Chapter 11

Transposons for Non-Viral Gene Transfer

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Additional information is available at the end of the chapter

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1. Introduction

DNA based transposon vectors offer a mechanism for non-viral gene delivery into mammalian and human cells. These vectors work via a cut-and-paste mechanim whereby transposon DNA containing a transgene(s) of interest is integrated into chromosomal DNA by a transposase enzyme. The first DNA based transposon system which worked efficienty in human cells was *sleeping beauty*. This was followed a few years later by the use of the *piggyBac* transposon system in mammalian and human cells. The advantages of transposon vectors include lower cost, less innate immunogenicity, and the ability to easily co-deliver multiple genes when compared to viral vectors. However, when compared to viral vectors, non-viral transposon systems are limited by delivery to cells, they are possibly still immunogenic, and they can be less efficient depending on the cell type of interest. Nonetheless, transposons have shown promise in genetic modification of clinical grade cell types such as human T lymphocytes, induced pluripotent stem cells, and stem cells. Recently generated hyperactive transposon elements have improved gene delivery to levels similar to that obtained with viral vectors. In addition, current research is focused on manipulating transposon systems to achieve user-selected and site-directed genomic integration of transposon DNA cargo to improve safety and efficacy of transgene delivery. DNA based transposon systems represent a powerful tool for gene therapy and genome engineering applications.

2. Transposons as gene delivery systems

Transposons or mobile genetic elements were first described by Barbara McClintock as "jumping genes" responsible for mosaicism in maize [1]. Transposons are found in the genome of all eukaryotes and in humans at least 45% of the genome is derived from such ele-

ments [2]. Transposons active in eukaryotes can work either by a "copy and paste" (Class I) or "cut and paste" (Class II) mechanism (Figure 1).

In the "copy and paste" mechanism, the transposon first makes a copy of itself via an RNA intermediate (hence also known as retrotransposons).Class II DNA-transposons work by a "cut and paste" mechanism in which the transposon is excised by the transposase upon expression and then relocates to a new locus by creating double strand breaks *in situ*. Most transposon systems used for gene delivery use a modified "cut and paste" system consisting of a transposon carrying the transgene of interest and a helper plasmid expressing the transposase (Figure 2). The "cut and paste" transposition mechanism involves recognition of the inverted terminal repeat sequences (IRs) by the transposase and excision of the transposon from the donor loci, usually a supplied plasmid. The two most commonly used transposon system for genetic modification of mammalian and human cells are *sleeping beauty* and *piggyBac*.

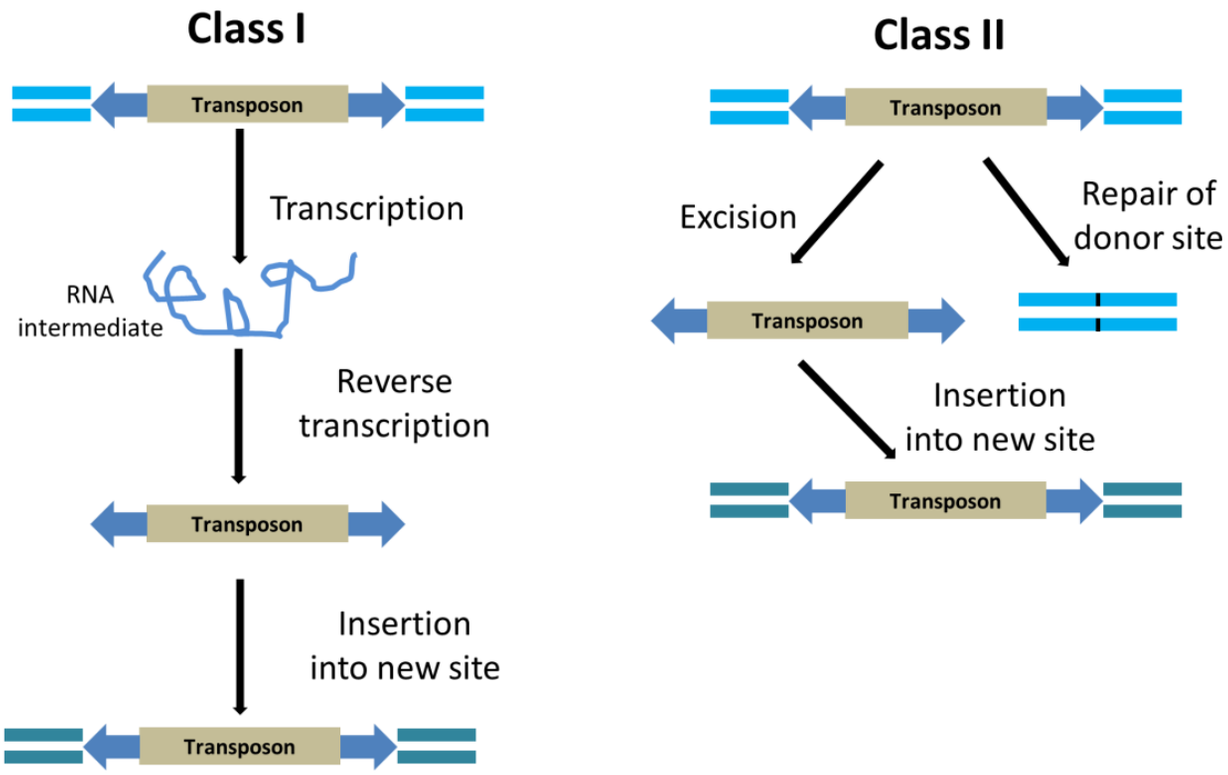

Figure 1. Class I and II transposons and mechanisms of integration.

The *sleeping beauty* (SB) transposon was reconstructed from the genome of salmonid fish using molecular phylogenetic data [3] and belongs to the Tc1/mariner superfamily of transposons. The sleeping beauty transposon is flanked by 230bp IRs which conatin within them non identical direct repeats (DRs).

The *piggyBac* transposon was isolated from cabbage looper moth *Trichoplusia ni*[4].One desirable feature of the *piggyBac* system is the precise excision of the transposon from the do-

nor site without leaving behind any footprints [5], making it an attractive feature for cellular reprogramming. Excision of the transposon from the donor site, creates complimentary TTAA overhangs which undergo simple ligation to regenerate the donor site bypassing DNA synthesis during transposition [6].

In "cis" delivery the transposase is carried by the same plasmid backbone as the transposn. In "trans" delivery it is delivered by a separate circular plasmid. For gene therapy purposes transposase and transposon are delivered either in "cis" or in "trans" (Figure 2). In "cis" delivery the transposase is carried on the same vector backbone as the transposon carrying the gene of interest (GOI). In the "trans" configuration, the transposase is delivered by a separate non integrating plasmid. The "cis" configuration has been shown to improve transposition efficiency [7], but there is a question of whether the linearized backbone carrying the transposase may also get integrated and lead to residual transposase expression. A comparison of the properties of *sleeping beauty* and *piggyBac* is described in Table 1.

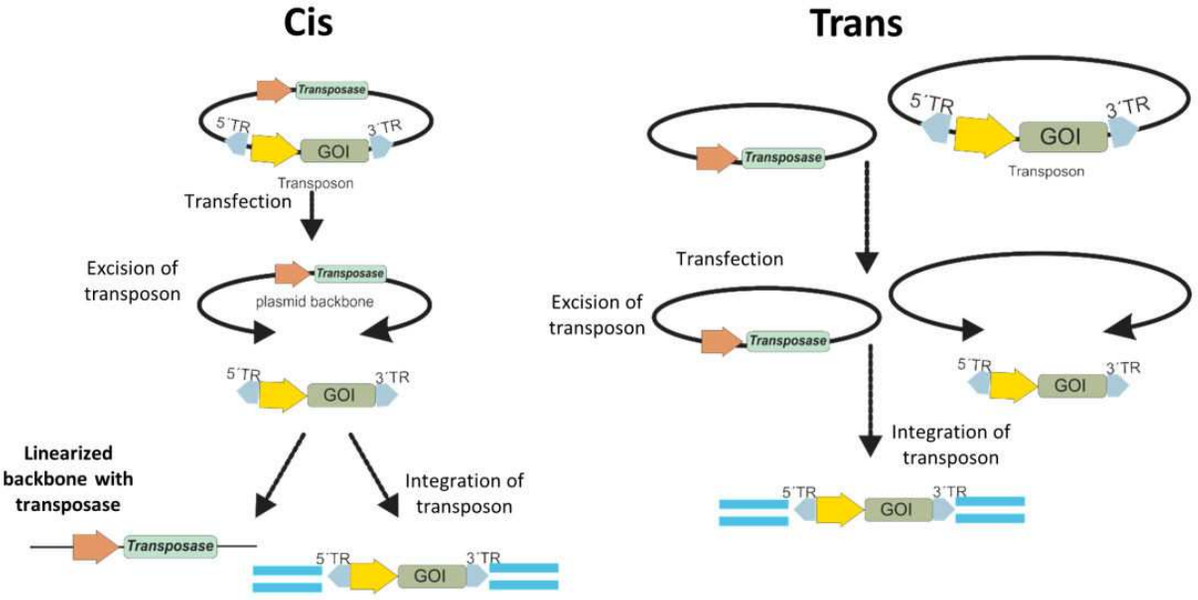

Figure 2. "Cis" and "Trans" transposon mediated gene delivery. GOI, gene of interest; 5'TR, 5' terminal repeat; 3'TR, 3' terminal repeat; the yellow and beige arrows indicate promoters to drive gene expression.

3. Advantages of transposon as gene delivery system

3.1. Lower cost compared to viral vectors

In spite of viral vectors having been successfully used in gene therapy clinical trials (e.g. generation of clinical grade T cells for immunotherapy [8], their use in extensive gene therapy regimens is constrained. Clinical grade viral vectors are very expensive to manufacture given the stringent regulatory oversight and limited number of GMP certified production fa-

cilities. A batch of clinical grade retroviral supernatant for treating patients costs between $400,000 to $500,000 (personal communciation, GMP facility director, Baylor College of Medicine). The production of clinical GMP (cGMP) grade viral supernatant is extremely time intensive as, in addition to optimization of culture conditions, the supernatant needs extensive testing for microbial contamination, presence of replication competent viral particles as well as validation of sequence and functionality. The entire production run and associated testing may require up to six months. These viral stocks also have limited shelf life. Upon release the desired cell type is transduced, selected and expanded which is then followed by quality assurance checks. This also requires extensive training of the personnel involved in production and testing and scaling up production as would be required for future gene therapy regimens will not be economical. In contrast, cGMP grade transposon plasmids can be manufactured more quickly. The production can be scaled up quickly and existing facilities can be upgraded and certified in a shorter time frame. The cost of manufacturing and release of cGMP grade plasmid DNA is between $20,000 and $ 40,000 [9]. The use of transposons drastically reduces both the time and cost of production of the gene delivery system. In the first clinical trial approved by the FDA for infusion of autologous *ex vivo sleeping beauty* modified T cells [10], the most time intensive step was the test for fungal and bacterial contamination (14 days).

	sleeping beauty	***piggyBac***
Cargo Capacity	~10 kb	>100 kb
Foot Print	Insertion site mutated upon excision	No "foot print" mutation
Needs titration for optimal activity	Yes	Yes
Hyper Active Versions	SB100X (most active SB version)	hyPBase
Effect of 'N' and 'C' terminal modifications	50% or more reduction in efficacy	No apparent reduction in efficiency
Integration site preference	More random	Slight increased preference for genes and TSS
Can be engineerd to bias integration sites	Yes	Yes

Table 1. Comparison of *sleeping beauty* and *piggyBac*properties. TSS, transcriptional start sites.

3.2. Delivery of large and multiple transgenes

Although retroviral and lentiviral vectors have been successfully used for delivering multiple transgenes, they are limited by their cargo capacity[11,12]. Both these vector systems can carry a limited cargo of up to 8kb which is limited by the packaging capacity of their capsid envelop [13]. Early reports demontrated the *sleeping beauty* system to have reduced efficiency beyond transposon size of 10kb [14]. In contrast the *piggyBac* system has been successful-

ly utilized to modify primary human lymphocytes with 15 kb transposon with an initial transfection efficiency of 20% which increased up to 90% upon selection and expansion [15]. The *piggyBac* system has been successfully used for mobilizing transposons as large as 100 kb in mouse embryonic stem (ES) cells [16]. An increased cargo capacity also imparts the ability to deliver multiple transgenes to the same cell. For example, using the *piggyBac* system, human cells were efficiently modified to express a three subunit functional sodium channel which retained its electro-physiological properties even after 35 passages [17].

3.3. Less immunogenicity

One of the major concerns for viral gene delivery system is the associated immunogenicity as evidenced by the death of a patient receiving liver targeted adenoviral gene therapy for partial ornithine transcarbamylase deficiency in 1999 [13].The systemic delivery of the viral particles initiated a cytokine storm leading to multiple organ failure within four days of administration of the vector [18]. Attempts have been made to reduce the immunogenicity of viral vectors by stripping them of all endogenous viral genes ('gutted' or 'helper-dependent' vectors) [19], but even the use of modified viral delivery systems are potentially immunogenic as evidenced by long term inflammation of rat brains injected with replication deficient adenoviral vectors [20].

Transposons are circular plasmid DNA molecules and do not contain a viral shell or viral antigens. The host response to non-viral vectors has not been well characterized. Toll-like receptor (TLR)-9 is known to recognize DNA with unmethylated CpG dinucleotides in the endosome- which can lead to signalling via MyD88 and production of inflammatory mediators such as TNF and IFN-α [21]. Other mechanisms of innate immune sensing of naked DNA include DNA-dependent activator of interferon (IFN)-regulatory factors (DAI) (also called Z-DNA-binding protein 1, ZBP1), RNA polymerase III (Pol III), absent in melanoma 2 (AIM2), leucine-rich repeat (in Flightless I) interacting protein-1 (Lrrfip1), DExD/H box helicases (DHX9 and DHX36), and most recently, the IFN-inducible protein IFI16 [22]. These molecules use independent and sometimes overlapping signalling pathways to elicit immune response to delivered DNA. Nonetheless, much remains to be discovered about host immune response to delivered DNA and how to overcome such an obstacle for effective gene therapy.

3.4. Less propensity for oncogenic mutations

Human immunodeficiency virus (HIV) has been shown to prefer genes for integration in SupT1 and Jurkat cells [23]. Murine leukemia virus (MLV) derived vectors have been used for stable gene transfer for therapy but they have been shown to prefer transcriptional start sites (TSS) for integration [24]. Integrations near the promoter of the LMO2 proto-oncogene has been associated with leukemia in the French X-SCID gene therapy trial [25]. The genome wide mapping of *sleeping beauty* transposons in mammals have revealed a modest bias towards transcriptional units and upstream regulatory sequences which varies between cell types [26]. The integration site profiling of both *piggyBac* in primary human cells and cell lines have revealed no preferred chromosomal hotspots [7,27]. It also has no preference for genomic repeat elements and known proto-oncogenes. *PiggyBac* has a preference for inte-

grating into RefSeq genes and near TSS and CpG enriched motifs although this may be influenced by the state of the cell or type of the cell. Both *sleeping beauty* and *piggyBac* are being engineered for site-directed gene delivery to improve the safety of gene transfer. True genotoxic risk for viral vectors was not discovered until they were used in humans. Transposons have not yet been used in humans, though one clinical trial has be approved.

4. Challenges of transposon as gene delivery system

Given the promise of transposons as gene delivery vehicle, it suffers from certain challenges e.g. reduced delivery, random integration profile and silencing of the integrated transgene.

4.1. Low delivery efficiency

Transposon systems are carried by naked DNA plasmids and there efficiency is limited to the efficiency of getting the plasmid into to the cell by chemical or physical means. Certain primary cells and cell lines are easy to transfect (e.g. HEK293, HeLa, Hepatocytes) and transposons have high transposition efficiency in these cells. But other clinically relevant cells (e.g. primary lymphocytes) are difficult to transfect. Often the method used for transfection (e.g. nucleofection and electroporation) is toxic to the cells and leads to excessive cell death thus reducing the efficiency of stable transfection. Efforts are on to circumvent these difficulties by developing novel delivery methods e.g. cell-penetrating peptides (CPP) –*piggyBac* fusions [28] or using polyethylenimine [29]. Some investigators have encapsulated transposon systems within viruses to use the virus to deliver the DNA from which transposition occurs [30-34] This may improve efficiency, however, the issues with immunogenicity of viruses remain.

4.2. Random integration profile

Transposons as described above have uncontrolled or relatively random integration preference with regards to genomic elements. This leaves the transposed transgene open to influence of the neighboring genomic region. Additional, uncontrolled or not site-directed integration increases the risk for possible genotoxicity.

4.3. Silencing of the integrated transgene

Gene silencing has been observed when using *sleeping beauty* in cultured cells [35]. Transgene silencing and epigenetic transgene modification has not been well studied with *piggyBac*.

5. Applications

Both *sleeping beauty* and *piggyBac* have demonstrated correct of disease phenotypes in animal models or in human cells (Table 2).

Disease	Transposon system	Reference
Hemophilia B	SB	[34,36]
Hemophilia A	SB	[37,38]
Tyrosinemia Type I	SB	[39]
JunctionalEpidermolysisBullosa	SB	[40]
Diabetes	SB	[41]
Huntington's disease	SB	[42]
Mucopolysaccharidosis I & VII	SB	[43,44]
α1-antitrypsin deficiency	PB	[45]

Table 2. List of diseases corrected with *Sleeping Beauty* (SB) and *piggyBac* (PB)

5.1. Genetic modification of human T lymphocytes

Peripheral blood and umbilical cord T cells have been extensively modified with both viral and non-viral gene delivery systems for immunotherapeutic purposes [10]. This therapeutic avenue has been successfully used for the treatment of viral infections and Epstein Barr virus (EBV) associated lymphoma post autologous bone marrow transplantation [46,47]. They also hold promise for treatment of other cancers [48-50]. But the use of of viral vectors for the generation of clinical grade T cells is expensive, time intensive and not free of risks. Non-viral gene delivery systems, including DNA transposons, are being increasingly explored as an alternative strategy.

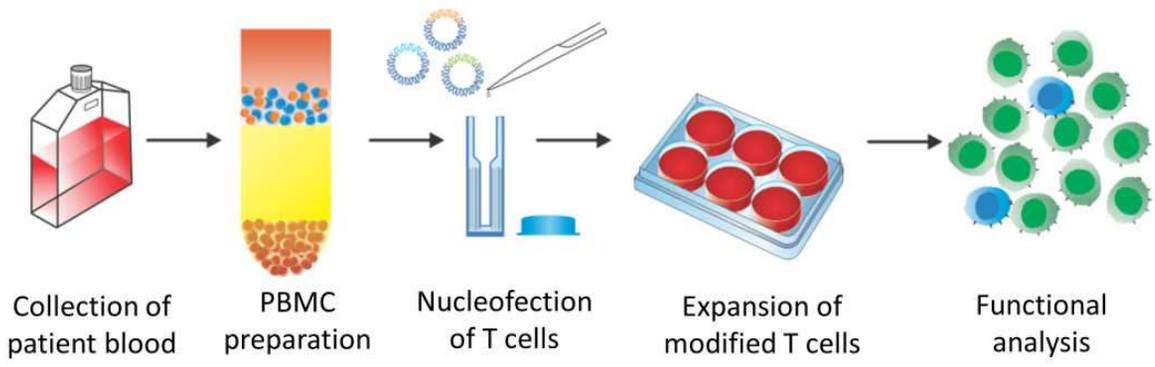

Figure 3. Schematic of transposon modificaiton of primary human T cells.

A schematic of how primary human T lymphocytes can be gene modified with transposons is shown in Figure 3. The *sleeping beauty* system was used to successfully modify peripheral blood mononuclear cells with a CD19-specific chimeric antigen receptor (CAR)[9]. These modified PBMCs were then used to generate CAR+ T cells which preserved their CD4+, CD8+, central memory and effector-effector cell phenotypes. The *piggyBac* system has also

been optimized to achieve stable transgene expression in human T lymphocytes [51]. Further, primary lymphocytes have been modified with multiple transgenes to redirect their specificity for CD19 and make them resistant to off target effects of chemotherapeutic drugs like rapamycin [15]. Cytotoxic T lymphocytes specific for Epstein Barr Virus (EBV) have also been successfully modified with human epidermal growth factor receptor-2 specific CAR (HER2-CAR)[52]. The first clinical trial involving transposon modified autologous T cells with a second generation CD19-specific CAR has been approved by the Food and Drug Administration[10]. This trial will involve the infusion of *ex vivo* expanded autologous T cells in patients undergoing autologous hematopoietic stem cell (HSC) transplantation with high risk of relapsed B-cell malignancies.

5.2. Generation of induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) generated from a patient's own differentiated somatic cells holds promise for regenerative medicine. Early successful attempts involved delivery of defined reprogramming factors using retroviral vectors [11,53]. Unfortunately 20% of the chimeric offspring obtained from germline transmission of retrovirally reprogrammed clones developed tumors due to reactivation of the c-myc oncogene [54]. In addition, ectopic expression of the reprogramming factor(s) has been linked to tumors and skin dysplasia [55-56]. One way to circumvent the use of viral delivery systems is to deliver the programming factors as recombinant proteins [57] or by repeated plasmid transfections [58], both of which have proven to be extremely slow and inefficient. The higher gene delivery efficiency of transposons together with their ability of being excised from the cells post reprogramming and differentiation make them an attractive choice for generating iPSCs.

Somatic cells have been transfected with *piggyBac* transposons carrying reprogramming factors and transposase. Reprogrammed iPSCs are selected and propagated to obtain individual iPSC clones. To generate transgene-free iPSCs, the transposase is re-expressed to remove the reprogramming factors followed by negative selection to identify transgene-free iPSCs (Figure 4).

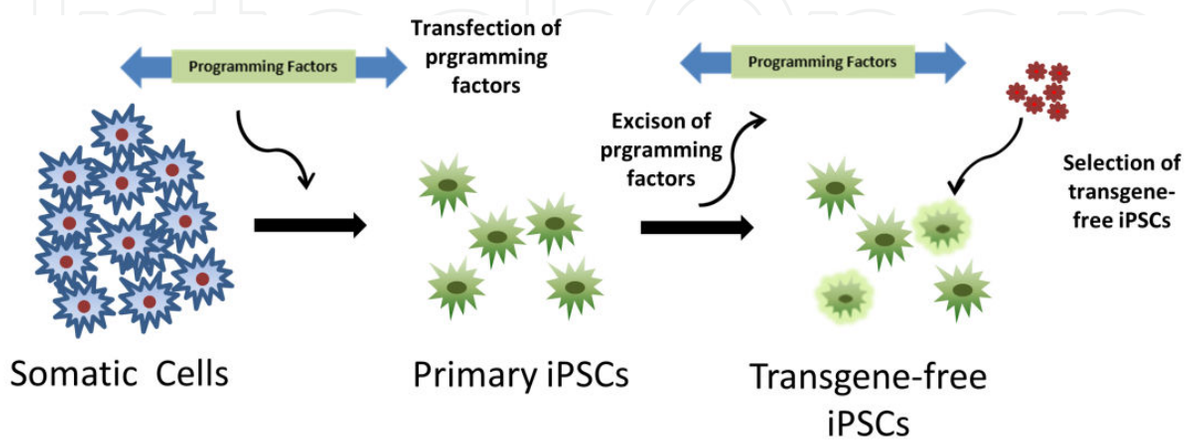

Figure 4. Generation of transgene-free iPSCs using the *piggyBac* system.

The *piggyBac* system seems to be ideally suited for this as it can undergo precise excision and does not leave behind "foot print" mutations [5]. In contrast, the *sleeping beauty* system has been shown to excise imprecisely leaving behind altered insertion sites [3]. The *piggyBac* system has been successfully used to generate transgene free iPSCs from both mouse and human embryonic fibroblasts with efficiency comparable to retroviral vectors [59-60]. *PiggyBac* has also been used to successfully reprogram murine tail tip fibroblasts into fully differentiated melanocytes which are more compatible with cell therapy regimens [61]. The use of a *piggyBac* based inducible reprogramming system also proved to be more stable and quicker than an inducible lentiviral system [62].

5.3. Genetic modification of stem cells

Transposons have been used for genetic modification of human embryonic stem cells [63]. More recently, transposons have been used to insert bacterial artificial chromosomes (BACs) in human ES cells [64]. Both *sleeping beauty* and *piggyBac* have been used to genetically modify hematopoietic stem cells [65]. Transposons provide an effective mechanism for permanent (or reversible in the case of *piggyBac*) genetic modification of a variety of stem cell types for eventual use in therapy.

6. Current hot topics and future directions

6.1. Generation of hyperactive transposon elements

SB100X and native *piggyBac* both have similar activity levels in human cells which is 100 fold more than the native *sleeping beauty*. The hyperactive *piggyBac* transposase (hyPBase) has been shown to have 2 to 3 fold more activity than SB100X or native PB [66] (Figure 5).

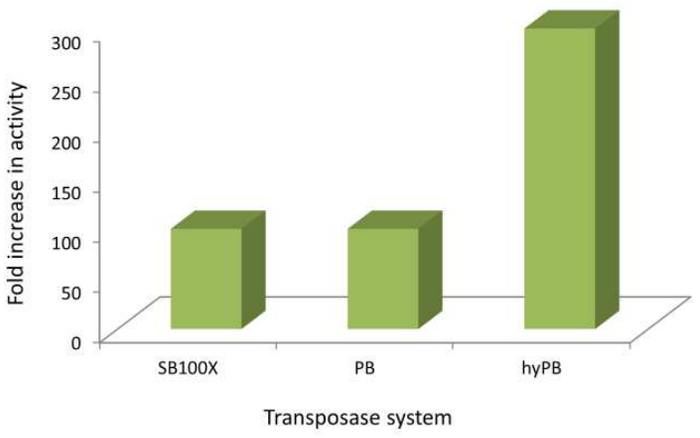

Figure 5. Comparison of transposase activity in human cells

Efficiency of transposition is perceived as a bottleneck to efficient gene delivery. Attempts to engineer hyperactive versions of transposase have resulted in versions with increasing transposition activity. Strategies employed include import of amino acids from related transposases [67], alanine scanning [68] and site directed mutagenesis [69]. The construction of the SB100X transposase with ~100 folds higher activity than the original *sleeping beauty* transposase employed a high throughput screen of mutant transposases obtained from DNA shuffling [70]. A hyperactive version of the *piggyBac* transposase (hyPBase) has also been engineered with 17-fold increase in excision and 9-fold increase in integration [71]. The hyPBase has 7 amino acid substitution identified from a screen of PBase mutants but none of the 7 substitutions are in the catalytic domain of the transposase. The hyPBase also has footprint mutation frequency (<5%) comparable to the wild type transposase and no apparent effect on genomic integrity. Unlike SB100X which showed a 50% reduction, the addition of a 24 kDa ZFN tag did not significantly alter transposition efficiency [66]. In vivo, a mouse codon optimized version of hyPBase showed 10-fold greater long term gene expression than both native *piggyBac* and SB100X.

6.2. Engineering transposon systems for site-directed integration

Random integration of transgene during delivery have resulted in adverse events including leukemia [25,72]. Integration of transgenes at other genetic loci may also affect expression of critical genes. Engineering transposon systems for site-directed integration would allow transgene delivery to sites in the genome resulting in improved gene expression, reduced positional effects at the site of integration, and improved safety. Most studies have utilized fusion of DNA-binding domains to the transposase to achieve site directed integration, beginning with the engineering of the *sleeping beauty* system. *Sleeping beauty* has been engineered to bias integration into plasmids containing target sites [73-74] and near selected elements and repeat elements in the genome [75-76]. The *piggyBac* system seems to be more suited for transposase modifications as the addition of additional domains to the transposase does not alter the systems efficiency [7,77-79]. A Gal4-*piggyBac* fusion transposase has been shown to bias integration near Gal4 sites in episomal plasmids [80] and the genome [81]. A chimeric transposase containing an engineered zinc finger protein (ZFP) fused to the native *piggyBac* transposase has also been successfully used to bias integration at the genomic level [79]. Researchers have also used transcription factor DNA binding domains fused to the *piggyBac* transposase to label nearby transcription factor binding sites in the genomes of cells [82]. Current approaches are hampered by the ability of the transposase to integrate on its own without the targeting machinery which can lead to off-target integration. Futher engineering modifications to both the transposase and transposon may overcome this limitation.

7. Conclusion

Transposon systems are well suited for *ex vivo* gene therapy and *in vivo* delivery to target organs may also become a reality in the future. The advantages of lower cost and more

widespread applicability than viral vectors, in combination with the potential for site-directed gene delivery, make transposons a promising non-viral gene delivery system as an alternative to viral vectors.

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