated by replacing the degenerated RPE cells with healthy RPE cells, which would synthesize and secrete the anti-angiogenic and neuroprotective pigment epithelium-derived factor (PEDF) *in vivo*. Administering recombinant PEDF is not feasible because of its short half-life, and transplantations of RPE or iris pigment epithelial (IPE) cells as substitutes for degenerated RPE cells have not led to significant improvements in the visual acuity of nvAMD patients[91-94]. This indicates that the replacement cells do not produce levels of anti-angiogenic factors that are sufficient to overcome the pathological overexpression of VEGF.

An alternative to frequent, life-long intravitreal injections would be a mode of treatment that provides the retina with an inhibitor of neovascularization that lasts for the lifetime of the patient. This would require a constant inhibition of VEGF and a proper balance between angiogenic and anti-angiogenic activities; in other words, the stable integration of a transgene and continuous PEDF expression are critical for re-acquiring vision. To avoid the risks accompanied by gene delivery mediated by viral vectors, the

TargetAMD consortium (an international consortium of universities, research institutes and commercial organizations funded by the European Commission) has been pursuing the use of the hyperactive SB100X system for efficient delivery of a human *PEDF* transgene cassette to cultured and freshly isolated RPE and IPE cells. Cells that have been transfected this way have been found to express recombinant PEDF over the entire duration of 16 months that the cells have been in culture[95]. The effectiveness of this strategy requires establishing whether RPE cells that express elevated levels of PEDF inhibit choroidal neovascularization. To determine this, 10000 rat RPE cells transfected with the *PEDF* gene using SB100X, which secreted approximately 2 ng PEDF/day, were transplanted to the subretinal space of rats, in which choroidal neovascularization had been induced by laser rupture of Bruch's membrane[96]. A marked reduction of neovascularization was observed at 7 and 14 days post-transplantation, with the area of neovascularization reduced by 50%[97].

TargetAMD is pursuing a strategy, by which genetically modified RPE or IPE cells that overexpress PEDF are transplanted into the subretinal space of the eye[50] (**Fig. 3**). Specifically, RPE or IPE cells isolated from the peripheral retina or obtained from an iris biopsy of a patient will be transfected with an SB transposon vector carrying a *PEDF* expression cassette. They will then be transplanted back into the same patient during one surgical session lasting about 60 minutes (**Fig. 3**). A standard operating procedure has been established that i) consistently shows highly efficient transfer of the *PEDF* gene in RPE and IPE cells obtained from donor eyes, ii) permits the expression of recombinant PEDF at high levels of recombinant protein in cultured *PEDF*-transfected cells, and iii) achieves the sustained expression of the transgene (for over one year that the cells have been in culture) in genetically engineered cells. The robustness of this procedure is coupled with salient safety features including a close-to-random transgene integration profile of the SB transposon in human IPE and RPE cells and a lack of

antibiotic resistance genes in the vector components. Based on the results described here and approval by the Swissmedic regulatory agency, TargetAMD will shortly begin patient recruitment for a Phase Ia/IIb clinical trial, and expects to launch the first European human clinical trial using SB transposon and pFAR technologies[50].

Cancer immunotherapy with tumor-reactive CAR T-cells

A new approach to the treatment of advanced malignancies is based on adoptive immunotherapy: gene transfer is used to engineer T cells to express a synthetic CAR that reacts to a tumor and uses the immune system to destroy it. CARs are designer molecules comprised of several components: an extracellular antigen binding domain, usually the variable light and heavy chains of a monoclonal antibody; a spacer and transmembrane region that anchors the receptor on the T-cell surface and provides the reach and flexibility necessary to bind to the target epitope; and an intracellular signaling module, most commonly CD3 zeta and one or more costimulatory domains that mediate T-cell activation after antigen binding[98, 99].

Currently the most advanced clinical development has been the use of CARs specific for the B-lineage marker CD19, which is expressed on B cells in acute and chronic lymphocytic leukemia and B-cell lymphomas. Several groups have administered patient-derived CD19-CAR T cells to achieve rates of complete remission of up to 90% in patients with chemotherapy- and radiotherapy-refractory B-cell acute lymphoblastic leukemia (ALL) and >60% complete remissions in patients with non-Hodgkin lymphoma (NHL)[100-106]. Many consider this a major medical breakthrough, given the advanced stage of the disease and the failure of conventional treatments in many of the patients included in these clinical trials. The side effects that have been reported for CD19-CAR T-cell therapies are a consequence of the strong anti-tumor immune response: they include tumor lysis syndrome due to the rapid destruction of a large number of tumor

cells, cytokine release syndrome due to the rapid release of cytokines by CAR T cells and other immune cells, and the depletion of normal B-cells due to their physiological expression of CD19.

The potential of SB transposition as a means of integrating the genetic information of the CAR into T cells was first explored by the group of Cooper *et al*. [77]. They demonstrated that functional CD19-CAR T cells can be produced by providing the SB transposase either as plasmid DNA or mRNA in combination with a plasmid-encoded CAR transposon and introduced into T cells by electroporation (**Fig. 2B**). Consistent with observations in other mammalian cell types, SB11 and hyperactive SB100X accomplished higher rates of gene transfer than the first-generation SB transposase[37, 107]. The same group has also provided the successful clinical debut of SB-engineered CD19-CAR T cells, and recently reported results of two pilot clinical trials in 26 patients with ALL and NHL who had undergone HSC transplantation (HSCT) based on autologous (n=7, ClinicalTrials.gov Identifier 00968760) or allogeneic (n=19, ClinicalTrials.gov Identifier NCT01497184) cells prior to CAR T cell therapy[27]. These clinical studies demonstrated that the administration of SB-engineered CD19-CAR T cells is safe and may provide additional tumor control in patients after HSCT. They are the first CAR T cell clinical trials that rely on non-viral SB-based gene transfer, and provide a proof-of-concept for the use of SB transposition in CAR T-cell engineering.

A clinical trial with CD19-CAR T cells engineered from MC vectors by SB transposition is in preparation at the Universitätsklinikum Würzburg, Germany, with the aim of obtaining a clinical proof-of-concept for this novel approach[50]. This trial will make use of a CD19-CAR construct that has been selected from pre-clinical analyses for its optimal anti-tumor functions. It has already been validated in clinical trials of a CD19-CAR therapy based on lentiviral gene transfer[100, 108]. An additional step will be the

formulation of cell products that contain equal proportions of CAR-modified $CD8^+$ killer and $CD4^+$ helper T cells, based on previous work showing that this has advantages over the use of cell products with random subset composition[109]. The key advantages of CAR T-cell products with defined subset compositions are that i) lower total numbers of CAR T cells are needed to have clinical efficacy, reducing the risk of side effects and shortening the manufacturing process; and ii) there is less product-to-product variability between patients, making the time and level of CAR T-cell engraftment and proliferation more consistent and predictable, and allowing the establishment of dose-response relationships as well as parameters for the immune pharmacokinetics and pharmacodynamics of CAR T cells as medicinal products.

Concluding Remarks and Future Perspectives

A number of studies have established that SB-mediated transposition provides long-term expression *in vivo*. Stable transgene expression from SB vectors has been observed in mice in the liver[11, 110-112], lung[113, 114], brain[115] and blood after hematopoietic reconstitution *in vivo*[28, 35]. The long-term expression of transgenes from SB transposon vectors can thus be achieved both *ex vivo* and *in vivo*, which is a crucial step in the use of the technology in clinical applications. The first such uses of the system are ongoing; T cells whose genes have been modified with SB vectors have been outfitted with a CAR to render the cells specifically cytotoxic toward $CD19^+$ hematologic tumors[27, 77, 78]. Lymphocytes represent a suitable initial platform for testing new gene transfer systems, as T cells can apparently be genetically modified using viral and non-viral approaches without leading to genotoxicity. There remains, however, a major hurdle in *ex vivo* delivery of transposon components into relevant primary cell types due to the toxicity of

contemporary transfection/electroporation protocols. This is a serious issue that may undermine clinical applications in situations where target cells are scarce and/or the culturing and expansion of the transfected cells is impossible or cannot be solved without compromising cell identity and grafting potential. There is hope: recent experimental data indicate that cellular toxicity can be reduced through the use of transposon cassettes vectorized as MCs and providing the transposase in the form of mRNA[43], followed by *in vivo* selective proliferation based on cytokine signaling[116]. This puts clinical applications with SB well within reach, at least in the area of T-cell engineering.

These developments are accompanied by rapid advances in alternative technologies for genetic engineering in clinically relevant cell types. 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, permitting the editing and addition of genes[117-120]. Designer nucleases have the particular feature of introducing a double-strand break (DSB) into the DNA, and are therefore highly efficient in mutagenizing a target site[121, 122]. However, adding a gene at the cut site requires the cellular process of homology-directed repair (HDR), whose efficiency is considerably lower than introducing the DSB in the first place[123]. In other words, using designer nucleases is far more efficient at knocking out a gene than inserting a gene into a specific site. In eukaryotic cells, DSBs can be repaired by at least two pathways: HDR and non-homologous end joining (NHEJ). HDR is strongly downregulated in most post-mitotic cells[124]; thus, gene addition and gene repair require target cells that divide. In sharp contrast to designer nucleases, gene integration is a fundamental step of the life cycle of integrating viruses and transposable elements, and they have evolved machineries to achieve that. That

means that vector systems based on such genetic elements exhibit a robust efficiency of gene insertion – a key requirement for medically relevant applications. An additional benefit is that some integrating vectors, particularly those based on transposons, can deliver their cargo into the genomes of non-dividing cells[11, 125].

The advantages of using the SB system for gene therapy include i) the ease and reduced cost of manufacturing of clinical-grade, plasmid-based vectors compared to recombinant viral vectors, ii) scalability: SB vectors can be manufactured in any quantity, iii) the ease of ensuring quality control for clinical use, and iii) indefinite storage with absolute fidelity. There is a rapidly growing interest in using the SB system for gene therapy and other applications. The unique and salient features of this gene vector system have led to the use of SB in 12 clinical trials worldwide at the moment, and has stimulated the formation of a number of companies devoted to refining and developing the system for further uses[126]. It is remarkable that in two decades, the SB system has been resurrected from its dormant state in vertebrates to become a powerful tool on the threshold of joining the clinical arsenal, with a potential to address diseases that have long resisted classical therapies.

ACKNOWLEDGEMENTS

The TargetAMD project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 305134. Z. Ivics has been funded by the Deutsche Forschungsgemeinschaft (DFG) under grants IV 21/11-1 and IV 21/13-1, and has been supported by the Center for Cell and Gene Therapy of the LOEWE (Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz) program in Hessen, Germany. We thank M. Kropp for the image presented in Fig. 3.

AUTHOR DISCLOSURE STATEMENT

Z. Izsvák and Z. Ivics are co-inventors on several patents in the area of *Sleeping Beauty* transposon-based gene delivery.

REFERENCES

1. Ivics Z, Hackett PB, Plasterk RH et al. Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. Cell 1997;91:501-510.
2. Ivics Z, Li MA, Mates L et al. Transposon-mediated genome manipulation in vertebrates. Nat Methods 2009;6:415-422.
3. Narayanavari SA, Chilkunda SS, Ivics Z et al. Sleeping Beauty transposition: from biology to applications. Critical reviews in biochemistry and molecular biology 2016:1-27.
4. Ivics Z, Izsvak Z. The expanding universe of transposon technologies for gene and cell engineering. Mob DNA 2010;1:25.
5. Ivics Z, Izsvak Z. Nonviral gene delivery with the sleeping beauty transposon system. Human gene therapy 2011;22:1043-1051.
6. Hackett PB, Largaespada DA, Cooper LJN. A transposon and transposase system for human application. Mol Ther 2010;18:674–683.
7. Hackett PB, Jr., Aronovich EL, Hunter D et al. Efficacy and safety of Sleeping Beauty transposon-mediated gene transfer in preclinical animal studies. Curr Gene Ther 2011;11:341-349.
8. Izsvak Z, Hackett PB, Cooper LJ et al. Translating Sleeping Beauty transposition into cellular therapies: victories and challenges. Bioessays 2010;32:756-767.
9. VandenDriessche T, Ivics Z, Izsvak Z et al. Emerging potential of transposons for gene therapy and generation of induced pluripotent stem cells. Blood 2009;114:1461–1468.
10. 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;15:255-265.
11. Yant SR, Meuse L, Chiu W et al. Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. Nat Genet 2000;25:35-41.
12. Zayed H, Izsvak Z, Walisko O et al. Development of hyperactive sleeping beauty transposon vectors by mutational analysis. Mol Ther 2004;9:292-304.
13. 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;16:359-369.
14. Moldt B, Yant SR, Andersen PR et al. Cis-acting gene regulatory activities in the terminal regions of sleeping beauty DNA transposon-based vectors. Human gene therapy 2007;18:1193-1204.
15. Ivics Z, Katzer A, Stuwe EE et al. Targeted Sleeping Beauty transposition in human cells. Mol Ther 2007;15:1137-1144.
16. Di Matteo M, Samara-Kuko E, Ward NJ et al. Hyperactive piggyBac transposons for sustained and robust liver-targeted gene therapy. Mol Ther 2014;22:1614-1624.
17. Loperfido M, Jarmin S, Dastidar S et al. piggyBac transposons expressing full-length human dystrophin enable genetic correction of dystrophic mesoangioblasts. Nucleic acids research 2016;44:744-760.
18. Cooney AL, Singh BK, Sinn PL. Hybrid nonviral/viral vector systems for improved piggyBac DNA transposon in vivo delivery. Mol Ther 2015;23:667-674.
19. Saha S, Nakazawa Y, Huye LE et al. piggyBac transposon system modification of primary human T cells. Journal of visualized experiments : JoVE 2012:e4235.

20. Manuri PV, Wilson MH, Maiti SN et al. piggyBac transposon/transposase system to generate CD19-specific T cells for the treatment of B-lineage malignancies. Human gene therapy 2010;21:427-437.
21. Fraser MJ, Brusca JS, Smith GE et al. Transposon-mediated mutagenesis of a baculovirus. Virology 1985;145:356-361.
22. Cui Z, Geurts AM, Liu G et al. Structure-function analysis of the inverted terminal repeats of the sleeping beauty transposon. Journal of molecular biology 2002;318:1221-1235.
23. Izsvák 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;277:34581-34588.
24. Yant SR, Park J, Huang Y et al. Mutational analysis of the N-terminal DNA-binding domain of sleeping beauty transposase: critical residues for DNA binding and hyperactivity in mammalian cells. Molecular and cellular biology 2004;24:9239-9247.
25. Wang Y, Pryputniewicz-Dobrinska D, Nagy EE et al. Regulated complex assembly safeguards the fidelity of Sleeping Beauty transposition. Nucleic acids research 2016.
26. Geurts AM, Yang Y, Clark KJ et al. Gene transfer into genomes of human cells by the sleeping beauty transposon system. Mol Ther 2003;8:108-117.
27. Kebriaei P, Singh H, Huls MH et al. Phase I trials using Sleeping Beauty to generate CD19-specific CAR T cells. The Journal of clinical investigation 2016.
28. Mátés 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;in press.
29. Ivics Z, Mates L, Yau TY et al. Germline transgenesis in rodents by pronuclear microinjection of Sleeping Beauty transposons. Nat Protoc 2014;9:773-793.
30. Ivics Z, Hiripi L, Hoffmann OI et al. Germline transgenesis in rabbits by pronuclear microinjection of Sleeping Beauty transposons. Nature protocols 2014;9:794–809.
31. Ivics Z, Garrels W, Mátés L et al. Germline transgenesis in pigs by cytoplasmic microinjection of Sleeping Beauty transposons. Nature protocols 2014;9:810–827.
32. Bevacqua RJ, Fernandez-Martin R, Canel NG et al. Assessing Tn5 and Sleeping Beauty for transpositional transgenesis by cytoplasmic injection into bovine and ovine zygotes. PloS one 2017;12:e0174025.
33. Garrels W, Talluri TR, Apfelbaum R et al. One-step Multiplex Transgenesis via Sleeping Beauty Transposition in Cattle. Scientific reports 2016;6:21953.
34. Katter K, Geurts AM, Hoffmann O et al. Transposon-mediated transgenesis, transgenic rescue, and tissue-specific gene expression in rodents and rabbits. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2013;27:930-941.
35. Xue X, Huang X, Nodland SE et al. Stable gene transfer and expression in cord blood-derived CD34+ hematopoietic stem and progenitor cells by a hyperactive Sleeping Beauty transposon system. Blood 2009;114:1319-1330.
36. Belay E, Matrai J, Acosta-Sanchez A et al. Novel hyperactive transposons for genetic modification of induced pluripotent and adult stem cells: a nonviral paradigm for coaxed differentiation. Stem cells (Dayton, Ohio) 2010;28:1760–1771.

37. Jin Z, Maiti S, Huls 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;18:849–856.
38. Abrusan G, Yant SR, Szilagyi A et al. Structural Determinants of Sleeping Beauty Transposase Activity. Mol Ther 2016;24:1369-1377.
39. Voigt F, Wiedemann L, Zuliani C et al. Sleeping Beauty transposase structure allows rational design of hyperactive variants for genetic engineering. Nature communications 2016;7:11126.
40. Wilber A, Frandsen JL, Geurts JL et al. RNA as a source of transposase for Sleeping Beauty-mediated gene insertion and expression in somatic cells and tissues. Mol Ther 2006;13:625-630.
41. 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;15:1280-1287.
42. Wiehe JM, Ponsaerts P, Rojewski MT et al. mRNA-Mediated Gene Delivery Into Human Progenitor Cells Promotes Highly Efficient Protein Expression. Journal of Cellular and Molecular Medicine 2007;11:521–530.
43. Monjezi R, Miskey C, Gogishvili T et al. Enhanced CAR T-cell engineering using non-viral Sleeping Beauty transposition from minicircle vectors. Leukemia 2016.
44. Sharma N, Cai Y, Bak RO et al. Efficient sleeping beauty DNA transposition from DNA minicircles. Molecular therapy Nucleic acids 2013;2:e74.
45. Darquet AM, Cameron B, Wils P et al. A new DNA vehicle for nonviral gene delivery: supercoiled minicircle. Gene therapy 1997;4:1341–1349.
46. Chabot S, Orio J, Schmeer M et al. Minicircle DNA electrotransfer for efficient tissue-targeted gene delivery. Gene Therapy 2013;20:62–68.
47. Izsvak Z, Ivics Z, Plasterk RH. Sleeping Beauty, a wide host-range transposon vector for genetic transformation in vertebrates. Journal of molecular biology 2000;302:93-102.
48. 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. The journal of gene medicine 2010;12:323-332.
49. Thumann G, Harmening N, Prat-Souteyrand C et al. Engineering of PEDF-Expressing Primary Pigment Epithelial Cells by the Sleeping Beauty Transposon System Delivered by pFAR4 Miniplasmids. Molecular therapy Nucleic acids 2017;in press.
50. Turchiano G, Latella MC, Gogol-Doring A et al. Genomic analysis of Sleeping Beauty transposon integration in human somatic cells. PloS one 2014;9:e112712.
51. Vink CA, Gaspar HB, Gabriel R et al. Sleeping beauty transposition from nonintegrating lentivirus. Mol Ther 2009;17:1197–1204.
52. Staunstrup NH, Moldt B, Mates L et al. Hybrid lentivirus-transposon vectors with a random integration profile in human cells. Mol Ther 2009;17:1205-1214.
53. Moldt B, Miskey C, Staunstrup NH et al. Comparative genomic integration profiling of Sleeping Beauty transposons mobilized with high efficacy from integrase-defective lentiviral vectors in primary human cells. Mol Ther 2011;19:1499-1510.
54. 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.

55. 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. Molecular therapy Nucleic acids 2016;5:e337.
56. 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.
57. Bowers WJ, Mastrangelo MA, Howard DF et al. Neuronal precursor-restricted transduction via in utero CNS gene delivery of a novel bipartite HSV amplicon/transposase hybrid vector. Mol Ther 2006;13:580-588.
58. de Silva S, Mastrangelo MA, Lotta LT, Jr. et al. Extending the transposable payload limit of Sleeping Beauty (SB) using the Herpes Simplex Virus (HSV)/SB amplicon-vector platform. Gene Ther 2010;17:424-431.
59. 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;19:844-851.
60. Turunen TA, Laakkonen JP, Alasaarela L et al. Sleeping Beauty-baculovirus hybrid vectors for long-term gene expression in the eye. The journal of gene medicine 2014;16:40-53.
61. Henssen AG, Henaff E, Jiang E et al. Genomic DNA transposition induced by human PGBD5. eLife 2015;4.
62. Ivics Z. Endogenous Transposase Source in Human Cells Mobilizes piggyBac Transposons. Mol Ther 2016;24:851-854.
63. Naldini L. Ex vivo gene transfer and correction for cell-based therapies. Nature reviews Genetics 2011;12:301-315.
64. Papapetrou EP, Lee G, Malani N et al. Genomic safe harbors permit high beta-globin transgene expression in thalassemia induced pluripotent stem cells. Nat Biotechnol 2011;29:73-78.
65. Sadelain M, Papapetrou EP, Bushman FD. Safe harbours for the integration of new DNA in the human genome. Nature reviews Cancer 2012;12:51-58.
66. Gogol-Doring 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.
67. Ivics Z, Izsvak Z. Transposons for gene therapy! Curr Gene Ther 2006;6:593-607.
68. Fernando S, Fletcher BS. Sleeping beauty transposon-mediated nonviral gene therapy. BioDrugs 2006;20:219-229.
69. Kovac A, Ivics Z. Specifically integrating vectors for targeted gene delivery: progress and prospects. CELL & GENE THERAPY INSIGHTS 2017;3:103-123.
70. Ren J, Stroncek DF. Gene therapy simplified. Blood 2016;128:2194-2195.
71. Richter M, Saydaminova K, Yumul R et al. In vivo transduction of primitive mobilized hematopoietic stem cells after intravenous injection of integrating adenovirus vectors. Blood 2016;128:2206-2217.
72. Hartman ZC, Appledorn DM, Amalfitano A. Adenovirus vector induced innate immune responses: impact upon efficacy and toxicity in gene therapy and vaccine applications. Virus research 2008;132:1–14.
73. Hausl MA, Zhang W, Muther 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.

74. Hollis RP, Nightingale SJ, Wang X et al. Stable gene transfer to human CD34(+) hematopoietic cells using the Sleeping Beauty transposon. Experimental hematology 2006;34:1333-1343.
75. Izsvak Z, Chuah MK, Vandendriessche T et al. Efficient stable gene transfer into human cells by the Sleeping Beauty transposon vectors. Methods 2009;49:287-297.
76. Sumiyoshi T, Holt NG, Hollis RP et al. Stable transgene expression in primitive human CD34+ hematopoietic stem/progenitor cells, using the Sleeping Beauty transposon system. Human gene therapy 2009;20:1607-1626.
77. Singh H, Manuri PR, Olivares S et al. Redirecting specificity of T-cell populations for CD19 using the Sleeping Beauty system. Cancer Res 2008;68:2961-2971.
78. Huang X, Guo H, Kang J et al. Sleeping Beauty transposon-mediated engineering of human primary T cells for therapy of CD19+ lymphoid malignancies. Mol Ther 2008;16:580-589.
79. 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;18:1803-1813.
80. Orban TI, Apati A, Nemeth A et al. Applying a "double-feature" promoter to identify cardiomyocytes differentiated from human embryonic stem cells following transposon-based gene delivery. Stem Cells 2009;27:1077-1087.
81. Izsvak Z, Ivics Z. Sleeping beauty transposition: biology and applications for molecular therapy. Mol Ther 2004;9:147-156.
82. Hackett PB, Ekker SC, Largaespada DA et al. Sleeping beauty transposon-mediated gene therapy for prolonged expression. Adv Genet 2005;54:189-232.
83. Essner JJ, McIvor RS, Hackett PB. Awakening gene therapy with Sleeping Beauty transposons. Curr Opin Pharmacol 2005;5:513-519.
84. Velez-Montoya R, Oliver SC, Olson JL et al. Current knowledge and trends in age-related macular degeneration: genetics, epidemiology, and prevention. Retina 2014;34:423-441.
85. Ohno-Matsui K, Morita I, Tombran-Tink J et al. Novel mechanism for age-related macular degeneration: an equilibrium shift between the angiogenesis factors VEGF and PEDF. Journal of cellular physiology 2001;189:323-333.
86. Nesmith BL, Ihnen M, Schaal S. Poor responders to bevacizumab pharmacotherapy in age-related macular degeneration and in diabetic macular edema demonstrate increased risk for obstructive sleep apnea. Retina 2014;34:2423-2430.
87. Saint-Geniez M, Maharaj AS, Walshe TE et al. Endogenous VEGF is required for visual function: evidence for a survival role on muller cells and photoreceptors. PloS one 2008;3:e3554.
88. Scott AW, Bressler SB. Long-term follow-up of vascular endothelial growth factor inhibitor therapy for neovascular age-related macular degeneration. Current opinion in ophthalmology 2013;24:190-196.
89. Boulanger-Scemama E, Querques G, About F et al. Ranibizumab for exudative age-related macular degeneration: A five year study of adherence to follow-up in a real-life setting. Journal francais d'ophtalmologie 2015;38:620-627.
90. Lad EM, Hammill BG, Qualls LG et al. Anti-VEGF treatment patterns for neovascular age-related macular degeneration among medicare beneficiaries. American journal of ophthalmology 2014;158:537-543 e532.
91. Lappas A, Foerster AM, Weinberger AW et al. Translocation of iris pigment epithelium in patients with exudative age-related macular degeneration: long-term results. Graefe's archive for clinical and experimental ophthalmology =

Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie 2004;242:638-647.
92. Binder S, Krebs I, Hilgers RD et al. Outcome of transplantation of autologous retinal pigment epithelium in age-related macular degeneration: a prospective trial. Investigative ophthalmology & visual science 2004;45:4151-4160.
93. Aisenbrey S, Lafaut BA, Szurman P et al. Iris pigment epithelial translocation in the treatment of exudative macular degeneration: a 3-year follow-up. Arch Ophthalmol 2006;124:183-188.
94. Falkner-Radler CI, Krebs I, Glittenberg C et al. Human retinal pigment epithelium (RPE) transplantation: outcome after autologous RPE-choroid sheet and RPE cell-suspension in a randomised clinical study. The British journal of ophthalmology 2011;95:370-375.
95. Johnen S, Izsvak Z, Stocker M et al. Sleeping Beauty transposon-mediated transfection of retinal and iris pigment epithelial cells. Investigative ophthalmology & visual science 2012;53:4787-4796.
96. Grossniklaus HE, Kang SJ, Berglin L. Animal models of choroidal and retinal neovascularization. Progress in retinal and eye research 2010;29:500-519.
97. Johnen S, Djalali-Talab Y, Kazanskaya O et al. Antiangiogenic and Neurogenic Activities of Sleeping Beauty-Mediated PEDF-Transfected RPE Cells In Vitro and In Vivo. BioMed research international 2015;2015:863845.
98. Sadelain M, Brentjens R, Riviere I. The basic principles of chimeric antigen receptor design. Cancer discovery 2013;3:388-398.
99. Srivastava S, Riddell SR. Engineering CAR-T cells: Design concepts. Trends in immunology 2015;36:494-502.
100. Turtle CJ, Hanafi LA, Berger C et al. CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. The Journal of clinical investigation 2016;126:2123-2138.
101. Turtle CJ, Maloney DG. Clinical trials of CD19-targeted CAR-modified T cell therapy; a complex and varied landscape. Expert review of hematology 2016;9:719-721.
102. Maude SL, Frey N, Shaw PA et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. The New England journal of medicine 2014;371:1507-1517.
103. Davila ML, Riviere I, Wang X et al. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. Science translational medicine 2014;6:224-225.
104. Lee DW, Kochenderfer JN, Stetler-Stevenson M et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet 2015;385:517-528.
105. Kochenderfer JN, Dudley ME, Kassim SH et al. Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2015;33:540-549.
106. Turtle CJ, Hanafi LA, Berger C et al. Immunotherapy of non-Hodgkin's lymphoma with a defined ratio of CD8+ and CD4+ CD19-specific chimeric antigen receptor-modified T cells. Science translational medicine 2016;8:355ra116.
107. Singh H, Figliola MJ, Dawson MJ et al. Manufacture of clinical-grade CD19-specific T cells stably expressing chimeric antigen receptor using Sleeping Beauty system and artificial antigen presenting cells. PloS one 2013;8:e64138.

108. Hudecek M, Sommermeyer D, Kosasih PL et al. The nonsignaling extracellular spacer domain of chimeric antigen receptors is decisive for in vivo antitumor activity. Cancer immunology research 2015;3:125-135.
109. Sommermeyer D, Hudecek M, Kosasih PL et al. Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor reactivity in vivo. Leukemia 2015.
110. Ohlfest JR, Frandsen JL, Fritz S et al. Phenotypic correction and long-term expression of factor VIII in hemophilic mice by immunotolerization and nonviral gene transfer using the Sleeping Beauty transposon system. Blood 2005;105:2691-2698.
111. Aronovich EL, Bell JB, Khan SA et al. Systemic correction of storage disease in MPS I NOD/SCID mice using the sleeping beauty transposon system. Mol Ther 2009;17:1136-1144.
112. 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. The Journal of clinical investigation 2009;119:2086–2099.
113. Belur LR, Frandsen JL, Dupuy AJ et al. Gene insertion and long-term expression in lung mediated by the Sleeping Beauty transposon system. Mol Ther 2003;8:501-507.
114. Liu L, Sanz S, Heggestad AD et al. Endothelial targeting of the Sleeping Beauty transposon within lung. Mol Ther 2004;10:97-105.
115. Ohlfest JR, Demorest ZL, Motooka Y et al. Combinatorial antiangiogenic gene therapy by nonviral gene transfer using the sleeping beauty transposon causes tumor regression and improves survival in mice bearing intracranial human glioblastoma. Mol Ther 2005;12:778-788.
116. Hurton LV, Singh H, Najjar AM et al. Tethered IL-15 augments antitumor activity and promotes a stem-cell memory subset in tumor-specific T cells. Proceedings of the National Academy of Sciences of the United States of America 2016;113:E7788-E7797.
117. Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 2014;346:1258096.
118. Urnov FD, Rebar EJ, Holmes MC et al. Genome editing with engineered zinc finger nucleases. Nature reviews Genetics 2010;11:636-646.
119. Ousterout DG, Gersbach CA. The Development of TALE Nucleases for Biotechnology. Methods Mol Biol 2016;1338:27-42.
120. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell 2014;157:1262-1278.
121. Hockemeyer D, Soldner F, Beard C et al. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nat Biotechnol 2009;27:851-857.
122. Hockemeyer D, Wang H, Kiani S et al. Genetic engineering of human pluripotent cells using TALE nucleases. Nat Biotechnol 2011;29:731–734.
123. Zou J, Maeder ML, Mali P et al. Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. Cell stem cell 2009;5:97–110.
124. Fung H, Weinstock DM. Repair at single targeted DNA double-strand breaks in pluripotent and differentiated human cells. PloS one 2011;6:e20514.
125. Walisko O, Izsvak Z, Szabo K et al. Sleeping Beauty transposase modulates cell-cycle progression through interaction with Miz-1. Proceedings of the National Academy of Sciences of the United States of America 2006;103:4062-4067.

126. Narayanavari SA, Izsvak Z. Sleeping Beauty transposon vectors for therapeutic applications: advances and challenges. CELL & GENE THERAPY INSIGHTS 2017;3:131-158.
127. Liu L, Mah C, Fletcher BS. Sustained FVIII Expression and Phenotypic Correction of Hemophilia A in Neonatal Mice Using an Endothelial-Targeted Sleeping Beauty Transposon. Mol Ther 2006;13:1006-1015.
128. Zhu J, Kren BT, Park CW et al. Erythroid-specific expression of beta-globin by the sleeping beauty transposon for Sickle cell disease. Biochemistry 2007;46:6844–6858.
129. Belcher JD, Vineyard JV, Bruzzone CM et al. Heme oxygenase-1 gene delivery by Sleeping Beauty inhibits vascular stasis in a murine model of sickle cell disease. J Mol Med (Berl) 2010;88:665-675.
130. Sjeklocha LM, Wong PY, Belcher JD et al. beta-Globin sleeping beauty transposon reduces red blood cell sickling in a patient-derived CD34(+)-based in vitro model. PloS one 2013;8:e80403.
131. Hyland KA, Olson ER, Clark KJ et al. Sleeping Beauty-mediated correction of Fanconi anemia type C. The journal of gene medicine 2011;13:462-469.
132. Verhenne S, Vandeputte N, Pareyn I et al. Long-Term Prevention of Congenital Thrombotic Thrombocytopenic Purpura in ADAMTS13 Knockout Mice by Sleeping Beauty Transposon-Mediated Gene Therapy. Arteriosclerosis, thrombosis, and vascular biology 2017;37:836-844.
133. Aronovich EL, Bell JB, Belur LR et al. Prolonged expression of a lysosomal enzyme in mouse liver after Sleeping Beauty transposon-mediated gene delivery: implications for non-viral gene therapy of mucopolysaccharidoses. The journal of gene medicine 2007;9:403-415.
134. Aronovich EL, Hall BC, Bell JB et al. Quantitative analysis of alpha-L-iduronidase expression in immunocompetent mice treated with the Sleeping Beauty transposon system. PloS one 2013;8:e78161.
135. Zayed H, Xia L, Yerich A et al. Correction of DNA protein kinase deficiency by spliceosome-mediated RNA trans-splicing and sleeping beauty transposon delivery. Mol Ther 2007;15:1273-1279.
136. Xiao J, Meng XM, Huang XR et al. miR-29 inhibits bleomycin-induced pulmonary fibrosis in mice. Mol Ther 2012;20:1251-1260.
137. Liu L, Liu H, Visner G et al. Sleeping Beauty-mediated eNOS gene therapy attenuates monocrotaline-induced pulmonary hypertension in rats. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2006;20:2594-2596.
138. Ortiz-Urda S, Lin Q, Yant SR et al. Sustainable correction of junctional epidermolysis bullosa via transposon-mediated nonviral gene transfer. Gene Ther 2003;10:1099-1104.
139. Latella MC, Cocchiarella F, De Rosa L et al. Correction of recessive dystrophic epidermolysis bullosa by transposon-mediated integration of COL7A1 in transplantable patient-derived primary keratinocytes. The Journal of investigative dermatology 2016.
140. Montini E, Held PK, Noll M et al. In vivo correction of murine tyrosinemia type I by DNA-mediated transposition. Mol Ther 2002;6:759-769.
141. Pan XJ, Ma ZZ, Zhang QJ et al. Sleeping Beauty transposon system is a reliable gene delivery tool for hereditary tyrosinaemia type 1 disease gene therapy: size of the foreign gene decides the timing of stable integration into the host chromosomes. The Journal of international medical research 2012;40:1850-1859.

142. He C-X, Shi D, Wu W-J et al. Insulin expression in livers of diabetic mice mediated by hydrodynamics-based administration. World journal of gastroenterology : WJG 2004;10:567–572.
143. Turunen TA, Kurkipuro J, Heikura T et al. Sleeping Beauty Transposon Vectors in Liver-directed Gene Delivery of LDLR and VLDLR for Gene Therapy of Familial Hypercholesterolemia. Mol Ther 2016;24:620-635.
144. Wang X, Sarkar DP, Mani P et al. Long-term reduction of jaundice in Gunn rats by nonviral liver-targeted delivery of Sleeping Beauty transposon. Hepatology 2009;50:815-824.
145. Chen ZJ, Kren BT, Wong PY et al. Sleeping Beauty-mediated down-regulation of huntingtin expression by RNA interference. Biochem Biophys Res Commun 2005;329:646-652.
146. Eyjolfsdottir H, Eriksdotter M, Linderoth B et al. Targeted delivery of nerve growth factor to the cholinergic basal forebrain of Alzheimer's disease patients: application of a second-generation encapsulated cell biodelivery device. Alzheimer's research & therapy 2016;8:30.
147. Muses S, Morgan JE, Wells DJ. Restoration of dystrophin expression using the Sleeping Beauty transposon. PLoS currents 2011;3:RRN1296.
148. Escobar H, Schowel V, Spuler S et al. Full-length Dysferlin Transfer by the Hyperactive Sleeping Beauty Transposase Restores Dysferlin-deficient Muscle. Molecular therapy Nucleic acids 2016;5:e277.
149. Peng PD, Cohen CJ, Yang S et al. Efficient nonviral Sleeping Beauty transposon-based TCR gene transfer to peripheral blood lymphocytes confers antigen-specific antitumor reactivity. Gene Ther 2009;16:1042-1049.
150. Deniger DC, Pasetto A, Tran E et al. Stable, Nonviral Expression of Mutated Tumor Neoantigen-specific T-cell Receptors Using the Sleeping Beauty Transposon/Transposase System. Mol Ther 2016;24:1078-1089.
151. 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;7:51581-51597.
152. Song J, Kim C, Ochoa ER. Sleeping Beauty-mediated suicide gene therapy of hepatocellular carcinoma. Bioscience, biotechnology, and biochemistry 2009;73:165-168.
153. Belur LR, Podetz-Pedersen KM, Sorenson BS et al. Inhibition of angiogenesis and suppression of colorectal cancer metastatic to the liver using the Sleeping Beauty Transposon System. Molecular cancer 2011;10:14.

Table 1. Preclinical studies with *Sleeping Beauty* gene transfer in disease models

Disease	Delivery	Site of delivery	Reference
Hematologic disorders			
Hemophilia A and B	Tail vein hydrodynamic injection of naked DNA	*In vivo*, mouse liver	[11, 110]
	Intravenous injection of DNA/polyethyleneimine (PEI) complexes	*In vivo*, mouse lungs	[127]
	Intravenous injection of nanocapsules	*In vivo*, mouse liver	[112]
	Intravenous injection of adenovirus/SB hybrid vector	*In vivo*, dog liver	[73]
Sickle cell disease	Transfection	*In vitro*, human cell lines	[128]
	Tail vain hydrodynamic injection of naked DNA	*In vivo*, mouse liver	[129]
	Transfection	*In vitro,* patient HSCs	[130]
Fanconi anemia	Transfection	*In vitro*, human cell lines	[131]
Congenital thromboticthrombocytopenic purpura	Tail vain hydrodynamic injection of naked DNA	*In vivo*, mouse liver	[132]
Lysosomal storage diseases			
Mucopolysaccharidosis	Tail vein hydrodynamic injection of naked DNA	*In vivo*, mouse liver	[111, 133, 134]
Immunologic diseases			
Severe combined immunodeficiency	Transfection	*In vitro*, human cell lines	[135]
Pulmonary disorders			
Fibrosis	Intravenous injection of DNA/polyethyleneimine (PEI) complexes	*In vivo*, mouse lungs	[136]
Pulmonary hypertension	Intravenous injection of DNA/polyethyleneimine (PEI) complexes	*In vivo*, rat lungs	[137]
Dermatologic disorders			
Junctional epidermolysis bullosa	Transfection	*Ex vivo*, patient epidermis	[138]
Dystrophic epidermolysis bullosa	Transfection	*Ex vivo*, human keratinocytes, followed by xenograft in mice	[139]
Metabolic disorders			
Tyrosinemia type I	Tail vain hydrodynamic injection of naked DNA	*In vivo*, mouse liver	[41, 140, 141]
Diabetes type I	Tail vain hydrodynamic injection of naked DNA	*In vivo*, mouse liver	[142]
Hypercholesterolemia	Tail vain hydrodynamic injection of naked DNA	*In vivo*, mouse liver	[143]
Crigler-Najjar syndrome type I (hyperbilirubinemia)	Intravenous injection of proteoliposomes	*In vivo*, mouse liver	[144]

Neurologic disorders			
Huntington disease	Transfection	*In vitro*, human cell lines	[145]
Alzheimer's	Transfection followed by encapsulated cell biodelivery	*In vitro*, human cell line, followed by graft in patient brain	[146]
Muscular dystrophy			
	Transfection	*In vitro*, mouse cell line, followed by transplantation into mice	[147]
	Transfection	*Ex vivo*, mouse myoblasts, followed by transplantation into mice	[148]
Cancer			
	Electroporation, TCR gene transfer	*Ex vivo*, human PBMCs or T cells	[149, 150]
	Electroporatiion, CAR gene transfer	*Ex vivo*, human PBMCs or T cells	[37, 77, 78, 151]
	Transfection	*In vitro*, hepatocellular carcinoma cell lines	[152]
	Intratumoral injection	*In vivo*, human glioblastoma xenografts in mice	[115]
	Tail vain hydrodynamic injection of naked DNA	*In vivo*, mouse liver	[153]

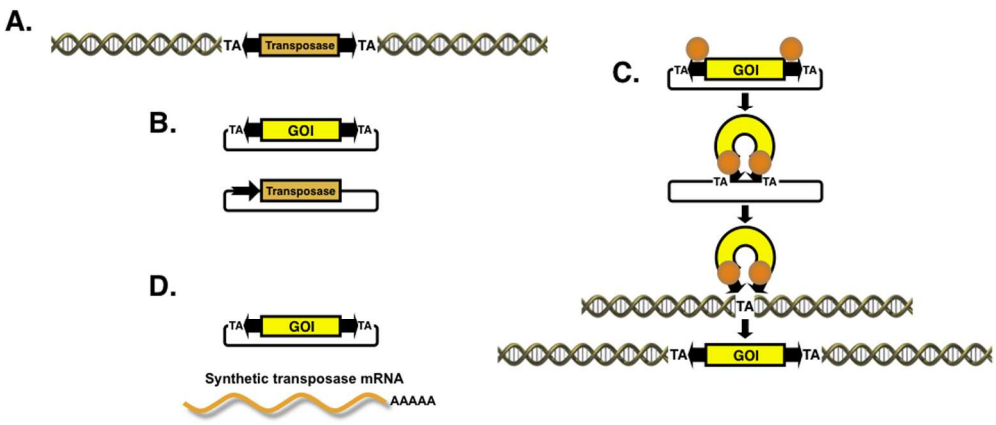

Figure 1

209x88mm (300 x 300 DPI)

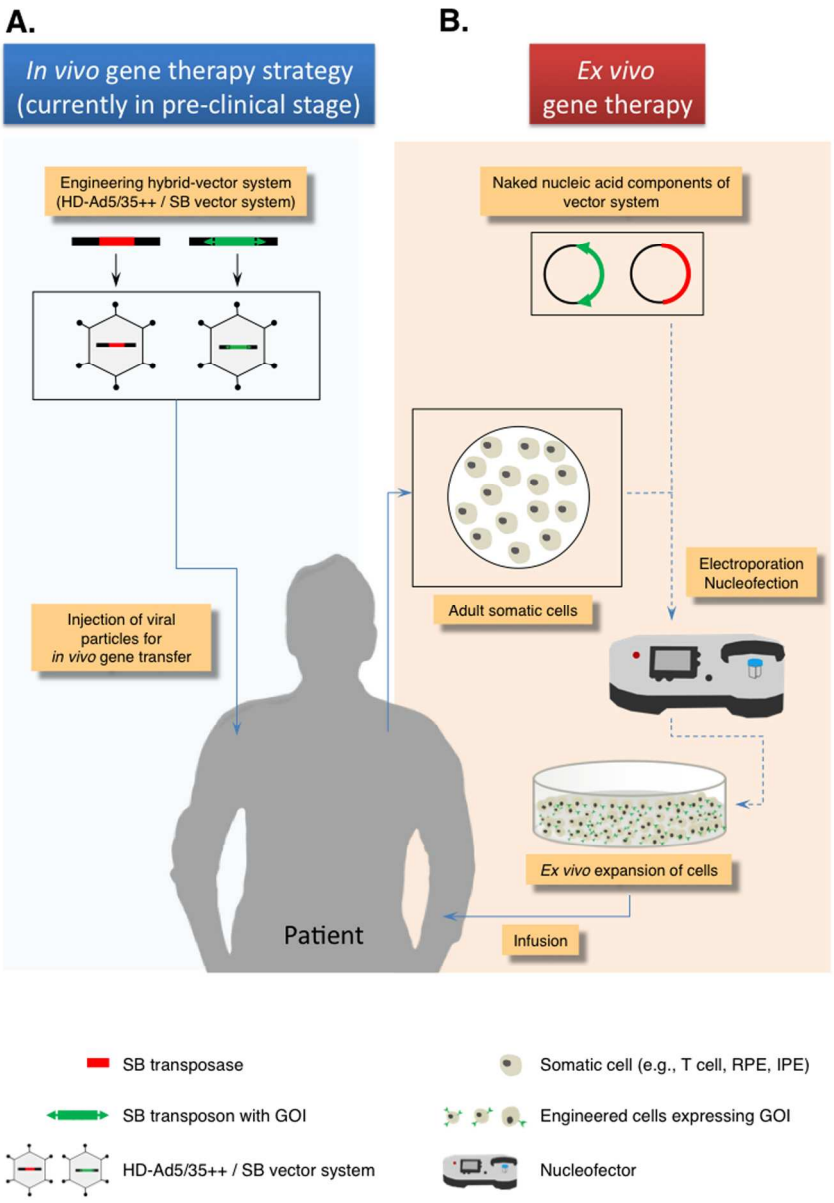

Figure 2

147x209mm (300 x 300 DPI)

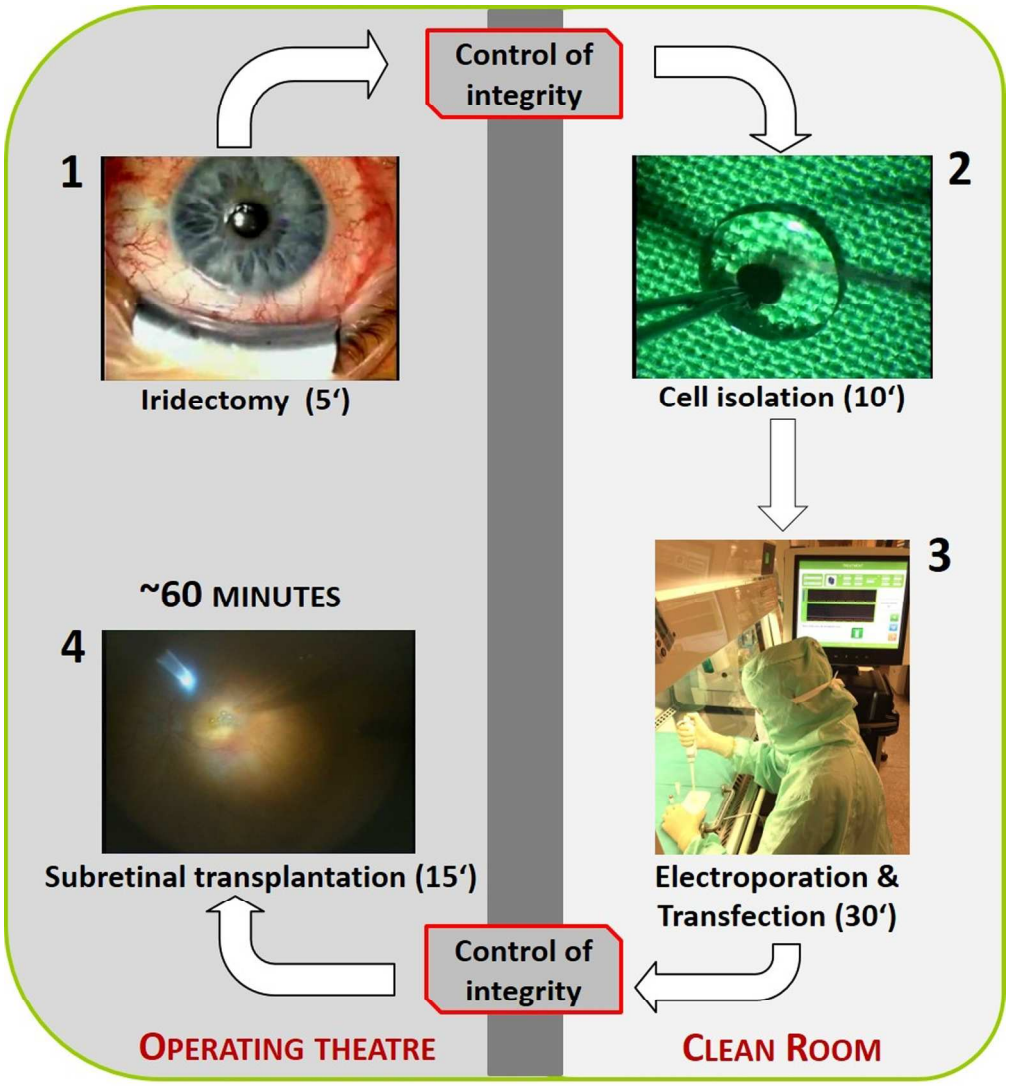

Figure 3

192x209mm (300 x 300 DPI)

Figure Legends

Figure 1. The *Sleeping Beauty* transposon system. A) Autonomous transposable elements consist of terminal inverted repeats (TIRs, black arrows) that flank the transposase gene (orange). The transposon is flanked by TA target site duplications. **B**) 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 TA site in the genome by the transposase. **D**) Plasmid-based transposon cassettes can be mobilized by transposase supplied as *in vitro*-transcribed mRNA.

Figure 2. Strategies for gene therapy. A) **Direct *in vivo* gene delivery.** A therapeutic gene vector, typically a viral vector, is directly inroduced into the patient's body. The example depicts the use of an integrating, hybrid adenovirus/SB transposon vector sytem, wherein an expression cassette producing the SB transposase (shown in red) and an SB transposon carrying a gene-of-interest (GOI) (shown in green) are packaged into helper-dependent adenoviral (HD-Ad5/35++) vectors. **B**) ***Ex vivo* gene therapy** involves isolation of somatic cells (such as T cells and RPE or IPE cells) from the patient's body (autologous cells) followed by genetic engineering by electrotransfer (e. g., nucleofection) of naked nucleic acid components of the SB vector system and reinfusion of the genetically modified cells into the patient. The example depicts transposition-mediated genetic engineering to stably express a CAR. Genetically engineered cells may or may not undergo a period of *ex vivo* expansion.

Figure 3. TargetAMD therapy protocol. Harvest (Step 1), isolation (Step 2), transfection (Step 3) and transplantation (Step 4) of autologous RPE or IPE cells will be accomplished in one surgical session. Autologous RPE or IPE cells overexpressing the antiangiogenic factor PEDF will be transplanted subretinally to deliver a life-long solution for the patient. Source: http://www.targetamd.eu/scientific-background/targetamd-surgery/.