Sleeping Beauty transposition: from biology to applications

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REVIEW ARTICLE

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ABSTRACT

Sleeping Beauty (SB) is the first synthetic DNA transposon that was shown to be active in a wide variety of species. Here, we review studies from the last two decades addressing both basic biology and applications of this transposon. We discuss how host–transposon interaction modulates transposition at different steps of the transposition reaction. We also discuss how the transposon was translated for gene delivery and gene discovery purposes. We critically review the system in clinical, pre-clinical and non-clinical settings as a non-viral gene delivery tool in comparison with viral technologies. We also discuss emerging SB-based hybrid vectors aimed at combining the attractive safety features of the transposon with effective viral delivery. The success of the SB-based technology can be fundamentally attributed to being able to insert fairly randomly into genomic regions that allow stable long-term expression of the delivered transgene cassette. SB has emerged as an efficient and economical toolkit for safe and efficient gene delivery for medical applications.

Introduction

Transposable elements (TEs) are repetitive sequences that are components of nearly all genomes (Huang et al., 2012). Approximately 50% of the human genome is derived from TEs (Cordaux & Batzer, 2009; Lander et al., 2001). The vast majority of TEs accumulate inactivating mutations over evolutionary time to give rise to a fraction of the genome that is often called "junk DNA". Recent studies suggest that the portion of the inactive mass is going through a recycling, "gain of function" process, yet to be fully deciphered (Lander et al., 2001; Prak & Kazazian, 2000). In an attempt to turn the "junk" into a "jewel", a reverse engineering approach was applied to eliminate the accumulated mutations of Tc1-family transposons in fish genomes, which resulted in a synthetic transposon system known as Sleeping Beauty (SB) (Ivics et al., 1997). SB not only represents the first DNA-based TE ever shown to be active in vertebrates, but the first functional gene ever reconstructed from an inactive, ancient genetic material, for which an active, naturally occurring copy either does not exist or has not yet been isolated. Currently, the hyperactive version of SB (SB100X) is one of the most active transposon in vertebrates (Mates et al., 2009). SB gave an opportunity to understand several aspects of transposon regulation. It is assumed that, unlike viruses, TEs and the host have coevolved in a way that permits propagation of the transposon, but minimizes damage to the host. The SB system is an excellent tool to model horizontal gene transfer or the establishment of a complex interaction network between a vertebrate host and a transposon. The accumulated knowledge of transposon biology was also applied to establish a transposon-based technology platform for genome engineering in vertebrate species, including cancer research, gene annotation, vertebrate transgenesis or gene therapy (Ivics et al., 2004).

The sleeping beauty transposon

SB belongs to the Tc1/mariner superfamily of DNA transposons (Plasterk et al., 1999) comprising a transposase gene flanked by inverted repeats (IRs) containing recognition sequences for transposase binding (Figure 1A).

Transposases of the Tc1/mariner superfamily, the bacterial insertion sequence (IS) elements, retroviral integrases and the V(D)J recombinase contain an evolutionarily and functionally conserved catalytic domain, having a DDE/D (Asp, Asp, Glu) motif. DDE/D

Figure 1. Structure of the *Sleeping Beauty* transposon system. (A) The *Sleeping Beauty* (SB) system. The transposase gene (yellow rectangle) is flanked by left and right inverted repeats (IRs) (arrows). Each IR contains two direct repeats (DR), an inner (DRI; orange) and an outer (DRO; brown) to which the transposase (yellow pie) binds at the respective core regions (gray thick line). (B) Domain organization of the transposase: The transposase consists of an N-terminal, DNA-binding domain (PAI + RED), a nuclear localization signal (NLS), an interdomain linker and a C-terminal, catalytic domain (CD). The CD has a clamp loop with a glycine strip (GGG) and three conserved catalytic residues (DDE). Both PAI and RED domains contain three alpha-helices and are separated from each other by a GRRR AT-hook motif. Numbers in the lowest panel represent residues that directly interact with the DNA. A color version of the figure is available online (see color version of this figure at www.informahealthcare.com/bmg).

recombinases catalyze a remarkably similar overall chemistry of DNA recombination (Craig, 1995; Ivics & Izsvak, 2015). Each IR unit of these elements is 200–250 bp in length, and possesses two transposase binding sites known as direct repeats (DRs) that are 15–20 bp in length. The left IR is not identical to the right IR, as the former contains an extra "half direct repeat (HDR)" of the transposase-binding site acting as a transpositional enhancer (Izsvak et al., 2002) and the untranslated regions (UTR) regions which appear to regulate transposition transcriptionally (Moldt et al., 2007; Walisko et al., 2008) (Figure 1A).

Mechanism of *sleeping beauty* transposition

During non-replicative, "cut and paste" transposition of SB, the transposase first binds to its recognition

sequences (DRs) of the transposon (Figure 2). During synaptic complex formation, the two transposon ends are brought together. The transposon is liberated by excision, and the excised molecule is mobilized between the donor and the reintegration loci. The transposase acts at least as a tetramer (Izsvak et al., 2002). Excision leaves a footprint (3 bp) at the donor site while integration results in target site duplications (Ivics et al., 1997) (Figure 2).

Structure of the *sleeping beauty* transposase

The SB transposase contains an N-terminal DNA-binding domain (DBD), a nuclear-localizing signal (NLS) involved in nuclear transport and a C-terminal catalytic domain (DDE) involved in the DNA cleavage and strand transfer reactions (Ivics et al., 1997)(Figure 1B). The bipartite DBD consists of two subdomains; namely, the PAI and RED (PAI + RED = PAIRED; connected by a linker), and shows similarity to the paired domain of the PAX transcription factor family (Czerny et al., 1993). Both the PAI and RED subdomains are predicted to possess three alpha helices, two of which form a helix-turn-helix (HTH), found in many DNA-binding proteins (Aravind et al., 2005). Similarly to other DDE recombinases, the catalytic domain is predicted to have an RNaseH-like fold (Hickman et al., 2010; Rice & Baker, 2001). Despite multiple attempts over the last decade, the crystal structure of the full-length SB transposase remains unknown. Alternatively, successful studies have been reported on solving the structure of functional domains separately (Carpentier et al., 2014; Voigt et al., 2016) (Figure 3). The closest structure to SB is Mos1 (Richardson et al., 2009). Mos1 is a *mariner*-family transposase that, similarly to SB, belongs to the Tc1/*mariner* superfamily.

The DNA-binding domain

The NMR structure of the PAI domain confirms previous predictions (Izsvak et al., 2002), in that it possesses three alpha helices (helix1 = 12–22; helix2 = 29–33; helix3 = 39–55) (Carpentier et al., 2014) (Figure 1B, Figure 3A). Although the structure of a PAI domain/DNA substrate complex is currently unknown, the residues involved in DNA binding were identified to be in the second alpha helix, connecting HTH loop and the third alpha helix (residues 28, 29,31, 33–36, 38–43, 47) (Figure 1B, Figure 3A). The PAI domain binds to the DR-core sequence located within the inner and outer DRs. Upon comparison of SB-PAI domain with other DBDs of the related Tc1/*mariner* family members (e.g. Tc3 and Mos1), the
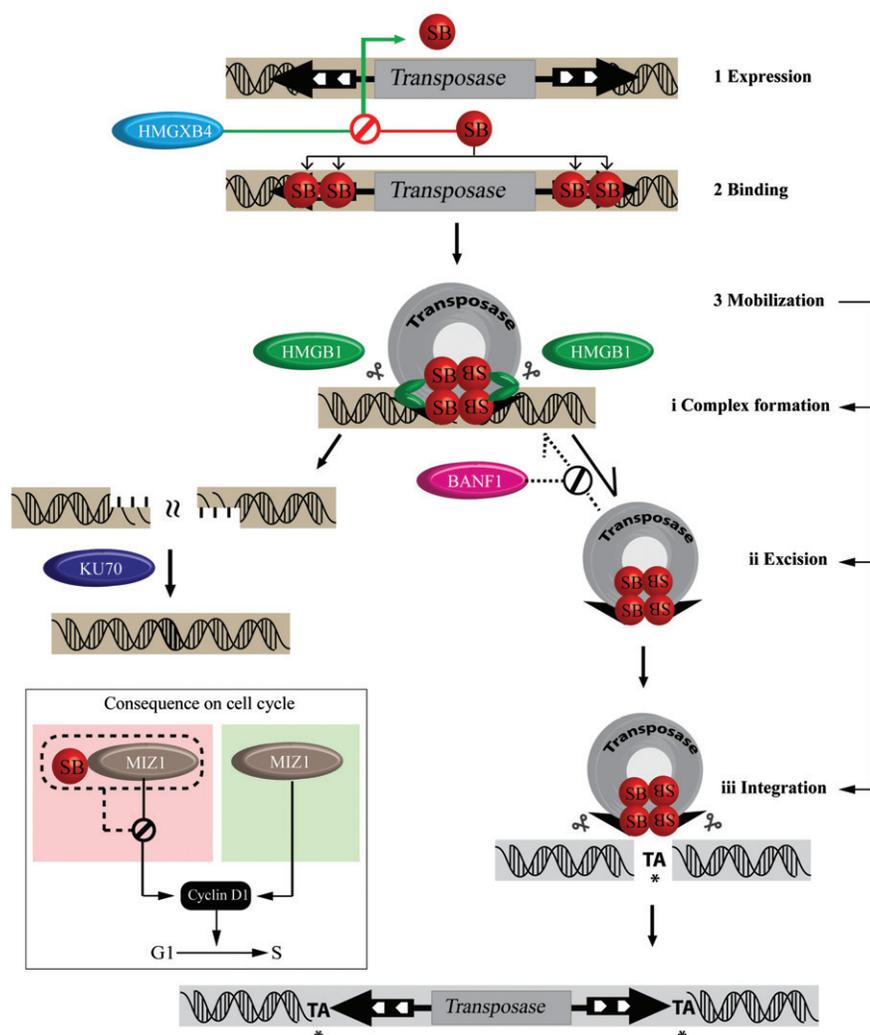
Figure 2. Host factors assist *Sleeping Beauty* transposition. Schematic representation of transposon mobilization: following expression (1) SB (red spheres) binds to the IRs (2) leading to the formation of a synaptic complex (3i) resulting in excision (3ii) from the donor DNA. The excised element then inserts into a TA dinucleotide (*) (3iii), which is duplicated following a successful integration. Excision would leave a 3-bp footprint behind. Role of host factors in regulating transposition: Transcriptional control: the high mobility group protein, HMGXB4 upregulates SB transcription (green arrow). Upon expression, the SB transposase antagonizes the effect of HMGXB4. The synaptic complex: SB recruits the high mobility group protein, HMGB1 (green ovals), which promotes synaptic complex assembly starting at the inner DRs. Prevention of autointegration: The host factor, barrier-to-autointegration factor (BAF1) prevents suicidal self-integration. Excision site repair: The Ku70/80 complex of the non-homologous end joining repair pathway (NHEJ) assists healing double stranded DNA damage generated upon transposon excision. Cell cycle modulation: SB modulates cell cycle transition via Miz1 by down regulating Cyclin D1 expression. A color version of the figure is available online (see color version of this figure at www.informahealthcare.com/bmg).

main differences are observed in the length and relative orientations of the alpha helices. As PAX5 has the highest amino acid sequence similarity to the SB-PAI than Tc3 or Mos1, we superimposed PAX5 and SB-PAI structures (Figure 3B). The PAI domain of SB differs from PAX5 with respect to Helix-2 in spite of high similarity at sequence level, and this could be attributed to the simple fact that the structure of SB's PAI was obtained without DNA whereas the structure of PAX5 was deciphered in-complex with DNA (Figure 3B).

The catalytic domain

The structure of the SB transposase catalytic domain along with the flexible inter-domain linker was recently crystalized and used to model the SB transposase/transposon complex (Voigt et al., 2016). As expected (Rice & Baker, 2001), the catalytic domain of the SB transposase contains a canonical RNaseH-fold, consisting of centrally located β-sheets (five-stranded) surrounded by α-helices (five) (Figure 3C). The catalytic residues (D153, D244 and E279) are assembled in close proximity establishing
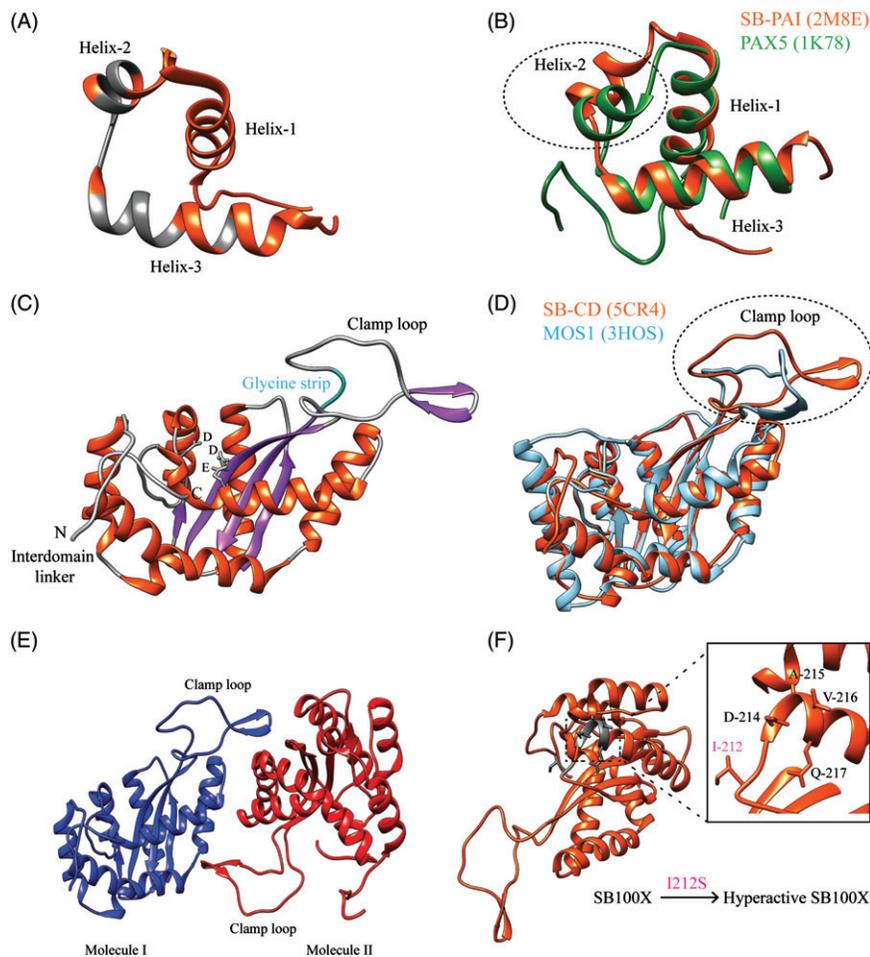
Figure 3. Structure of the *Sleeping Beauty* transposase. (A) Structure of the PAI domain as deciphered by NMR. The structure consists of three α helices (in orange; PDB 2M8E). Amino acids involved in DNA binding are highlighted in gray. (B) Superimposition of PAX5 (in green; PDB 1K78) with the PAI domain. The differences are highlighted by a dotted circle. (C) Structure of the catalytic domain (CD; in orange; PDB 5CR4) showing the N-terminal interdomain linker in gray, α helices in orange, β sheets in purple. The glycine strip (shown in cyan) is part of the clamp loop. The three catalytic residues, DDE are shown in gray. (D) Superimposition of the catalytic domain (CD) of SB (red) on MOS1 structure (cyan; PDB 3HOS). Note that main differences among them are located in the clamp loop (circled dotted line). (E) The potential role of the clamp loops in SB dimerization. (F) The mutation (I212S) further improves the hyperactivity of SB100X (Voigt *et al.*, 2016). The DAVQ stretch is used as a reference to show the position of I-212 (refer the adjoining inlet). The structural analysis was done by the Chimera version of 1.10.2 (Pettersen *et al.*, 2004). A color version of the figure is available online (see color version of this figure at www.informahealthcare.com/bmg).

an active site conformation, similarly to the one observed in the crystal structure of the MOS1 transposase (Richardson *et al.*, 2009). In addition, the catalytic domain also contains a flexible "clamp loop" having a glycine-rich strip (PTVKHGGG; with three consecutive glycine residues), which is inserted within the RNaseH-fold (Figure 1B, Figure 3C). The SB-RNaseH superimposes closely (rmsd of 1.97 Å) with MOS1, except in the linker and the clamp loop domains (Figure 3D). The clamp loop assumes an unusually bent conformation (at three consecutive glycine residues) that is predicted to form a protein–protein interface between protein monomers (Figure 3E). As in MOS1 (Richardson *et al.*, 2009), this interface might bring two catalytic domains

into close proximity with their active sites facing each other during synaptic assembly. The clamp loops of both protomers form reciprocal interactions with the RNaseH core of the partner molecule (Figure 3E). The clamp loop also contains two short anti-parallel β-strands, forming a β-hairpin that interacts with the main chain of the interdomain linker of the partner molecule (Figure 3E). Due to the lack of the availability of a co-crystal containing the substrate DNA with transposase, the authors generated an *in silico* transposon capture complex (TCC) model. The TCC model contains the full-length transposase complexed with target DNA. In the proposed model, the positively charged groove of the catalytic domain can accommodate the bent

target DNA. The model is in conjunction with earlier studies showing that SB prefers bendable, even severely distorted target sites (Vigdal et al., 2002). Using the TCC model, it was also possible to rationalize previous hyperactive mutations (SB100X), and also design novel hyperactive variants (Voigt et al., 2016). The structure-based engineering might pave the way for the future development of designer transposases with differential activities and target site specificities.

Transposon–host interactions

SB is a synthetic transposon, mimicking an ancestral element that was highly successful in the genomes of various fish species >10 million years ago (Ivics et al., 1997). Inactive SB-like elements are widespread in various fish species, are also present in certain amphibian species but never colonized other vertebrates (Ivics et al., 1996). Thus, no SB-like sequences are present in mammals, including humans. In fact, the human genome consists of around 3% inactivated DNA transposons (Cordaux & Batzer, 2009; Lander et al., 2001). The most closely related sequences to SB are the human mariner elements, belonging to the same Tc1/mariner superfamily of transposons. While no active DNA transposon is present in the human genome, certain copies have been "recycled" in a domestication process, and gained a novel cellular function. For example, specific mariner loci gave rise to microRNA (miRNA) genes (Borchert et al., 2011; Smalheiser & Torvik, 2005), and five piggyBac-derived elements (PGBD1–5) are under selection presumably to perform novel, yet to be characterized cellular activities. Importantly, the SB transposase is highly specific with respect to the sequences that it can mobilize, and does not cross-mobilize related Tdr1 elements in the zebrafish genome (Ivics et al., 1997; Izsvak & Ivics, 2004). Surprisingly, by contrast, PGBD5 has been reported to be capable of mobilizing the insect-derived gene transfer tool, piggyBac (Henssen et al., 2015; Ivics, 2016).

Despite being resident only in fish, the transposition reaction catalyzed by SB is supported in all Chordata species tested (Izsvak et al., 2000), including the non-vertebrate Ciona intestinalis (Hozumi et al., 2013). The only report where SB was shown to successfully transpose outside chordates was an invertebrate, the Black Legged Tick (Ixodes scapularis, Phylum Arthropoda) (Kurtti et al., 2008). Remarkably, the insect I. scapularis seems to be able to cross the Chordata-specific barrier as an animal/human pathogen (e.g. Lyme disease).

Compared to SB, piggyBac, originating from an insect (Trichoplusia ni) (Lobo et al., 2006) has a relatively loose host requirement, because it can transpose even in human cells. In contrast, the P element has an extreme species-specificity, and it is active only in a single species (Drosophila melanogaster). Previously, it was generally accepted that a transposon capable of transposing in vitro does not require host-encoded factors. Furthermore, transposons with wide host range were assumed to require no host factors (Raz et al., 1998; Vos et al., 1996). Both assumptions are proved to be false. First, although the nematode Tc1 is capable of performing transposition in vitro, it is not able to support precise transposition in a phylogenetically distant host (Schouten et al., 1998; Vos et al., 1996). Second, the vertebrate-specific SB transposition requires a number of host factors, but these host-encoded proteins are evolutionary conserved in vertebrates. The strategy of recruiting phylogenetically conserved cellular factors could help to establish a stable host–transposon relationship upon colonizing a related naïve genome.

A delicate host–transposon relationship is important for stabilizing long-term transposon–host coexistence. Via its host factors, SB is able to sense and react to various signaling processes, and participates in complex interactive regulatory processes involving evolutionary conserved cellular mechanisms (Figure 3). Host-encoded factors might enable the transposon to sense and react to spatio-temporal cellular signals and help to filter out aberrant transposition products (Wang et al., 2014c). The transposition reaction might not work at all or would lose fidelity under conditions, where host factors are not accessible. Indeed, when a transposon is transferred too far from its original host, the conditions in a new environment could be suboptimal, and the fidelity of the reaction could be compromised. For example, the nematode Tc3 catalyzed aberrant transposition reactions in zebrafish (Raz et al., 1998). Another example is the piggyBac that in comparison to SB performs more frequent, aberrant transposition products in mammalian cells (Wang et al., 2014c). Understanding the delicate mechanisms of transposon–host coexistence can help us to design gene transfer tools with minimal genotoxicity. The accessibility of a host factor in a given cell type is likely to affect the efficacy of the reaction. Indeed, it has been observed that the frequency of SB transposition varies in different vertebrate cells (Copeland & Jenkins, 2010; Dupuy et al., 2001; Dupuy et al., 2002; Fischer et al., 2001; Horie et al., 2001; Luo et al., 1998; Yant et al., 2000; Yusa et al., 2004). The host-encoded factors of SB transposition represent a variety of cellular processes, and are involved at various steps of the reaction (Figure 3).

Transcriptional regulation by HMGXB4

A component of the Wnt signaling pathway (Yamada et al., 2003), HMGXB4 (also known as HMG2l1), functions as a transcription factor of SB transposase expression (Walisko et al., 2008). HMGXB4 accesses the 5'-UTR region of the transposase coding sequence, and promotes its transcription. Besides acting as a transcription factor, HMGXB4 is able to physically associate with the transposase protein. Sequestering HMGXB4 not only abolishes HMGXB4-mediated transcriptional activation, but has a repressing effect on transcription by the 5'-UTR (Walisko et al., 2008). Thus, SB transposase can exert a negative feedback regulation on its own expression with the transposase/HMGXB4 complex acting as a transcriptional repressor (Figure 3).

DNA methylation enhances sleeping beauty transposon excision

DNA methylation plays an important role in regulating the activity of TEs. DNA methylation triggers the formation of heterochromatin, and is associated with transcriptional repression. Curiously, SB excision is significantly ($\sim$100X) enhanced upon CpG methylation of the SB transposon (Yusa et al., 2004). Similar enhancement was also observed with other members of the IR/DR subfamily of transposases, including *Frog Prince*, and *Minos* elements (Jursch et al., 2013), but not with simple-IR transposons. According to a model, CpG methylation and subsequent chromatin condensation promotes synaptic complex formation. The chromatin condensation helps to bring the distantly located recognition sequences (DRs) of the IR/DR structure into a close proximity, and results in enhanced transposon excision (Jursch et al., 2013). This feature might help the SB transposon to escape CpG methylation-based epigenetic repression. Importantly, although SB excision might be enhanced by a condensed chromatin structure, transposon integrations are not enriched in heterochromatic regions (Gogol-Doring et al., 2016; Huang et al., 2010).

HMGB1 assists pre-cleavage complex formation

Another high-mobility group protein, HMGB1, a highly conserved DNA bending protein was identified as a cofactor of SB transposition (Zayed et al., 2003). It has been postulated that HMGB1 might induce conformational changes in the transposon DNA, promoting efficient synaptic complex formation. HMGB1 stimulates preferential binding of the transposase to the inner DRs, located distantly from the cleavage site, indicating

that HMGB1 might be involved in a regulatory checkpoint to enforce synapsis prior catalysis. It is interesting to note that, HMGB1 has also been shown to be assisting in V(D)J recombination process, by its DNA bending activity during paired end complex formation (van Gent et al., 1997) (Figure 3).

Assistance from the host DNA repair to seal sleeping beauty inflicted DNA damage

In contrast to bacterial DDE/D transposition or V(D)J recombination, members of the Tc1/*mariner* superfamily, including SB, do not use a hairpin intermediate to liberate the transposon (Izsvak et al., 2004; Richardson et al., 2009). The SB transposase cleaves both strands of the DNA, thereby leaving a double strand break (DSB) at the excision site (Izsvak et al., 2004), while transposon integration is assumed to generate single stranded gaps. The transposase is not able to seal the gap alone, and recruits Ku70, along with the DNA-dependent protein kinase (DNA-PKcs), the key factors of the non-homologous end joining (NHEJ) pathway of DSB repair to repair the excision sites (Izsvak et al., 2004; Yant & Kay, 2003) (Figure 3). DNA-PKcs is a limiting factor of SB transposition, as its cellular level affects the frequency of the reaction. Potential host-encoded factors involved at the integration site repair are yet to be identified. However, the dependence of SB transposition on NHEJ to seal the gaps is not absolute, NHEJ and HR both contribute to the repair of SB-induced DSBs in mammalian somatic cells (Figure 3).

Modulation of the cell cycle

Myc-interacting protein zinc finger 1 (Miz1) transcription factor was identified as an interacting partner of the SB transposase in a yeast two-hybrid screen (Walisko et al., 2006). Sequestration of Miz1 results in a down-regulation of Cyclin D1 expression, which eventually leads to a temporary cell cycle arrest in G1 by interfering with the G1/S transition (Figure 3). Curiously, a temporary G1 arrest enhances transposition suggesting that SB transposition might be favored in the G1 phase of the cell cycle where the NHEJ pathway of DNA repair is preferentially active (Walisko et al., 2006) (Figure 3). The SB transposase-induced G1 slowdown by interfering with cell cycle represents a common strategy shared by selfish genetic elements (Walisko & Ivics, 2006). Stable overexpression of SB has also been reported to induce a G2/M arrest and apoptosis (Galla et al., 2011). Importantly, the apoptosis is not associated with transposase-induced DNA damage, but most probably a

response to accumulated transposase proteins in the nucleus due to overexpression.

Protection from a self-destructive autointegration process

During the excision process of transposition, the excised transposon could be re-inserted into its own genome in a self-disruptive process, known as suicidal autointegration. This phenomenon has been widely associated with various retroviruses, but is also observed to affect transposition, including SB (Benjamin & Kleckner, 1989; Garfinkel et al., 2006; Maxwell et al., 1987; Shoemaker et al., 1981; Wang et al., 2014b). Intriguingly, barrier-to-autointegration factor (BANF1 also known as BAF1), a cellular co-factor of certain retroviruses was detected in higher-order protein complexes containing the SB transposase. Thus, similarly to certain viruses the SB transposon/transposase is able to recruit the phylogenetically conserved cellular protein BANF1 to avoid suicidal autointegration. BANF1 is presumably acting by compacting the excised, extracellular transposon genome to be a less accessible target for autointegration, thereby promoting productive chromosomal integration (Wang et al., 2014b) (Figure 3).

Integration site distribution

Sequence analysis of SB integration sites from mammalian cells showed that integration occurs exclusively into TA dinucleotides (Ivics et al., 1997) (Figure 2). Target site selection of SB is rather structure- than sequence-specific, and AT-rich palindromes around the central TA nucleotide help to form a preferred bendable DNA structure (Vigdal et al., 2002).

While remobilization of the SB transposon from a genomic locus exhibits the "local hopping" phenomenon, resulting in de novo integrations in the neighborhood of the donor site within few megabase window (Keng et al., 2005; Kokubu et al., 2009; Luo et al., 1998; Yant et al., 2005). In contrast, the integration profile is fairly random with a small bias toward certain repetitive elements when initiated from an ectopic molecule. It was observed that SB targets microsatellite DNA, MIR-type SINEs (short interspersed elements) more frequently, while avoids long terminal repeat (LTR) elements and LINE-1 repeats (Yant et al., 2005). The close-to-random integration profile of SB has no overt bias for integrating into genes or near transcriptional regulatory regions of genes (Gogol-Doring et al., 2016; Yant et al., 2005). Thus, SB integrations display only a minor enrichment near transcription start sites (TSSs) or near transcription-associated histone modifications, including mono-methylated H3K4 (a marker for enhancer regions) and tri-methylated H3K4 (associated with promoters of active genes). Integration is not favored either in regions rich in H3K27me3 (a histone modification typically associated with transcriptionally repressed heterochromatin) (Gogol-Doring et al., 2016; Huang et al., 2010).

This close-to-random integration profile of SB was confirmed by multiple studies, and was reported from various organisms and cell types (Ammar et al., 2012a; Geurts et al., 2006; Gogol-Doring et al., 2016; Huang et al., 2010; Liu et al., 2005; Moldt et al., 2011; Vigdal et al., 2002; Voigt et al., 2012; Yant et al., 2005). Nevertheless, the fairly random integration of SB seems to be rather exceptional among transposons and integrating viruses in mammalian cells (Figure 4). The Tol2 transposon (also of fish origin) exhibits a preferential integration into transcription start sites and transcriptional regulatory regions (Ammar et al., 2012a; Grabundzija et al., 2010; Huang et al., 2010). PiggyBac has also a biased insertion pattern, and has a pronounced preference for integrating into the 5′-transcriptional regulatory regions of genes (Huang et al., 2010;
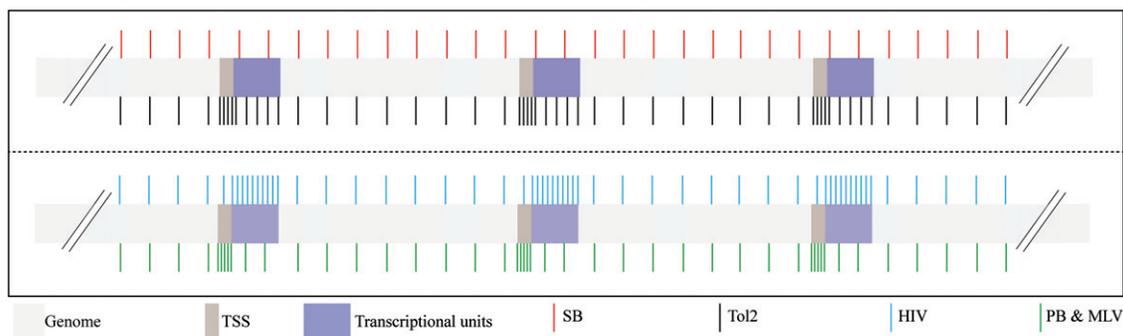
Figure 4. Integration profile of various integrating vectors. SB (red vertical lines) has a fairly random integration profile when compared to other DNA transposons like piggyBac (PB) (green vertical lines) and Tol2 (black vertical lines) and viral systems like HIV (cyan vertical lines) and MLV (green vertical lines). TSS: transcription start site. A color version of the figure is available online (see color version of this figure at www.informahealthcare.com/bmg).

Gogol-Doring et al., 2016). Curiously, the *piggyBac* transposon displays an integration profile highly similar to that of the MLV retrovirus (de Jong et al., 2014; Wu et al., 2003), and both MLV integrase and the *piggyBac* transposase interact physically with BET proteins for guided integrations to specific chromatin environments (de Rijck et al., 2013; Gogol-Doring et al., 2016; Gupta et al., 2013; Sharma et al., 2013a). The HIV-1 integrase also uses a host tethering complex LEDGF/p75/PSIP to localize genomic targets (Ciuffi et al., 2005), and exhibits a strong preference for actively transcribed genes (Schroder et al., 2002). Whether the close-to-random integration profile of SB is guided by a cellular factor is yet to be explored.

Regulating transcription from sleeping beauty by RNA interference

Numerous studies indicate that the host-encoded RNA interference (RNAi) mechanism regulates transposition by suppressing transposase expression (Robert et al., 2004; Sijen & Plasterk, 2003; Vastenhouw & Plasterk, 2004; Vastenhouw et al., 2003). In *C. elegans*, where Tc1 transposition is active, three main mechanisms were proposed to generate double stranded RNAs (dsRNAs) that are eventually fed into RNAi pathway of silencing. Besides bidirectional transcription from the transposon, dsRNA can also be derived from read-through transcription from neighboring genes that can form hairpin structures of dsRNA (Sijen & Plasterk, 2003). Alternatively, RNA-directed RNA polymerases can copy Tc1-transcripts into dsRNA (Sijen & Plasterk, 2003). Transposase-mediated integration avoids the formation of multiple, tandem integrations, which could trigger RNAi-mediated heterochromatin formation and silencing. Nevertheless, bidirectional (convergent) transcription driven by the IRs of SB may lead to the formation of dsRNA, which can serve as templates for the RNAi machinery (Moldt et al., 2007; Rauschhuber & Ehrhardt, 2012; Walisko et al., 2008). The distance between the IRs, e.g. the size of the transposon influences the frequency of the dsRNA production. Interestingly, *mariner* elements frequently give rise to short internally deleted transposon variants, known as miniature inverted repeat elements (MITEs). Certain *mariner*-derived MITEs function as regulatory miRNA genes in the human genome (Piriyapongsa & Jordan, 2007).

Overproduction inhibition is autoregulatory

The efficiency of transposition reaction is limited by a phenomenon termed overproduction inhibition (OPI). OPI is a well-known observation among Tc1/*mariner* elements, in which the transposase appears to inhibit transposition above a certain dosage (Bouuaert et al., 2014; Grabundzija et al., 2010). Although OPI is involved in maintaining a stable co-existence between the transposon and its host, it is assumed to be an autoregulatory mechanism. The current explanation behind this phenomenon lies in the observation that excess transposase molecules can saturate the binding sites on the IRs before productive synapse could take place, thereby poisoning the reaction (Claeys Bouuaert et al., 2013). Alternatively, it is also plausible to assume that improperly folded and/or truncated transposase molecules inhibit transposition by competing with the active transposase molecules (reviewed in (Izsvak & Ivics, 2004)).

Translating sleeping beauty transposition biology

Although SB-like transposons have been inactivated millions of years ago, the synthetic SB transposon has started a new "career" in synthetic biology. Importantly, the accumulated knowledge of the mechanism of its transposition and its interaction with a vertebrate host enables us to modulate the transposition reaction for various applications (Figure 5).

Compared to viral vectors that have been engineered over decades for their use in gene therapy, the SB system was established relatively recently. Since its discovery, it has been used in translational biology, and proved to be tremendously successful for delivery of gene cassettes for addressing a variety of questions, under various molecular contexts and for various applications both in conjunction with other genetic manipulation tools and as a standalone entity (Figure 5). Its success in all tested scenarios can be fundamentally attributed to its exquisite features; first, having an ability to insert fairly randomly, second, being able to insert into regions that allow long-term expression of the delivered cassette and third, allowing low cost vector production.

The two-component transposon vector system

The basis of using SB as a tool was establishing a two-component transposon system. As the SB transposase recognizes the IRs in *trans*, it was possible to physically separate the transposase coding sequence from the recognition motifs for the transposase (DRs). This gene transfer system is suitable to mobilize a gene of interest (transgene) flanked by the IRs. The two-component system can be placed on various carriers, such as plasmid DNA (non-viral vector) or can be combined with various
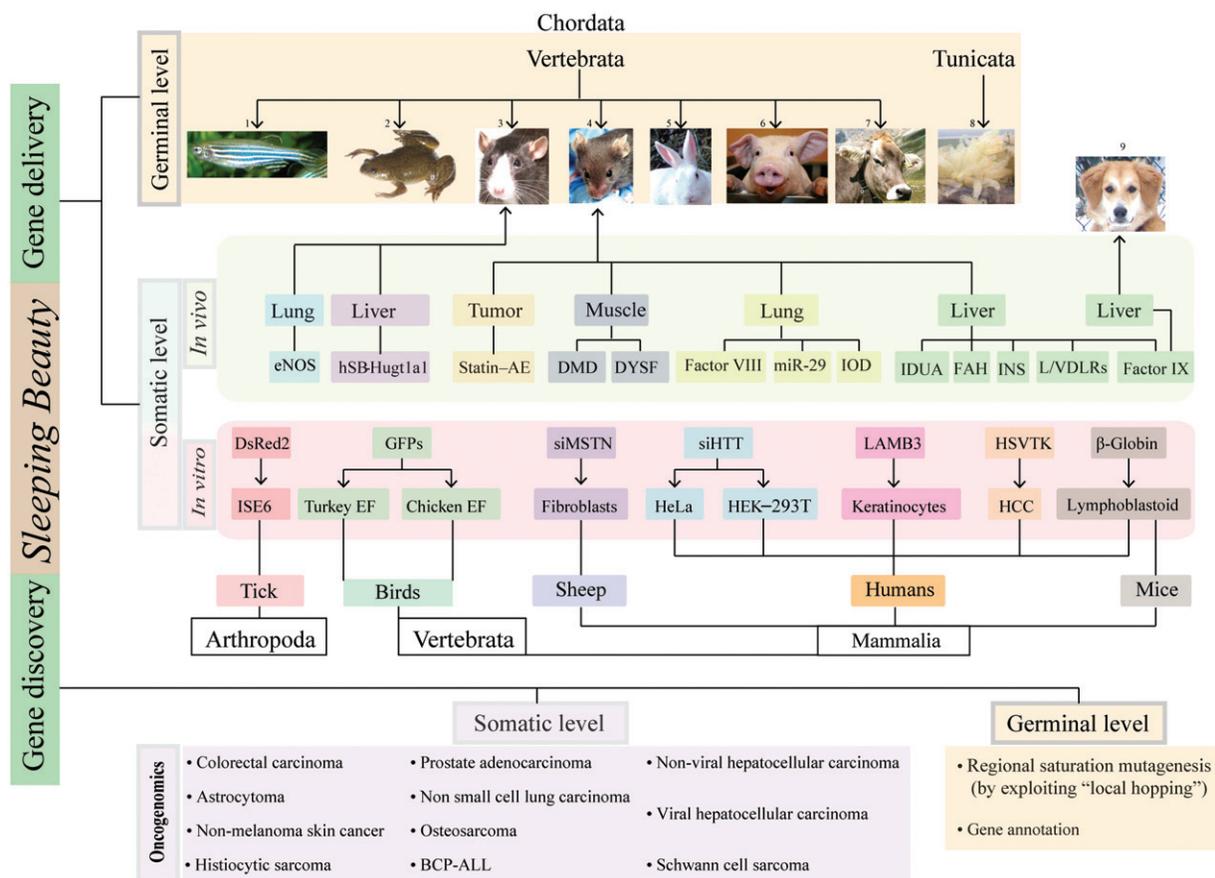
Figure 5. *Sleeping Beauty* transposon-based applications. SB was successfully used for germline transgenesis, in various models (fish[1], frog[2], rat[3], mouse[4], rabbit[5], pig[6], cow[7] and sea squirt[8]). The SB system has been employed for somatic gene delivery in various vertebrates, but also in a tick (insect) cell line, ISE6. SB-based gene delivery has been used in several preclinical animal models. Alternatively, the mutagenic version of the SB can be employed in functional genomics. Insertional mutagenesis screens can be used to annotate genes in somatic cells (oncogenomics) or in the germline. Abbreviations: eNOS: endothelial nitric oxide synthase; hUGT1A1: human uridinediphosphoglucuronate glucuronosyltransferase-1A1; statin-AE: angiostatin–endostatin fusion cassette; DMD: Duchenne muscular dystrophy; DYSF: dysferlin; IDUA: α-L-iduronidase; FAH: fumarylacetoacetate hydrolase; INS: insulin; L/VDLRs: low-density lipoprotein and very-low-density lipoprotein receptors; miR–29: micro RNA 29; IOD: indoleamine-2,3 dioxygenase; DsRed2: red fluorescent protein 2; GFPs: green fluorescent proteins; siMSTN: siRNA against myostatin; siHTT: siRNA against Huntington; LAMB3: laminin subunit beta-3; HSVTK: herpes simplex virus thymidine kinase type 1 gene; BCP-ALL: B cell precursor acute lymphoblastic leukemia). A color version of the figure is available online (see color version of this figure at www.informahealthcare.com/bmg).

viruses (hybrid transposon/virus vectors). The transposase can be also supplied in the form of an mRNA.

It is widely believed that naturally occurring transposons have not been selected for the highest possible activity, and are strongly down regulated. Since transposons co-exist with their hosts, transposition activity is regulated in order to avoid insertional inactivation of essential genes. Low intrinsic activity, self-regulation and interaction with cellular host factors appear to allow wild type transposons to persist in the host without producing serious levels of genetic damage. To derive an optimal molecular tool, it was necessary to optimize both components of the SB system.

Optimization efforts generated several hyperactive variants of the SB transposase (Baus *et al.*, 2005;

Geurts *et al.*, 2003; Mates *et al.*, 2009; Voigt *et al.*, 2016; Yant *et al.*, 2004; Zayed *et al.*, 2004) (Figure 6). Molecular evolution was used to generate the hyperactive SB100X (Mates *et al.*, 2009) (Figure 6). SB100X is now a component of the first plasmid-based vector system that is able to overcome the efficacy problem of non-viral vectors, and can transfer genes at an efficiency comparable to integrating viral systems even in stem and progenitors cells (Mates *et al.*, 2009). SB100X carries a combination of nine amino acid substitutions, scattered along the coding sequence of the transposase (Figure 6). Curiously, the individually hyperactive single amino acid changes cannot be freely combined, and only a fraction of hyperactive mutations are compatible with each other ("friendly" mutations). In principle,
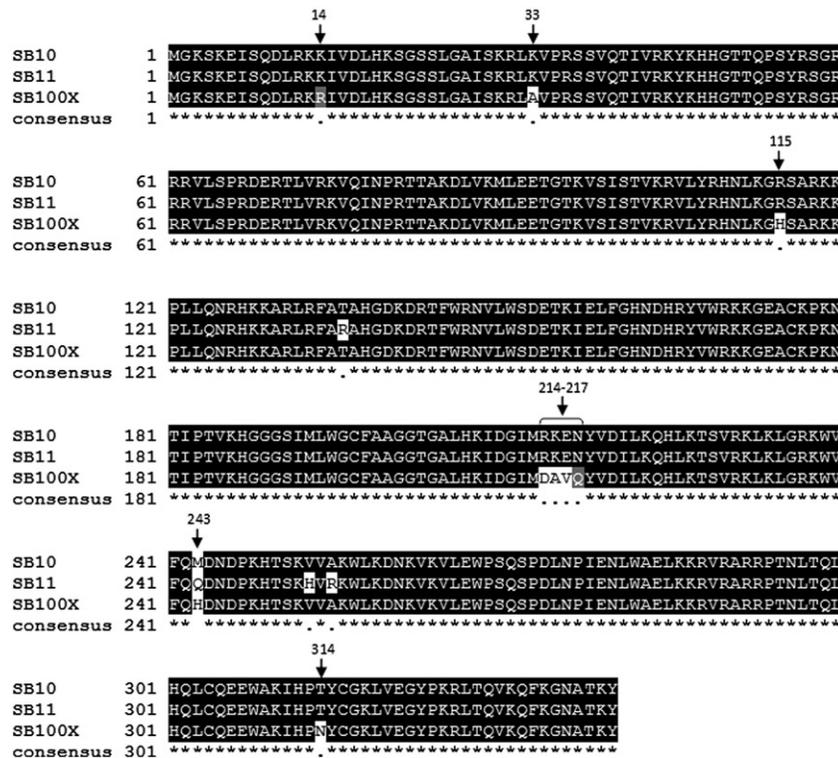
Figure 6. Multiple sequence alignment of *Sleeping Beauty* transposase sequences. Hyperactive amino acid mutations (in white and/or gray) compared to SB10 the original version of the SB transposase, SB10. Hyperactive SB11 and SB100X are targeted for clinical applications. Multiple sequence alignment was performed using EBI Clustal omega (Sievers *et al.*, 2011) and shading was performed using BOXSHADE server version 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

hyperactivity might interfere with the fidelity of the transposition reaction, and generate more frequent aberrant transposition products, as it was recently shown for certain *mariner* elements (Bouuaert *et al.*, 2014; Liu & Chalmers, 2014). Importantly, despite its hyperactive nature, SB100X catalyzes a faithfully precise transposition reaction. In most of its features, SB100X does not significantly differ from first-generation SB transposase, including its stability, OPI profile and affinity to the transposon IRs. In contrast, SB100X is less sensitive to heat shock-induced aggregation, suggesting that the hyperactive mutagenesis affected the folding of the transposase in a positive way (Mates *et al.*, 2009). Perhaps the hyperactivity of SB100X could be further improved. Indeed, the recently solved crystal structure of the catalytic domain of the transposase could be a useful resource of structure-based engineering of tailored SB transposases (Voigt *et al.*, 2016).

The transposon DNA sequences have also undergone stepwise optimization. First, a minimal vector was constructed, where the regulatory sequences, including promoter/enhancer was removed from the transposon (Cui *et al.*, 2002). This step created a transcriptionally neutral vector. Second, transposon excision was improved by correcting/optimizing the recognition sequences (IR/DR) of the minimal vector (Cui *et al.*, 2002; Izsvak *et al.*, 2002). Third, the neighboring sequence of the IRs was changed to mimic a natural target site of SB (Cui *et al.*, 2002). This minimal SB system is highly flexible and can be combined with many additional features. The basic rule is that the IRs should flank the cargo. The cargo can be a single or multiple expression cassettes (Kowarz *et al.*, 2015). An advantageous feature of SB, compared to retroviral vectors, is that it does not require reverse transcription, thus it is able to express transgenes of highly complex structure (e.g. repeated motifs, multiple genes, various regulatory sequences, etc.).

SB-mediated integration supports highly efficient transgene integration in various cell types (Izsvak *et al.*, 2000). The list includes somatic or germ cells, differentiated or stem cells essentially in all vertebrate species (Figure 5). SB is suitable for genetic modification of both overexpressing and knocking down (Hu *et al.*, 2011) transgene expression, and can be combined with other recombination techniques (Grabundzija *et al.*, 2013) or delivery approaches (non-viral/viral). Indeed, in the last decade a whole technology platform, a

"transposon toolbox", has been established around SB (for review see (Ammar *et al.*, 2012b)). SB can be optimized for stable transgene expression (gene/cell therapy, germ line transgenesis). Alternatively, the vector can be developed as a highly potent mutagenic agent for gene annotation (somatic/germ line mutagenesis) (Figure 5).

Sleeping beauty for biotechnology

The SB system has been extensively applied in routine cell culture (for creating transgenic cell lines and for gene knockdowns). This plasmid-based system can be combined with any conventional non-viral delivery technique (e.g. electroporation, essentially all commercially available transfection reagents, etc.).

Sustainable long-term expression of transgenes remains a significant challenge for large-scale biotechnological applications, especially when antibiotic selection is not applicable. For such a purpose, SB represents an attractive transgene expression vector because of its ability to promote efficient genomic integration in a variety of mammalian cell types. Transposons, including *SB*, *piggyBac* and *Tol2* are suitable for generating polyclonal cell pools or clonal cell lines for large scale (industrial) production of recombinant proteins in Chinese hamster ovary (CHO) cells (Balasubramanian *et al.*, 2016).

The characterized expression cassette can be exchanged relatively simply when the SB cassette is combined with other molecular engineering tools (Grabundzija *et al.*, 2013; Petrakis *et al.*, 2012). A powerful strategy could be used to express a series of expression cassettes from the same genomic locus by cassette exchange using Cre or FLP recombinases (Garrels *et al.*, 2016a; Grabundzija *et al.*, 2013).

Recently, a protein-based sensor system was also developed and delivered using SB system for pharmacokinetic and pharmacodynamics characterization of various synthetic analogs of vitamin D3 (Staunstrup *et al.*, 2011).

Sleeping beauty to generate induced pluripotent stem cells

Reprogramming of somatic cells provides an opportunity to generate patient/disease-specific pluripotent stem cells that can be genetically modified, expanded and differentiated into multiple cell types for gene therapy applications. Although retroviral and lentiviral vector systems have been used for generation of induced pluripotent stem cells (iPSCs), they are associated with increased prevalence of tumor formation

(Okita *et al.*, 2007). Attempts have been made to generate iPS cells using non-viral approaches such as transposon-based systems (Grabundzija *et al.*, 2013; Kaji *et al.*, 2009; Muenthaisong *et al.*, 2012; Woltjen *et al.*, 2009; Yusa *et al.*, 2009). The SB system is simple, economical and convenient. The reprogramming efficiency is similarly effective compared to viral vectors (Grabundzija *et al.*, 2013). Indeed, the SB system has been successfully used for the production of iPSCs in various organisms and models (Davis *et al.*, 2013; Fatima *et al.*, 2016; Grabundzija *et al.*, 2013; Kues *et al.*, 2013; Muenthaisong *et al.*, 2012; Talluri *et al.*, 2015).

After generating iPSCs, the expression of reprogramming factors needs to be modulated or shut down for further differentiation or genetic modifications. For personalized gene or cell therapy, the patient-derived iPSCs need to be genetically modified with the therapeutic gene of interest followed by differentiation into a specific lineage and transplantation of these corrected cells back to the patients. To achieve these goals, SB transposon was engineered to carry both reprogramming cassette and recognition motifs for the Cre recombinase (Grabundzija *et al.*, 2013). After successful reprogramming, the reprogramming cassette could be either excised or exchanged with a therapeutic gene construct. This strategy would allow reprogramming and phenotype correction in a single step. Overall, the SB system represents a non-viral methodology for the generation of therapeutically safe pluripotent stem cells.

Enriching for human naïve-like pluripotent stem cell populations

The ability to derive and stably maintain ground-state human pluripotent stem cells (hPSCs) that resemble the cells found *in vivo* in the inner cell mass has the potential to be an invaluable tool for researchers developing stem cell-based therapies. Considerable efforts have been made to isolate and enrich cells with ground-state pluripotency *in vitro*. To date, derivation of human naïve-like pluripotent stem cell lines has been limited to a small number of lineages, and their long-term culturing remains problematic (for review see (Izsvak *et al.*, 2016)). The SB system has been successfully used for genetic and phenotypic tagging, selecting and maintaining naïve-like hPSCs (Wang *et al.*, 2014a; Wang *et al.*, 2016). hPSCs were tagged by GFP driven by the LTR7 of HERVH endogenous retrovirus promoter. By using the reporter, homogeneous hPSC cultures can be derived, characterized and maintained long term by repeated re-sorting and re-plating steps. In this setup,

SB is used to screen for transcriptionally permissive genomic loci in pluripotent stem cells. In addition, SB supports stable expression of the LTR7-GFP reporter, and enables to optimize long-term culturing. This strategy has been reproduced with multiple hPSC lines, including embryonic and induced pluripotent stem cells (Wang et al., 2016).

Optimizing *sleeping beauty* for gene therapy

The advantages of using the SB system lays in its simplicity, unbiased integration pattern, low post-integration silencing of the transgene and its high cargo capacity. For therapeutic applications, the SB system has been further improved addressing efficacy and safety issues (for reviews (Boehme et al., 2015; Di Matteo et al., 2012; Hackett et al., 2013; Hou et al., 2015; Ivics & Izsvak, 2006; Ivics & Izsvak, 2010; Izsvak et al., 2010; Singh et al., 2015).

An optimal vector for the cargo

Beside sequence and conformation, SB transposition was shown to be sensitive to the size of the DNA cargo between the IRs (Izsvak et al., 2000). Bringing the two IRs in a physical proximity (∼300 bp) enhances transposon excision (Izsvak et al., 2000). Thus, combining SB with miniplasmid technologies, such as the minicircle or the free of antibiotic resistance marker donor molecules (pFAR) (Marie et al., 2010), improves the rate of SB transposition (Sharma et al., 2013b), and address safety issues in therapeutic applications as these miniplasmids lack bacterial and antibiotic sequences.

A general feature of DNA transposons is their sensitivity to the size of the cargo. Similarly to other elements (e.g. *piggyBac*), the efficacy of transposition declines over ∼6 kb of cargo size (Wang et al., 2014c). However, in contrast to viruses, these transposons do not have an absolute limit regarding their cargo capacity. Indeed, both SB and *piggyBac* were reported to be capable of mobilizing giant molecules of DNA, such as bacterial artificial chromosomes (BACs) (Li et al., 2011; Rostovskaya et al., 2012). The strategy of mimicking naturally occurring configurations of certain bacterial transposons that are adapted to carry extra sequences (e.g. antibiotic resistance genes) could significantly increase the cargo capacity of SB (Zayed et al., 2004). In the "sandwich" (SA) configuration, the cargo DNA to be mobilized is flanked by two complete SB elements arranged in an inverted orientation (Zayed et al., 2004). The SA transposon was demonstrated to have the ability to transpose up to 18 kb transgenes (Turchiano et al., 2014).

Targeting transposon integration to specific genomic loci – on the way to create a safe vector for gene therapeutic applications

Compared to other integration vectors, SB has a superb chance to integrate into "genomic safe harbor" loci (Gogol-Doring et al., 2016), representing currently a safest integrating gene transfer vehicle for application in human gene therapy. Still, the genotoxic risk is not zero, and it would be desirable to direct integration to a "safe harbor" region. For targeted transposon insertion, at least one component of the transposon system, either the transposon vector DNA or the transposase needs to be tethered to defined sites in the human genome. Proof of principle studies exist demonstrating that it is possible to direct transposon integration into predetermined genomic loci by coupling a site-specific DNA binding domain (DBD) to the SB transposase (Ammar et al., 2012a; Ivics et al., 2007; Voigt et al., 2012; Yant et al., 2007) (Figure 7).

Although SB transposase is sensitive for tagging, it was possible to fuse various DBDs to the N-terminus of the transposase (Figure 7A). The first demonstration that the targeting strategy works was using the TetR/TRE system. This highly specific DNA-recognition system consists of the bacterial tetracycline repressor (TetR) that binds to the tetracycline response element (TRE). Intriguingly, SB transposon integration could be enriched by 10^7-fold to an artificially generated TRE genomic locus by using a TetR-SB fusion protein in human HeLa cells (Ivics et al., 2007). The targeting was region-specific as it occurred in a 2.5 kb window around the targeted sequence (Ivics et al., 2007).

Fusion of the SB transposase with the GAL4 DBD also showed an enrichment of transposon insertions in ∼400-bp window around the targeted sites in cultured human cells (Yant et al., 2007). Transposon targeting was also successful using a polydactyl Zn-finger DBD, E2C, specifically recognizing a unique sequence on human chromosome 17 (Voigt et al., 2012; Yant et al., 2007) (Figure 7A).

Another attempt was taking advantage of the locus-specific integration system of adeno-associated virus (AAV). The AAV Rep protein binds to naturally existing recognition sequences (RRSs) in the human genome, and mediates viral integration into nearby sites. A fusion protein consisting of the N-terminal DBD of Rep and the SB transposase was generated. Remarkably, the Rep-SB yielded a 15-fold enrichment of transposition near the targeted RRS when supplied in the form of plasmid DNA (Ammar et al., 2012a) (Figure 7A).

Although it is a real challenge to target a single genomic locus, it might be a feasible approach to target a
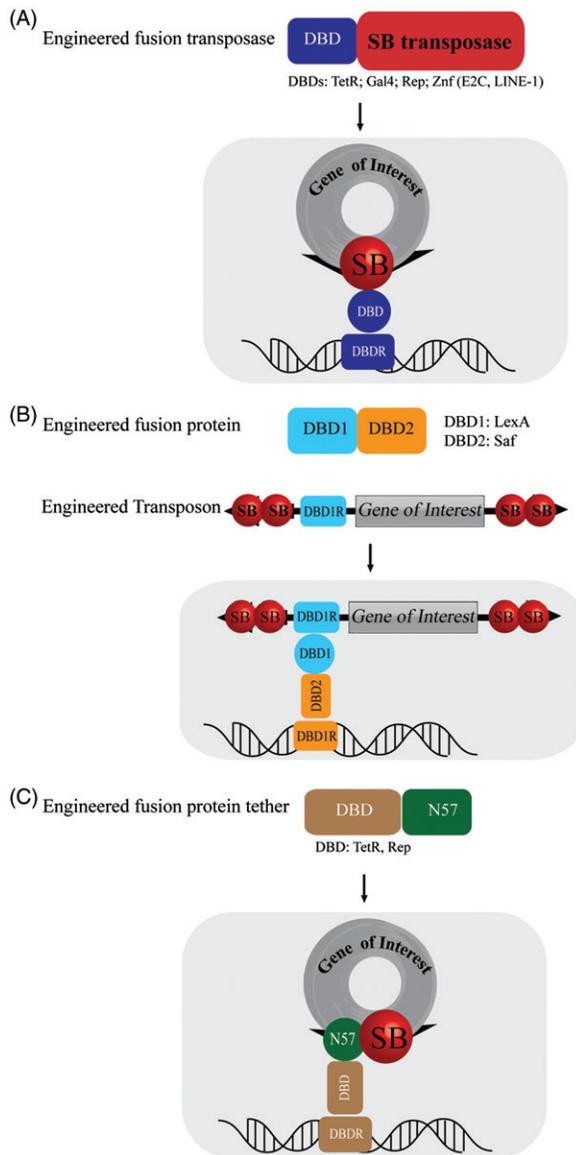
Figure 7. Modulating *Sleeping Beauty* target site specificity. Strategies to increase target site specificity of *Sleeping Beauty* integration. (A) Fusing a DNA-binding domain (DBD) (dark blue) to the N-terminus of the SB transposase (red). The affinity of DBD to DNA-binding domain region (DBDR) could direct transposon integration. (B) Co-delivery of a fusion construct with two DBDs, where DBD1 (cyan) binds an engineered region in the transposon (DBD1R, cyan), while DBD2 (orange) recognizes a genomic target sequence (DBD2R, orange). (C) Alternatively the SB system can be co-delivered with a a fusion protein, where a DBD (brown) is fused to an N-terminal, SB transposase derived peptide, N57 (green), a natural interaction partner of the full-length SB transposase. A color version of the figure is available online (see color version of this figure at www.informahealthcare.com/bmg).

repetitive region in the human genome. Zn-finger DBD was designed and fused to the SB transposase in a strategy to target multiple genomic loci simultaneously, recognizing LINE-1 sequences that occur repetitively in the human genome (Voigt *et al.*, 2012). Approximately, a four-fold enrichment of targeted SB insertions was achieved by targeting LINE-1 repeats, yielding ∼45% overall frequency of insertion into LINE-1 genome wide (Figure 7A).

Targeted transposition events into a chromosomally integrated TRE or into endogenous matrix attachment regions (MARs) were recovered by employing targeting fusion proteins (LexA-Saf) that bind to the engineered transposon DNA (Ivics *et al.*, 2007) (Figure 7B).

Intriguingly, targeted transposition could also be achieved without fusing DBD directly to the full-length SB transposase. Indeed, targeted transposition could be achieved at similar (or even better) frequencies by fusing the DBD (TetR, Rep) to the N-terminal HTH domain of the SB transposase, spanning 57 amino acids (N57) (Ammar *et al.*, 2012a; Ivics *et al.*, 2007; Voigt *et al.*, 2012) (Figure 7C). N57 was previously shown to mediate protein–protein interactions between transposase subunits, and is assumed to bind the HDR (the enhancer sequence in the IR) (Izsvak *et al.*, 2002). Presumably, binding of N57 to HDR occurs in the pre-integration protein-DNA complex. The most significant advantage of such a molecular design is that the transposase itself does not need to be engineered. Thus, the negative effects of direct DBD-transposase fusion on transposition activity can be eliminated (Figure 7C).

The above studies indicate the feasibility of directed transposon integration and highlight potential means for future development. In all these approaches, the high specificity of the DNA-recognition domain is crucial. Furthermore, ideally, the sequence of the selected target site should resemble an SB target site. In comparison to site-specific genome editing performed by a ZF-nuclease, the mechanism of transposon targeting is principally different. In contrast to the nuclease-coupled genome-targeting approaches, the transposition would integrate the transposon/cargo cassette into the genome without generating unwanted DSBs in the genome as a side effect of the reaction (off-target effect). On the other side, targeting efficacy might not reach 100%, still transposon targeting could significantly enrich integration in close proximity of the target site. Furthermore, in contrast to genome-editing techniques, transposons are suitable to deliver large cargos in a large variety of cells. In addition, compared to targeted nuclease approaches, transposition does not depend on the relatively inefficient homology-dependent DSB repair of the cell.

To ensure stability of the integration, the remobilization (re-hopping) frequency of the integrated cassette should be negligible. The frequency of SB

remobilization was estimated to be relatively low (Riordan *et al.*, 2014). Nevertheless, manipulating the half-life of the transposase by targeting it to cellular protein degradation pathways could further improve the safety of SB-mediated gene delivery in clinical applications.

Sleeping beauty-based transposon-viral hybrids combine viral delivery with unbiased-random integration profile

As SB is a non-viral system, the efficacy of its nuclear delivery is a challenge. Importantly, combining the transposon vector with cutting-edge non-viral delivery, like nucleofection and lipofection (for review see (Izsvak *et al.*, 2010)) has been optimized. Delivery using nucleofection has been approved for clinical application (Maiti *et al.*, 2013). Nanotechnology seems to open novel opportunities in efficient non-viral delivery, and can be combined with cell type specific targeting (Kren *et al.*, 2009; Wang *et al.*, 2015). In an alternative approach, several laboratories have generated transposon-viral hybrid vectors (Table 1). Viral vectors are excellent gene delivery vehicles due to their intrinsic ability in transporting

genetic cargos from outside to inside of the cell by crossing membrane barriers. The transposon-viral hybrids were constructed with the expectation that the effectiveness of viral delivery can be combined with the safety features of SB integration (Figure 8) (Table 1).

Recently, advanced deep sequencing technology coupled with bioinformatic analysis was utilized to characterize the integration profiles of various vectors (Berry *et al.*, 2006; de Jong *et al.*, 2014; Gogol-Doring *et al.*, 2016) (Figure 4) (Table 2). Such studies can help to forecast the potential risk of using different vectors. Integrating vectors based on both Moloney murine leukemia virus (MLV) have been reported to cause severe adverse effects after gene transfer in clinical trials. Besides biased integrations, these vectors could cause problems by transactivating cellular promoters (e.g. oncogenes), resulting in clonal expansion of engineered cells. For instance, the mutagenic potential of MLV-based vectors have been reported in multiple clinical gene therapy trials: SCID-X1 (Hacein-Bey-Abina *et al.*, 2003), (Deichmann *et al.*, 2007; Hacein-Bey-Abina *et al.*, 2008; Howe *et al.*, 2008; Thrasher *et al.*, 2006), X-CGD (Stein *et al.*, 2010) and WAS (Braun *et al.*, 2014). Furthermore, recent analyses also demonstrate that HIV

Table 1. Various sleeping beauty-viral hybrid technologies.

Hybrid technology	Delivering vehicle	Integration machinery	Advantages	References
Adeno/SB	Recombinant adenovirus	SB transposase	• High transduction efficiency • SB-mediated unbiased, random integration profile • Stable long term expression • Capable of integrating large genetic cargos	(Yant *et al.*, 2002)
AAV/SB	Recombinant AAV			(Zhang *et al.*, 2013)
HSV-1 amplicon/SB	HSV-1			(Bowers *et al.*, 2006; de Silva *et al.*, 2010a; de Silva *et al.*, 2010b; Peterson *et al.*, 2007)
Baculo/SB	Baculovirus			(Luo *et al.*, 2012; Turunen *et al.*, 2014)
IDLV/SB	IDLV			(Moldt *et al.*, 2011; Staunstrup *et al.*, 2009; Vink *et al.*, 2009;)
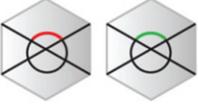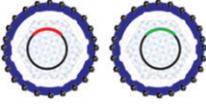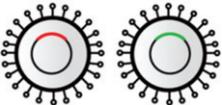
Adeno: adenovirus; AAV: adeno associated virus; IDLV: integrase defective lentivirus; HSV-1: herpes simplex virus 1 amplicon; baculo: baculovirus
Note: The transposase (highlighted in green) and the transposon (highlighted in red) plasmids can be packaged into various recombinant viruses. A colored version is available online (www.informahealthcare.com/bmg).
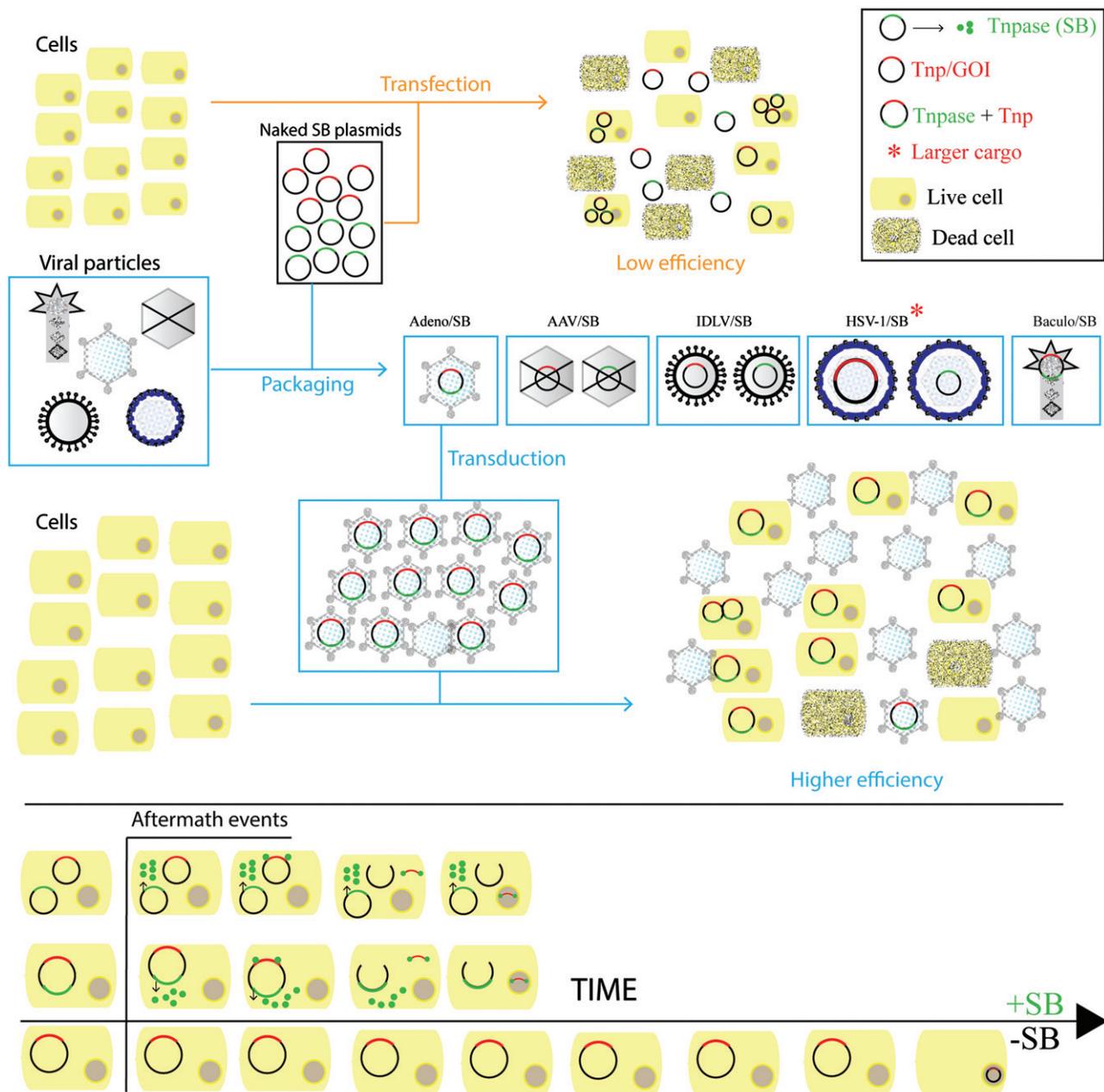
Figure 8. *Sleeping Beauty* transposon-based hybrid vectors. To circumvent the hindrance associated with transfection efficiency of naked DNA plasmids, the SB system, including both the transposase (Tnpase/SB – green dots for protein) and the transposon (Tnp/GOI – red) can be packaged into various recombinant viruses like Adeno, AAV, IDLV, HSV-1 and Baculovirus for delivery (by transduction) into the cytoplasm. The delivery is followed by quick and stable genomic integration of the cargo/GOI, mediated by the SB system as shown in follow-up qualitative time plot. Abbreviations: GOI: gene of interest; adeno: adenovirus; AAV: adeno associated virus; IDLV: integrase defective lentivirus; HSV-1: herpes simplex virus 1 amplicon; baculo: baculovirus. A color version of the figure is available online (see color version of this figure at www.informahealthcare.com/bmg).

integration sites can play a critical role in clonal expansion and persistence of HIV-infected cells (Maldarelli et al., 2014; Wagner et al., 2014), pinpointing to the risk of clinical application of HIV-based (lentiviral) vectors. Importantly, based on the frequencies of integration into genome safe harbors (GSHs), SB ranks top among four integrating genetic elements (SB, MLV, HIV,

piggyBac) in the context of human applications (Gogol-Doring et al., 2016).

In an attempt to modulate the integration profile of a lentiviral gene delivery system, the viral integration machinery was replaced with the SB transposase (Staunstrup et al., 2009; Vink et al., 2009). The employment of the integrase-defective lentiviral vectors (IDLVs)

Table 2. Different types of viral vectors used for gene therapy.

Vector	Safety	Genetic material	Packaging capacity	Tropism	Inflammatory potential	Vector genome forms	Remarks	Benefits
Enveloped								
Retrovirus	BSL-1 or if amphotropic BSL-2/2+	RNA	8 kb	Dividing cells only	Low	Integrated	• Risk of insertional mutagenesis • Biased Integration pattern	Persistent gene transfer in dividing cells
Lentivirus	BSL-2	RNA	8 kb	Broad	Low	Integrated		Persistent gene transfer in most tissues
HSV-1	BSL-2	dsDNA	Up to 150 kb	Strong for neurons	High	Episomal	Risk of Inflammation	Large packaging capacity
Baculovirus	BSL-1	dsDNA	38 kb	Broad	High	Episomal	Inactivated by serum complement	low cytotoxicity
Non-enveloped								
Adenovirus	BSL-2	dsDNA	up to 80 kb	Broad	High	Episomal	Risk of Inflammation	Efficient transduction for most tissues
AAV	BSL-1	ssDNA	<5 kb	Broad with exception[a]	Low	Episomal (>90%)	Small packaging capacity	• Non-inflammatory • Non-pathogenic

[a]Hematopoietic cells are an exception.

in combination with the SB system made it possible to alter the biased viral integration profile (Figure 8). The hybrid vector-mediated transposition reduced the number of insertions within genes to 30%, overriding the biased integration profile of the viral vector, and thus promoting a safer integration profile of SB100X (Moldt et al., 2011). These data clearly indicate that the unbiased integration profile of SB can be maintained independently of the virus used for delivery.

The adeno/transposon system consists of the two-component transposon system (transposon and SB100X transposase) delivered by adenoviral vectors (Figure 8). While adenoviral delivery is efficient, transgene expression is transient, due to the episomal nature of the adenovirus (Table 2). The hybrid adeno/transposon vector enables the integration of the gene of interest, thereby providing a sustainable expression of the therapeutic gene (Yant et al., 2002) (Table 1). This hybrid system has been proved to stably express Factor IX as a treatment for hemophilia B in dogs with negligible toxicity (Hausl et al., 2010). The adeno/transposon hybrid vector has several attractive features. First, it is capable of integrating large cargos (over 10/20 kbs). Second, due to the required low amount of therapeutic vector, the toxicity (provoked immune response) of the administration is low, and there in no requirement for repeated administration. Furthermore, by developing viruses of different tropism, the adenovirus has a premise to be suitable of targeting different cell types.

AAV vectors have been shown to be one of the most promising vectors for therapeutic gene delivery, and were involved in several clinical trials, primarily in slowly dividing cells (Mingozzi & High, 2011). Due to the stable integration, the hybrid AAV/transposon (SB100X) provides sustained gene expression (Zhang et al., 2013), that generally declines in fast dividing cells, as the simple AAV vector remains episomal (Table 2). While AAV vectors have limited cargo capacity, several serotypes are available. Importantly, the hybrid adeno/transposon and AAV/transposon vectors exhibit SB100X-directed, random integration profile.

SB-based hybrid vectors were also developed using herpes simplex virus (HSV) vectors (Bowers et al., 2006; de Silva et al., 2010a; de Silva et al., 2010b; Peterson et al., 2007) (Figure 8). Again, the hybrid system exhibits unbiased integration profile and a premise to efficiently deliver and support stable expression in neuronal cells. The HSV/SB vector has opened up the possibility of treating early-onset neurological disorders with its capacity to integrate a therapeutic gene within a neuronal precursor cell population within the fetal brain.

Baculovirus mediates short-term expression in the transduced cells as a result of the non-replicative and non-integrative nature of the baculovirus (Chen et al., 2015; Kost et al., 2005) (Table 2). To circumvent these problems, a hybrid baculovirus/SB vector was generated for efficient mammalian cell transduction and sustained transgene expression (Luo et al., 2012; Turunen et al., 2014) (Figure 8 and Table 1). The baculovirus-SB vector combines the efficient baculovirus transduction with SB-mediated gene expression, alleviating the shortcoming of conventional baculovirus vectors. The integration preferences for the baculovirus/SB hybrid vector systems are yet to be determined.

Awakening sleeping beauty in the clinic

SB has proven successful as a gene delivery agent and the technology reached clinical level just about a decade following its initial conception (Williams, 2008). The SB system has met success as a therapeutic vector to support sustainable, long-term expression in individual organs (e.g. liver (Wang et al., 2009), lung (Belur et al., 2003; Liu et al., 2006a), muscle (Escobar et al., 2016; Muses et al., 2011)) and cells (e.g. retinoid cells (Johnen et al., 2012), keratinocytes (Ortiz-Urda et al., 2003), primary T cells etc. (reviewed in (Singh et al., 2015)) of choice. The clinically relevant cell types tested include dividing- and non-dividing cells, stem cells (Marg et al., 2014; Mates et al., 2009; Xue et al., 2009), including iPSCs (Belay et al., 2010), somatic stem cells and differentiated ones as well.

SB has been systematically tested in several preclinical models (for review see (Izsvak et al., 2010)). Importantly, no biologically or clinically relevant immunogenicity has ever been reported that significantly affects its applicability. The preclinical models included a wide range of metabolic disorders, degenerative diseases and cancer.

Several metabolic disorders have been successfully modeled in rodents using the SB system. For example, it was possible to ameliorate the clinical symptoms manifested in lysosomal storage disease (Aronovich et al., 2007; Aronovich et al., 2009), Crigler–Najjar syndrome (Wang et al., 2009), type–I Tyrosinemia (Montini et al., 2002; Pan et al., 2012; Wilber et al., 2007), type–I diabetes (He et al., 2004) and hypercholesterolemia (Turunen et al., 2016). It was possible to achieve long-term expression of human uridinediphosphoglucuronate glucuronosyltransferase-1A1 (pSB-hUGT1A1) in Gunn rats leading to a significant reduction in serum bilirubin levels (Wang et al., 2009). Similarly, phenotypic correction was achieved by expressing fumarylacetoacetate hydrolase (FAH) (Montini et al., 2002), human insulin gene precursor (He et al., 2004) and LDLR and VDLR genes (Turunen et al., 2016) in mouse models. By using

the SB system, it was also possible to achieve significant reduction of pulmonary arterial pressure by delivering endothelial nitric oxide synthase (eNOS) into a rat model of pulmonary hypertension (Liu *et al.*, 2006a). It was possible to witness phenotypic correction by long-term stable expression of Factor-VIII (Kren *et al.*, 2009; Liu *et al.*, 2006b; Ohlfest *et al.*, 2005b).

Regarding muscular dystrophies, SB-mediated delivery of dystrophin gene into muscle cells lines followed by transplantation into mouse muscles resulted in normal myogenic properties without transforming into tumors (Muses *et al.*, 2011). Restoration of dysferlin expression and with signs of muscle regeneration was also observed (Escobar *et al.*, 2016).

The low immunogenicity of the SB system provides an opportunity to develop strategies to treat degenerative diseases associated with aging. The SB system was successfully used to deliver shRNAs against selected mutant exons of Huntington gene (*htt*), resulting in up to 90% repression of the mutant gene (Chen *et al.*, 2005) in a Huntington disease model. Therapeutic SB vectors expressing microRNA-29 (miR-29) were also capable of preventing inflammatory macrophage infiltration in a mouse model of pulmonary fibrosis (Xiao *et al.*, 2012).

Various therapeutic strategies to monitor (Ohlfest *et al.*, 2004; Ohlfest *et al.*, 2009) and counter tumor growth using SB have also been explored. Anti-angiogenic therapy yielded promising results as it was possible to achieve tumor regression by delivering endogenous angiogenesis inhibition factors i.e. an angiostatin–endostatin fusion cassette into mice bearing a human xenografted glioblastoma tumor (Ohlfest *et al.*, 2005a) and metastasized colorectal carcinoma tumors (Belur *et al.*, 2011). In a suicide gene approach, overexpressing HSV thymidine kinase type 1 (HSV-TK) under the hTERT promoter yielded successful induction of apoptosis and significant inhibition of tumor growth (Song *et al.*, 2009). Employing the SB system in T cell adoptive immunotherapy has a great promise in treating hematological malignancies (Magnani *et al.*, 2016).

In vivo, the delivery of the therapeutic gene is frequently performed by hydrodynamic tail vein injections of plasmids (Belur *et al.*, 2003; Carlson *et al.*, 2005; Ohlfest *et al.*, 2005b). By employing an adeno/SB hybrid vector in a canine model, it was possible to demonstrate that the technology was transferable from the small to large animal (dog) model (Hausl *et al.*, 2010). Alternatively, the SB system is used to engineer cells *ex vivo* that are administered/transplanted to the patient to treat the disease. Remarkably, this approach has already has been approved for a Phase 1, 2 clinical trials to fight against cancer by using engineered T cells

(Deniger *et al.*, 2016; Krishnamurthy *et al.*, 2015; Magnani *et al.*, 2016; Singh *et al.*, 2013; Singh *et al.*, 2015), and to treat age-related macular degeneration (AMD, TargetAMD (http://www.targetamd.eu/)). In sum, SB combines the integrating abilities of viral gene therapy vectors needed for stable and long-lasting transgene expression with the advantageous properties of easy production, simpler handling and potentially safer chromosomal integration profiles.

Sleeping beauty is a simple, unbiased and efficient tool to gene function annotation

SB has also proved to be an invaluable tool in gene annotation. By exploiting the local hopping feature of the SB system, it was possible to decipher the genomic neighborhood of a particular integration site by remobilizing the transposons from individual chromosomes (Keng *et al.*, 2005; Kokubu *et al.*, 2009). The greatest success of SB has been in its use for phenotype-driven genetics (Carlson *et al.*, 2003; Elso *et al.*, 2015; Horie *et al.*, 2003; Ivics & Izsvak, 2011; Izsvak *et al.*, 2010; Moriarity & Largaespada, 2011). Unlike a candidate-based strategy, SB transposon-based gene trapping, enhancer trapping and unbiased mutagenesis approach is suitable to identify genes with unexpected phenotypes (Balciunas *et al.*, 2004; Lu *et al.*, 2007; Song & Cui, 2013).

Germline transgenesis

In the last two decades, the applicability of the SB system as a transgenic agent has been systematically tested in almost all of the existing animal model systems. SB was successfully used as a delivery vehicle for germline transgenesis in a variety of vertebrate species like in fish, including zebrafish (*Danio rerio*) (Balciunas *et al.*, 2004; Davidson *et al.*, 2003), Nile tilapia (*Oreochromis niloticus*) (He *et al.*, 2013), medaka (*Oryzias latipes*) (Grabher *et al.*, 2003), in the amphibian *Xenopus laevis* (Sinzelle *et al.*, 2006), in rodents (Garcia Diaz *et al.*, 2016; Garrels *et al.*, 2016c; Ivics *et al.*, 2014c), rabbits (Ivics *et al.*, 2014b), swine (Garrels *et al.*, 2011; Ivics *et al.*, 2014a) and cattle (Alessio *et al.*, 2016; Garrels *et al.*, 2016b; Yum *et al.*, 2016).

Pronuclear injection of the transposase in the form of mRNA, the integration of the transgene flanked by recognition sequences for the transposase is faster, and can occur before the first cell division. This feature of the SB system is highly beneficial in animals, possessing a series of fast cell division that cannot be challenged by using conventional non-viral or lentiviral vectors (e.g. rabbits) (Ivics *et al.*, 2014b; Katter *et al.*, 2013). The SB system has proven to be highly efficient to generate

transgenic animals with a robust, whole-body transgene expression, with no significant transgene silencing. This feature also helps to establish tissue-specific transgenic models (Katter et al., 2013). Combining SB transgenesis with recombinase-mediated cassette exchange could be employed to test multiple expression cassettes at a single, well-characterized genomic locus (Garrels et al., 2016a). Transgenic animals stably expressing specific markers could be used to monitor dynamic processes and establish various drug-testing schemes (Szebenyi et al., 2015). Although transgenic techniques were traditionally aimed at mice, the SB system could offer a feasible solution to generate transgenic versions in a large variety of animal models, and also in livestock (reviewed in (Bosch et al., 2015)). Indeed, the employment of the SB system could have a positive impact on livestock production by facilitating an easy-to-use protocol to generate transgenic farm animals (Hu et al., 2011).

Sleeping beauty for functional oncogenomics

Oncogenomic screens applying SB-mediated insertional mutagenesis have been highly productive (Figure 9; for review see (Mann et al., 2014; Moriarity et al., 2015; Tschida et al., 2014)). For the oncogenic screens, the SB system is modified to be a highly mutagenic agent. The mutagenic SB transposons were designed to induce either gain-of-function (GOF) or loss-of-function (LOF) mutations when inserted in or near a gene based on its genetic cargo. It is an effective method for candidate gene discovery that can aid in distinguishing driver from passenger mutations in cancer. This system has been adapted for unbiased screens to identify drivers of multiple cancer types. For example, these screens identified novel genes and pathways that were causative of non-melanoma skin cancer (Quintana et al., 2013), colo-rectal carcinoma (Starr et al., 2009; Starr et al., 2011), non-viral hepato-cellular carcinoma (Keng et al., 2009), leukemia and lymphoma (Collier et al., 2009), B-cell precursor acute lymphoblastic leukemia (BCP-ALL) (van der Weyden et al., 2011), high grade astrocytoma (Bender et al., 2010), Schwann cell sarcoma – in particular, malignant peripheral nerve sheath tumor (Rahrmann et al., 2013), hepatitis B virus-induced hepato-cellular carcinoma (Bard-Chapeau et al., 2014), histiocytic sarcoma (Been et al., 2014), osteosarcoma (Moriarity et al., 2015), prostate adenocarcinoma (Rahrmann et al., 2009) and non-small cell lung carcinoma (Dorr et al., 2015). Recently oncogenomic screens have also been adapted for functional annotation of cancer genomes by enabling insertional mutagenesis screens in higher eukaryotes that are not amenable to germline transgenesis, including humans (Chen et al., 2016;

Molyneux et al., 2014). These cancer screens apart from revealing previously implicated genes have also led to the discovery of several new potential drivers of cancers thereby providing new targets for chemical and genetic therapies.

Future directions

The future challenges of the SB-mediated therapeutic approaches include a further improvement of safety and efficacy. A tighter regulation of transposase expression would be necessary to guarantee the stability of the transgene expression. Targeted gene insertion into predetermined safe loci in the human genome is quite ambitious, but might be feasible in the near future. The efficacy of delivery is still a bottleneck problem of SB-mediated applications. Coupling SB to cutting edge non-viral delivery techniques should be further explored (flow-through electroporation, nanotechnology, etc.). Alternatively, coupling of SB for its safety features with viral vectors for their efficient delivery could be an ideal combination. The continuously accumulating knowledge on host–transposon interactions could further fuel applications by lifting existing limitations.

In this revolutionary era, it became possible to genetically engineer a number of organisms. The field of genome engineering is developing fast, and offers more and more sophisticated tools to modify the genome. Depending on the aim, the evaluation of pros and cons of any particular engineering tool helps to identify the most appropriate strategy. Importantly, the tools can be combined with each other or with other recombination systems. Remarkably, now it is possible to precisely edit the genome (ZF-/TALEN-nucleases, CRISPR-Cas9 system). For therapeutic applications, safety and feasibility still remain the key issues. With respect to efficiency, a targeted nuclease would be an ideal tool to knockout or engineer a short specific DNA region. However, transposons might be a better choice to deliver large cargos in a variety of cell types. This is because in contrast to targeted nuclease systems, transposons are not dependent on the homology-dependent repair pathways of the host, whose efficiency represents a bottleneck of targeted genome-modifications in certain cell types. Finally, while nuclease-coupled targeting systems can precisely edit a desired genomic locus, they can generate undesired double stand breaks in the genome as off-target events. On the other hand, transposons do not damage the genome with unwanted DSBs, but their untargeted integration can be potentially mutagenic in clinical applications. Targeting transposition to safe harbor genomic location could be feasible. For functional genomic application (phenotypic screens), transposons
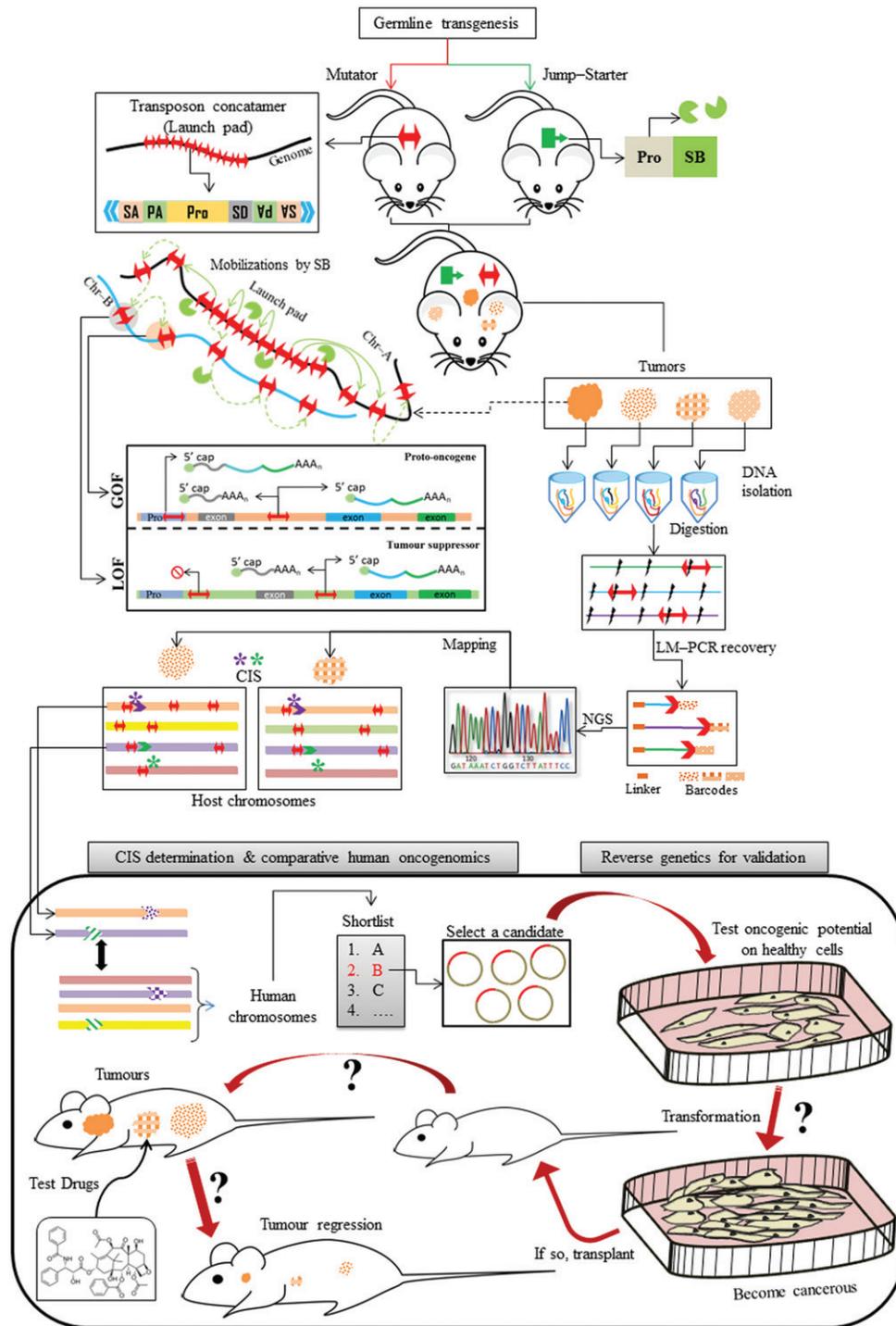
Figure 9. *Sleeping Beauty* transposon-based functional oncogenomics. An overall scheme depicting key steps for employing the SB system for oncogenomics. The conditional insertional mutagenesis begins with the generation of transgenic lineages of the chosen model system, popularly referred to as the mutator (transposon) and jump-starter (transposase) lineages. The mutator lineage is transgenic for a custom-engineered transposon (red double-headed arrow) that can be mobilized by the SB transposase (green pie). The mutator transposon is designed so that by virtue, it can promote, alter or even terminate expression of endogenous reading frames upon SB mediated insertion in either orientations eventually leading to gain and/or loss of functions. This entire scenario can be rendered "conditional" by restricting the activity of SB to specific tissues or organs of choice by placing it under the command of appropriate promoter. The double-transgenic animals are aged for development of relevant cancer phenotypes. The next step involves the identification of the transposon integration sites that very likely resulted in the observed phenotypes. This primarily involves extraction and digestion of the DNA from which these sites are recovered by LM-PCR, barcoded and sequenced by next generation sequencing. The recovered sites are mapped on the genome, and subjected to a statistical analysis to identify common integration sites (CISs) that were present in majority of the tumors. Shortlisted candidates are further validated by reverse genetic approach as illustrated in the figure. Abbreviations: SA: splice acceptor; PA: poly-A tail; pro: promoter; SD: splice donor; GOF: gain of function; LOF: loss of function; Chr: chromosome; LM-PCR: linker-mediated PCR; NGS: next generation sequencing; CIS: common integration sites. A color version of the figure is available online (see color version of this figure at www.informahealthcare.com/bmg).

have the advantage of being unbiased with respect to the sequences they mutagenize, whereas gene-editing tools are biased by their very nature. It is likely, that transposons will continue to uniquely contribute to the genome-engineering toolbox for many years to come.

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Disclosure statement

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