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## Going non-viral: the *Sleeping Beauty* transposon system breaks on through to the clinical side

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### ABSTRACT

Molecular medicine has entered a high-tech age that provides curative treatments of complex genetic diseases through genetically engineered cellular medicinal products. Their clinical implementation requires the ability to stably integrate genetic information through gene transfer vectors in a safe, effective and economically viable manner. The latest generation of *Sleeping Beauty* (SB) transposon vectors fulfills these requirements, and may overcome limitations associated with viral gene transfer vectors and transient non-viral gene delivery approaches that are prevalent in ongoing pre-clinical and translational research. The SB system enables high-level stable gene transfer and sustained transgene expression in multiple primary human somatic cell types, thereby representing a highly attractive gene transfer strategy for clinical use. Here we review several recent refinements of the system, including the development of optimized transposons and hyperactive SB variants, the vectorization of transposase and transposon as mRNA and DNA minicircles (MCs) to enhance performance and facilitate vector production, as well as a detailed understanding of SB's genomic integration and biosafety features. This review also provides a perspective on the regulatory framework for clinical trials of gene delivery with SB, and illustrates the path to successful clinical implementation by using, as examples, gene therapy for age-related macular degeneration (AMD) and the engineering of chimeric antigen receptor (CAR)-modified T cells in cancer immunotherapy.

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chimeric antigen receptor;  
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### Introduction: vectors in gene therapy and genetic engineering

The ability to deliver natural or synthetic genes into human somatic cell types provides the technological basis for gene therapy to treat inherited and acquired genetic diseases, and enables genetic engineering of cellular medicinal products that are endowed with novel properties and functions for use as diagnostic and therapeutic tools in medicine. The vast majority (~70%) of gene delivery systems used in ongoing clinical trials of gene therapy are based on viral vectors, and only a small proportion of trials utilizes non-viral delivery systems, mainly non-integrating naked plasmid DNA, that only provide transient gene expression (Gene Therapy Clinical Trials Worldwide, <http://www.abedia.com/wiley/vectors.php> (Figure 1(A,B)).

This article will focus on non-viral stable gene transfer through the *Sleeping Beauty* (SB) transposon as a viable and ready-to-use alternative to viral gene transfer vectors. We will review basic principles, and feature the key scientific and technologic advances that have been made to position SB transposition as a preferred gene delivery system in translational and clinical medicine. The conceptual appeal of SB transposition for gene delivery is illustrated by examples of clinical applications in gene therapy and genetic engineering, and complemented by a concise review of regulatory aspects for clinical implementation.

### Viral gene transfer vectors

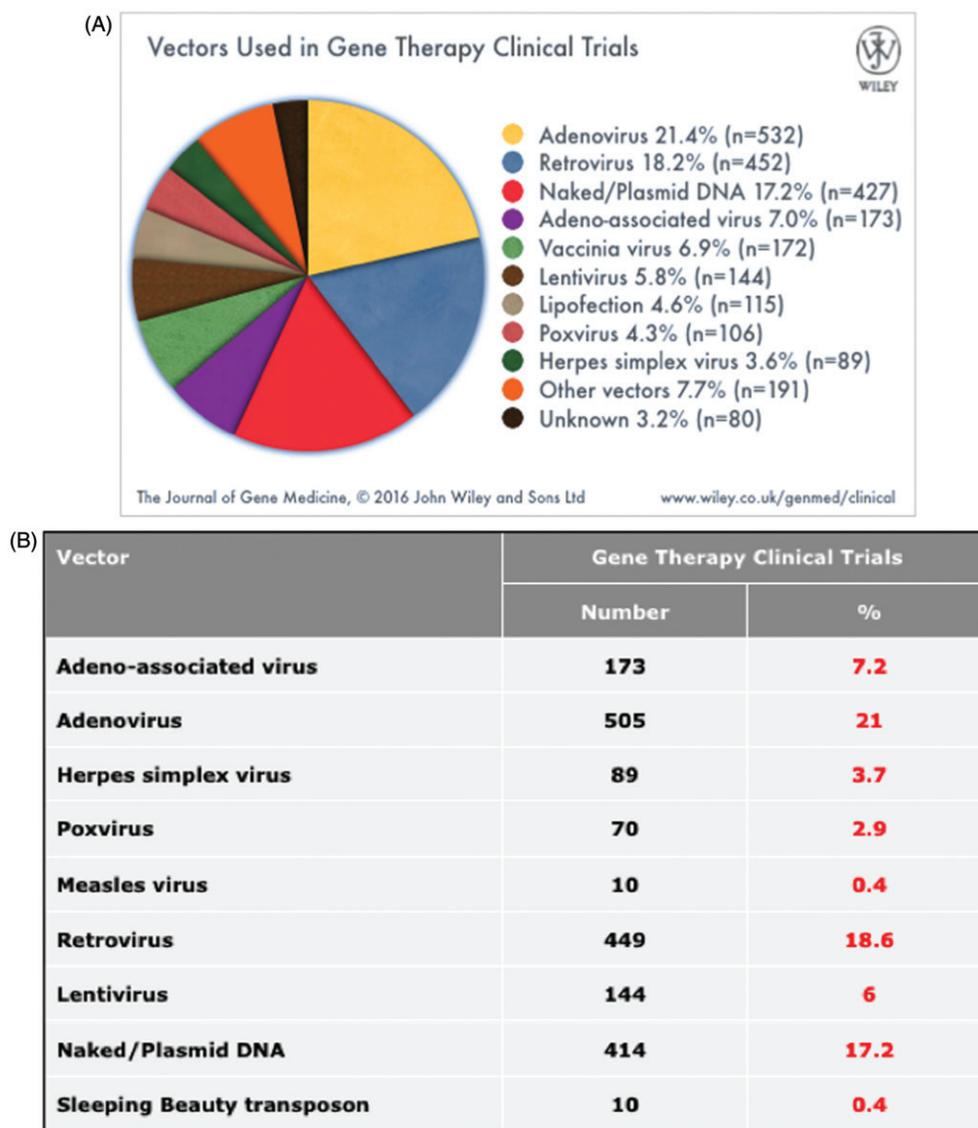
Viral vector systems are attractive for gene delivery, because viruses have evolved the ability to cross

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**Figure 1.** Viral and non-viral vectors in currently running gene therapy clinical trials. A) Percent contribution of different vector systems of currently running clinical trials. B) List of some of the most popular vector systems employed in clinical trials. The list also contains the currently running trials with the *Sleeping Beauty* transposon (see colour version of this figure at [www.tandfonline.com/ibmg](http://www.tandfonline.com/ibmg)).

through cellular membranes by infection, thereby delivering nucleic acids to target cells. However, some viral vectors, including those derived from adenoviruses or adeno-associated viruses (AAVs), are not equipped for chromosomal integration, and thus remain largely episomal. Especially in cycling cells, episomal DNA is gradually lost requiring re-administration *in vivo* in order to sustain a desired level of transgene expression over time. However, repeated delivery can provoke immune responses against vector-encoded proteins [reviewed in (Hartman *et al.*, 2008)], which can manifest as loss of the desired therapeutic effect and potentially therapy-related adverse events. In contrast, retroviral vectors integrate their therapeutic cargo into the genome, and thus have the potential to confer long-term transgene

expression. Indeed, hematopoietic stem cell (HSC)-based gene therapy with integrating viral vectors has clearly provided therapeutic benefit in primary immunodeficiencies (including SCID-X1, ADA-SCID), thalassemia and leukodystrophies (Aiuti *et al.*, 2013a; Biffi *et al.*, 2013; Cartier *et al.*, 2009; Cavazzana-Calvo *et al.*, 2010). A concern with retroviral vectors is that chromosomal integration may be associated with genotoxicity and mutagenic effects elicited by insertion of the vector into or near proto-oncogenes (Baum *et al.*, 2004; Deichmann *et al.*, 2007; Hacein-Bey-Abina *et al.*, 2003; Hacein-Bey-Abina *et al.*, 2008; Kustikova *et al.*, 2005). Such risk is especially pronounced with gammaretroviral vectors based on the murine leukemia virus (MLV) that preferentially integrate into transcriptional regulatory

elements of active genes (Cattoglio *et al.*, 2010; Cavazza *et al.*, 2013; De Ravin *et al.*, 2014); in fact, severe adverse events associated with vector integration have been observed in clinical trials for SCID-X1 (Hacein-Bey-Abina *et al.*, 2003; Hacein-Bey-Abina *et al.*, 2008; Howe *et al.*, 2008; Thrasher *et al.*, 2006), X-CGD (Ott *et al.*, 2006) and WAS (Braun *et al.*, 2014).

Furthermore, HIV-derived lentiviral vectors are potential mutagens due to their biased insertion into transcription units (Cavazza *et al.*, 2013). Although lentiviral vectors appear to be safer than gammaretroviral vectors in gene therapy, recent studies indicate that some HIV integrations into genes associated with cancer or cell cycle regulation may confer a survival advantage of HIV-infected cells, and thus a clonal imbalance of HIV integrations in AIDS patients (Maldarelli *et al.*, 2014; Wagner *et al.*, 2014). A potential technical hurdle that can set limitations to vector design for clinical use of retroviral vectors is that large transgenes may inhibit viral reverse transcription and packaging. Finally, the high costs associated with manufacture of clinical-grade retroviral vector batches seem incompatible with their use as gene delivery systems in large patient cohorts in routine medical practice.

As a result, significant efforts have been made to craft novel gene transfer vectors that exceed the qualities of currently available viral vectors in: i) efficacy: i.e. achieve high-level stable gene transfer at low toxicity to the host cell; ii) safety: i.e. induce low levels of genotoxicity and possess a “safe” integration profile, with a high proportion of integrations into genomic safe harbors (GSHs); iii) economic viability: i.e. are associated with acceptable cost per treatment, and scalable/exportable vector production to serve large numbers of patients.

### Non-viral gene transfer vectors

Non-viral vector systems, including nucleic acid vectors such as plasmid DNA, generally suffer from inefficient cellular delivery, pronounced cellular toxicity and limited duration of transgene expression due to the lack of genomic insertion and resulting degradation and/or dilution of the vector in transfected cell populations. Recent developments of non-viral delivery techniques, including liposomal formulations, nanoparticles, advanced electroporation methods such as nucleofection and cell-penetrating peptides can significantly enhance transfer of nucleic acids into therapeutically relevant cell types. However, just like non-integrating viral systems, non-viral vectors do not provide long-term nuclear maintenance and transgene expression in dividing cell types such as stem cells. One class of non-

viral vector system that unites favorable characteristics of integrating viral vectors (i.e. stable chromosomal integration and long-lasting transgene expression) with those of non-viral delivery systems (i.e. lower immunogenicity, enhanced safety profile and reduced costs of GMP manufacture) is transposon-based gene delivery systems.

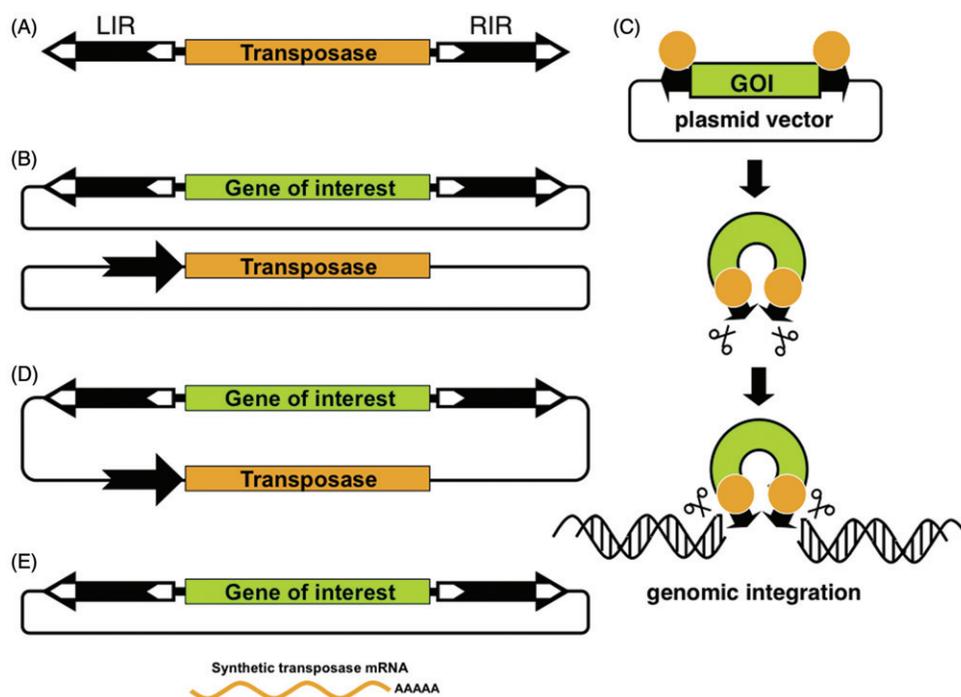
## Non-viral gene transfer using the *Sleeping Beauty* transposon

### *Jumping genes: transposon basics*

DNA transposons are genetic elements with the ability to change their positions within the genome. The prevalent mode of transposition in the genome is *via* a cut-and-paste mechanism. In nature, these elements exist as mobile (“jumping”) units of DNA containing a transposase gene flanked by terminal inverted repeats (TIRs) that carry transposase binding sites (Figure 2(A)). Importantly, it is possible to separate the two functional components of the transposon (the TIRs and the transposase) in the form of bi-component vector systems. Transposon-based vectors enable incorporation of virtually any DNA sequence of interest between the transposon TIRs and mobilization by *trans*-supplementing the transposase in the form of an expression plasmid (Figure 2(B)), very much like non-autonomous viral vectors are produced in packaging cell lines. In the transposition process, the transposase enzyme mediates the excision of the element from its donor plasmid, followed by reintegration of the transposon into a chromosomal locus (Figure 2(C)). This feature makes transposons natural and easily controllable DNA delivery vehicles that can be used as tools for versatile applications in genetic engineering, including gene therapy.

### *Discovery of the Sleeping Beauty transposon*

Based on ancient, inactive transposon sequences isolated from fish genomes, an active transposon was reconstructed, and named SB after the Grimm brothers’ famous fairy tale, because it was literally awakened after a long evolutionary “sleep” (Ivics *et al.*, 1997). SB was the first transposon ever shown capable of efficient transposition in vertebrate cells, thereby enabling new avenues for genetic engineering, including gene therapy [reviewed in (Boehme *et al.*, 2015; Hackett *et al.*, 2010; 2011; Ivics *et al.*, 2009; Izsvak *et al.*, 2010; Ivics & Izsvak, 2010, 2011; Narayanavari *et al.*, 2016; Vandendriessche *et al.*, 2009)].



**Figure 2.** The *Sleeping Beauty* transposon system. A) Autonomous transposable elements consist of terminal inverted repeats (LIR = left inverted repeat; RIR = right inverted repeat, black arrows) that flank the transposase gene (yellow). B) Bi-component, *trans*-arrangement transposon vector system for delivering transgenes that are maintained in plasmids. One component contains a gene of interest (green) 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 gene of interest (GOI) is excised from the donor plasmid and is integrated at a chromosomal site by the transposase. D) One-vector or *cis*-arrangement, in which the transposase expression cassette and the GOI are located on the same plasmid. E) Plasmid-based transposon cassettes can be mobilized by transposase supplied as mRNA (see colour version of this figure at [www.tandfonline.com/ibmg](http://www.tandfonline.com/ibmg)).

The advantage of SB transposon-based gene delivery is that it combines the favorable features of viral vectors with those of naked DNA molecules. Namely, owing to permanent genomic insertion of transgene constructs (Figure 2(C)), transposition-mediated gene delivery can lead to sustained and efficient transgene expression in preclinical animal models (Hackett *et al.*, 2010). However, in contrast to viral vectors, transposon vectors can be maintained and propagated as plasmid DNA (Figure 2), which makes them simple and inexpensive to manufacture, an important consideration for implementation and scale-up in clinical practice. Further advantages of SB as gene-transfer system compared to viral vectors include its lower immunogenicity *in vivo* (Yant *et al.*, 2000), the greater capacity for genetic cargo (Zayed *et al.*, 2004) and its superior biosafety profile (Ivics *et al.*, 2007; Moldt *et al.*, 2007; Vandendriessche *et al.*, 2009; Walisko *et al.*, 2008). Because transposition proceeds through a cut-and-paste mechanism that only involves DNA, transposon vectors are not prone to incorporating mutations by reverse transcription [that are generated in retroviral stocks at reasonable frequencies (Menendez-Arias, 2009)], and can tolerate larger and more complex transgenes.

### Performance facts of *Sleeping Beauty* transposition

#### Cargo capacity

There is an inverse correlation between the size of the SB transposon unit and the efficiency at which transposition takes place (Fischer *et al.*, 1999; Izsvák *et al.*, 2000; Karsi *et al.*, 2001; Lampe *et al.*, 1998), and such effect was also observed with the insect *piggyBac* (PB) transposon (Wang *et al.*, 2014) and with the bacterial transposons IS1 (Chandler *et al.*, 1982) and Tn10 (Morisato *et al.*, 1983). There are at least two molecular mechanisms that can explain this phenomenon. First, in order to form a catalytically primed transposon/transposase complex (also called transpososome), the two ends of the transposon must be held together in close physical proximity by transposase molecules in a complex often called a synaptic complex or a paired-end complex (PEC) (Ivics & Izsvák, 2015). Formation of the PEC is a strict requirement for transposon excision (Wang *et al.*, 2016b). It is conceivable that there might be a limitation in PEC formation in case the transposon ends are far apart, which can manifest in less efficient transposition

of larger transposons. A strategy to partially rescue transposition of large transgene constructs is to shorten the length of DNA that connects the transposon ends *outside* the transposon (Izsvák *et al.*, 2000). In case of a plasmid vector carrying the transposon unit, this means shortening the plasmid backbone. By placing the transposon TIRs next to one another, bacterial artificial chromosomes (BACs) over 100 kb in length have been demonstrated to transpose at reasonable efficiencies in human ES cells (Rostovskaya *et al.*, 2012; Rostovskaya *et al.*, 2013). The second mechanism limiting transposition of large constructs is a suicidal transpositional mechanism called autointegration (Wang *et al.*, 2014). Autointegration means that following excision the transposon ends attack a TA dinucleotide *inside* the transposon, as opposed to a TA site in the target genome. This will abort the reaction and effectively kill the transposing element. Since larger transposons contain more potential target sites, they could be particularly attractive targets for autointegration. Indeed, increasing size was found to sensitize SB transposition for autointegration (Wang *et al.*, 2014).

Although larger transposons are expected to transpose less efficiently, there does not seem to exist an absolute upper size limit for transposition, or at least this limit has not been experimentally reached. This is in sharp contrast to popular viral gene delivery systems, where packaging into infectious viral particles sets a strict limitation to the size of cargo to be incorporated. Indeed, recombinant vectors based on AAV are not competent to package vector genomes >5 kb (Hirsch *et al.*, 2016; Salganik *et al.*, 2015), and both retroviral and lentiviral vectors undergo a severe loss of titer beyond a vector size of ~8–10 kb (Matrai *et al.*, 2010; Sinn *et al.*, 2005).

### Transgene expression level and stability

Any transgene vector system should ideally provide long-term expression of transgenes. By using classical, plasmid-based, non-viral delivery approaches, expression from the extrachromosomal plasmid rapidly declines following delivery. Transgenes delivered by non-viral approaches often form long, repeated arrays (concatemers) that are targets for transcriptional silencing by heterochromatin formation. In addition, long-term expression of transgenes delivered by retroviruses has been shown to be compromised by transcriptional silencing (Jahner *et al.*, 1982; Pannell & Ellis, 2001). It was shown that the zinc finger protein ZFP809 bridges the integrated proviral MLV genome and the tripartite motif-containing 28 transcriptional co-repressor in embryonic stem cells (Wolf & Goff, 2009). Thus,

sequence elements in the vector itself can predispose the cargo for silencing. Cut-and-paste DNA transposition results in a single copy of the transgene per insertion locus; thus, concatemer-induced gene silencing is by definition a non-issue. Indeed, Grabundzija *et al.* found that transposon insertions delivered by the SB system only rarely (<2% of all insertions) undergo silencing in HeLa cells (Grabundzija *et al.*, 2010), indicating that it is unlikely that certain sequence motifs in the transposon vector are recognized by mediators of silencing in the cell.

An additional factor that may provoke transgene silencing is the cargo DNA, particularly the type of promoter used to drive expression of the gene of interest. Indeed, it was previously shown that transgene constructs delivered into mouse cells using SB transposition can be subject to epigenetic regulation by CpG methylation. A determinant of epigenetic modifications of the integrating transposon vector was found to be the cargo transgene construct itself, with viral promoter elements playing a major role (Garrison *et al.*, 2007). However, with careful promoter choice, several studies have established that SB-mediated transposition provides long-term expression *in vivo*. Notably, stable transgene expression from SB vectors was seen in mice after gene delivery in the liver (Aronovich *et al.*, 2009; Kren *et al.*, 2009; Ohlfest *et al.*, 2005b; Yant *et al.*, 2000), lung (Belur *et al.*, 2003; Liu *et al.*, 2004), brain (Ohlfest *et al.*, 2005a) and blood after hematopoietic reconstitution *in vivo* (Mátés *et al.*, 2009; Xue *et al.*, 2009). Thus, it appears that SB transposon vectors have the capacity to provide long-term expression of transgenes both *in vitro* and *in vivo*.

### Sleeping Beauty transposition in pre-clinical models

In *ex vivo* gene delivery, the therapeutic gene vector is introduced into a selected cell population that had been isolated from a donor, and the genetically engineered cells are transplanted into a patient. Depending on whether the donor is the patient itself or another person, we differentiate between autologous or allogeneic cell products, respectively. Unlike viruses, transposons are not equipped to cross cell membranes through infection. Thus, it is necessary to combine the transposon vectors composed of naked nucleic acids (DNA and mRNA) with technologies capable of efficient delivery of these non-viral vectors into cells. Since the efficiency of transposition is dependent on the efficiency of uptake of the introduced nucleic acids into the cell nuclei, delivery is a rate-limiting factor in transposition, and is thus of paramount importance. In

principle, any technology developed for transferring nucleic acids into cells can be combined with transposon vectors. Unfortunately, there is no generally applicable method, and procedures must be established for each cell type. In hard-to-transfect cells, including primary human cell types, delivery of transposon-based vectors can be significantly facilitated by nucleofection, a procedure based on electroporation that transfers nucleic acids directly into the nucleus. Indeed, nucleofection facilitated transposition in CD34<sup>+</sup> HSCs (Hollis *et al.*, 2006; Izsak *et al.*, 2009; Mátés *et al.*, 2009; Sumiyoshi *et al.*, 2009; Xue *et al.*, 2009), primary T cells (Gogol-Doring *et al.*, 2016; Huang *et al.*, 2008, 2010; Singh *et al.*, 2008) and human embryonic stem cells (Orban *et al.*, 2009; Wilber *et al.*, 2007). Importantly, in the context of the hematopoietic system, this *ex vivo* gene delivery procedure apparently did not compromise the potential of transposon-marked CD34<sup>+</sup> cells to differentiate normally into the erythroid, megakaryocytic, granulocyte/monocyte/macrophage (Mátés *et al.*, 2009) as well as into the CD4<sup>+</sup>CD8<sup>+</sup> T, CD19<sup>+</sup> B, CD56<sup>+</sup>CD3<sup>-</sup> NK, and CD33<sup>+</sup> myeloid lineages (Xue *et al.*, 2009). The robustness and feasibility of this non-viral, transposon-based procedure significantly facilitates clinical realization of *ex vivo* stem cell therapy for the treatment of hematopoietic disorders and cancer, and is already successfully applied in humans (Kebriaei *et al.*, 2016).

### Refinement of *Sleeping Beauty* for clinical applications

There were three major areas of development and refinement of SB technology bearing paramount importance for clinical translation: i) increasing gene transfer rate and lowering toxicity; ii) defining suitable vectors to encode transposase and transposon; iii) establishing a biosafety profile that satisfied requirements for clinical use.

### Enhancing *Sleeping Beauty's* performance index

#### Hyperactive SB transposases

In evolutionary terms, the SB transposon was a successful element with the ability of colonizing several fish genomes millions of years ago (Ivics *et al.*, 1996). However, even successful transposons have not been selected for the highest possible activity. On the contrary, there is likely a strong selective pressure to avoid insertional mutagenesis of essential genes of their host. In an attempt to derive hyperactive transposase variants for advanced genetic engineering, amino acid substitutions

spanning almost the entire SB transposase polypeptide have been screened for eliciting a change in catalytic activity. These amino acid replacements were conducted either by systematic alanine-scanning (Wang *et al.*, 2016b; Yant *et al.*, 2004), by “transplanting” single amino acids or small (2–7 aa) blocks of amino acids from related transposases (Baus *et al.*, 2005; Geurts *et al.*, 2003; Zayed *et al.*, 2004), and by replacement of selected amino acid residues based on charge (Zayed *et al.*, 2004). These approaches generated transposase variants with i) no change in activity; ii) reduced activity or iii) a relatively modest increase of transposition activity. A second-generation SB transposase called SB11 contains five amino acid replacements (selected based on a phylogenetic comparison to active Tc1/*mariner* transposases) over the first-generation transposase (Geurts *et al.*, 2003). SB11 is about 3-fold more active than the first-generation SB transposase, and has been primarily employed in currently running clinical trials based on CAR-engineered T cells (Kebriaei *et al.*, 2016) (Figure 1(B)).

Surprisingly, some combinations of hyperactive mutations were found to result in a significant reduction of activity. Nevertheless, screening of a library of possible combinations yielded SB transposase variants with significantly enhanced activities (Mátés *et al.*, 2009). The most hyperactive SB transposase version currently available, SB100X, displays a ~100-fold hyperactivity when compared to the originally resurrected transposase (Mátés *et al.*, 2009). The hyperactivity of SB100X cannot be explained by altered transposase stability, nor by increased binding to the transposon TIRs; instead, the particular combination of mutations in SB100X appears to affect the folding properties of the transposase (Mátés *et al.*, 2009). The SB100X transposase enables highly efficient germline transgenesis in relevant mammalian models, including mice, rats, rabbits and pigs (Ivics *et al.*, 2014a, 2014b, 2014c). Moreover, the use of the SB100X system yielded robust gene transfer efficiencies into human HSCs (Mátés *et al.*, 2009; Xue *et al.*, 2009), mesenchymal stem cells, muscle stem/progenitor cells (myoblasts), iPSCs (Belay *et al.*, 2010) and T cells (Jin *et al.*, 2011). These cells are relevant targets for stem cell biology and for regenerative medicine and gene- and cell-based therapies of complex genetic diseases. Thus, the SB100X hyperactive transposase holds great promise for of *ex vivo* and *in vivo* gene therapies.

#### Optimized transposons and transposon donor vectors

The first-generation SB transposon vector (called pT) was based on a naturally occurring sequence originally

isolated from the *Tanichthys albonubes* genome (Ivics *et al.*, 1997). As with the transposase, although to a lesser extent, mutagenesis of the SB TIR sequences has been undertaken with the aim to increase the efficiency of transposition. For example, flanking transgenes by two left TIRs of SB (instead of the canonical arrangement of one left TIR (LIR) and one right TIR (RIR) (Figure 2(A))) was shown to enhance transposition by  $\sim 3$ -fold, likely due to the presence of a transpositional enhancer sequence located in LIR (Izsvák *et al.*, 2002). Replacement of four base pairs in RIR and flanking both TIRs by a doublet of TAs (i.e. by TATA sequences as opposed to the canonical TA sites) resulted in  $\sim 3$ -fold increase in transposition over pT (Cui *et al.*, 2002). Incorporation of a multi-cloning-site consisting of several unique restriction enzymes sites available for cloning genes of interests into the optimized transposon sequences led to the pT2-generation SB transposon vector, which has become the most popular SB transposon vector in the scientific community. A combination of the 2-left-TIR and 2-TA arrangements led to a vector called pT3 (Yant *et al.*, 2004). Finally, optimization of SB transposase binding to its internal binding sites within the transposon TIRs by cyclic amplification and selection of targets (CASTing) recently yielded pT4 with modest hyperactivity (Wang *et al.*, 2016b). In addition to enhancing transposition *per se*, SB vectors of enhanced utility have been devised, for example by including cassettes for constitutive or inducible expression of any gene of interest in combination with different antibiotic selection markers and fluorescent reporters (Kowarz *et al.*, 2015).

### Vectorization of Sleeping Beauty components

#### Plasmid DNA to encode SB transposase and transposon

The typical setup for delivery of the SB transposon system into cells is supplying the two components of the vector system (i.e. the engineered transposon carrying a gene of interest and a transposase source) as conventional plasmids. Although both components can be placed on a single plasmid (Mikkelsen *et al.*, 2003) (Figure 2(D)), this arrangement has not become popular, mainly because it does not allow careful titration of transposase expression required for optimal transposition at a certain transposon dose. Because optimal transposon-to-transposase ratio can substantially vary dependent on cell type, most investigators opt for the use of the 2-plasmid vector system (Figure 2(B)). Two recent developments that

address both the efficiency and safety of SB gene delivery are the use of mRNA-encoded SB100X and minicircle (MC) vectors to encode transposase/transposon. Both are key milestones toward the clinical use of SB transposition.

#### mRNA as a transient source of SB transposase

The transposase expression plasmid that is typically used to provide a transposase source in cultured cell lines can be replaced by mRNA synthesized in *in vitro* transcription reactions (Figure 2(E)). Co-delivery of SB transposase-encoding mRNA with an SB transposon plasmid to somatic cells was originally tested in a mammalian cell line *in vitro* and in the mouse liver *in vivo*, using an early version of the SB transposase, SB11 (described above) (Wilber *et al.*, 2006, 2007). By applying mRNA for intracellular delivery in therapeutically relevant cells *ex vivo*, some hurdles of gene transfer typical for DNA-based vectors can be avoided. For example, nucleofection of primary human cells, including HSCs and T cells, with mRNA was shown to cause significantly reduced cellular toxicity as compared to nucleofection with plasmid DNA (Monjezi *et al.*, 2016; Wiehe *et al.*, 2007). Second, upon transfection, mRNA translocates into the cytoplasm where it is readily available for the host translational machinery and protein production. Finally, the implementation of an mRNA source for transient delivery of the SB transposase increases the biosafety of this approach, as mRNA does not bear the risk of chromosomal integration. In contrast, it is known that transfection of plasmid DNA is associated with a small, but non-negligible chance of spontaneous, illegitimate vector integration into the host genome (Wang *et al.*, 2004). Genomic integration of the SB transposase coding sequence into the genome represents a finite risk in a gene therapy application, because such event could lead to genomic instability due to prolonged and uncontrollable transposase expression resulting in continuous remobilization of the already integrated SB transposon.

#### Minicircle DNA – minimalistic transposase and transposon expression cassettes

A recent addition to the development of SB vectors with enhanced utility for clinical applications is the use of MC vectors as carriers of the SB transposon components. The MC technology allows for a significant reduction of SB vector size by removing most of the backbone sequences from parental plasmids (Sharma *et al.*, 2013). The presence of bacterial backbone elements is typically required for vector propagation in

*Escherichia coli*, but completely redundant or even undesired for clinical applications. The first evident advantage of using MC vectors over plasmids is related to increased cell survival rates following nucleofection of human T cells (Monjezi *et al.*, 2016) (described in detail below in “Cancer immunotherapy with tumor-reactive CAR T-cells”). Unmethylated CpG motifs, which are highly enriched in the bacterial backbone of exogenously delivered plasmids, were shown to trigger strong inflammatory responses through toll-like receptor-9 (Chuang *et al.*, 2002; Hemmi *et al.*, 2000) and/or interferon induction (Huerfano *et al.*, 2013). Activation of these cellular sensors might be a conceivable explanation for the observed cytotoxicity and thus for the loss of a large fraction of cells transfected with plasmid DNA from the rest of the proliferating cell culture. Indeed, removal of CpG motifs from plasmid DNA vectors was demonstrated to reduce inflammatory responses upon pulmonary gene delivery (Hyde *et al.*, 2008), and vector CpG methylation was able to lower immune responsiveness towards “non-self” DNA and led to delayed clearance of transfected cells *in vivo* (Reyes-Sandoval & Ertl, 2004). Along with decreased levels of cytotoxicity, nucleofection of T cells with SB transposon components supplied as MCs resulted in enhanced transient gene delivery and more efficient stable genome modification as compared to conventional plasmid vectors (Monjezi *et al.*, 2016) (described in detail below in “Cancer immunotherapy with tumor-reactive CAR T-cells”).

The MC components are likely more efficient in transfection than plasmids because, due to their smaller size, they cross cellular membranes more efficiently than plasmids (Chabot *et al.*, 2013; Darquet *et al.*, 1997). Second, MC vectors may support enhanced transcription of transgene cassettes. Indeed, enhanced and sustained transgene expression has been seen in episomal gene therapy applications with MC vectors with concomitant gene silencing commencing rapidly after hydrodynamic injection of parental plasmid vectors *in vivo* (Chen *et al.*, 2003; Osborn *et al.*, 2011). Covalent linkage of bacterial DNA sequences to a eukaryotic expression cassette has been suggested to facilitate the spreading of repressive chromatin formed primarily on the bacterial backbone, leading in turn to rapid loss of transgene expression from plasmid DNA vectors (Chen *et al.*, 2004, 2008). Finally, the elevated levels of transposition observed with MC vectors are likely supported, at least in part, by the relatively short, ~200 bp distance between the SB transposon ends in the MC-based transposon vector, owing to the depletion of the bacterial plasmid backbone. Indeed, SB transposition was shown to be far more efficient when the length of DNA sequence outside the transposon unit was shortened,

likely by aiding transposon/transposase complex formation, as discussed above (Izsvák *et al.*, 2000). In addition to efficacy, the MC technology also offers biosafety advantages over conventional plasmid DNA vectors. Namely, the absence of bacterial plasmid backbone elements in therapeutic vectors is highly relevant in clinical applications, because antibiotic resistance genes included in a therapeutic cell product may raise safety concerns.

### Establishing a biosafety profile

#### Genome-wide distribution of Sleeping Beauty insertions

With any vector that integrates into chromosomes in a semi-random manner comes the potential risk of insertional mutagenesis leading to transcriptional activation or inactivation of cellular genes (Baum *et al.*, 2004). Such genotoxic effects can have devastating consequences for the cell and the whole organism, including the development of cancer, as discussed above in “Viral gene transfer vectors”.

SB displays considerable specificity in target site selection at the primary DNA sequence level in that TA dinucleotides are near-obligate target sites (Vigdal *et al.*, 2002). A palindromic AT-repeat consensus sequence with bendability and hydrogen bonding potential was found to constitute preferred target sites (Vigdal *et al.*, 2002). It was later shown that a characteristic deformation of the DNA sequence may be a recognition signal for target selection (Liu *et al.*, 2005). Although characterization of the target site selection properties of different vector systems still falls short of predicting the *actual* risk of insertional oncogenesis in a clinical trial, it is highly useful for ranking the different vector types and designs according to their genotoxic potential (Naldini, 2011). We have previously undertaken a comparative study addressing target site selection properties of the SB and PB transposons as well as MLV-derived gammaretroviral and HIV-derived lentiviral systems in primary human CD4<sup>+</sup> T cells. Our bioinformatic analyzes included mapping against the T cell genome with respect to proximity to genes, transcriptional start sites (TSSs), CpG islands, DNaseI hypersensitive sites, chromatin marks and transcriptional status of genes. The SB transposon displayed the least deviation from random with respect to genome-wide distribution: no apparent bias was seen for either heterochromatin marks or euchromatin marks and only a weak correlation with transcriptional status of targeted genes was detected (Gogol-Doring *et al.*, 2016). Our compiled datasets also allowed us to rank these vector systems with respect to

their projected relative “safety” based on the frequencies of integration into GSHs that were previously proposed to satisfy five criteria: (i) distance of at least 50 kb from the 5′-end of any gene, (ii) distance of at least 300 kb from any cancer-related gene, (iii) distance of at least 300 kb from any microRNA (miRNA), (iv) location outside a transcription unit and (v) location outside ultraconserved regions of the human genome (Papapetrou *et al.*, 2011; Sadelain *et al.*, 2012). Our analyses collectively established a favorable integration profile of the SB transposon, suggesting that SB might be safer for therapeutic gene delivery than the integrating viral vectors that are currently used in clinical trials. Importantly, no SB-associated adverse effects have been observed in preclinical animal studies (Fernando & Fletcher, 2006; Hackett *et al.*, 2010; Ivics & Izsvak, 2006; Izsvak *et al.*, 2010).

### Vector-associated transcriptional activity

Integration of a vector into a gene or its regulatory elements can knock out the gene, overexpress the gene or alter its spatio/temporal expression pattern. The major underlying mechanism, by which insertional mutagenesis manifests in gene therapy appears to be transcriptional *trans*-activation of oncogenes (Baum *et al.*, 2004; Deichmann *et al.*, 2007) by the retroviral long terminal repeats (LTRs) that carry strong enhancer/promoter elements. Due to their intrinsic preference for integrating into TSSs (see above), MLV-derived gammaretroviral vectors are especially prone to transcriptionally upregulate endogenous genes. Indeed, it has been demonstrated that MLV-based gammaretroviral insertions, although they target GSHs >2-fold more frequently than HIV-based lentiviral vectors (Gogol-Doring *et al.*, 2016), were approximately 3-fold more likely to trigger transformation of primary HSCs in a cell-based immortalization assay (Modlich *et al.*, 2009). This suggests that an MLV insertion next to a TSSs tends to be more genotoxic than an HIV insertion in a gene body, in line with the expectation that gain-of-function due to oncogene upregulation has a more profound effect on cellular homeostasis than loss-of-function due to knockout mutations (unless the affected gene is haplo-insufficient). To ameliorate vector-induced insertional oncogenesis, second-generation, self-inactivating (SIN) vectors have been developed for both gammaretroviral and lentiviral vectors. These vectors lack transcriptional enhancer/promoter elements in their LTRs (Schambach *et al.*, 2006, 2007), and therefore they are expected to have an enhanced safety profile in clinical applications. Indeed, SIN vectors result in reduced

clonal immortalization in cell-based genotoxicity assays (Modlich *et al.*, 2009).

Unlike the LTRs of retroviruses, the TIRs of SB vectors have negligible enhancer/promoter activity (Moldt *et al.*, 2007; Walisko *et al.*, 2008). The left TIR of SB is separated from the transposase coding sequence by a 5′-UTR of 160 bp stretch of DNA with no apparent function in the transposition reaction (Zayed *et al.*, 2004). As measured by transient reporter assays, transcription driven by the 5′-UTR of SB is ~18-fold higher than transcription of a promoter-less sequence, and ~5-fold higher than transcription driven by a TATA-box minimal promoter (Walisko *et al.*, 2008). Importantly, the 5′-UTR is not present in SB transposon vectors (because it is dispensable for transposition). Thus, contemporary SB vectors are transcriptionally neutral, and therefore it is expected that their mutagenic potential will largely depend on their cargo (including the transcriptional regulatory elements that drive transgene expression).

Beyond the genomic integration pattern and the transcriptional activities associated with a given vector, a third determinant of risk stemming from vector integration is the type of cell, in which therapeutic gene transfer is executed. In contrast to HSC-based gene therapy, leukemia was never observed in preclinical animal models or clinical trials involving gene transfer into peripheral blood-derived T lymphocytes (Newrzela *et al.*, 2008; Recchia *et al.*, 2006). Thus, mature T cells seem to be less susceptible to transformation by genotoxic events than are HSCs. It is likely that other types of terminally differentiated cells, including retinal pigment epithelium (RPE) cells isolated from the eye are also more refractory to oncogenic transformation than stem cells (Balaggan *et al.*, 2012), thereby presenting attractive targets for Phase I clinical trials.

## Clinical application of Sleeping Beauty gene delivery

### Overview

The past few years have seen a steadily growing interest in applying SB transposition in gene therapy to provide innovative and potentially curative treatments for genetic disorders [reviewed in (Boehme *et al.*, 2015; Essner *et al.*, 2005; Hackett *et al.*, 2005, 2010, 2011; Ivics & Izsvak, 2006, 2011; Izsvak & Ivics, 2004; Izsvak *et al.*, 2010)]. Prime examples for the use of SB in gene therapy include the treatment of hematologic disorders, lysosomal storage diseases, pulmonary disorders, dermatologic diseases, a variety of metabolic disorders, neurologic disorders, muscle disorders and cancer (Table 1).

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

Indication	Method of delivery	Site of delivery	References
<b>Hematologic disorders</b>			
Hemophilia A and B	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	(Yant <i>et al.</i> , 2000; Ohlfest <i>et al.</i> , 2005b)
	Intravenous injection of DNA/polyethyleneimine (PEI) complexes	<i>In vivo</i> , mouse lungs	(Liu <i>et al.</i> , 2006b)
	Intravenous injection of nanocapsules	<i>In vivo</i> , mouse liver	(Kren <i>et al.</i> , 2009)
	Intravenous injection of adenovirus/SB hybrid vector	<i>In vivo</i> , dog liver	(Hausl <i>et al.</i> , 2010)
Sickle cell disease	Transfection	<i>In vitro</i> , human cell lines	(Zhu <i>et al.</i> , 2007)
	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	(Belcher <i>et al.</i> , 2010)
<b>Lysosomal storage diseases</b>			
Mucopolysaccharidosis	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	(Aronovich <i>et al.</i> , 2007, 2009)
<b>Pulmonary disorders</b>			
Fibrosis	Intravenous injection of DNA/polyethyleneimine (PEI) complexes	<i>In vivo</i> , mouse lungs	(Xiao <i>et al.</i> , 2012)
Pulmonary hypertension	Intravenous injection of DNA/polyethyleneimine (PEI) complexes	<i>In vivo</i> , rat lungs	(Liu <i>et al.</i> , 2006a)
<b>Dermatologic disorders</b>			
Junctional epidermolysis bullosa	Transfection	<i>Ex vivo</i> , patient epidermis	(Ortiz-Urda <i>et al.</i> , 2003)
Dystrophic epidermolysis bullosa	Transfection	<i>Ex vivo</i> , human keratinocytes, followed by xenograft in mice	(Latella <i>et al.</i> , 2016)
<b>Metabolic disorders</b>			
Tyrosinemia type I	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	(Montini <i>et al.</i> , 2002; Wilber <i>et al.</i> , 2007; Pan <i>et al.</i> , 2012)
Diabetes type I	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	(He <i>et al.</i> , 2004)
Hypercholesterolemia	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	(Turunen <i>et al.</i> , 2016)
Crigler-Najjar syndrome type I (hyperbilirubinemia)	Intravenous injection of proteoliposomes	<i>In vivo</i> , mouse liver	(Wang <i>et al.</i> , 2009)
<b>Neurologic disorders</b>			
Huntington disease	Transfection	<i>In vitro</i> , human cell lines	(Chen <i>et al.</i> , 2005)
Alzheimer's	Transfection followed by encapsulated cell biodelivery	<i>In vitro</i> , human cell line, followed by graft in patient brain	(Eyjolfsson <i>et al.</i> , 2016)
<b>Muscular dystrophy</b>			
	Transfection	<i>In vitro</i> , mouse cell line, followed by transplantation into mice	(Muses <i>et al.</i> , 2011)
	Transfection	<i>Ex vivo</i> , mouse myoblasts, followed by transplantation into mice	(Escobar <i>et al.</i> , 2016)
<b>Cancer</b>			
	Electroporation, TCR gene transfer	<i>Ex vivo</i> , human PBMCs or T cells	(Peng <i>et al.</i> , 2009; Deniger <i>et al.</i> , 2016)
	Electroporation, CAR gene transfer	<i>Ex vivo</i> , human PBMCs or T cells	(Huang <i>et al.</i> , 2008; Singh <i>et al.</i> , 2008; Jin <i>et al.</i> , 2011, Magnani <i>et al.</i> , 2016)
	Transfection	<i>In vitro</i> , hepatocellular carcinoma cell lines	(Song <i>et al.</i> , 2009)
	Intratumoral injection	<i>In vivo</i> , human glioblastoma xenografts in mice	(Ohlfest <i>et al.</i> , 2005a)
	Tail vein hydrodynamic injection of naked DNA	<i>In vivo</i> , mouse liver	(Belur <i>et al.</i> , 2011)

In addition, important steps have been made toward SB-mediated gene transfer in the eye to treat neovascular age-related macular degeneration (AMD), which will be discussed in detail in "Sleeping Beauty non-viral gene delivery for gene therapy of neovascular age-related macular degeneration", as an example for successful clinical translation. Further, a medical revolution is currently taking place in the field of hematology and oncology with the successful clinical use of genetically modified tumor-reactive T cells that are equipped with synthetic chimeric antigen receptors (CARs) or transgenic T-cell receptors (TCRs). Clinical-proof-of-concept

has been obtained with both CAR-modified T cells in advanced hematologic malignancies and TCR-transgenic T cells in hematologic and solid tumors (Davila *et al.*, 2014; Kochenderfer *et al.*, 2015; Maude *et al.*, 2014; Morgan *et al.*, 2006; Rapoport *et al.*, 2015, Turtle *et al.*, 2016a, 2016b; Turtle & Maloney, 2016). Intriguingly, the concept of endowing T cells with a novel specificity through CAR- and TCR-gene transfer is already being adopted to also treat infections and autoimmune diseases and will establish novel treatment paradigms in medicine. As a prime example, the use of SB-mediated gene transfer to engineer CAR-modified T cells for anti-



**Figure 3.** Neovascular age-related macular degeneration (nvAMD). In nvAMD choroidal neovascularization (CNV) due to an imbalance of pro- and anti-angiogenic factors like VEGF and PEDF leads to severe hemorrhages damaging the retinal pigment epithelium (RPE) and the neural retina. The funduscopic image illustrates such a bleeding (arrow) in advanced nvAMD (see colour version of this figure at [www.tandfonline.com/ibmg](http://www.tandfonline.com/ibmg)).

cancer therapy will be discussed in detail in “Cancer immunotherapy with tumor-reactive CAR T-cells”.

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

TargetAMD is an international consortium of universities, research institutes and commercial organizations funded by the European Commission to execute a Phase Ia/Ib clinical trial for the treatment of neovascular AMD by transplantation of genetically modified, autologous retinal pigment epithelial (RPE) or iris pigment epithelial (IPE) cells that overexpress pigment epithelial-derived factor (PEDF) to the subretinal space of the eye. 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 and transplanted back into the same patient during one surgical session lasting about 60 min.

### **AMD is a major cause of acquired irreversible blindness in adults**

AMD refers to the degeneration of RPE cells in the macula area (Figure 3), which is essential for central vision. With over 13 million people affected, AMD is the fourth most common cause of blindness after cataract,

retinopathy of prematurity, and glaucoma in the world and the leading cause of irreversible blindness in people aged >50 years in developed countries (Velez-Montoya *et al.*, 2014). There are two forms of AMD: avascular AMD (avAMD) or geographic atrophy (GA) and neovascular AMD (nvAMD), which is characterized by the subretinal invasion of choroidal vessels. Whereas avAMD is a slow progressing disorder, in which photoreceptor degeneration follows RPE cell degeneration, nvAMD progresses rapidly to blindness following RPE cell degeneration. No treatments are available for avAMD.

### **Limited clinical benefit of AMD from antibody-mediated VEGF blockade**

Since overexpression of vascular endothelial growth factor (VEGF) has been shown to be responsible for the development of subretinal neovascularization underlying the development of nvAMD (Ohno-Matsui *et al.*, 2001; Velez-Montoya *et al.*, 2014), its current treatment, which is effective in 30–40% of patients, is based on monthly, life-long, intravitreal injections of inhibitors of VEGF. The three most commonly used anti-VEGFs are bevacizumab (Avastin<sup>®</sup>, Genentech, San Francisco, CA), its Fab fragment ranibizumab (Lucentis<sup>®</sup>, Genentech) and aflibercept (Eylea<sup>®</sup>, Regeneron, Tarrytown, NY), which is a recombinant fusion protein consisting of the extracellular binding domains of human VEGF receptors 1 and 2 fused to the Fc portion of the human IgG1 immunoglobulin.

The two major limitations of anti-VEGFs are the lack of improvement of vision in 60–70% of patients and adverse effects (Nesmith *et al.*, 2014) including more rapid progression of GA (Enslow *et al.*, 2016), increased intraocular pressure, retinal detachment, endophthalmitis, photoreceptor cell death and thinning of the inner neuronal layer of the retina (Saint-Geniez *et al.*, 2008; Scott & Bressler, 2013). In addition to the adverse effects, the issues associated with logistics of blind or low-vision patients traveling to a clinic on a monthly basis result in a significant number of patients discontinuing treatment, which has been reported to be from 57% over five years (Boulanger-Scemama *et al.*, 2015) to as high as 71% within 24 months (Lad *et al.*, 2014). Therefore, it is evident that new approaches are required for the efficient treatment of nvAMD.

### **One-shot AMD treatment: Sleeping Beauty-based PEDF gene therapy**

The evidence that nvAMD is caused by RPE cell degeneration has engendered the hypothesis that the condition could be treated by replacing the degenerated RPE

cells with healthy RPE cells, which synthesize and secrete the anti-angiogenic and neuroprotective factor pigment epithelium-derived factor (PEDF) *in vivo*. Administration of recombinant PEDF is not feasible because of its short half-life, and transplantation of RPE or IPE cells, as a substitute for degenerated RPE cells, has not resulted in significant improvement in visual acuity in nvAMD patients (Aisenbrey *et al.*, 2006; Binder *et al.*, 2004; Falkner-Radler *et al.*, 2011; Lappas *et al.*, 2004), indicating that the replacement cells did not express anti-angiogenic factors at sufficient levels to overcome the pathologic overexpression of VEGF.

Therefore, a desirable alternative to overcome the difficulties associated with frequent, life-long intravitreal injections would be a treatment modality that introduces an inhibitor of neovascularization to the retina that would last for the life of the patient. To meet such treatment modality, Campochiaro and colleagues (Campochiaro *et al.*, 2006) delivered a *PEDF* transgene cassette to the retina of nvAMD patients using an adenoviral vector, and reported significant improvement in 25% of patients after 12 weeks and no harmful side effects. However, no follow-up to the trial has been reported. Currently, four clinical trials are ongoing ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)): i) trial NCT01494805 (Avalanche Biotechnologies, Menlo Park, CA), phase I/II, that employs a recombinant adeno-associated viral (rAAV) vector encoding sFlt-1, a splice variant of VEGF receptor 1, by subretinal delivery to nvAMD patients; ii) trial NCT01024998 (Genzyme, Cambridge, MA), phase I, that delivers an rAAV vector encoding sFLT01, a fusion protein that consists of the VEGF binding domain of sFlt-1 fused to the Fc portion of human IgG1, by a single intravitreal injection in nvAMD patients; iii) trial NCT01301443 (Oxford Biomedica, Oxford, UK), phase I, that delivers a lentiviral vector encoding endostatin and angiostatin, both inhibitors of VEGF (RetinoStat<sup>®</sup>), subretinally to nvAMD patients; and iv) trial NCT01678872 (Oxford Biomedica), phase I, that is a 15-year trial to determine safety and efficacy of subretinal RetinoStat<sup>®</sup> encoded in a lentiviral vector in nvAMD patients enrolled in the NCT01301443 trial.

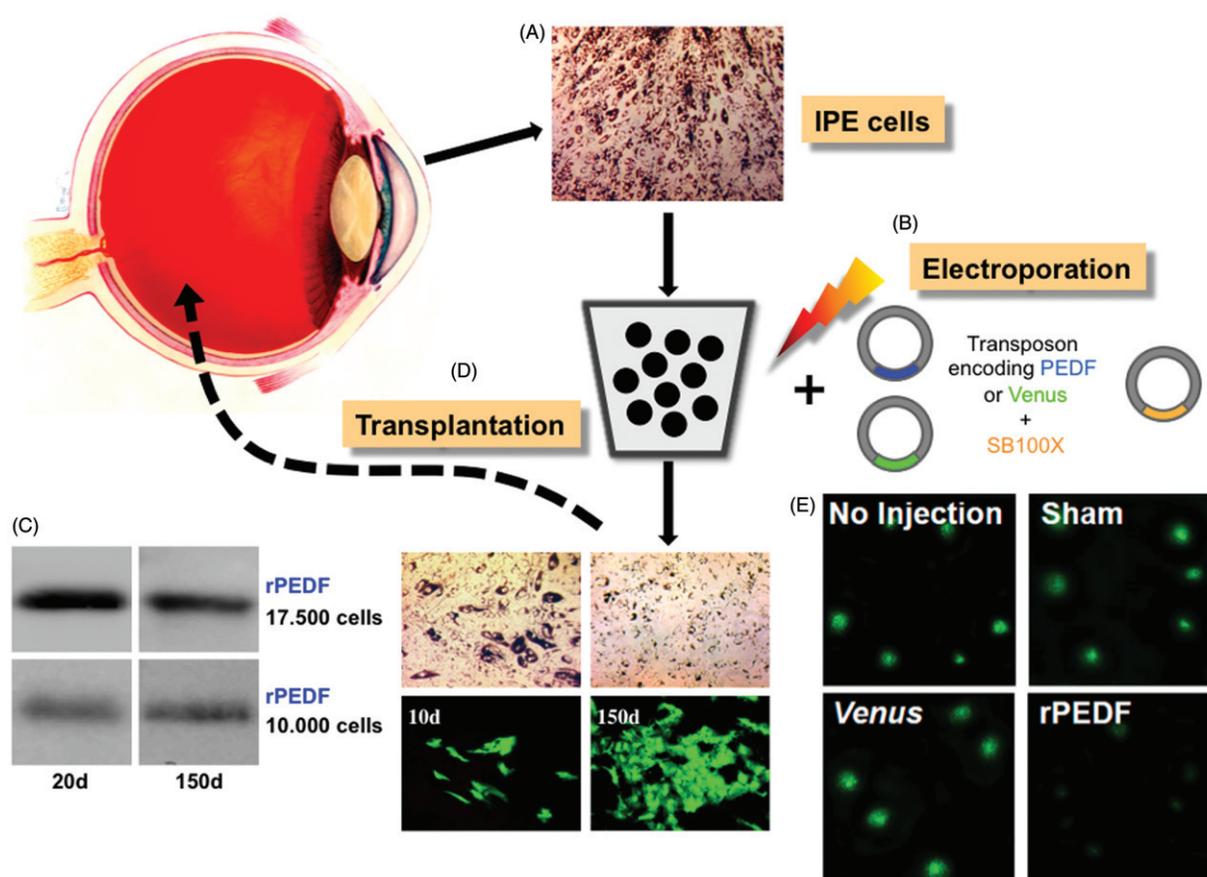
As discussed earlier, viral vectors, although efficient at gene delivery, have limitations in human use; since adenoviral and rAAV vectors remain episomal and readministration may elicit immune responses (Basner-Tschakarjan & Mingozzi, 2014; Dijkhuizen *et al.*, 1998; Hermens & Verhaagen, 1997; Tse *et al.*, 2015), while lentiviral and gammaretroviral vectors have a preference for transgene integration into transcriptionally active genomic regions that could result in insertional mutagenesis and aberrant expression of endogenous genes (Cesana *et al.*, 2012; Hacein-Bey-Abina *et al.*, 2008;

Hargrove *et al.*, 2008) with potentially detrimental consequences for the patient.

In nvAMD, choroidal blood vessel growth into the subretinal space not only disorganizes the normal architecture of the retina, but leads to RPE cell degeneration and loss of vision. The use of vectors to deliver inhibitors of neovascularization to the subretinal space of nvAMD patients will benefit only those patients that have normally functioning RPE cells. However, due to advanced RPE cell degeneration, 60–70% of nvAMD patients do not benefit from anti-VEGF treatment, and thus replacement of the degenerated RPE cells appears essential to regain vision. Since transplantation of RPE or IPE cells does not have a significant effect on choroidal neovascularization (CNV), the hallmark of nvAMD, and does not improve vision in nvAMD patients, we have hypothesized that transplantation of genetically modified RPE or IPE expressing PEDF at supra-physiological levels would not only replace the degenerated cells, but at the same time could inhibit CNV, prevent the continued degeneration of RPE cells, and restore vision in nvAMD patients (Figure 4).

#### Local delivery of PEDF through SB-engineered autologous RPE/IPE cells

Because the inhibition of VEGF must be constant and the proper balance between angiogenic and anti-angiogenic activities must be maintained for the life of the patients for an effective gene therapy of nvAMD, stable transgene integration 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 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 (Figure 4(A,B)). Importantly, cells transfected with a PEDF-transposon and the SB100X transposase have been found to express recombinant PEDF long-term for the duration of 16 months that the cells have been in culture (Figure 4(C)) (Johnen *et al.*, 2012). Genome-wide insertion site analysis in human RPE cells established a close-to-random insertion profile of SB and a frequency of inserting into GSHs similar to that found in human T cells (Gogol-Doring *et al.*, 2016; Monjezi *et al.*, 2016; Thumann *et al.*, 2017). Importantly, no insertions were observed in genes critical to RPE cell function, including the angiogenic factor VEGF-A, the visual cycle protein CRALBP, the lysosomal enzyme cathepsin D, the tight-junction protein ZO-1 and the cytokeratin KRT8. SB100X-mediated stable gene transfer of PEDF in the human RPE cell line ARPE-19 cells, followed by subconjunctival



**Figure 4.** Gene therapy to treat neuroretinal degeneration. A) The anatomy of the human eye is shown. Image source: National Eye Institute, NIH. A small biopsy is taken from the patient's iris for the isolation of  $\sim 10,000$  IPE cells that are taken in culture in an appropriate buffer. B) The IPE cells are electroporated *ex vivo* with components of the SB transposon system: a transposon carrying the therapeutic *PEDF* transgene or a *Venus* reporter and the SB100X transposase. C) Stable transfection and gene expression in primary human IPE cells. For the analysis of *Venus* reporter gene expression, human IPE cells were isolated *post mortem*, and taken in culture for four weeks before transfection with the transposon system components. For the analysis of *PEDF* expression, human IPE cells were isolated from donor tissue, and taken in culture for 8–12 weeks before transfection with the transposon system components. Expression of *Venus* (micrographs) and secretion of human *PEDF* (Western blot) at early (10 and 20 days) and late (150 days) indicate stable gene transfer and transgene expression. D) Genetically engineered IPE cells expressing and secreting human *PEDF* are grafted onto the patient's retina. E) *PEDF*-transfected cells significantly reduced CNV in rats *in vivo* and corneal neovascularization in rabbits (Johnen *et al.*, 2015; Kuerten *et al.*, 2015). Shown here are retinal flat mounts of rats treated with laser to provoke CNV. Lesions are stained with FITC-conjugated isolectin B4 at day 14 post-transplantation. In non-injected (No Injection), sham-treated (Sham) animals and after transplantation of *Venus*-transfected cells (*Venus*) lesions are unaltered, whereas after transplantation with *PEDF*-transfected cells (*rPEDF*) lesion size was significantly reduced (see colour version of this figure at [www.tandfonline.com/ibmg](http://www.tandfonline.com/ibmg)).

transplantation in a rabbit model significantly suppressed and prevented neovascularization (Kuerten *et al.*, 2015). To establish whether RPE cells expressing elevated levels of *PEDF* inhibit CNV, 10,000 rat RPE cells transfected with the *PEDF* gene using SB100X and secreting approximately 2 ng *PEDF*/day were transplanted to the subretinal space of rats in which CNV was induced by laser rupture of Bruch's membrane (Grossniklaus, *et al.*, 2010). A marked reduction of neovascularization was observed at 7 and 14 d post-

transplantation, with the area of neovascularization reduced by 50% (Figure 4(E)) (Johnen *et al.*, 2015).

It has been demonstrated that, by using SB transposon plasmids in conjunction with the SB100X transposase, the *PEDF* gene can be efficiently delivered to IPE cells; however, although safer than viral vectors, plasmids carry antibiotic resistance genes, e.g. the pT2SB transposon vector (Cui *et al.*, 2002) carries an ampicillin-resistance gene, which is necessary for plasmid maintenance in *E. coli*, and thus for efficient

manufacturing of the plasmid vector. The presence of antibiotic resistance markers in gene therapy vectors is a matter of concern, since there is a risk that the antibiotic resistance genes could elicit resistance to pathogenic bacteria by horizontal gene transfer and residues of antibiotics could contaminate the final product, placing patients with severe hypersensitivity to antibiotics at risk (Solensky, 2003). In fact, regulatory agencies recommend that antibiotic resistance genes be eliminated from gene therapy treatments whenever possible. Thus, the TargetAMD consortium resorted to apply a variant of MC technology in free of antibiotic markers (pFAR) miniplasmids (Marie *et al.*, 2010). Like MCs, pFAR-based vectors lack antibiotic-resistance genes, thereby significantly enhancing the safety profile of nonviral gene delivery in clinical settings.

#### **TargetAMD: Phase I clinical trial of SB-based PEDF gene therapy launched in 2017**

The ultimate goal of TargetAMD is to perform a Phase Ia/IIb clinical trial in which IPE cells will be isolated from an iris biopsy of a nvAMD patient, transfected with the *PEDF* gene and transplanted to the subretinal space of the same patient during a single surgical session lasting approximately one hour (Figure 4). Since the isolation of IPE or RPE cells from a patient's biopsy yields a limited number of cells, and since safety is a major concern of any therapy, and of gene therapy in particular, we have developed a protocol for the efficient delivery of the *PEDF* gene in as few as 5,000–10,000 freshly isolated IPE and RPE cells from human donor eyes by vectorizing the *PEDF*-encoding SB transposon and the SB100X transposase in pFAR miniplasmids (Marie *et al.*, 2010; Thumann *et al.*, 2017). Using this protocol, 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) enables expression of recombinant *PEDF* at high levels of recombinant protein in cultured *PEDF*-transfected cells, and iii) allows for sustained transgene expression (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 the lack of antibiotic resistance genes in vector components. Based on the results described here and approval by the Swissmedic regulatory agency, TargetAMD will begin patient recruitment for the phase Ia/IIb clinical trial in the spring of 2017 and expects to complete the first European in-man clinical trial using SB transposon and pFAR technologies.

#### **Cancer immunotherapy with tumor-reactive CAR T-cells**

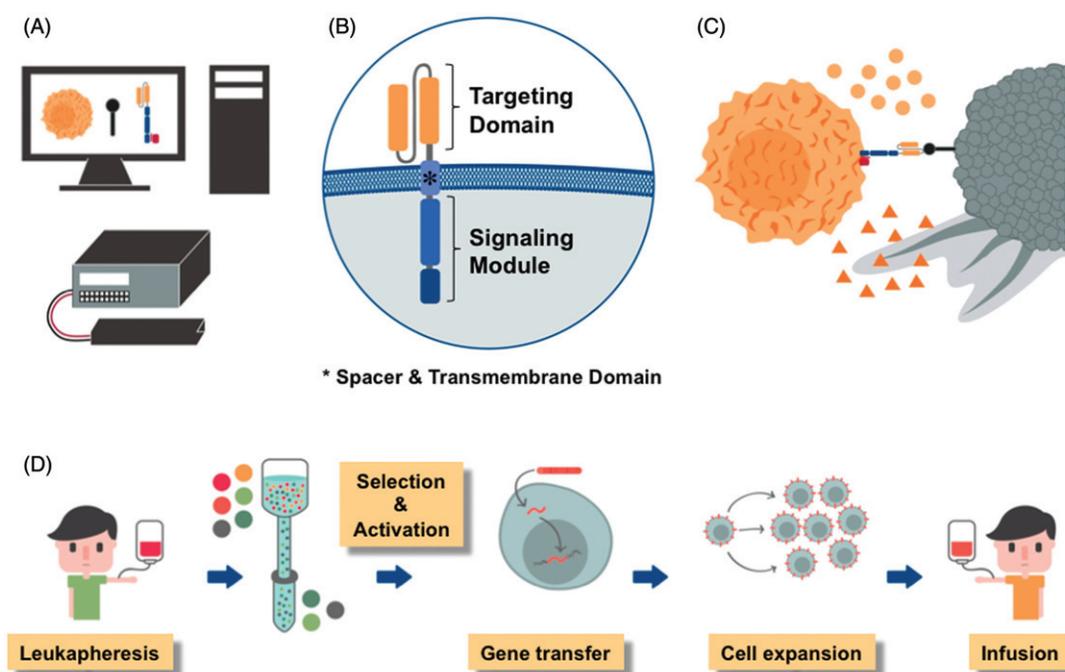
Adoptive immunotherapy with tumor-reactive T cells that are engineered by gene transfer to express a synthetic CAR is emerging as an effective and potentially curative treatment for advanced malignancies. CARs are designer molecules comprised of an extracellular antigen binding domain, most commonly 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 reach and flexibility for binding 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 (Sadelain *et al.*, 2013; Srivastava & Riddell, 2015) (Figure 5). The genetic information required for encoding a CAR is ~1–2 kb depending on binding domain configuration, spacer length and the number of costimulatory moieties.

#### **A medical revolution: CAR T-cell immunotherapy for B-cell malignancies**

The most advanced clinical development is the use of CARs specific for the B-lineage marker CD19 that is expressed on B-cell acute and chronic lymphocytic leukemia and B-cell lymphoma. Several groups have reported up to 90% complete remissions in patients with chemotherapy- and radiotherapy-refractory B-cell acute lymphoblastic leukemia (ALL) and >60% complete remissions in patients with non-Hodgkin lymphoma (NHL) after administration of autologous (i.e. patient-derived) CD19-CAR T cells (Davila *et al.*, 2014; Kochenderfer *et al.*, 2015; Lee *et al.*, 2015; Maude *et al.*, 2014; Turtle *et al.*, 2016a, 2016b; Turtle & Maloney, 2016) (Table 2). These results are considered by many to be a medical breakthrough, given the advanced disease stage and failure of conventional treatments in many patients that were included in these clinical trials. Side effects of CD19-CAR T-cell therapy are a consequence of the strong anti-tumor immune response and 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 physiologic expression of CD19.

#### **Viral gene transfer vectors dominate pre-clinical and clinical studies with CAR T cells**

The overwhelming majority of pre-clinical work and clinical trials that reported efficacy of CD19-CAR T-



**Figure 5.** Chimeric antigen receptors (CARs) are synthetic designer molecules. A) The CAR is a synthetic molecule that can be designed using specialized software, a gene encoding the desired amino acid sequence synthesized and introduced into cells by an electroporation unit. B) CARs consist of an extracellular antigen binding domain (e.g. the variable light and heavy chains of a monoclonal antibody shown in yellow), a spacer and transmembrane region and an intracellular signaling domain (e.g. CD3 zeta and one or more costimulatory domains shown in blue). C) Expression of the CAR enables T cells (yellow) to recognize surface molecules on tumor cells (grey) and exert their anti-tumor effector function such as the release of cytolytic granules (triangles) and cytokines (circles). D) To manufacture a CAR T-cell product, white blood cells are obtained from the patient or a matched donor, CD8<sup>+</sup> killer and CD4<sup>+</sup> helper T cells isolated, the genetic information for the CAR introduced, CAR-modified T cells expanded and administered to the patient (see colour version of this figure at [www.tandfonline.com/ibmg](http://www.tandfonline.com/ibmg)).

**Table 2.** Clinical trials with CD19-CAR T cells.

CAR targeting domain/co-stim/vector	T-cell dose/kg	Entity	CR rate	References
FMC63/4-1BB LV	$0.8 \times 10^6$ – $21 \times 10^6$	ALL	90% (27/30)	(Maude <i>et al.</i> , 2014)
SJ25C1/CD28 RV	$3 \times 10^6$	ALL	88% (14/16)	(Davila <i>et al.</i> , 2014)
FMC63/4-1BB LV	$2 \times 10^5$ – $2 \times 10^6$	ALL	86% (25/29)	(Turtle <i>et al.</i> , 2016a)
FMC63/4-1BB LV	$2 \times 10^5$ – $2 \times 10^6$	NHL	50% <sup>a</sup> (10/20)	(Turtle <i>et al.</i> , 2016b)

CAR: chimeric antigen receptor; LV: lentivirus; RV: retrovirus; ALL: acute lymphoblastic leukemia; NHL: non-Hodgkin lymphoma; CR: complete remission

<sup>a</sup>In patients who received a lymphodepleting preparative regimen with fludarabine/cyclophosphamide, the CR rate at the maximum tolerated CAR T-cell dose level was 64%.

cell therapy has employed gammaretroviral and lentiviral vectors to stably integrate the genetic information of the CD19-CAR in patient's T cells (Table 2). As a consequence, the CAR is not only expressed in T cells that are modified and infused to the patient, but also in subsequent generations of daughter T cells after cell division and propagation. The use of stable gene transfer systems is consistent with the concept and conducive to the ambition of CAR T-cell immunotherapy that administration of a small number of patient-derived CAR T cells is sufficient to achieve engraftment and proliferation *in vivo* until all tumor cells have been cleared and persistence as

memory CAR T cells that protect the patient from tumor relapse long-term.

The potential to achieve transient CAR expression in T cells through transfection of mRNA has been demonstrated and results in CAR surface expression for up to several days which can be sufficient to induce an anti-tumor effect, especially when high doses of CAR T cells are administered (Beatty & Moon, 2014; Caruso *et al.*, 2016). However, because of the rapid decline in CAR expression, multiple subsequent administrations are required to sustain the therapeutic effect. A problem with administering sequential doses of CAR T cells is the induction of immune responses to immunogenic

epitopes that may be harbored in the targeting domain (that are often of murine or other “foreign” origin), and fusion sites in the synthetic CAR molecule. Such immune responses may lead to rapid and sometimes fulminant CAR T-cell rejection with loss of the therapeutic effect (Maus *et al.*, 2013).

### *Sleeping Beauty and CAR T cells: clinical proof-of-concept obtained*

The potential to use SB transposition to integrate the genetic information of the CAR into T cells has first been explored by the group of Cooper *et al.* (Singh *et al.*, 2008). It was demonstrated that SB transposase can be provided either as plasmid DNA or mRNA in combination with a plasmid-encoded CAR transposon and introduced into T cells by electroporation to yield functional CD19-CAR T cells. Consistent with observations in other mammalian cell types, the use of SB11 and hyperactive SB100X accomplished higher rates of gene transfer than first-generation SB transposase (Jin *et al.*, 2011; Singh *et al.*, 2013). 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 autologous ( $n = 7$ , ClinicalTrials.gov Identifier 00968760) or allogeneic ( $n = 19$ , ClinicalTrials.gov Identifier NCT01497184) HSC transplantation (HSCT) prior to CAR T-cell therapy (Kebriaei *et al.*, 2016). From these clinical studies, it was concluded that the administration of SB-engineered CD19-CAR T cells is safe, and may provide additional tumor control in patients after HSCT. The SB transposition strategy pursued in this trial was relatively “basic”, and comprised the nucleofection of plasmid-encoded SB11 transposase and a plasmid-encoded, pT-based CAR transposon, followed by *ex vivo* propagation of CAR-modified T cells for ~28 days (four stimulation cycles with artificial antigen presenting cells). These studies are the first CAR T cell clinical trials that rely on non-viral SB-based gene transfer, and provide proof-of-concept for the utility of SB transposition in CAR T-cell engineering. However, because CD19-CAR T cells were administered as an adjuvant therapy after HSCT, the presented data are somewhat less spectacular than the dramatic and durable anti-tumor responses with sometimes year-long persistence of CD19-CAR T cells that have been reported in other clinical trials that used viral gene transfer vectors and administered CD19-CAR T cells outside the HSCT setting (Maude *et al.*, 2014; Turtle *et al.*, 2016a, 2016b). We would like to point out, however, that comparisons between clinical trials of CAR

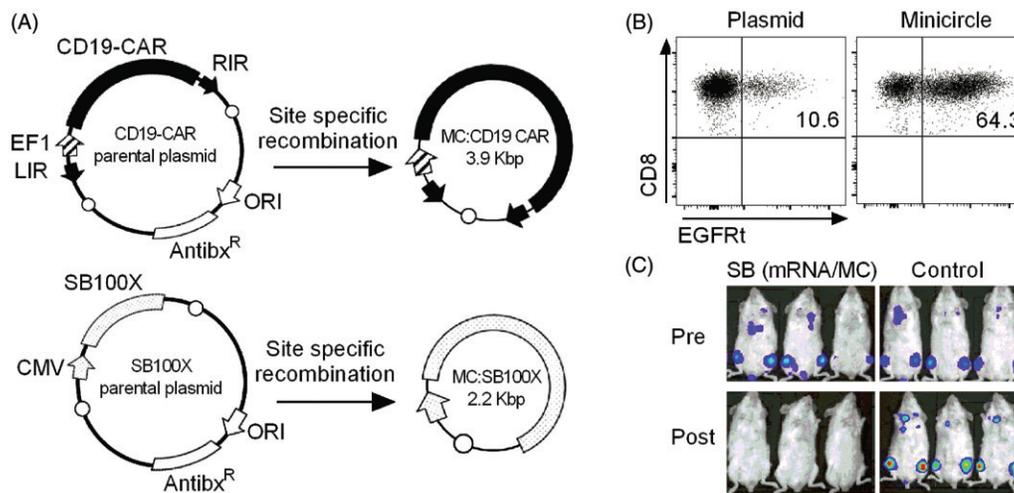
therapy conducted by different groups are difficult due to multiple variations in study design and clinical parameters of their patient population. Further, there are significant differences between trials in the specific design of CD19-CAR constructs, the CAR T-cell manufacturing process, CAR T-cell dose and subset composition, all of which are factors that we and others have shown significantly affect clinical safety and efficacy (Hudecek *et al.*, 2015; Sommermeyer *et al.*, 2015).

In our view, the use of SB transposition is a preferable gene transfer strategy for CAR T-cell engineering. The clinical trials reported by Kebriaei *et al.* (2016) provide a first benchmark; however, manufacturing and clinical performance of SB-engineered CD19-CAR T-cell products may be enhanced by the use of: i) an SB transposase (SB100X) and transposon (pT2) with enhanced transposition rate (see above in “Enhancing Sleeping Beauty’s performance index”); ii) improved vectorization and transfection techniques that achieve higher gene transfer rates at lower toxicity, and enable iii) a short *ex vivo* manufacturing process with optimal fitness of CAR T cells at the time of infusion.

### *Enhanced CAR T-cell engineering with minicircle-encoded SB100X and CAR transposon*

We have recently shown that vectorization of SB100X and a pT2-based CAR transposon as MC DNA (Figure 6(A)) dramatically improves transposition efficacy (Monjezi *et al.*, 2016). Our experience with CD19-CAR T cells is that the gene transfer rate is ~5-fold higher with the use of MCs compared to conventional plasmids (Figure 6(B)). Importantly, despite higher transposition rates, the toxicity of transfecting MCs into T cells is lower compared to standard DNA plasmids (Monjezi *et al.*, 2016). As a consequence, therapeutic doses of CD19-CAR T cells from  $1 \times 10^6$  input T cells can be obtained within as short as 14 days with our MC-based SB approach, and the manufacturing process can be further trimmed to only a few days if higher input T-cell numbers are utilized. Similarly effective, and preferable from a regulatory point of view, is the use of mRNA-encoded SB100X in combination with a MC-encoded CAR transposon. CD19-CAR T cells that we engineered with the mRNA-MC (and the MC-MC) combination confer potent anti-tumor responses *in vitro* and *in vivo* (Figure 6(C)), and are equally functional as CD19-CAR T cells prepared by lentiviral transduction (Monjezi *et al.*, 2016).

We have undertaken a careful analysis of genomic insertion sites and found a close-to-random insertion profile of CAR transposons after mobilization from MCs, consistent with previous studies where SB transposition



**Figure 6.** Anti-tumor function of CD19-CAR T cells engineered by *Sleeping Beauty* transposition. A) Schematic of minicircle (MC) DNA vectors encoding SB100X transposase and CD19-CAR transposon. MCs are prepared from parental conventional plasmids through site-specific intramolecular recombination. MCs contain exclusively the transgene and its promoter, but no bacterial origin of replication and antibiotic resistance genes. EF1, elongation factor-1 alpha promoter; CMV, cytomegalovirus promoter; ORI, bacterial origin of replication; Antibx<sup>R</sup>, antibiotic resistance gene; LIR, left inverted repeat; RIR, right inverted repeat; open circle = recombination site. B) CAR expression in CD8<sup>+</sup> killer T cells after transfection of MC- vs. plasmid-encoded SB100X transposase and CD19-CAR transposon. Flow cytometric analysis was performed on day 14 and shows a significantly higher percentage of CAR-modified T cells after transfection of MCs compared to plasmids. C) Anti-tumor efficacy of CD19-CAR T cells prepared by SB-transposition in a murine xenograft model of systemic B-cell lymphoma. Immunodeficient NSG mice were inoculated with firefly-luciferase expressing Raji lymphoma cells and treated with  $5 \times 10^6$  CD19-CAR T cells (1:1 ratio of CD8<sup>+</sup> and CD4<sup>+</sup> T cells,  $2.5 \times 10^6$  each) that had been generated by transfection with SB100X mRNA and CD19-CAR MC or control unmodified T cells. Bioluminescence images were obtained before T-cell infusion (pre, upper row) and 7 days after T-cell infusion (post, lower row) and show complete lymphoma eradication in the SB CD19-CAR T-cell group, whereas control mice present with progressive disease. A–C) modified from Monjezi *et al.* (2016) (see colour version of this figure at [www.tandfonline.com/ibmg](http://www.tandfonline.com/ibmg)).

had occurred from conventional pT2 transposon donor plasmids (Gogol-Doring *et al.*, 2016). A substantial fraction and indeed higher number of CAR transposon insertions compared to lentiviral integrants had occurred in GSHs of the T-cell genome that are neither expected to cause genotoxicity, nor to induce malignant transformation of their host cells as has been described for gammaretroviral vectors after gene therapy in HSCs (Hacein-Bey-Abina *et al.*, 2003; Monjezi *et al.*, 2016; Sadelain *et al.*, 2012). Importantly, no case of malignant transformation has thus far been reported in clinical trials of CD19-CAR T-cell therapy that employed viral (and non-viral) gene transfer vectors. However, in some patients that achieved remission of their disease, the therapeutic effect was apparently mediated by single CD19-CAR T-cell clones that expanded *in vivo* to comprise a significant proportion of the patient's peripheral T-cell repertoire. Based on the available data, the potential for malignant transformation after CAR gene transfer in human T cells is anticipated to be very low. However, especially once larger cohorts of patients are treated, non-viral SB transposition may be preferable to gammaretroviral and

lentiviral gene transfer vectors due to its 'safer' genomic integration profile.

#### *Clinical trial with CAR T cells engineered by Sleeping Beauty transposition from minicircle vectors*

A clinical trial with CD19-CAR T cells that are engineered by SB transposition from MC vectors is in preparation at the Universitätsklinikum Würzburg, Germany, to obtain clinical proof-of-concept for this novel approach. In this trial, a CD19-CAR construct will be employed that has been selected from pre-clinical analyzes due to optimal anti-tumor function, and has been validated in clinical trials of CD19-CAR therapy that relied on lentiviral gene transfer (Hudecek *et al.*, 2015; Turtle *et al.*, 2016a). Further, cell products that contain equal proportions of CAR-modified CD8<sup>+</sup> killer and CD4<sup>+</sup> helper T cells will be formulated, based on our previous work which showed this is advantageous to the use of cell products with random subset composition (Sommermeyer *et al.*, 2015). The key advantages of CAR T-cell products with defined subset composition are that i) lower total numbers of CAR T cells are sufficient for clinical efficacy

which reduces the risk for side effects and shortens the manufacturing process; and ii) because of less product-to-product variability between patients, the time to and level of CAR T-cell engraftment and proliferation is more consistent and predictable, and allows establishing dose-response relationships as well as immune pharmacokinetic and pharmacodynamic parameters for CAR T cells as medicinal products. We have determined the copy number of the CD19-CAR transposon in both CD8<sup>+</sup> and CD4<sup>+</sup> T cells, and found it to be in an acceptable range ( $n = 6$  and  $n = 5$ , respectively), similar to that obtained with gammaretroviral and lentiviral vectors (Monjezi *et al.*, 2016).

For future clinical trials, there is a pre-clinical development pipeline with CARs specific for target antigens in other hematologic and solid tumors, including a SLAMF7-specific CAR in multiple myeloma, and a ROR1-specific CAR in lymphoma, breast and lung cancer (Berger *et al.*, 2015; Hudecek *et al.*, 2013). Importantly, the MC-encoded SB transposon can accommodate additional genetic cargo without compromising transposition efficacy. Our CAR transposon includes an EGFRt marker that was developed in Mike Jensen's laboratory and serves multiple functions, e.g. to select CAR-modified T cells to further refine the infusion product, and to detect CAR T cells in blood and tissue samples (Wang *et al.*, 2011). Further, CD19-CAR\_EGFRt T cells can be depleted through administration of an anti-EGFR antibody to mitigate toxicity or terminate the therapeutic effect after tumor clearance. We have recently shown in a pre-clinical model that depletion of CD19-CAR\_EGFRt T cells after lymphoma eradication reverses B-cell aplasia as the major long-term side effect and adds another layer of safety to CD19-CAR T-cell therapy (Paszkiwicz *et al.*, 2016).

### Regulatory considerations for clinical trial approval

Currently, the main focus of clinical development of the SB transposon/transposase system is on *ex vivo* gene delivery into primary cells. Thus, from a regulatory point of view, the actual investigational medicinal product (IMP) administered to humans are the genetically modified cells, which in the European Union (EU) are classified as gene therapy medicinal product (GTMP) in most cases. In the EU, GTMPs belong to the group of advanced therapy medicinal products (ATMPs). Basic guidance on the regulatory requirements for this type of products regarding quality and non-clinical aspects, as well as on clinical development is given in the "Guideline on quality, non-clinical and clinical aspects of

medicinal products containing genetically modified cells" (EMA/CAT/GTWP/671639/2008), which was released in 2012. However, in this guideline only limited information is provided on the new technologies for genetic modification of cells, such as the transposon/transposase system.

For genetically modified cells, both the cells to be modified as well as the vector encoding the transposon and the therapeutic gene are considered to be starting materials, and need to be manufactured according to the principles of GMP. However, in case of primary cell donation, procurement and testing of the cells can be performed outside of a GMP environment. The principles of testing quality parameters during manufacturing and for release of the final product are, for the cellular component, similar to most cell therapies, irrespective whether the cells are genetically modified or not, but may depend on the cell type employed.

One important aspect that is specific for transposon-based genetic modification of cells is to monitor the integration profile of the transposon and the number of vector copies integrated into the host genome. As discussed above, genomic integration may have functional consequences, and such impact on the genetic stability of the cells and their proliferation and differentiation capacity needs to be analyzed and documented. In addition, potential toxic effects by the transposase need to be taken into account. In many cases the potency of such products is directly linked to the number or percentage of gene-modified cells as well as to the levels of expression of the therapeutic sequence. It is important to determine these parameters already in early clinical phases as release criteria for each batch. Increasing clinical experience would then allow the development of biological assays indicative for the functional activity of the therapy and related to the clinical efficacy.

In contrast to classical integrating vectors such as retro- or lentiviral vectors, which deliver and insert the therapeutic sequence into the host genome in a "one-shot" process, the transposon in theory is able to be repeatedly excised and integrated at different loci in case of persistent transposase activity. Therefore, a critical issue is the presence of the transposase at the time of administration of the IMP. Delivery of the transposase as protein might thus be preferable to delivery via transposase-encoding mRNA or DNA; however, this will most probably be at the expense of efficacy of the genetic modification. Currently, transcriptionally regulated transposase expression systems are under evaluation, to allow for highly efficient delivery of the transposase gene and concurrent switch-off of gene expression prior to administration of the cell to the patient (Cocchiarella

*et al.*, 2016). In such a case, full function of the system including control of leakage needs to be demonstrated before clinical application.

As for all other gene modifying systems, the risk of insertional oncogenesis due to semi-random genomic integration is an important regulatory concern prior to human use. Transposons integrate their payload, similar to retro- and lentiviruses, in a more or less random fashion bearing the risk of activating a proto-oncogene or inactivating a tumor suppressor gene, as discussed above. However, additional factors may contribute to the risk of oncogenesis, such as the presence and strength of transcriptional regulatory elements, the therapeutic gene, the vector dose, the target cell population, and the indication to be treated (Aiuti *et al.*, 2013b). Thus, the need and, if so, the extent of integration studies, genotoxicity and tumorigenicity studies strongly depends on the above parameters employed in context of the transposon/transposase delivery system. The current clinical database available for retro- or lentiviral vector-transduced T cells used in cancer immunotherapy, for example, justifies that integration studies for this type of therapy are omitted, taking into account also the life-threatening nature of the disease with poor treatment alternatives. For the transposon/transposase system, clinical experience is more limited, and thus a decision on the need for integration and/or tumorigenicity studies is on a case-by-case basis, and should follow a risk-based approach. In any case, use of transient delivery of the transposase in the form of mRNA or recombinant protein is preferred over transposase coding sequences in the form of DNA to limit transposase activity to reduce the risk of insertional mutagenesis.

### **Perspective: *Sleeping Beauty* – a possible new gold standard for gene transfer!**

The first clinical application of the SB system is currently ongoing using autologous T cells gene-modified with SB vectors carrying a CAR to render the T cells cytotoxic specifically toward CD19-positive lymphoid tumors (Huang *et al.*, 2008; Kebriaei *et al.*, 2016; Singh *et al.*, 2008). Lymphocytes represent a suitable initial platform for testing new gene transfer systems, as T cells can be genetically modified using viral and non-viral approaches without apparent resulting genotoxicity. The advantage of using the SB system for genetic modification includes i) the ease and reduced cost associated with manufacturing of clinical-grade, plasmid-based vectors compared with recombinant viral vectors, ii) scalability: SB vectors can be manufactured in any

quantity, iii) easy quality control for clinical use, and iii) indefinite storage with absolute fidelity.

A major hurdle in *ex vivo* delivery of the transposon components into relevant primary cell types is the toxicity of contemporary transfection/electroporation protocols. In situations, where target cells are scarce and/or culturing and expansion of the transfected cells is impossible or cannot be solved without compromising cell identity and grafting potential, cytotoxicity of the transfection procedures is a serious issue that may undermine clinical applications. However, recent experimental data indicate that by employing transposon cassettes vectorized as MCs and by providing the transposase in the form of mRNA cellular toxicity can be reduced (Monjezi *et al.*, 2016), thereby positioning clinical applications with SB well within reach, at least in the area of T cell engineering.

Alternatively, the development of hybrid vector systems combining the natural ability of viruses to traverse cell membranes with efficient genomic insertion mediated by the SB system can be a promising strategy. Indeed, components of the SB transposon have been incorporated into integrase-defective lentiviral particles that showed efficient gene transfer in a range of human cell types and an insertion profile favorable to conventional lentiviral vectors (Moldt *et al.*, 2011; Staunstrup *et al.*, 2009; Vink *et al.*, 2009). Hybrid adenovirus/SB vectors (Boehme *et al.*, 2016; Yant *et al.*, 2002) have been used to efficiently deliver SB transposon vectors expressing FIX into the liver in a hemophilic dog model (Hausl *et al.*, 2010). Recently, a new approach was established based on a hybrid adenovirus/SB vector targeted to CD46, a receptor expressed on HSCs. The system has been shown to yield up to 10% stable gene transfer in HSCs following *in vivo* transduction, thereby alleviating the need for myeloablation and transplantation (Richter *et al.*, 2016). Retroviral vectors disabled in generating a cDNA copy of the retroviral vector have been shown to deliver the SB transposase mRNA into target cells with an impressive efficiency (Galla *et al.*, 2011). Herpes simplex virus vectors with a tropism to infect neural progenitor cells have been used to target SB insertions in the central nervous system in an *in utero* gene delivery system in the mouse (Bowers *et al.*, 2006; de Silva *et al.*, 2010). Finally, similar to adenovirus, baculoviral vectors can efficiently transduce many cell types, but can only provide transient expression in the absence of stable genomic integration. Hybrid baculovirus/SB vectors have been shown to confer prolonged transgene expression in human cells both *in vitro* and *in vivo* in intratumoral injections in xenograft mice (Luo *et al.*, 2012), as well as *in vivo* in the mouse eye (Turunen *et al.*, 2014). Note that, although these hybrid virus/SB

transposon vectors may provide advantage from a technical point of view (non-toxic delivery of the SB vector components into target cells), their application would lose all the advantages of a fully non-viral system that we outlined above.

*Ex vivo* modification of T cells is currently limited to a small number of centers with the required infrastructure and expertise in the logistics of cell isolation, activation, gene transfer, expansion and cryopreservation steps. To simplify procedures and widen applicability for clinical therapies, automation of these procedures is being developed. The CliniMACS Prodigy<sup>®</sup> (Miltenyi Biotec, Bergisch Gladbach, Germany) device delivers automated cell processing in a closed GMP-compliant system, and has recently been adapted for lentiviral transduction of T cells (Mock *et al.*, 2016). The advantage of the system is that it can standardize the complete manufacturing process of cellular products. Future generations of the system adapted to non-viral vector delivery may advance manufacturing of SB-engineered cellular products.

Alternative technologies for genetic engineering in clinically relevant cell types are rapidly advancing. Designer nucleases, including zinc finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas nucleases are excellent tools for genome engineering and gene editing (Doudna & Charpentier, 2014). The common advantage of these technologies is that they enable site-specific engineering of the genome, thereby allowing introduction of facile and highly specific changes in the genetic code. These technologies are making their way toward therapeutic applications (Yu *et al.*, 2016); for example, site-specific gene addition at reasonable efficiencies has recently been demonstrated by mRNA encoding ZFN and a homology template delivered as AAV in both HSCs (Wang *et al.*, 2015) and T cells (Wang *et al.*, 2016a). One key difference that sets designer nucleases apart from a transposon is that they are specialized and highly active at cutting the genome at a specific sequence, but transgene addition at the cut site is dependent on double strand break repair mechanisms of the cell. In contrast, transposons are gene integration “machines” that evolved to insert themselves into the genome; an attribute that forms the basis of applying them for robust gene transfer.

Finally, the sequence “space” of the SB transposase remains largely unexplored for hyperactive phenotypes. Importantly, the crystal structure of SB100X’s catalytic domain has recently been solved (Voigt *et al.*, 2016). The structure was highly informative with respect to rationalizing the biochemical basis of hyperactivity of already existing SB mutants, and can likely be used in

the future for structure-based design of next generation SB transposases for therapeutic gene delivery.

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M.H., Z.Iz. and Z.Iv. are inventors on patents related to *Sleeping Beauty* gene transfer technology.

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