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

Russ Hodge¹, Suneel A. Narayanavari¹, Zsuzsanna Izsvák¹ 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

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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 tale¹. 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 dinucleotides¹) leads to sustained and efficient transgene expression in preclinical animal models⁶. 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 vectors¹¹; it can deliver larger genetic cargoes¹², 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 therapy¹⁶⁻²⁰ is the *piggyBac* (PB) transposon originally isolated from the cabbage looper moth²¹.

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 binding²²⁻²⁵. In addition, the so-called “sandwich” vector architecture (containing two full-length copies of SB flanking a gene-of-interest) was developed to aid transposition of large transgenes¹².

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 SB11²⁶ 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 underway²⁷. Further improvements produced SB100X, the most hyperactive SB transposase version currently available, whose activity is ~100-fold that of the originally resurrected transposase²⁸. SB100X transposase enables highly efficient germline

transgenesis in relevant mammalian models, including mice, rats, rabbits, pigs, sheep and cattle²⁹⁻³⁴. 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), iPSCs³⁶ and T cells³⁷, 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 modeling³⁸ and crystallography³⁹ 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 plasmids⁴⁴. The first evident advantage of MC vectors over plasmids has to do with the fact that they increase the survival rates of human T cells following nucleofection⁴³. Alongside a lower cytotoxicity, stable genome modification with MCs was more efficient than with conventional plasmid vectors in T cells⁴³. 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 easier⁴⁷.

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 technologies⁴⁹, and a Phase Ia/IIb clinical trial to treat age-related macular degeneration based on this approach is planned in the near future⁵⁰ (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}, AAV⁵⁶, 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 process²⁵ 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 cells⁶¹. 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 PGBD5⁶².

To estimate the genotoxic potential of different vector types and designs, it is important to characterize the properties, which influence their selection of target sites⁶³. 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 genes⁶⁶. 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 integration⁶⁹.

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 vector⁵⁴ and the hyperactive SB100X transposase⁵⁵ (**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 mice⁷¹ and in dogs⁷³.

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 trials²⁷, 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 countries⁸⁴. 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 effects⁸⁶ 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 years⁸⁹ to as high as 71% within 24 months⁹⁰. 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 patients⁹¹⁻⁹⁴. 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 culture⁹⁵. 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 membrane⁹⁶. 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 eye⁵⁰ (**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 technologies⁵⁰.

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 therapy²⁷. 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 approach⁵⁰. 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 composition¹⁰⁹. 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}, brain¹¹⁵ 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 mRNA⁴³, followed by *in vivo* selective proliferation based on cytokine signaling¹¹⁶. 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 genes¹¹⁷⁻¹²⁰. 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 place¹²³. 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 cells¹²⁴; 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 uses¹²⁶. 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.

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AUTHOR DISCLOSURE STATEMENT

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

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

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

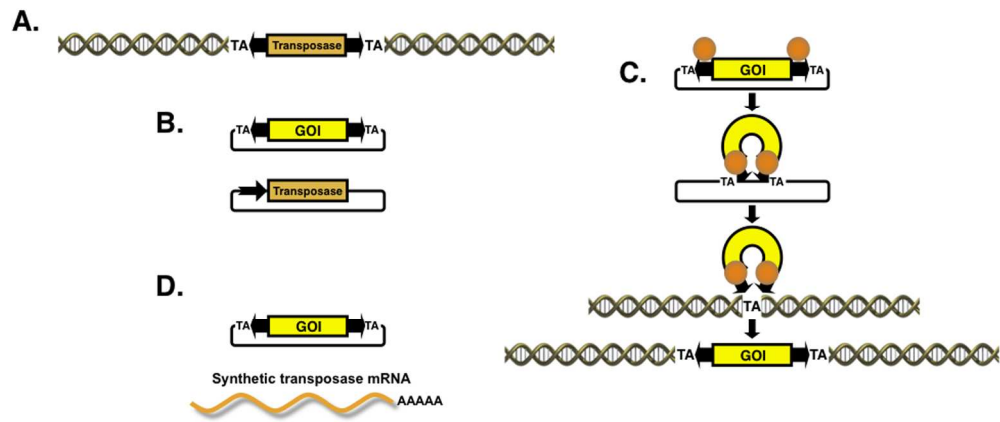


Figure 1

209x88mm (300 x 300 DPI)

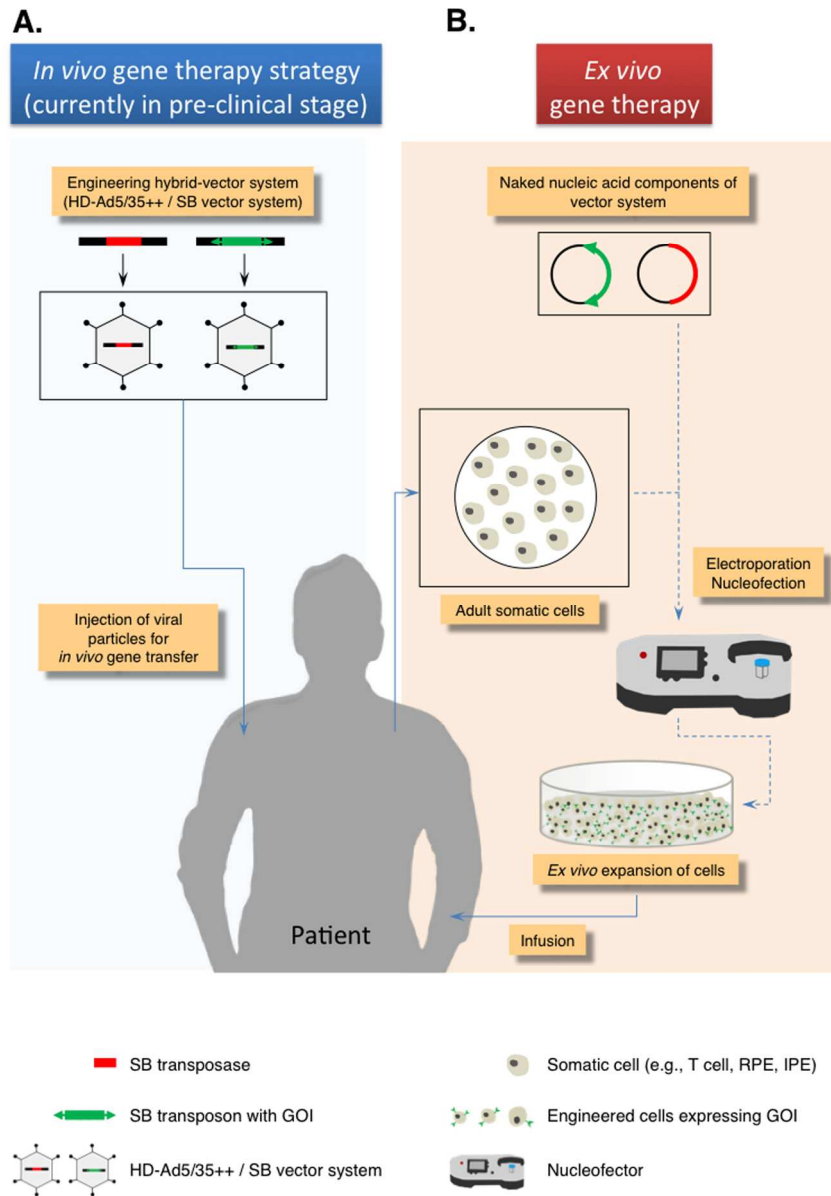


Figure 2

147x209mm (300 x 300 DPI)

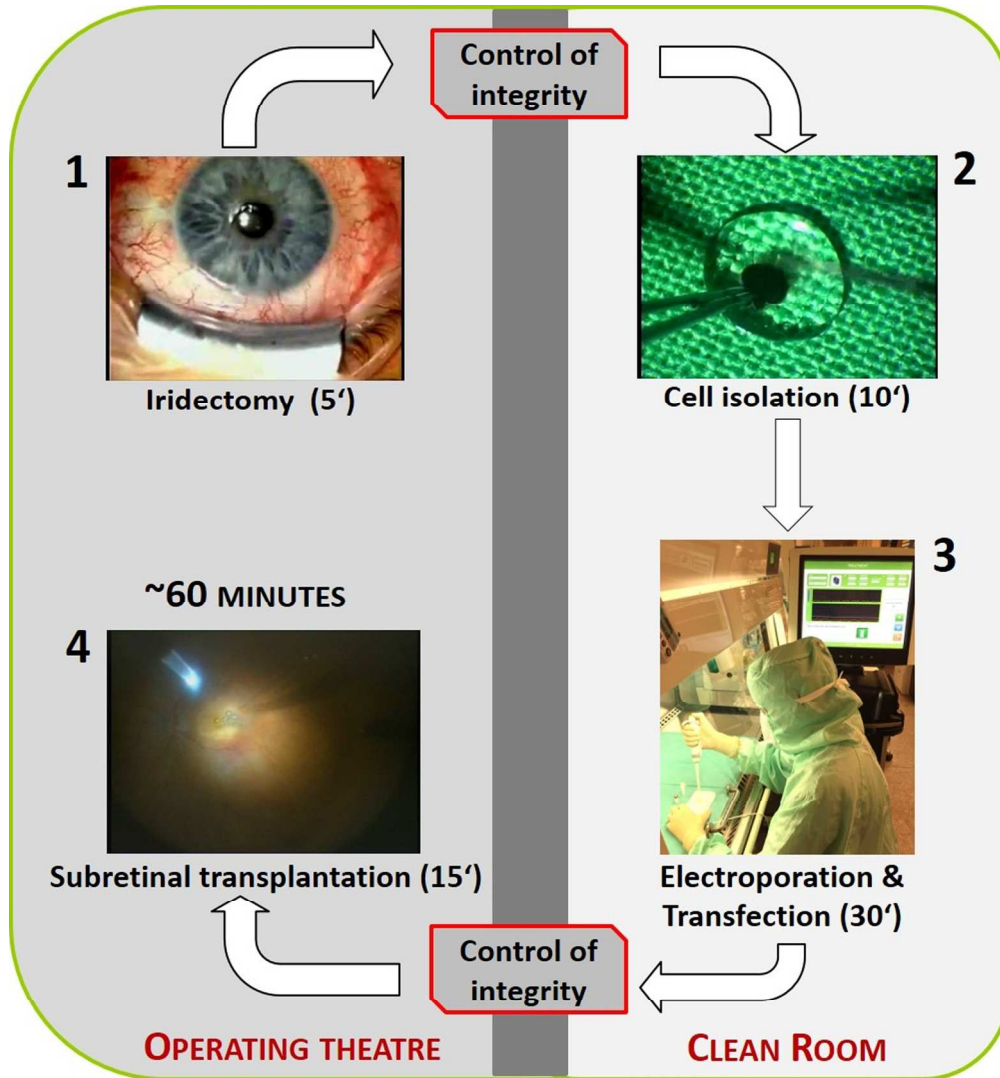


Figure 3

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 introduced into the patient's body. The example depicts the use of an integrating, hybrid adenovirus/SB transposon vector system, 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/>.