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***piggyBac*-ing models and new therapeutic strategies**

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Abstract

DNA transposons offer an efficient non-viral method of permanently modifying the genomes of mammalian cells. The *piggyBac* transposon system has proven effective in genomic engineering of mammalian cells for pre-clinical applications including gene discovery, simultaneous multiplexed genome modification, animal transgenesis, gene transfer *in vivo* achieving long-term gene expression in animals, and for genetic modification of clinically relevant cell types including induced pluripotent stem cells and human T lymphocytes. *piggyBac* has many desirable features including seamless excision of transposons from the genomic DNA and the potential to target integration events to desired DNA sequences. This review explores these recent applications and also highlights the unique advantages of using *piggyBac* for developing new molecular therapeutic strategies.

Keywords

piggyBac; transposon; iPSC; transgenesis; cell therapy; immunotherapy

***piggyBac*: a versatile tool for biotechnology**

DNA-based transposon systems first emerged as efficient tools for genome engineering of mammalian cells after the *Sleeping Beauty* transposon system was resurrected from the genome of the medaka fish [1]. Transposon DNA vectors can be engineered for a variety of purposes including transgenesis, gene therapy, gene trapping, or insertion of other DNA elements into the genomes of cells (see **Glossary**). The *piggyBac* transposon system is naturally active and was first discovered in insect cells while propagating Baculovirus in the TN-386 cell line, from the cabbage looper moth *Trichoplusia ni* of the order Lepidoptera [2, 3]. The cause of unexpected viral plaques was the insertion of novel Class II Mobile DNA elements in the Baculovirus. The inserted mobile DNA was being carried “piggyback” by the virus, so it was named *piggyBac*; the capitalized and shortened “Bac” part of the name signifies its Baculovirus-related discovery. Since its discovery in 1983, *piggyBac* (formerly

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named IFP-2) was studied mostly in insects. *piggyBac* was first shown to be efficient in gene transfer in mice in 2005 [4]. Since that time, it has been used for genomic modification of human cells [5] and for a variety of applications. These include mammalian transgenesis, mutagenesis, *ex vivo* modification of clinically relevant cell types, and gene transfer in mammals *in vivo*. Such applications have opened new areas of research that will hopefully lead to new therapeutic strategies for human disease.

piggyBac-based gene transfer or mobilization is carried out through a “cut and paste” mechanism (**Box 1**). For most applications, the *piggyBac* transposase and *piggyBac* transposon are carried on two separate plasmids (*trans*). It is also possible to deliver the transposase and transposon on the same plasmid (*cis*) with the transposase gene encoded outside of the transposon inverted terminal repeat elements (IRs). When the *piggyBac* transposase protein is expressed in mammalian cells, it binds to the inverted repeats of the transposon, nicking the DNA and freeing a 3' hydroxyl group at both ends of the transposon. This results in hydrophilic attack of the flanking TTAA sequence and hairpin formation (**Box 1**), freeing the transposon from its plasmid backbone [6]. The plasmid backbone is then repaired by host cell factors by ligation of the complementary TTAA overhangs. *piggyBac* transposase locates TTAA sequences in the genomic DNA of the mammalian cells. Through hairpin resolution of the transposon and hydrophilic attack of the genomic DNA by 3' hydroxyl groups on the transposon, a staggered 4-bp cut in the genomic DNA is produced, creating a transient double-strand break with TTAA overhangs on both sides of the break. The transposon is then inserted into the genomic DNA at the TTAA site, resulting in a duplication of this TTAA such that a TTAA is found on both sides of the transposon. Upon excision of the transposon by *piggyBac* transposase, which can be induced and selected for to rid the cells of the transgene, the single stranded TTAAs are religated to reform a single TTAA. Thus, the unique mechanism of *piggyBac* transposition results in a unique advantage: seamless excision of the transposon sequence (**Box 2**). This phenomenon has been put to great advantage already to generate transgene-free induced pluripotent stem (iPS) cells [7, 8]. The *piggyBac* transposon system has proven important for a number of versatile biotechnology applications (Figure 1). *piggyBac* enables greater cargo capacity compared to commonly used viral vectors for gene transfer and it has a different genomic target sequence, a higher likelihood of hitting genes, and does not suffer from overproduction inhibition as compared to *Sleeping Beauty* (Table 1).

Gene discovery via insertional mutagenesis

piggyBac has been used for insertional mutagenesis to evaluate various pathways both in tissue culture *ex vivo* and in live animals *in vivo*. Transposon integrations can be recovered and mapped to identify genes that have been misregulated (either activated or inactivated), resulting in the phenotype of the mutant cell or organism [9]. As *piggyBac* is known to have different genomic target site preferences than viruses or other transposon systems, it has proven to be a new valuable tool for gene discovery [9]. The ability to turn on mutagenesis in transgenic animals with exquisite control over the timing and cell type of interest has led to the discovery of many new oncogenes and tumor suppressors [9].

Insertional mutagenesis is a powerful way to elucidate genes involved in a variety of pathways in cultured cells. *In vitro*, *piggyBac* has been used for gene discovery in both mouse neural stem cells [10] and human pancreatic cells [11]. Studies in mouse embryonic stem (mES) cells have included genetic manipulation of *Blm*-deficient mES cells [12] and inducible genomic transposition in mES cells for gene trapping [13].

piggyBac transposons were used to insert *loxP* sites into the mouse genome whereby subsequent expression of the Cre recombinase would result in large chromosomal deletions in intact animals [14]. Reporter-gene trapping mouse lines [15] and cancer gene discovery lines [9] have been generated to aid in gene discovery, identifying genes that were not previously identified by retroviral or *Sleeping Beauty* transposon screens. Additionally, *piggyBac* and *Sleeping Beauty* may be used in a combinatorial manner if desired as transposons contain both sets of inverted repeats [9]. Recently, a conditional *piggyBac* system was employed to identify novel genes in pancreatic cancer, revealing the power of the versatile transposon system in identifying new cancer genes that could be applied to a variety of tissues and cancer types [16].

Generation of transgenic animals

Animal transgenesis represents an important methodology for discovering and evaluating disease processes and establishing pre-clinical models of human diseases. One of the limiting factors for generation of traditional transgenic animals is the low efficiency of generating them. The increased efficiency of transgenesis after employing *piggyBac* can make such manipulations more efficient and affordable. *piggyBac* was used to make transgenic mice that expressed reporter genes [4]. Subsequent studies have also used *piggyBac* to genetically modify mouse embryonic (ES) cells [12, 17, 18]. Rat induced pluripotent stem cells [19] have also been modified by *piggyBac* to make transgenic animals [20]. Pig cells have also been gene-modified using *piggyBac* to make transgenic animals [21, 22]. Animal transgenesis remains an important research endeavor for modelling human disease.

Engineering stable cell lines

piggyBac offers a versatile plasmid based system for stable cell line generation, especially for generation of cultured cells stably expressing one or more recombinant gene sequences for biomedical research, biotechnology, or drug development. More traditional methods of transfecting plasmids and using antibiotic selection are not efficient (Figure 2) and have severe limitations especially when engineering cells to co-express multiple transgenes or multi-protein complexes. Multiplexed *piggyBac* transposon delivery was by co-expressing large transgenes encoding a multi-subunit neuronal voltage-gated sodium channel (SCN1A) containing a pore-forming subunit and two accessory subunits, plus two additional genes for selection and obtained robust sodium current through 38 passages suitable for use on an automated high-throughput electrophysiology platform [23]. Three large (up to 10.8 kb) *piggyBac* transposons were co-delivered, thereby generating a heterozygous SCN1A stable cell line with robust functional expression. Others have also generated vectors capable of

modular assembly such that multiple transgenes can be co-integrated into cells using a single *piggyBac* vector [24].

Investigators have demonstrated the ability of *piggyBac* to generate stable cell lines for large-scale protein production [25, 26]. Engineered doxycycline inducible stable cells lines have proven capable of reproducible protein production in the absence of selection, even the ability to produce 750 mg of an endoplasmic-reticulum-resident fucosyltransferase. Investigators have also used *piggyBac* transposons with inducible expression for myogenic differentiation of human iPSCs including human a human myopathy [27] and to reliably incorporate BAC transgenesis in human embryonic stem cells [28]. Therefore, the plasmid based *piggyBac* system offers a capable transgene delivery system for single or simultaneous multi-gene modification of cultured cells for a variety of research applications, including potential new therapies (**Box 3**).

Modification of clinically relevant cells

Cell therapy strategies are emerging for a variety of human diseases. *piggyBac* has been used to generate mouse and human induced pluripotent stem cells (iPSC) and to modify human iPSCs, human embryonic stem (hES) cells, human hematopoietic stem cells (HSCs), and human T lymphocytes.

Cellular reprogramming of iPS cells

iPSCs were first generated by using a combination of transcription factors to reprogram mammalian cells to a stem cell-like phenotype [29, 30]. The factors were discovered by narrowing a pool of 15 retroviral vectors, each carrying a different transcription factor, down to the four factors responsible for reprogramming. However, retroviral insertions were variable in number for each factor when introduced in this manner. To better control copy number in the genome, all four factors could be introduced on one vector using a 2A protease cleavage sequence to separate the different genes [31, 32]. Using a non-viral method simplified and reduced the cost of producing the four-factor vector. Additionally, transgene-free iPS cells would be more desirable than those still harboring the transgene insertions because reactivation of the inserted genes could result in oncogenic transformation [33]. *piggyBac* has been demonstrated to have a particular advantage in this area because the reprogramming factors, when introduced on a transposon, can be precisely excised from the iPSCs by re-expression of the *piggyBac* transposase. iPSC clones from which the reprogramming factors were excised were found to have an un-manipulated genome lacking any apparent mutations from the initial four-factor gene insertion [7, 8, 34]. Such “transgene-free” iPSCs will likely be needed for eventual therapeutic application, as accidental activation of the inserted transgenes could result in oncogenic transformation of differentiated iPSCs. *piggyBac* has successfully been used to generate iPSCs from a variety of species [7, 20, 34–36]. Recently, *piggyBac* was used to excise the selection component of a gene-targeting cassette in human iPSCs derived from patients with α 1-antitrypsin deficiency. Zinc-finger nucleases were used to target integration of a therapeutic cassette into the α 1-antitrypsin locus. A *piggyBac* transposon used for selection was subsequently excised, leaving behind gene repair with precise removal of the selection cassette [36].

Modification of HSCs

piggyBac has also been used for genetic modification of HSCs. Grabundjia et al. compared native *piggyBac* to *SB100X*, currently the most hyperactive version of the *Sleeping Beauty* transposase [37]. Although *SB100X* appeared more active in this cell type, both systems were capable of modifying HSCs [37]. Recently, a hyperactive *piggyBac* transposase has been generated which appears more active than *SB100X* in other cell types [38] but a comparison of hyperactive *piggyBac* to *SB100X* in HSCs has not yet been reported.

Transposon-modified T cells

Human T lymphocytes are an attractive cell type for adoptive immunotherapy for cancer. Retroviruses have been most widely used for genetic modification of T cells for adoptive transfer in clinical trials. Transposon-mediated gene modification of human T cells was first reported using *Sleeping Beauty* [39]. Recently, a human clinical trial has been approved for *Sleeping Beauty* transposon modification of T cells to target CD19-positive lymphoma [40]. The *piggyBac* transposon system has also been successfully utilized for gene-modification of human T cells. Stable transgene expression of up to ~40% without selection was achieved using *piggyBac* followed by multiple logs of expansion of primary T-cells in culture [41]. *piggyBac* could simultaneously express multiple transgenes from two-independent transposons in combination with magnetic beads-selection for the transgenic surface marker, truncated CD19, in ~85% T cells for over 9 weeks [41]. We used *piggyBac* to deliver a large 14 kb transposon in T-cells and to deliver a non-immunogenic suicide gene, inducible caspase 9 [41]. In human T-cells, integration site mapping showed that *piggyBac* did not preferentially integrate into or near known proto-oncogenes [42]. Hypothetically, *piggyBac* should be safer than the widely used retroviruses because *piggyBac* has less of a preference for proto-oncogenes and there have been no genotoxic events observed when using retroviruses in humans in T cell modification. Human T-cells that were modified using *piggyBac*-transposons to express a chimeric antigen receptor targeting the CD19 antigen effectively killed CD19-expressing human lymphoma cell lines, demonstrating the functional activity of *piggyBac*-modified T-cells [43].

Virus-specific T cells have been shown to persist long-term in humans after genetic modification *ex vivo* followed by infusion [44]. *piggyBac* was used to genetically modify Epstein Barr Virus-specific cytotoxic T lymphocytes to target HER2 expressing cancer cells both *in vitro* and *in vivo* [45]. Additionally, *piggyBac* has recently been used to engineer T-cells to be resistant to rapamycin via delivery of a large mTOR transgene, a strategy which could be used in combination with rapamycin therapy for lymphoma [46]. Therefore, *piggyBac* has enabled high gene transfer efficiency in human T-cells and the ability to expand cells to clinical numbers. The unique features of *piggyBac* allow us to transfer large segments of DNA and to co-deliver and co-express multiple transgenes. The ability to deliver a suicide gene that can be selectively activated for ablation of all of the introduced *ex vivo*-modified cells should improve safety. In pre-clinical models, *piggyBac* gene-modified human T-cells performed targeted killing of cancer cells *in vitro* and *in vivo*. *piggyBac* seems to be a promising non-viral gene delivery system for modifying T cells for cancer immunotherapy in a future clinical trial.

***In vivo* gene transfer**

Transposon systems have been used for *in vivo* gene transfer in mice, including correction of inherited diseases [47]. Hydrodynamic tail vein injection, which targets mouse liver, has been used to deliver *piggyBac* transposons containing reporter genes *in vivo* (Figure 2) [48, 49]. Long-term inducible gene expression *in vivo* has been observed after a single hydrodynamic injection of transposon DNA [49]. In addition to hydrodynamic injection, *piggyBac* transposons have been delivered to the mouse lung in polyethylenamine complexes and achieved long-term gene expression from the *piggyBac* transposons [49]. By modifying hydrodynamic injection to specifically target the kidneys of live mice, multiple transgenes have been delivered to the kidney [50, 51]. Kidney-targeted delivery of *piggyBac* transposons carrying either glutathione S-transferase A4 [51] or insulin-like growth factor-1 receptor [50] gave significant protection against obstruction-induced renal fibrosis.

More recently, investigators have demonstrated phenotypic correction of hemophilia A [52] and B [53] by *piggyBac*-mediated gene transfer to mouse liver. One report coupled the use of *piggyBac* with novel synthetic cell-type-specific promoters to improve efficiency of gene expression *in vivo* [53]. Others have created hybrid adenovirus and adeno-associated virus (AAV) *piggyBac* vectors capable of *in vivo* gene transfer [54, 55]. Such vectors can overcome the limitations of delivering plasmid DNA into tissues by harnessing viruses to gain entry into the desired cell-type of interest.

Special considerations of *piggyBac*

***piggyBac* vs nuclease platforms**

Given the recent advances of precision genome engineering including zinc finger nucleases, transcription activator-like effector nucleases (TALENs) and Cas9:sgRNA endonucleases [56], why would one consider using *piggyBac* for genome modification? All nuclease platforms appear to require homologous recombination for gene repair or transgene insertion. Homologous recombination usually has poor efficiency for gene transfer or modification in adult somatic quiescent tissues because the cellular homologous recombination machinery is not present. In adult tissues, non-homologous end-joining is preferred instead, resulting in disruptive mutations that result in loss of gene function, making gene addition strategies for somatic gene therapy perhaps unattainable with the nucleases.

piggyBac transposase is an enzyme which actively integrates DNA cargo without the need for cellular cofactors. However, integration of therapeutic genes inserted in the genome can potentially cause mutagenesis and impair the integrity of the genome. The level of gene expression in a gene-addition strategy may exceed endogenous levels, though cell-type-specific promoters can be used. Nuclease-based homologous recombination can edit the genome at a precise location to attain restored gene expression under endogenous regulation. However, nuclease platforms must be carefully titrated to avoid toxicity and off-target effects. For example, Cas9:sgRNA has been shown to result in chromosomal translocations in some instances [57]. *piggyBac* offers an adaptable system for co-delivery of one or multiple transgenes including other elements such as those required for inducible

expression. There are also clear advantages of *piggyBac* for gene discovery or to generate stable cell lines.

Precise transposon excision

The *piggyBac* transposon system is unique in that it performs precise excision, leaving behind no footprint mutation. Precise excision was first demonstrated in insect cells [58] and subsequently in mammalian and human cells [5]. This characteristic of *piggyBac* has been used to generate transgene-free iPSCs, whereby transgenes are delivered for reprogramming on *piggyBac* transposons [7, 8, 34]. The *piggyBac* transposase can be re-expressed after reprogramming has occurred and the transgene cassettes can be excised from the genome, leaving behind no trace of the reprogramming elements or other genomic alterations. Other transposon systems such as *Sleeping Beauty* leave behind a footprint mutation [1, 59]. For gene-correction in human iPSCs, investigators have used a *piggyBac* selection transposon to enable selection of cells corrected by zinc finger nucleases. Subsequently, the *piggyBac* selection transposon was precisely excised, thereby removing the selection cassette at the site of gene repair [36]. Investigators have created a *piggyBac* transposase capable of excision but lacking integration (Exc+/Int–, Table 1) properties for seamless excision and removal of integrated *piggyBac* transposons [60].

piggyBac fusion proteins

The *piggyBac* transposase appears to be more amenable to protein-domain addition than the *Sleeping Beauty* transposase. This was initially tested by adding a hemagglutinin epitope tag for detection of expression which revealed no altered activity with *piggyBac* compared to *Sleeping Beauty* [5]. This flexibility of the transposase has been utilized to create an inducible transposase enzyme whereby transposition is regulated by tamoxifen [61]. *piggyBac* has also been fused to DNA binding domains to the transposase enzyme with the goal of redirecting *piggyBac* integration at the genomic level [62, 63], including to identify transcription factor binding sites in cultured cells [64]. Maintenance of transposase activity may be dependent on the terminus and linker(s) used to create the fusion protein, as well as the protein to be fused, so creation of novel fusions requires rigorous validation that they are functional [65].

The ability to direct transposon integration into user-defined chromosomal locations should provide more specificity in gene transfer experiments and improve safety in gene therapy experiments. Investigators first demonstrated the ability to target *piggyBac* integrations in a plasmid-based assay in mosquito embryos [66]. Subsequent investigations have demonstrated the ability of *piggyBac* to achieve site-directed integration in plasmid assays in human cells and in the human genome [62, 67]. *piggyBac* has shown biased integration into artificial target loci integrated in human cells [62]. Fusion of the *piggyBac* transposase to a Gal4 DNA binding domain biased *piggyBac* integration within 0.8 kb of the 56,898 putative Gal4 DNA binding sites in the human genome at a rate of 24% compared to 5% of native *piggyBac* integrations within the same window [67]. Recently, investigators have fused the transposase to a TAL domain and isolated single-copy integration clones with directed transposition at the CCR5 genomic locus observing an integration rate of between 0.01–0.015% depending on the TAL utilized [68]. The ability of a DNA binding domain to

redirect *piggyBac* has also been used to “tag” transcription factor binding sites in mammalian cells [64]. Although the *piggyBac* transposase can be modified to achieve site-directed integration, there is room for improvement. The transposase can still integrate transposon DNA at sites that are independent of the attached DNA-binding domain. Mutagenesis strategies aimed at making transposase integration activity more dependent on fused DNA binding domains may improve specificity.

Control over transposon copy number

The *piggyBac* transposon system appears titratable, whereby the user can change the amounts of transposon and transposase plasmids provided to achieve as few (as little as one) or as many integrations per cell as is desired [18]. Naturally, if plasmid amounts are lowered to achieve fewer integrations per cell, this will likely result in a lower percentage of total cells transfected. On the opposite end of the spectrum, the ability to achieve multiple integrations per cell has been exploited for multiplexed gene delivery. *piggyBac* was used to simultaneously deliver ~28 kb of DNA on three different transposons creating stable cell lines expressing functional human brain sodium channels with the goal of using these cells for drug discovery [23]. The ability to integrate multiple transposon cargos simultaneously has enabled the creation of stable cell lines that could not be created previously and should open new avenues of research evaluating multi-protein complexes or signaling pathways.

Improved *piggyBac* mutants

Hyperactive *piggyBac* elements have also been generated. Investigators have improved activity through both transposase and transposon modifications. *epiggyBac* was generated by transposon inverted repeat mutation [69]. More recently, *piggyBac* was shown to be able to transpose in yeast and this was exploited to use random mutagenesis to create hyperactive transposases (HyPBBase) in yeast [70]. HyPBBase contains seven mutations and had increased transposition in mouse ES cells. A follow up study showed that HyPBBase increased transposition activity in human cells, *in vivo* in mice, and in primary human T cells, and this head-to-head comparison with *SB100X* found HyPBBase to be more active in human cells and *in vivo* in mice [38].

Unanswered questions

Will *piggyBac* prove safe enough for clinical gene transfer applications?

Insertional gene transfer carries with it the possible risk of insertional mutagenesis. Investigators have evaluated the potential for clonal outgrowth in long-term cultures of both *piggyBac*-modified human T lymphocytes [45] and human foreskin fibroblasts [71] and no clonal expansion has been observed. Additionally, there has been no observable tumor formation in the livers of wild-type mice modified with *piggyBac* out to one year in a small number of animals [49]. Nonetheless, with appropriate engineering of the transposon vector, insertional mutagenesis can be used to discover novel genes involved in a particular biological pathway. Long-term *in vivo* experimentation is necessary to determine the potential for genotoxicity when using *piggyBac* for potential gene therapy.

piggyBac has different genomic target selectivity when integrating into genomic DNA when compared to other transposon systems such as *Sleeping Beauty* [17, 72–74]. It has been demonstrated that *Sleeping Beauty* exhibits a more random integration pattern whereas *piggyBac* exhibits more of a preference for gene containing regions and transcriptional start sites. Therefore, *piggyBac* may result in more reliable transgene expression; however, intragenic integrations could be detrimental to the host genome. Some investigators have altered *piggyBac* integration site selectivity at the genomic level by mutagenesis of the transposase [75]. This altered integration site selectivity at the genomic level was revealed by deep sequencing of thousands of recovered integration sites [75]. Additionally, further refinements could alter the genomic integration pattern of *piggyBac* to make it safer.

Does plasmid backbone integration occur?

Recently, a relatively high rate of plasmid backbone DNA integration when using *piggyBac* for genetic modification of human embryonic kidney (HEK-293) cells [71]. As *piggyBac* is highly active in HEK-293 cells, it remains to be determined if this phenomenon occurs in clinically relevant cell types such as human T lymphocytes or *in vivo* such as in mouse liver. The potential safety concern of the plasmid backbone randomly integrating into the host DNA remains to be determined, but can be nearly eliminated by cell sorting to remove cells expressing GFP from the integrated backbone [71]. Nonetheless, it remains to be determined if plasmid backbone integration occurs in clinically relevant cell types or *in vivo* animal models.

Will targeting of *piggyBac* improve selectivity and efficiency enough for gene therapy?

Investigators have demonstrated the ability to bias *piggyBac* integrations into user-defined genomic elements by fusing the transposase to ZFP or TAL proteins [62, 67, 68]. However, thus far, a high degree of specificity and selectivity in targeting integrations with *piggyBac* has not been demonstrated. Perhaps further refinement of the transposase or transposon could improve targeting specificity and efficiency. Targeting *piggyBac* integrations would greatly improve the safety of using this vector system for therapeutic gene transfer applications, especially for inherited diseases which will likely require a high degree of specificity in genome alteration with regards to safety.

Concluding remarks and future perspectives

The non-viral *piggyBac* transposon system has opened new areas of research with clinical implications. Undoubtedly, more studies will continue to be reported using *piggyBac* for gene discovery in cancer and other fields of research. The ability to create transgene-free iPSCs should improve the safety of their use in clinical practice. The use of *piggyBac* to efficiently modify human T cells may make immunotherapy applications more widely available due to its reduced cost as compared with viral vectors. Newer hyperactive *piggyBac* elements could increase efficiency even further. Finally, improving the targeting of *piggyBac* elements to user-defined chromosomal locations should improve safety and specificity in gene transfer approaches. *piggyBac* vector improvements and new delivery methodologies, whether viral or non-viral, should permit new biotechnology applications for transposon technology for pre-clinical and clinical applications.

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Glossary

Transposon	transposons are self-mobilizing pieces of nucleic acids. In this article we are referring to a specific subclass of transposons, the DNA transposons that employ a “cut and paste” non-replicating mechanism of transposition. When the transposase is expressed, the transposon is excised from its current location (plasmid, virus, or genomic DNA harboring the transposon) and moves (or transposes) to a new location. Therefore, transposons can be used to transfer genes into a cell or tissue (by using plasmid or viral DNA to carry the transposon into the cell) or to move transposons throughout the genome (such as in gene discovery applications).
Transgene	the transposon can be engineered to carry one or more gene(s) of interest which can be inserted into the genomic DNA. The transgene typically carries its own promoter. Alternatively, the transgene can be replaced with a gene trapping cassette for gene discovery. A wide variety of DNA sequences can be inserted into the transposon sequence for multiple different applications.
Seamless excision	after <i>piggyBac</i> excises the transposon from DNA, it seamlessly generates the original <i>piggyBac</i> target site more than 95% of the time. This characteristic of <i>piggyBac</i> has allowed it to be used to integrate DNA sequences (such as those harboring antibiotic resistance or transcription factors) which can then be removed by re-expression of the transposase.
Targeted integration	efforts are underway to target <i>piggyBac</i> integration into user-defined chromosomal loci. Native <i>piggyBac</i> integration is not targeted or site-specific. Targeting <i>piggyBac</i> integration would improve its safety and efficiency in gene transfer applications.

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Box 1: The mechanism of *piggyBac* transposition

For illustration purposes, transposition is demonstrated from plasmid DNA into the genome, although it could also be from one genomic locus to another. Once transposase is expressed (red circles) it binds to the *piggyBac* inverted repeats (IRs) and induces nicking and 3' hydrophilic attack of the TTAAs ends. Hairpin formation occurs with transposon excision. The transposon is then integrated and joined into genomic DNA at a TTAAs nucleotide sequence resulting in TTAAs target site duplication at the genomic locus. If precise excision is desired, transposase can be re-expressed and the transposon can be excised, thereby recreating the original TTAAs target site at the original locus.

Box 2: Seamless excision of *piggyBac* transposon, a unique tool for genetic manipulation

The *piggyBac* transposon integrates into a TTAA nucleotide sequence >95% of the time. Upon integration, target site-duplication occurs with a TTAA flanking both ends of the transposon DNA. Upon excision, the original TTAA target site is reformed >95% of the time. This seamless excision activity has led to in some clever genome engineering approaches. *piggyBac* can integrate transcription factor cassettes for cellular reprogramming into iPS cells. These factors may later be removed after re-expression of the transposase resulting in iPS cells with “unaltered” genomic DNA [8]. Others have performed gene editing of a disease locus in human cells and were able to select genome-modified cells with a *piggyBac* transposon selection cassette [30]. Once cells were selected and verified, the *piggyBac* transposon was excised, resulting in corrected human cells without additional genome modifications such as a remnant selection cassette.

Box 3: Generation of stable cell lines for drug discovery

Integrating viral vectors, such as retroviruses and lentiviruses, have limited cargo capacity (Table). In contrast, plasmid-based *piggyBac* has a large cargo capacity up to several hundred kilobases [76]. Multiplexed gene integration with *piggyBac* permits the design of novel cell-based assays for drug discovery, whether the target is a multi-protein complex such as a G-protein coupled receptor or a multi-subunit ion channel complex [23]. The ability to use a plasmid-based system to create stable cell lines expressing multiple transgenes should improve upon current drug discovery methodology. One can more easily generate stable cell lines expressing one or more transgenes which can be evaluated in high throughput screening assays to derive novel targets for potential therapies. *piggyBac* has also facilitated inducible large-scale protein production from human cells [25] for production of therapeutic enzymes, hormones, or antibodies.

Highlights

- The *piggyBac* transposon system offers a versatile genome modification system for biotechnology applications.
- *piggyBac* can be used for gene discovery or transgene insertion *in vitro* or *in vivo*.
- Stable cell lines can be more easily generated with *piggyBac* as compared to other gene delivery systems.
- New therapeutic strategies should result from *piggyBac*-related gene transfer research.

Figure 1.

The versatility of *piggyBac* in biotechnology applications for modification of the mammalian genome. *piggyBac* has been effective for mutagenesis in both cell lines and mice, transgenesis in a variety of species, modification of clinically relevant cell types, and *in vivo* gene transfer. The transposase is also amenable to modification to create an inducible transposon system or fusion to DNA binding domains or other protein sequences.

Figure 2.

piggyBac activity in cultured cells and *in vivo*. (A) Left, HEK-293 cells were transfected with a plasmid containing an antibiotic selection transposon without (left) or with (right) transposase. Two days after transfection, cells were split into the antibiotic for selection and cultured for 2 weeks and finally fixed and stained with methylene blue for visualization. The left plate shows the traditional way of making stable cell lines (very inefficient). The right plate demonstrates the efficiency of *piggyBac* in making stable cell lines (highly efficient). Right, a colony count on antibiotic resistant cells derived from a dilution of the plates on the left (figures adapted from [23]). (B) Hydrodynamic tail vein injection was used to deliver luciferase transposons to the livers of mice without (left) or with (right) transposase. *in vivo* imaging was then used to image luciferase expression at day 300 post gene transfer. Therefore, a single injection of *piggyBac* transposon DNA led to luciferase expression almost 1 year post gene transfer in a live animal (figure adapted from [49]).

Comparison of the *piggyBac* transposon system to the *Sleeping Beauty* transposon system and commonly used viral vectors.

Table 1

	Adenovirus	Adeno-associated virus	Retrovirus	<i>Sleeping Beauty</i>	<i>piggyBac</i>
Vector/nucleic acids	Viral/dsDNA	Viral/dsDNA	Viral/RNA	Non-viral/dsDNA	Non-viral/dsDNA
Maintenance	Episomal	wtAAV – integration rAAV-episomal	Integration	Integration	Integration
Cargo size	<8 kb	<5 kb	<8 kb	Increased cargo size decreases efficiency [77], has integrated a BAC[28]	Less cargo size limitation compared to SB, has integrated 100kb[76] and a BAC[28]
Integration mechanism		wtAAV – Rep dependent	Reverse transcriptase and integrase dependent (copy-and-paste)	Cut-and-paste	Cut-and-paste
Target site				TA	TTAA
Overproduction inhibition				Consistently reported	Rarely reported
Notable mutated variants				SB100X[78]	HyPBase[70] Exc+/Int–[60]
Integration profile		wtAAV – chromosome 19 rAAV – DNA breaks	Bias towards transcription units	Random	Bias towards transcription units
Genotoxicity		Insertional mutagenesis possible	Insertional mutagenesis possible	Insertional mutagenesis possible though more random integration pattern	Insertional mutagenesis possible
Enhancer/promoter activity		Weak	Strong	Minimal	Minimal
Immune response	+	+	+	+/-	+/-