Transposon-based Vector Systems for Gene Therapy Clinical Trials: Challenges and Considerations

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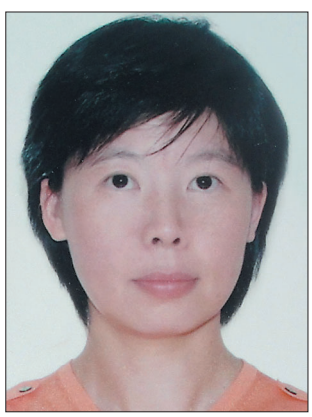
Much progress has been made in gene therapy, but significant challenges remain. One is development of a range of different tools that can be used for different therapeutic purposes. Another is site-specific gene targeting for safe and faithful therapeutic gene expression. Viruses have long been considered the most promising tools for human gene therapy. However, fatal side effects associated with viral vectors have hampered their clinical application. DNA transposons, widely utilized for decades as genetic tools in plants and insects, are now emerging as viable vectors for gene therapy. In this article, we will give a brief review of the adverse effects associated with virus-based gene therapy followed by a glimpse of the adeno-associated virus vector system, which is currently the most promising viral vector for gene therapy. The development of DNA transposon-based gene delivery systems and the advantages and limits of the most commonly used DNA transposon systems, *Sleeping Beauty, Tol2,* and *piggyBac,* will be extensively discussed Finally, we will focus on the most promising transposon system for gene therapy, *piggyBac*. Challenges and considerations for advancing *piggyBac* for therapeutic application will be critically addressed. *(Chang Gung Med J 2011;34:565-79)*



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Gene therapy is the insertion of genetic materials into an individual's cells and/or tissues to treat diseases. For many years, gene therapy has been championed for treating monogenetic inherited diseases such as cystic fibrosis and Duchenne's muscular dystrophy. More recently it has been explored for treating acquired diseases and polygenetic conditions including trauma tissues, cancers and diabetes. By correcting genetic defects causing diseases via genome manipulation, gene therapy can truly revolutionize medical intervention.

Gene therapy can be administered *in vivo* or *ex vivo* depending on the nature of a disease. A successful gene therapy system must perform several functions. In the simplest case, *ex vivo* gene therapy where isolated cells are treated *in vitro*, the therapeutic gene must first be delivered across the cell membrane, which is a significant barrier. Once delivered inside the cells, the therapeutic gene may exist episomally or be integrated into the host chromosome depending on the nature of the gene transfer vector. The latter is usually more desirable in treating inher-

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ited diseases, since it ensures faithful replication and segregation of the therapeutic gene during cell division to maintain long-lasting therapeutic gene expression. *In vivo* gene therapy is certainly the most challenging and may involve injecting the therapeutic vector system into target tissues or blood vessels close to the target area. *In vivo* gene therapy has been explored for treating cancers as well as inherited diseases like cystic fibrosis.[1,2] Currently, viral vector systems provide the most complete and efficient set of tools for these purposes. Non-viral systems, such as DNA transposons, have been actively developed as additional tools. Non-viral systems however lack mechanisms for crossing the cell membrane and rely on chemical transfection, physical devices, or a combination of these for gene delivery.

The first human gene therapy trial was initiated in 1989, and by 2007 over 1340 clinical trials in 28 countries had been completed.[3,4] Viruses are currently the vector of choice and yet very few phase II or III clinical trials are being conducted.[4] This reflects the need to explore new avenues and find new routes for efficient, safe gene delivery. Here we present an overview of the challenges and considerations involved in the development of non-viral DNA transposon-based systems, and focus on *piggyBac*-based vectors to achieve ultimate success in gene therapy.

Adverse effects associated with virus-based gene therapies

Virus-based vectors are the most commonly used gene delivery systems in gene therapy because of their highly efficient infection rate and ability to integrate therapeutic genes into the host chromosome to ensure stable and long term gene expression. As of 2007, 68% of gene therapy clinical trials completed were virus-based.[4] However, the initial enthusiasm for the use of viruses in gene therapy has diminished in the light of virus- associated fatal adverse events. The first occurred in 1999 when a young man had a fatal systemic inflammatory response syndrome following adenoviral gene transfer to treat ornithine transcarbamylase deficiency.[5] Gene therapy seemed more promising with the first successful treatment of children suffering from X-linked severe combined immunodeficiency (SCID-X1) in France in 2000.[6] However, a major setback occurred in 2002, when two of the ten children treated with SCID gene therapy developed T-cell leukemia, and one died.[7] The incidents were subsequently shown to be due to insertional mutagenesis caused by retroviral vector insertion leading to the activation of LMO-2, which is known to play a role in leukemia.[8]

Adeno-associated virus, the most promising viral vector for gene therapy

Up to 2007, adenoviruses and retroviruses were the two most popular virus vectors used for gene therapy.[4] Owing to their association with fatal adverse effects, enormous effort has focused on the development of the much safer virus-based vector, the adeno-associated virus (AAV). AAV is a parvovirus with a 4.7 kb single-stranded DNA genome and depends on a helper virus, usually an adenovirus, to proliferate.[9] It is capable of infecting both dividing and non- dividing cells. In the absence of a helper virus, it integrates into a specific point of the host genome (19q 13-qter) at a high frequency without known genotoxicity to the host.[10-12] Wild-type AAV is nonpathogenic with low immunogenicity. Recombinant AAV vectors have been used safely in gene therapy trials for genetic diseases, Parkinson's disease, Alzheimer's disease and cancers. Two recent AAV-based gene therapy clinical trials for Leber's congenital amaurosis, a common cause of blindness in infants and children, resulted in improved vision in response to treatment without serious adverse effects.[13,14] The fatal adverse effects associated with adenoviruses and retroviruses and the success of nonpathogenic AAV in recent trials have resulted in a dramatic change in the landscape of vectors used in gene therapy. AAV, a once unpopular vector for gene therapy, is emerging as one of the major vector systems in gene therapy clinical trials (3.5% as of 2007).[4] Although AAV is promising, its limited cargo capacity with a maximum of 4.9 Kb makes it difficult to insert a large genes into the host genome. Additionally, even though AAV is low in immunogenicity, AAV neutralizing antibodies prevalent in humans can hamper the clinical success of this approach. This reflects the need to explore new avenues for safe and efficient therapeutic gene delivery.

DNA transposon-based vectors

Because of their low immunogenicity and a low risk of causing insertional mutagenesis, naked DNA and plasmids remain popular as vectors for gene

therapy today. However, their episomal feature resulting in transient gene expression makes them unsuitable gene therapy vectors when long-term therapeutic gene expression is needed for treatment. DNA transposons have the desired features possessed by naked DNA and plasmids as well as the ability to insert transgenes into host chromosomes for long-term transgene expression, and are therefore gaining momentum as gene delivery vectors for gene therapy. DNA transposons are natural genetic elements residing in the genome as repetitive sequences that move through a direct cut-and-paste mechanism. A simple transposon is organized by terminal inverted repeats embracing a gene encoding transposase, an enzyme required for its relocation (Figure).

The cut-and-paste process, called transposition,

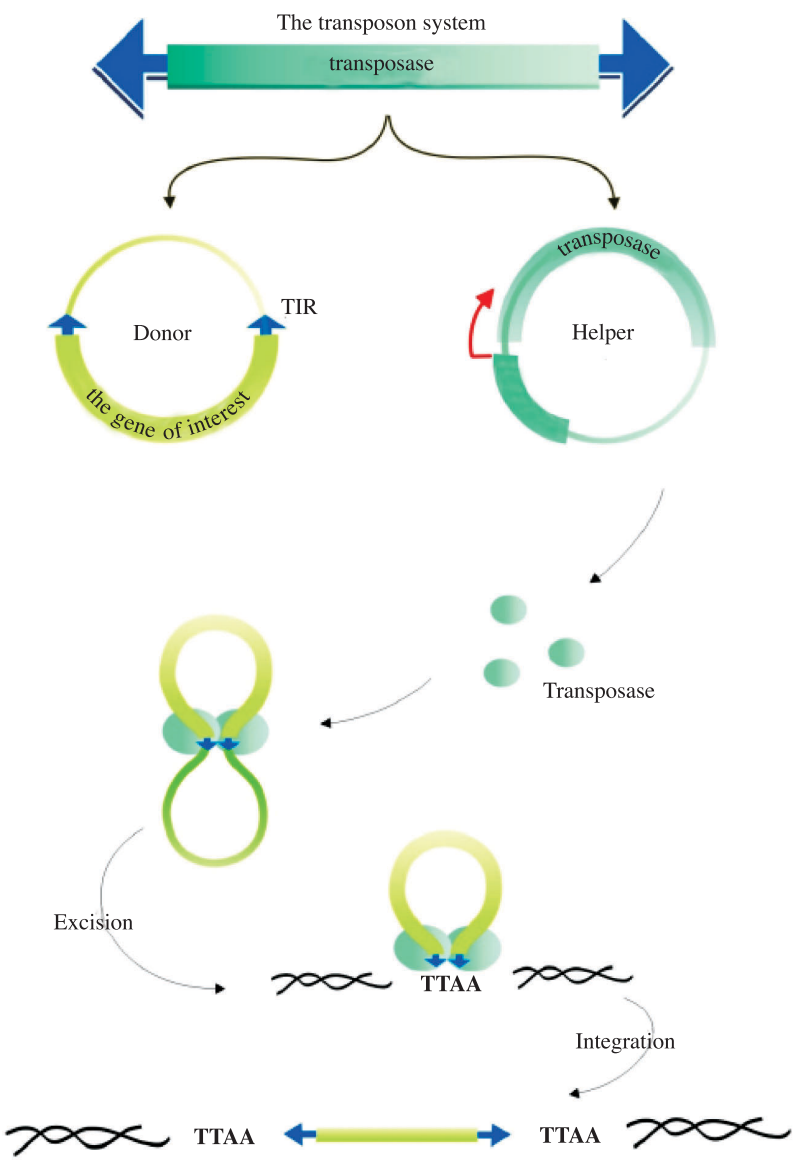

Figure Cut-and-paste transposition of a two-plasmid transposon system. As a genetic manipulation tool, a transposon is divided into two parts, a donor and a helper plasmid. The donor plasmid contains genes of interest embraced by terminal inverted repeats (TIRs). The helper plasmid expresses transposase which binds to the TIRs and excises the transposon from the donor plasmid. The excised transposon is then brought to the target site by the transposase, followed by integration.

makes DNA transposons particularly attractive as gene delivery tools. To turn DNA transposons into a gene delivery tool, a two-plasmid system, consisting of a helper plasmid expressing the transposase and a donor plasmid with the terminal repeat sequences embracing genes of interest, has been developed (Figure). Using this system, transposons have been utilized extensively as genetic tools in invertebrates and in plants for transgenesis and insertional mutagenesis.[15-20] Such tools, however, were not available for genome manipulations in vertebrates or mammals until the reactivation of a *Tc1*/*mariner*-like element, *Sleeping Beauty*, from fossils of the salmonid fish genome.[21,22] Subsequently, several transposons, some which exist naturally, such as Medaka fish *Tol2,* a member of the hAT family and cabbage looper moth *piggyBac,* the founding member of the *piggyBac* family, and some which have been reconstructed from humans, such as *Tc1*/mariner-like Frog Prince from *Rana pipiens*, were shown to effectively transpose in mammalian cells.[23-28] Among them, *Sleeping Beauty, Tol2,* and *piggyBac*, have been extensively evaluated for gene therapy.[29-32] We will discuss the pros and cons of these three transposon systems for gene therapy, focusing on efficiency, cargo capacity, stable gene expression, genotoxicity, and potential application in induced pluripotent stem cell (iPSC) technologies.

Efficiency

Previously we demonstrated that *piggyBac* is a more active transposon system in various mammalian cell types compared with *Tol2* and *SB11*, a hyperactive version of *Sleeping Beauty*.[27] A new hyperactive *Sleeping Beauty* transposase, SB100X, with 100-fold enhancement in efficiency compared with first-generation transposase, was recently identified and shown as effective as *piggyBac* under non-restrictive conditions.[33,43] mPB, a mouse codon-optimized version of the *piggyBac* transposase coding sequence, has been shown to provide transposition levels greater than native *piggyBac*.[34] A recent genetic screening in yeast further yielded a hyperactive *piggyBac* transposase with a nine-fold increase in transposition efficiency compared with *mPB*.[35]

While *Tol2* and *piggyBac* were highly active in all mammalian cell types tested, *SB11* displayed marginal activity in HeLa cells and no activity in the human lung carcinoma cell line, H1299[27] and the mouse neural stem cell- like cell line, C17.2. Meir Y.-J. et al., cell-type dependent activity of *Sleeping Beauty* suggests the involvement of host factors in *Sleeping Beauty*-mediated transposition. Indeed, HMGB1 has been identified as a cofactor for *Sleeping Beauty* transposition.[36] *Sleeping Beauty* also interacts with the transcription factor Miz-1 and the Ku protein, a component involving in the nonhomologous end-joining pathway of double-strand DNA break repair.[37,38]

Stable gene expression

Any transgene introduced into the host genome is a potential target for the position effect which causes transgene silencing. This phenomenon is often observed when the transgene is inserted into the host genome by viral vectors or by a random integration that results in a high copy number of the transgene arranged as a tandem array. The vector backbone (normally derived from bacteria) which is co-integrated into the host genome with the transgene, is also shown to contribute to transgene silencing. Since transposons are natural genetic elements existing in the mammalian genome, they may be less prone to being silenced than viral vectors. Furthermore, by embracing the transgene only inside its terminal repeats, a transposon can insert the transgene into the host genome without unwanted genetic materials that may induce transgene silencing. A recent study by Ivics et al demonstrated that SB100X, *Tol2* and *mPB* all showed low levels (1.7-3.8%) of transgene silencing compared with the control group (26.5%) with clones derived from transposon- independent random integrations.[46] However, using a non-selective fluorescence-activated cell sorter-based method, an earlier study by Garrison et al. reported that *Sleeping Beauty*-mediated transposition events can be subjected to progressive postintegrative gene silencing.[39] We recently observed a post-integration transgene silencing rate of up to 63% for *Sleeping Beauty* followed by 46.6% for *Tol2* and 19.9% for *piggyBac* (Meir Y.-J. et al., unpublished data). For therapeutic purposes, transgene delivery without drug selection is often a more desirable approach. In this regard, *piggyBac* may be superior to *Tol2* and *Sleeping Beauty* in therapeutic applications. Indeed, a recent study by Huang et al. demonstrated that *piggyBac* is the most efficient of the three transposon systems in mediated stable gene

transfer in human peripheral blood and umbilical cord blood- derived T cells.[40]

Cargo capacity

Limitation of cargo size is often observed in a *Tc1/mariner*-type transposon such as *Sleeping Beauty*. The wild-type *Sleeping Beauty* is 1.7 kb and every 1 kb increment results in 30% reduction of transposition.[41] *Tol2* and *piggyBac* transposon vectors are, however, able to integrate up to 11 and 9.1 kb of foreign DNA, respectively, into the host genome without significantly reducing transposition activity.[26,42] A recent study further demonstrated that the *Tol2* transposon has a surprisingly large cargo capacity that was used to precisely deliver single copies of a ~70 kb BAC transgene to zebrafish and mouse genomes.[43] A large cargo capacity is desired for therapeutic application. In this aspect, *piggyBac* and *Tol2* would be more suitablethan *Sleeping Beauty* for therapeutic gene delivery.

Target site preferences

Given the lethal adverse effects from a single chromosomal integration by a retroviral vector in SCID patients receiving gene therapy, it is important to fully investigate the targeting profile of various transposon systems in order to evaluate their potential clinical applications. Recent studies, including ours, have reported genome-wide targeting profiles of *piggyBac*, *Tol2*, *and Sleeping Beauty* in various human cell lines and human primary cells.[44-49] The findings of these studies are summarized and discussed, focusing on (1) global targeting profiles, (2) target site sequence preferences, and (3) risk of targeting to or near cancer genes.

Global targeting profiles

Although some discrepancies (Table 1 and 2) can be seen between different studies of the same transposon, the following consensus findings can be concluded from these studies: (1) *piggyBac* displays a much stronger tendency than *Tol2* and *Sleeping Beauty* in targeting to intragenic regions; (2) both *Tol2* and *piggyBac* show much more striking preferences for CpG islands than *Sleeping Beauty*; (3) *Tol2* is the most efficient and *piggyBac* the least efficient transposon system in targeting to repeats in the human genome; (4) in sharp contrast to *piggyBac* and *Tol2, Sleeping Beauty* avoids targeting to Short Interspersed and shows particular favor to simple repeats; and (5) the three transposon systems display distinct preferences in targeting to various types of repeats, with DNA transposons targeted least frequently by *Tol2*, and microsatellite DNA targeted most frequently by *Sleeping Beauty*. Additionally, our genome-wide analyses of *Tol2* and *piggyBac* targets in the HEK 293 genome revealed distinct targeting profiles which stand in sharp contrast to the targeting profiles reported previously (Table 1 and 2).[46,47] Differences in strategies and targeting preferences of *piggyBac* and *Tol2* in various cell types may account for these differences. The data sets used in other studies were obtained by retrieving targets from a heterogenous population or by a PCR-based strategy. Both approaches inevitably introduce bias. However, in our study, the *piggyBac* and *Tol2* target

Table 1. Analysis of *Sleeping Beauty, Tol2,* and *piggyBac* Integration Sites in the Human Genome

Genomic location	*Sleeping Beauty*		*Tol2*			*piggyBac*			
	Huh-7[49]	PBL/ UCB-T cells[48]	HEK293[45]	HeLa[46]	PBL/UCB derived T cells[48]	HEK293[45]	HeLa/ HEK293[44]	Human T cell[47]	PBL/UCB-T-cells[48]
Intergenic	60.9	~58	61.6	52.2	~54	48.4	51.2	48.1	~46
Intron	ND	ND	23.5	45.1	ND	38.6	ND	50.9	ND
Exon/ORF	ND	ND	15/3	3.5/ND	ND	2.4	ND	ND/0	ND
5'-UTR	ND	ND	5.6	ND	ND	2.4	ND	1.3 (3/228)	ND
3'-UTR	ND	ND	6.8	ND	ND	10.2	ND	0.4 (1/228)	ND
CpG±5kb	11.2	~11*	29.1	16.8	~24*	35.8	7.7	17.8	~24*

Abbreviations: ND: not determined; ORF: open reading frame; UTR: untranslated region; *: estimated percentage.

Table 2. Target Preferences of *Sleeping Beauty*, *Tol2*, and *piggyBac* to Repeats in the Human Genome

Repeat type	*Sleeping Beauty*	*Tol2*		*piggyBac*		
	Huh-7[49]	HEK293[45]	HeLa[46]	HEK293[45]	HeLa /HEK293[44]	Human T cell[47]
Repeats	41	48.5	44.5	31.5	ND	ND
LINE	13.1	18.8	16	9.4	12.7	9.9
SINE	1.6	12.6	10.1	11	6	4.9
LTR	13.1	7.1	9.2	3.9	6.8	5.4
DNA transposon	3.6	0.8	ND	4.7	4	15
Simple	11.2	1.3	2.5	0.8	ND	ND
Satellite	2.5	0.8	ND	0	ND	ND

Abbreviation: LINE: long interspersed nuclear element; SINE: short interspersed nuclear element; LTR: long terminal repeat; ND: not determined.

sequences were retrieved from each individual targeted clone. Consequently, the data set established in our study should be more reliable than previous ones.[45] Nevertheless, the differences in approaches used for establishing the data set should only partially contribute to the discrepancy in targeting preferences seen in separate studies. Even if one approach is less biased than the other, a certain degree of overlapping in *Tol2* or *piggyBac* target distributions should still be detected in different human cell types. However, no identical targets were detected in two separate studies, suggesting that the differences in cell types used for these studies contributed substantially to these discrepancies.[44,45]

Target site sequence preferences

An investigation of insertion site sequence preferences detected a TA dinucleotide and a TTAA tetranucleotide core consensus targeting sequence for *Sleeping Beauty* and *piggyBac,* respectively.[21,26] On the contrary, no obvious consensus sequence was found at *Tol2* target sites. Since our *piggyBac* and *Tol2* target sequences were retrieved from each individual targeted clone rather than from a heterogenous population, each sequence represents an independent target event. Hence these sequences provide reliable data sets allowing in-depth sequence analyses of *Tol2* and *piggyBac* target preferences without ambiguity. Our analyses revealed the following important features of *Tol2* and *piggyBac* target preferences.[45] First, *Tol2* targets in a selective manner to the host genome, despite the fact that no distinct features of *Tol2* target sequences can be readily identified. Second, only the TTAA tetranucleotide in a particular sequence context can be targeted by *piggyBac*, as opposed to arbitrary TTAA sites. Third, *piggyBac* and *Tol2* hotspots are not necessarily located in regions with high gene activity, although they both exhibit striking preference in targeting to the CpG island. Last, at least the first 100 nucleotides on either side of *piggyBac* target sites seem to be important for *piggyBac* target selections, and a subtle change in the primary sequence within this 200 bp interval may result in loss of potential for *piggyBac* targeting. A strong preference for localized regions of higher AT content containing a symmetric palindromic core sequence [5'-RCAYA(TA)TRTGY-3'] centered at the insertion site was detected for *Sleeping Beauty*.[49] Furthermore, *Sleeping Beauty* also does not preferentially target actively transcribed genes. Taken together, these data suggest that, in sharp contrast to the vast majority of integrating viral vectors, all of the three transposons here display no significant bias in targeting toward active genes in the host genome.[50-54]

Risks in targeting to cancer genes

As mentioned previously, random insertion mutagenesis is a real threat to gene therapy.[8] The mutagenic potential caused by random insertions of any transposon remains the greatest concern for their advancement in clinical application. Several studies including ours have tackled this issue by assessing the potential of transposons to induce oncogenesis. It was reported that the frequency of *piggyBac* integrations in or within 50 kb of the transcription start

site of 888 known proto-oncogenes (derived from the Sanger Cancer Gene Census) was only 2.3 or 3.1%, respectively in human primary T cells.[47] Our analyses, however, revealed that the frequency of targeting to sites within either a 400-kb or 1000-kb distance from 2,075 cancer-related gene in the CancerGene database was significantly higher for *piggyBac* than *Tol2*.[45] However, the frequency of targeting within a cancer-related gene was higher for *Tol2* (9.4%) than *piggyBac* (6.6%).[45] Most importantly, our study revealed that *piggyBac* targets twice to the same site within gephyrin, a cancer- related gene implicated in colon cancer and adult T-cell leukemia.[45,55-57] A risk evaluation for *Sleeping Beauty* in targeting to or near cancer-related genes is lacking. However, a recent study in human primary cells showed that *Tol2* and *piggyBac* appear more likely to promote clonal expansion than *Sleeping Beauty*, which may be due in part to the dysregulated expression of cancer-related genes near the insertion site.[48]

Potential application in iPS therapy

With their remarkable pluripotency resembling embryonic stem cells, iPSCs are promising adult stem cells for cell/gene therapy in treating degenerative diseases. The generation of iPSCs from somatic cells requires the exogenous expression of a set of defined factors, such as four Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc).[58] Currently, most protocols for generating iPSCs only demonstrate efficiencies between 0.01 and 0.1%. The majority of these protocols use retroviral vectors to deliver iPSC inducers. However, chimeric mice derived from retroviral-generated iPSCs developed tumors, probably due to the reactivation of the c-Myc oncogene. Additionally, ectopic expression of any one of these iPSC inducers may have devastating consequences. For instance, the ectopic expression of Oct4 in the skin and intestine leads to the tumor formation. To solve this problem, the Cre-lox system was incorporated into the retroviral vector for removal of iPSC inducers from iPSCs.[59] However, the remains of vector backbones may still cause mutagenesis and/or alter the expression of neighboring genes. For further therapeutic applications, foreign DNA-free iPSCs are necessary. Methods to address this issue, such as adenoviral transduction, protein-based induction, and repeated plasmid transfection, have been reported.[60] Although these methods proved successful in generating transgene-free iPSCs, their efficiencies were 2 to 3 orders lower than those achieved by retroviral-based nuclear reprogramming. To achieve this goal, *piggyBac* appears to be an ideal alternative because of its high transposition rate and unique property of transgene removal without leaving footprints. Recent studies using *piggyBac* to deliver iPSC inducers demonstrated largely enhanced efficiency of generating iPSCs s from adult mouse fibroblasts.[61,62] In addition, transgene-free iPSCs with wild-type genomic sequences can be subsequently obtained by removing *piggyBac*-carrying transgenes without leaving any footprint. Hence, *piggyBac* is likely the most superior tool of all genetic modifiers for generating transgene-free iPSCs with a high efficiency to achieve this goal.

PiggyBac, the most promising DNA transposon for gene and stem cell therapy

Genome-wide target preference analyses have clearly demonstrated that the three transposon systems display lower frequency in targeting to or near cancer-related genes and less bias in targeting to active genes compared with viral-based vectors. Because of these properties along with their lower immunogenicity and the fact that they are less prone to gene silencing, than viral vectors, they are a more desired therapeutic gene delivery system than viral vectors. *Sleeping Beauty* targets much less frequently to the CpG island and is less prone to clonal expansion in human primary T cells than *Tol2* and *piggyBac*. One may consider *Sleeping Beauty* a safer therapeutic gene delivery vector than the other two transposon systems. However, fatal genotoxicity caused by a single integration event with the retroviral vector in SCID patients receiving gene therapy highlights that no wild type DNA transposon is considered safe for gene therapy because of their random transgene insertion nature. Most mammalian genome manipulating enzymes, including viral integrases and DNA transposases, must therefore be molecularly modified to achieve the ultimate goal in gene therapy, namely safe, site-specific therapeutic gene targeting. To tackle this possibility, several studies including ours have molecularly engineered either one or more of these three transposases.[27,58] Results of these studies demonstrated that *piggyBac* transposase can be molecularly modified without substantially losing its activity, whereas any modifi-

cation done on *Tol2* and *Sleeping Beauty* transposases drastically reduces or completely eliminates their enzymatic activity.[63] Collectively, *piggyBac* is currently the most promising DNA transposon for gene and stem cell therapy because of its highly effective transposition activity, large cargo capacity, ability of stably expressing transgene, capability to be molecularly engineered to achieve site-specific gene targeting, and the unique feature of generating foreign DNA-free iPSCs.

Challenges and considerations

Although the future of gene therapy is promising, it is currently still in its infancy. Ultimate success in this field relies on a highly efficient gene delivery system that is capable of targeting the therapeutic gene to a predefined safe location in the host genome where the transgene can be stably and faithfully expressed without disturbing global gene expression. As concluded above, *piggyBac* is currently the most promising DNA transposon to achieve this ultimate goal. In this section, we will discuss the challenges and considerations in advancing *piggyBac* for clinical application.

Delivery strategies

Despite its effective chromosomal integration ability, *piggyBac* exists as a plasmid form and lacks a mechanism for delivery across the cell membrane. To achieve the status of true gene therapy vectors, it must first be combined with transfection devices and/or chemical reagents or be packaged into viral capsids to facilitate access to the cell. Gene therapy administered *in vivo* is much more challenging than that administrated *in vitro*. Many advances have been made in the field of non-viral gene delivery *in vivo*. In this section, we will briefly review the current state of the art in this field.

Liposomes and polymers

Non-viral nanovehicles with sizes ranging from a few to 1000 nm have been proposed for gene therapy. These nanovehicles include different polymeric and metal nanoparticles, liposomes, niosomes, solid lipid particles, micelles, quantum dots, microcapsules, cells, cell ghosts, and lipoproteins, and different nanoassemblies.[64] Among them, cationic liposomes and cationic polymers are by far the most widely utilized nanocarriers for gene and oligonucleotide delivery. The current advances of these two vector systems in gene therapy will be briefly discussed here.

Liposomes have long been viewed as promising biocompatible drug delivery systems because of their resemblance to cell membranes. Because of their opposite surface charge, cationic liposomes are commonly utilized for gene transfer by forming a complex, called lipoplexes, with negatively charged DNA molecules. Lipoplexes have been shown to transfect the airway epithelial cells and endothelial cells of mouse lungs in reasonably high efficiencies.[65,66] Gene expression and appropriate physiological effects have been observed following lipoplex-mediated administration of the cystic fibrosis transmembrane receptor gene into the nose and lungs of cystic fibrosis patients.[67,68] Cancer gene therapeutic strategies involving the use of cationic liposomes have recently progressed to Phase II clinical studies.[69,70] Recent studies have demonstrated that lipoplexes with high lipid to DNA charge ratios are capable of overcoming the inhibitory effects of serum on liposome-mediated gene delivery (lipofection).[71,72]

Complexes of polymers with DNA are called polyplexes. Most polyplexes consist of cationic polymers. Over the years, a significant number of cationic polymers in linear or branched configurations have been developed.[73] Polymers display striking advantages as vehicles for gene delivery. They can be specifically tailored for various therapeutic needs with the choice of appropriate molecular weights, coupling of cell or tissue- specific targeting moieties and / or other modifications to acquire specific physiological and physiochemical properties. Production in large quantities is also rather easy. Frequently used cationic polymers for non-viral DNA delivery include branched polyethylenimine (PEI) (e.g. PEI Aldrich 25 Kda), linear PEI (ExGen™ 500), poly L-lysine, dendrimers (e.g. Superfect™), imidazole modified poly L-lysine, and chitosan.[73] These polymers, however, are nondegradable. Consequently, there is a risk associated with their accumulation in the body, particularly after repeated administration. To solve this problem, biodegradable carrier systems including those based on water-soluble cationic polymers and on micro- and nanoparticles have recently been developed.[74] Currently, the effectiveness of polymers as gene therapy vehicles remains orders of

magnitude poorer than that of viral vectors. With the growing understanding of polymer gene delivery mechanisms and the continued efforts of creative polymer chemists, it is likely that polymer-based gene delivery systems will become an important tool in human gene therapy.

In vivo electroporation

Over the past decade, physical methods of gene delivery have revolutionized the efficiency of nonviral gene transfer to a clinically meaningful level. Among them, *in vivo* electroporation is gaining momentum in physical-based gene delivery.[75-77] It has proven to be an efficient system to introduce genes to a wide variety of tissues, including skeletal muscle, tumors, kidney, liver and skin. The first clinical study was initiated in 2004 with the primary objectives of determining the toxicity and maximum tolerated dose of intralesionally electroporated plasmids carrying human IL-12 cDNA. Other clinical trials have since commenced. However, the significant tissue damage related to harsh electroporation conditions raises serious safety concerns with its use in healthy tissues. Hence its current applications are mainly limited to nonhealthy tissues such as tumors. DNA formulations designed to minimize tissue damage or enhance expression at weaker electric pulses have been examined to address these concerns. One of the most recent studies provides further evidence that *in vivo* electroporation with an optimized protocol is a safe and effective tool for nonviral gene delivery to the beating heart.[78]

Ultrasound microbubbles

Microbubbles comprise spherical cavities filled by a gas encapsulated in a shell. The shells, usually 2 to 8 microns, are made of phospholipids, surfactant, denatured human serum albumin or synthetic polymers. Microbubbles have been developed for diagnostic imaging and used as ultrasound contrast agents. Ultrasound causes bubble destruction which decreases the threshold of ultrasound energy for cavitation. This results in microstreaming and an increase in permeability of cell membranes. Hence, ultrasound-mediated microbubble destruction has been proposed as an innovative method for noninvasive delivery of drugs and genes to tissues.[79,80] Destruction of the bubbles results in local release of their contents. The use of ultrasound microbubble-mediated targeted DNA delivery was first reported in 1996.[81] Subsequently, several studies have confirmed its efficacy in drug and gene delivery, both *in vitro* and *in vivo*. Shohet *et al*. demonstrated for the first time with an adenovirus vector that ultrasound-mediated disruption of gas-filled microbubbles could be used to direct transgene expression to the heart *in vivo*.[82] Using the same model, the authors confirmed that plasmid transgene expression can be directed to the heart, with an even higher specificity than viral vectors.[83] Since then, substantial efforts have tackled the therapeutic efficacy of ultrasound microbubble-mediated gene delivery for treating diseases using animal models. Recent studies further proved the therapeutic success of ultrasound microbubble gene therapy in treating cancers in animals.[84]

Adeno-transposon hybrid system

Despite great progress in the field of non-viral gene transfer, viral transduction remains the most effective way to deliver therapeutic genes inside cells. Among the viral-based vector systems currently available, recombinant adenovirus vectors, such as gutless adenovirus, are attractive vehicles for therapeutic gene transfer because of their high transduction rate, broad tropism, large cargo capacity, ability to transduce both dividing and non-dividing cells, and minimal immunogenicity owing to the elimination of all viral coding sequences.[85] However, a major limitation of the gutless adenoviral system is the instability of gene expression on account of the episomal nature of adenovirus vectors. To overcome this problem, Yant *et al*. incorporated the *Sleeping Beauty* integration machinery into gutless adenovirus vectors to combine the advantages of each system.[86] Utilizing this adenovirus-transposon hybrid system to deliver human coagulation factor IX, they demonstrated that somatic integration was sufficient to maintain therapeutic levels of the gene for more than six months in mice undergoing extensive liver proliferation.[86] Given the fact that *piggyBac* is more efficient than the version of *Sleeping Beauty* utilized in the aforementioned adeno-transposon system, development of a highly efficient mammalian genetic tool by combining the *piggyBac* integration system with the gutless adenovirus transduction system may be possible.

Site-specific therapeutic gene insertion

To make gene therapy an off-the-shelf solution for treating inherited and acquired diseases, the therapeutic gene must be integrated into a predefined safe site where it can be faithfully expressed without disturbing global gene expression. *piggyBac* is by far the most promising transposon for achieving this goal because its transposase can be molecularly engineered. Interestingly, some modifications done on *piggyBac* transposase enhance its activity as seen in a few *piggyBac* chimeras generated in our laboratory, including GAL4-*piggyBac* (Meir Y.-J. et al., unpublished observations).[27] Interplasmid site-directed integration by GAL4-*piggyBac* can be achieved in *Aedes aegypti* embryos.[87] Chromosomal site-directed integration by chimeric *piggyBac* transposase with a DNA binding domain, however, remains a challenging issue. Given that *piggyBac* only targets the TTAA tetranucleotide with a specific sequence context and likely displays cell type- specific targeting profiles, the following approaches should be helpful in addressing this issue. First, genome-wide *piggyBac* target profiling on therapeutically relevant stem cell types should be performed to identify universal safe hotspots for *piggyBac*. Accordingly, a functional *piggyBac* chimera with a designed zinc finger DNA binding domain which specifically recognizes the defined sequence in the vicinity of the universal, safe *piggyBac* hotspot should then be engineered.

Cytotoxicity and genome instability

Even though the goal of site-directed chromosomal integration by *piggyBac* can be achieved, unseen risks associated with *piggyBac*-mediated gene therapy still remain. In particular, the behavior of *piggyBac* remains largely unknown in different cell types. *Sleeping Beauty* displays cell type- dependent activity and has been shown to interact with human factors such as HMGB1 and Miz-1.[33,35] Our recent studies suggest that over- expression of *Sleeping Beauty* transposase exhibits great cytotoxicity which contributes substantially to the overproduction inhibition (OPI) of *Sleeping Beauty* (Meir Y.-J. et al., unpublished observations). Unlike *Sleeping Beauty, piggyBac* transposition appears to be cell -type independent. However, *piggyBac* also displays OPI, although to a lesser extent.[27,35] Hence, we can not exclude the possibility that *piggyBac* transposase might also impose cytotoxicity on the host by directly interacting with cellular factors.

One safety concern applied specifically to *piggyBac* is the existence of a *piggyBac*-like element in the human genome. Unlike *Tc1*-like elements, such as *Sleeping Beauty*, which have has no close relatives in the human genome, the *piggyBac* superfamily is well represented in the human genome (approximately 2,000 *piggyBac*-like elements have been detected).[88] Despite the great evolutionary distance between humans and moths, we cannot exclude the possibility that an exogenous source of *piggyBac* transposase could mobilize some of these *piggyBac*-like elements in the human genome during the process of genetic engineering.

The longer the foreign sequences introduced into the host genome, the greater the probability of evoking adverse consequences, such as therapeutic gene silencing and dysregulation of the endogenous genes nearby. Hence, another concern of *piggyBac*-mediated gene therapy is the potential existence of sequences in the *piggyBac* terminal repeats that may be susceptible to epigenetic silencing and / or possess enhancer or silencer activities. It has been reported that over time, *Sleeping Beauty* undergoes additional postintegrative gene silencing that is influenced by DNA methylation and histone deacetylation.[39] These observations implicate the existence of a postintegrative gene silencing network that efficiently targets invading transposons and silences transposon-mediated transgene expression in mammalian cells. Recently, we generated a *piggyBac* cis-element, designated micro-PB, with 40 bp and 67 bp replacing 245 bp and 311 bp of 3' and 5'-TRD, respectively, in the commonly used *piggyBac* (mini-PB).[45] Micro-PB displayed 1.5-fold higher transposition activity than mini-PB.[45] This observation implicates the possible interaction between epigenetic silencing factors and sequences present in mini-PB but not micro-PB. Another important feature of our micro-PB was the lack of a majority of activator sequences that are located within 3'-TRD of mini-PB have been shown to influence neighboring gene expression in *D. melanogaster*.[89] Collectively, micro-PB may be the ideal cis-*piggyBac* element for therapeutic applications. Future research should focus on addressing whether micro-PB exhibits any enhancer/silencer activity and whether it is susceptible to epigenetic silencing in therapeutic stem cells.

Conclusions

Although successes in gene therapy have been limited, the future still seems overwhelmingly promising. The DNA transposon *piggyBac* is currently the most promising gene delivery vector to achieve ultimate success in gene therapy, mainly because of its highly effective gene integration and the amenability of its transposase to molecular engineering to achieve site-directed therapeutic gene targeting. With ever innovative and improving technologies in the field of non-viral gene delivery, *piggyBac*-mediated gene transfer is expected to achieve therapeutic efficacy in the near future. Meanwhile, substantial efforts should be devoted to the identification of safe *piggyBac* hotspots in the genome of therapeutic stem cells and subsequently to the engineering of Zinc-finger DNA binding protein -*piggyBac* transposases to achieve safe, site-specific therapeutic gene insertion.

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跳躍基因躍身爲基因治療臨床試驗載體：挑戰及考量

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直至今日，基因治療長足地進步和發展值得喝彩。然而基因治療仍面臨著許多的挑戰。其中之一即是如何發展一套載體系統以適用並可滿足於各種不同基因治療策略之所需。除此之外，另一項挑戰是爲如何植入治療基因於特定之染色體位址中，達到治療基因長期且穩定表達之最終目標。長久以來，病毒被視爲最具潛力、並可成功地施行於人類基因治療的載體工具。然而病毒載體其致命性的副作用，嚴重地阻礙了更進一步持續於臨床醫療上的運用。相對地，廣泛運用於植物及昆蟲遺傳操作達數十年之久的 DNA 跳躍基因，現已躍昇爲具有成效性的基因治療載體。在本文中，我們將簡述過去運用病毒載體於基因治療上所造成之不良反應，同時介紹現今最新並具潛力可運用於基因治療之 AAV 病毒載體。緊接著將介紹以 DNA 跳躍基因爲基礎的基因載體，並探討目前常用的 *Sleeping Beauty*、*Tol2*、和 *piggyBac* 等跳躍基因運用於基因治療之優缺點。最後，我們將會著重於現今最具潛力，以跳躍基因爲基礎的 *piggyBac* 載體系統。於本文中，對 *piggyBac* 載體系統其未來所面對的挑戰、及運用於臨床試驗的考量，亦將深入地探討。(長庚醫誌 2011;34:565-79)

關鍵詞：基因治療，非病毒載體，*piggyBac* 跳躍基因

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