The Sleeping Beauty transposon system: a non-viral vector for gene therapy

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Over the past decade, the *Sleeping Beauty* (SB) transposon system has been developed as the leading non-viral vector for gene therapy. This vector combines the advantages of viruses and naked DNA. Here we review progress over the last 2 years in vector design, methods of delivery and safety that have supported its use in the clinic. Currently, the SB vector has been validated for *ex vivo* gene delivery to stem cells, including T-cells for the treatment of lymphoma. Progress in delivery of SB transposons to liver for treatment of various systemic diseases, such as hemophilia and mucopolysaccharidoses types I and VII, has encountered some problems, but even here progress is being made.

INTRODUCTION

As reviewed by several others in this issue, there has been considerable progress made over the past decade in developing viral vectors for human gene therapy. However, there are complications with viruses as gene-delivery vectors including their integration-site preferences that may increase chances of adverse effects (1,2), the need for extensive purification and quality control to prevent replication-competent virus and the costs associated with their production and handling (3,4). Advantages of non-viral vectors include the ease and relatively low cost of producing sufficient amounts required to meet the entire patient population, stability during storage and lack of immunogenicity once inside host cells (4–6). There are two major problems with non-viral gene therapy approaches that are almost always based on delivery and expression of genes carried on an engineered plasmid produced in *E. coli*. First, expression of the transgene in most mammalian cells is brief due to intracellular breakdown and epigenetic responses that recognize the prokaryotic origin of the plasmid. Secondly, delivery of DNA molecules into cells of a specific organ is inefficient. Because of these problems, non-viral vectors have not yet been tested for therapy of systemic inherited diseases. However, application of the *Sleeping Beauty* transposon system (SBTS) has changed this view (7). This non-viral vector, which combines the advantages of viruses and naked DNA (8), has experienced the most rapid of development from birth to application in humans of all vectors now in clinical trials (Fig. 1). The SBTS consists of two components: (i) a

transposon containing a gene-expression cassette and (ii) a source of transposase enzyme. By transposing the expression cassette from a plasmid into the genome, sustained transcription of a transgene can be achieved (Fig. 2).

The power of the SB system to treat disease models was first demonstrated by Yant *et al.* (9), who showed sustained expression of α1-antitrypsin in normal mice and of clotting Factor IX (FIX) in FIX-deficient hemophilic mice. This achievement was followed by successful treatment of other mouse models of genetic disease, including inherited tyrosinemia (10), hemophilia A (11–13) and mucopolysaccharidosis (MPS) types I and VII (14,15). In addition, SB transposons have been used to treat epidermolysis bullosa (16), glioblastoma multiforme (17), sickle cell anemia (18) and B-cell lymphoma (19–21). In rats, SB transposons have been used to treat pulmonary hypertension (22) and jaundice (23).

Thus, early proof-of-principle studies stressing efficacy of sustained gene expression have demonstrated the significant potential of the SBTS for gene therapy. Other important considerations with respect to gene therapy include efficacy in larger animals and safety. Here, we review these issues and recent progress in turning the SBTS from a gene transfer vector into a gene therapy vector that can be used in the clinic during the era of personalized medicine.

Improvements have involved all aspects of the SBTS that are necessary for its use in the clinic (Fig. 3). These include (i) efficacy—transpositional efficiency and engineered cassettes to achieve appropriate expression of therapeutic or

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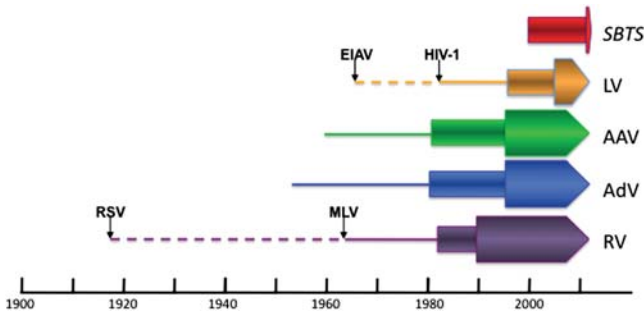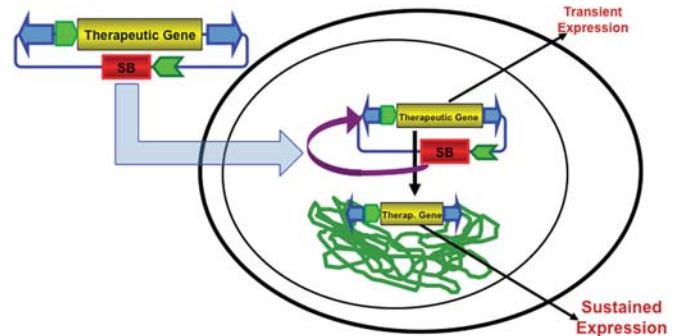
Figure 1. History of some vectors used for human gene therapy. For each type of vector, the lines indicate the period between its discovery and its use for gene transfer (in the case of retroviruses and lentiviruses, the initial virus discovered and the virus actually used for gene therapy are indicated by dashed and solid lines, respectively). Rectangles show the period of use for each vector as a gene transfer agent and the arrowheads indicate its use in humans. SB transposons were synthetically made and therefore validated by their use for gene transfer into cultured cells. Hence, they have no associated line; the first clinical trial has been initiated. LV, lentiviruses; AAV, adeno-associated viruses; AdV, adenoviruses; RV, retroviruses.


Figure 2. SB transposon-mediated gene transfer into chromosomal DNA for long-term expression of a therapeutic gene. An SB transposon (the inverted arrowheads) in a plasmid provides only transient expression of a transgene from a promoter unless transposed into a host genome. There are several methods for delivery of the transposon system into a cell based on whether the cell is in culture (electroporation or transfection) or in a tissue (e.g. hydrodynamic injection). In most studies, the source of the SB transposase is a gene on either the same (shown here) or a different plasmid as that harboring the transposon.

reporter genes, as well as SB transposase; (ii) delivery—methods and routes of plasmid delivery as well as cell-culture conditions used for *ex vivo* gene transfer to therapeutic target cells and (iii) safety—the integration preferences of SB transposase and duration of the transposition reaction in transduced cells. Along the way, we have gained insights into the effect of genetic background on transposition, targeting tissues, gender effects on gene expression and transposon dose–response. Some of these issues are specific to the SBTS (e.g. over-production inhibition), while others are associated with all non-viral vectors (e.g. efficiency of delivery), with integrating vectors in general (e.g. insertional mutagenesis and post-integrative promoter silencing), or with any type of gene therapy vector (e.g. host immune responses to transgenes and/or their products).

EFFICACY: IMPROVEMENT OF THE SBTS

The issue of low transposition levels has been addressed over the past decade by modifying the transposon (24,25) and the transposase. Hyperactive transposases have been derived from the original SB10 by mutagenesis of the catalytic and DNA-binding domains (26–29). SB100X, engineered using a combination of *in vitro* molecular evolution and selection (30), is the most active of the transposases generated so far, and was named molecule of the year for 2009 (http://iscspm. webnode.com/). This powerful transposase directs the highest levels of transposon integration yet demonstrated, presumably due to increased stability (30,31).

Appropriate expression cassettes are critical for attaining high and sustained levels of transgene expression in a target organ such as the liver. Tissue-specific transcriptional regulators are thought to help counteract host immune responses (32) by preventing transgene expression in antigen-presenting cells. Accordingly, the choice of transcriptional motifs for driving both therapeutic transgene and transposase expression should be considered to reduce or prevent silencing of the therapeutic transgene, allow expression of the transposase for only a very

short period of time while avoiding over-production inhibition of transposition that can occur at excessive transposase levels (26,33). There are multiple implications of improvements in the expression cassette. If the goal is to achieve strong, long-term expression of the transgene, promoter strength is not the only issue to consider. Gender can influence gene expression from some promoters, as we unexpectedly found in experiments using the CAGS (sometimes called CAGGS) promoter to drive human α-L-iduronidase (IDUA): males expressed transgenic IDUA at 30–50-fold higher levels than females (15). On the other hand, promoter silencing can be usefully employed in the SBTS; the cytomegalovirus early promoter is shut down within a few days in many mammalian cells including liver (34) and therefore is a good choice for transient expression of SB transposase to prohibit sustained re-mobilization of transposons after their integration into the genome (35).

Immune responses often curtail gene expression (32). Host immune responses can be expected, particularly when there is over-expression of a secretable transgenic protein. For example, after hydrodynamic tail vein injection 99% of the transgene expression is observed in the liver (15,36). However, antigen-presenting cells both in the liver and in the spleen may express the foreign protein. Substituting a hepatocyte-specific promoter, such as ApoEhAAT (37,38), for the ubiquitously expressed mini (m)CAGGS promoter to regulate IDUA resulted in prolongation of IDUA expression as well as a lack of significant gender bias in C57BL/6 mice (39). Substitution of the SB100X transposase (30) for SB11, in combination with the liver-specific promoter to drive IDUA expression and transient immunosuppression with cyclophosphamide resulted in significantly prolonged expression of transgenic IDUA at levels $\sim$100-fold higher than the endogenous level, which is therapeutically relevant for treating MPS I (40).

In addition to regulating desired levels of transgene expression, different promoters have been found to demonstrate unexpected features. For example, the CAGGS promoter yielded an exceptionally high level of eGFP expression
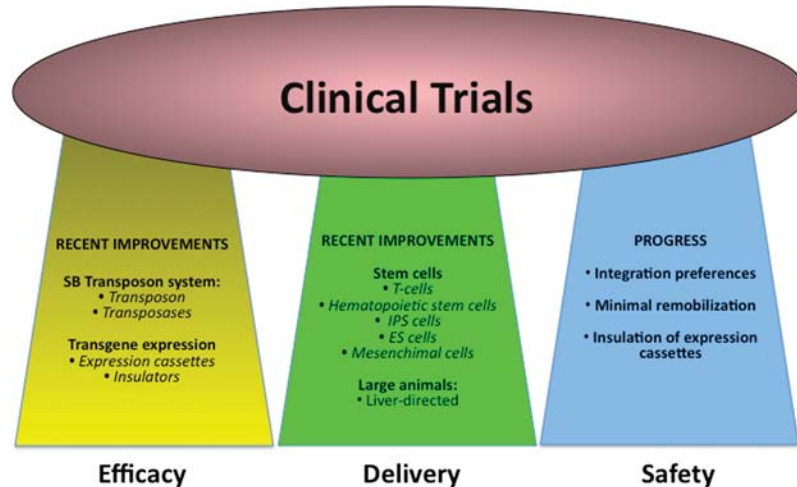
Figure 3. Success in clinical trials depends on three aspects of a vector such as its efficacy, its delivery and its safety. Recent developments in each of these areas support the application of the SBTS in clinical trials.

specifically in differentiated cardiomyocytes following SB-mediated gene transfer to human embryonic stem cells (41). Moreover, in stem cells, the transcriptional motifs may not be equally active following differentiation (42,43).

DELIVERY *IN VIVO* TO THE LIVER AND *EX VIVO* TO HEMATOPOIETIC CELLS

Although a variety of different DNA-conjugating materials and delivery vehicles have been explored for the purpose of promoting non-viral gene transfer into different animal tissues, the most effective method to be developed is the hydrodynamics-based procedure (44–46), which targets the liver for sustained gene expression. At present, this is the most efficient method of plasmid delivery, when a liver-directed approach is an option for therapy. The hydrodynamics-based procedure has successfully been adapted to rats (47). We have shown that with this method, transgene expression 2 weeks following hydrodynamic delivery to mouse liver is 100–1000-fold higher than after delivery using polyethylenimine–DNA conjugate (36,48). If the transgene expresses a secretable protein at a high level, a low percentage of genetically modified cells can provide therapeutic levels of gene product sufficient to treat an entire animal.

Based on its success in treating mouse models of hemophilia (9,11,12), we used this approach in gene therapy studies of the lysosomal storage diseases (14,15). Using the improved pT2 transposon and hyperactive transposase SB11, we demonstrated adequate delivery of therapeutic transgenes to mouse liver for treatment of MPS I and VII, systemic metabolic diseases caused by deficiency of the lysosomal enzymes IDUA and β-glucuronidase, respectively. In our experience, the efficiency of hydrodynamic delivery in mice is ∼10% of that reported for retroviral delivery (14,49). In the case of MPS I, sustained IDUA activity in the liver after hydrodynamic infusion was ∼100 times higher than wild-type levels, which was necessary to restore enzymatic activity in other organs by enzymatic cross-correction. IDUA levels and sustainability of enzymatic activity were comparable with those observed with viral vectors and, as a consequence, several

clinical manifestations of the disease, including skeletal malformations, were significantly ameliorated (15).

When adapting the hydrodynamic delivery method to larger animal models, several parameters must be considered: DNA dose, injection volume, rate of injection and species-specific variations in the anatomy of the target organ. From the outset, it was clear that in animals larger than 1 kg, hydrodynamic infusion would have to be limited to an isolated organ or tissue region because injections of >100 ml (10% of the animal's weight) in <10 s would not be practical. Catheter-mediated infusions to liver and other tissues, often employing balloons to limit direct hydrodynamic effects, have been conducted in larger animals, including rabbits (50,51), swine (52–59) and dogs (60,61,62,63). Although we have recently been able to achieve extended expression after hydrodynamic injection of SB transposons into dog liver using balloon catheters (64), reporter gene (canine secreted alkaline phosphatase) expression nonetheless was extinguished after 6 weeks. In mice, we have observed more effective expression when the DNA solution is injected with greater rapidity. Based on these studies, we have hypothesized that *impulse*, rather than pressure, may be the critical determinant for effectiveness of hydrodynamic delivery (65); this has several implications in the details of hydrodynamic delivery in larger animals.

An alternative delivery approach that combines the power of viruses to penetrate cellular membranes and SB transposons to deliver defined expression cassettes more randomly in the genome has been developed using chimeric viruses containing SB transposons. Chimeras include lentivirus/SB (66–68), adenovirus/SB (69) and herpes simplex virus/SB (70). A hybrid adenovirus/SB vector has been used for canine FIX gene delivery to a hemophilia B dog (69). However, the benefit provided by the hybrid in terms of delivery must be weighed against the loss of simplicity, low cost of production, long and stable shelf life, as well as other aspects involved with storage and quality control of purified DNA compared with virus preparations.

A primary target for integrative gene transfer has been hematopoietic stem cells (HSC), for which retroviruses, and more recently lentiviruses, have been the vector of choice

for *ex vivo* gene transfer (71). Gene transfer into HSC requires stable integration, and the advent of hyperactive SB transposases in combination with efficient plasmid DNA loading by electroporation has resulted in effective transposition of primary human cord blood CD34$^+$ cells that can be engrafted with the potential for differentiation into multiple cell lineages *in vivo* for non-viral gene therapy of blood disorders (43,72).

The most advanced use of the SBTS for gene therapy has been to transduce progenitor T-cells *ex vivo* to treat lymphoma. Retroviruses, including lentiviruses and spumaviruses, have been the preferred vector of choice for *ex vivo* gene transfer. Until recently, any non-viral gene therapy approach, including *ex vivo* transfer of the SBTS to stem cells, was hampered by low efficacy and/or high toxicity of transfection, lipofection or electroporation in combination with inefficient gene transfer catalyzed by the original SB10 transposase. However, non-viral, *ex vivo* gene delivery is now feasible with improved transposases and expression cassettes, refined methods of electroporation of appropriate ratios of transposon-to-transposase genes and advanced cell-culture conditions. As a result, genes can be introduced into primary human CD34$^+$ cells to obtain populations of stem cells that can be engrafted and differentiated into multilineage cell types *in vivo* for non-viral gene therapy of HSC (43,72). Stable transposition has been achieved in induced pluripotent stem cells (iPS), which retained their ability to differentiate along neural, cardiac and hepatic lineages without causing cytogenetic abnormalities (42). Efficient SB-mediated integration and expression in human embryonic stem cells has been achieved while preserving both the undifferentiated state and the potential to be directed down specific pathways of differentiation (41,73). The SBTS was employed to insert T-cell receptor genes into the genomes of peripheral blood lymphocytes for targeting against antigens presented on melanoma cells. Transgene expression and anti-tumor activity were comparable with those attained by γ-retroviral transduction in a clinical trial (74).

The first clinical application of the SBTS will be to provide a new specificity for T-cells to treat B-lineage malignancies (6). The SBTS has been used effectively to generate T cells that express a chimeric antigen receptor recognizing CD19, a lineage-specific tumor antigen (19,20). When the genetically altered T-cells are expanded on CD19$^+$ artificial antigen-presenting cells, there is a rapid outgrowth of CD4$^+$ and CD8$^+$ T-cells that express the anti-tumor chimeric antigen receptor. An early-phase human trial has been approved to assess the safety and feasibility of this approach.

SAFETY

There are several safety issues facing the SBTS for gene therapy. The first is common to all integrating vectors, insertional mutagenesis. The SBTS has the most random integration preference of the vectors currently in use for gene therapy (2,75,76). Loci into which the transposon integrates can influence the expression of the gene it carries, often leading to transcriptional silencing (77–79). Likewise, there is the possibility that the promoters in the transgenic expression cassette will influence genes in the vicinity of the integration site. Accordingly, some SB transposons have been engineered to contain expression cassette flanked by insulators such as the cHS4 locus control region. This results in a significant increase in stable transfection rates *in vitro* (80) as well as theoretical prevention of accidental transactivation of genes residing close to the transposon insertion site (81).

A second safety issue is the presence of the SB transposase gene and the potential for remobilization of transposons already sited in the recipient genome. We have found that by using a promoter that is active only over a few days, the chances of remobilization are below minimal detection (35). Moreover, were any transposon to remobilize, it would not have any increased probability of causing an adverse event per insertion than the original integration. Nevertheless, these two aspects represent fields for future development, such as the use of RNA as a source of SB transposase (82,83).

Increasing safety of the SBTS by targeting 'safe havens' in the genome is a prime future goal. The first attempts to combine the targeted integration ability of zinc fingers with SB transposons consisted of adding a zinc finger DNA-binding domain to SB transposase (84,85). These approaches resulted in diminished SB activity and marginal targeting. However, other strategies remain untested and represent one of the most important future directions to pursue.

CONCLUDING REMARKS

Several other DNA integrase/transposon systems providing efficient integration in mouse and human genomes have been developed during the past decade. One of these, the *piggyBac* (PB) transposon of insect origin (86), has shown promise for regenerative medicine as a tool for reprogramming iPS (87). Side-by-side comparison of SB and PB (31) showed that the SBTS has several advantages for human gene therapy application. SB100X has superior transduction rates for human hematopoietic cells (31,72). The near-random integration profile of SB would seem safer than that of PB, which tends to target genes and their upstream regulatory regions (88,89). Moreover, SB vectors have negligible inherent enhancer/promoter activity compared with PB transposons (8). However, PB is less sensitive than SB to over-production inhibition (31), which suggests greater care must be taken with the SBTS to achieve optimal rates of gene transfer. In contrast to SB and PB transposons, which integrate throughout the genome, the gene transfer system derived from phiC31 bacteriophage has an advantage of semi-targeted integration (90). However, phiC31-mediated recombination is not always precise and is associated with chromosomal rearrangements (91–93).

In summary, although the efficiency of naked DNA delivery is sometimes an issue, the SBTS has particular advantages for treatment of large populations of patients in terms of its relatively low cost and near-random integration profile, especially for treatment of lymphoma by modification of T-stem cells. However, there remain significant hurdles for the SBTS, primarily efficient delivery and maintenance of gene expression in the liver, before it will be an efficacious vector to treat hemophilia and select metabolic diseases.

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