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Hyperactive *piggyBac* Gene Transfer in Human Cells and *In Vivo*

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Abstract

We characterized a recently developed hyperactive *piggyBac* (pB) transposase enzyme [containing seven mutations (7pB)] for gene transfer in human cells *in vitro* and to somatic cells in mice *in vivo*. Despite a protein level expression similar to that of native pB, 7pB significantly increased the gene transfer efficiency of a neomycin resistance cassette transposon in both HEK293 and HeLa cultured human cells. Native pB and SB100X, the most active transposase of the *Sleeping Beauty* transposon system, exhibited similar transposition efficiency in cultured human cell lines. When delivered to primary human T cells *ex vivo*, 7pB increased gene delivery two- to threefold compared with *piggyBac* and SB100X. The activity of hyperactive 7pB transposase was not affected by the addition of a 24-kDa N-terminal tag, whereas SB100X manifested a 50% reduction in transposition. Hyperactive 7pB was compared with native pB and SB100X *in vivo* in mice using hydrodynamic tail-vein injection of a limiting dose of transposase DNA combined with luciferase reporter transposons. We followed transgene expression for up to 6 months and observed approximately 10-fold greater long-term gene expression in mice injected with a codon-optimized version of 7pB compared with mice injected with native pB or SB100X. We conclude that hyperactive *piggyBac* elements can increase gene transfer in human cells and *in vivo* and should enable improved gene delivery using the *piggyBac* transposon system in a variety of cell and gene-therapy applications.

Introduction

DNA-Based Transposon Systems are gaining wider acceptance for genome engineering, and multiple investigators have modified such systems to increase activity. Transposons are of interest for a range of applications due to their ability as a nonviral system to insert DNA cargo efficiently into the genome. Transposon vectors have been used for the generation of transgenic mice (Dupuy *et al.*, 2002; Ding *et al.*, 2005), genetic manipulation of embryonic stem cells (Wilber *et al.*, 2006; Cadinanos and Bradley, 2007; Chen *et al.*, 2010), generation of induced pluripotent stem cells (Woltjen *et al.*, 2009; Yusa *et al.*, 2009), efficient genetic modification of human T lymphocytes (Nakazawa *et al.*, 2009; Huang *et al.*, 2010), and long-term gene expression *in vivo* after somatic cell gene transfer in mice (Yant *et al.*, 2000; Wilson *et al.*, 2007). Recently, transposons have been approved for human application in the setting of a clinical trial of immunotherapy for CD19 malignancies (Hackett *et al.*, 2010).

Although DNA transposons in nature are a self-contained unit capable of catalyzing their own cut-and-paste movement around the genome, transposon systems as used for research applications and potential gene therapy are two-part systems consisting of transposase, the enzyme that catalyzes transposition, and transposon DNA containing the gene(s) of interest. The transposase is usually expressed from a nonintegrating vector, whereas the gene of interest, flanked by the recognition sites for transposase excision and integration, is expressed from the transposon.

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The transposon systems being most actively investigated for mammalian applications are *Sleeping Beauty* and *piggyBac*. *Sleeping Beauty* is a member of the Tc1/*mariner* family of transposases that was reconstructed from a salmonid genome and was the first transposon system to be used in human cells (Ivics *et al.*, 1997). The system has been modified extensively, leading to the development of a number of hyperactive elements (Yant *et al.*, 2004; Zayed *et al.*, 2004; Baus *et al.*, 2005; Mates *et al.*, 2009). The most active variant of the *Sleeping Beauty* transposase, SB100X (Mates *et al.*, 2009), reportedly increases transposition 100-fold compared with the original *Sleeping Beauty* transposase and has been shown to improve gene delivery in a variety of human cells (Xue *et al.*, 2009; Grabundzija *et al.*, 2010; Jin *et al.*, 2011) and *in vivo* in mice (Mates *et al.*, 2009).

The *piggyBac* transposon system is a naturally active transposon system derived from the cabbage looper moth, which was originally discovered in mutant baculovirus strains (leading to the name "*piggyBac*") (Fraser *et al.*, 1995). *PiggyBac* has been shown to be capable of catalyzing transposition in a variety of organisms, including yeast (Mitra *et al.*, 2008), *Drosophila* (Thibault *et al.*, 2004), mouse somatic cells (Saridey *et al.*, 2009; Nakanishi *et al.*, 2010), mouse and human embryonic stem cells (Wang *et al.*, 2008; Chen *et al.*, 2010), and a variety of human somatic cell types (Wilson *et al.*, 2007; Nakazawa *et al.*, 2009; Woltjen *et al.*, 2009). *PiggyBac* has a number of characteristics that make it flexible compared with other transposon systems, including its activity in a wide range of organisms, its ability to integrate multiple large transgenes with high efficiency (Kahlig *et al.*, 2010), the ability to add domains to the transposase without loss of activity (Cadinanos and Bradley, 2007; Wilson *et al.*, 2007), and excision from the genome without leaving a footprint mutation (Elick *et al.*, 1996). Recent research with *piggyBac* has led to the development of an improved transposase that exhibited increased transposition activity in yeast and mouse embryonic stem cells (Yusa *et al.*, 2011).

SB100X has shown the ability to achieve stable transfection of difficult-to-transfect cell types, such as modifying $CD34^+$ cells for transplantation and achieving persistence of genetically modified cells *in vivo* (Mates *et al.*, 2009). This could not be accomplished with earlier, less active versions of the *Sleeping Beauty* transposase. As more hyperactive enzymes open new avenues of research and new potential therapeutic applications, it is imperative to compare hyperactive systems in regard to their ability to mediate gene transfer to human cells and *in vivo.*

In this study, we tested a recently developed hyperactive *piggyBac* transposase (7pB) previously characterized in yeast and mouse embryonic stem cells (Yusa *et al.*, 2011) for gene transfer in human cells and *in vivo* in somatic cells in mice. We first compared the activity of this transposase to wild-type *piggyBac* and SB100X in cultured human cells and examined its ability to increase gene delivery to primary human T cells. We also evaluated the flexibility of these various transposases by evaluating the ability of each transposase to function after addition of an N-terminal protein domain. Next, we examined the integration characteristics of the hyperactive *piggyBac* transposase in comparison with wild-type *piggyBac*. Finally, we compared the ability of the transposases to deliver and mediate long-term gene expression *in vivo* in livers of mice using hydrodynamic tail-vein injection of transposase and transposon plasmid DNA.

Materials and Methods

Plasmid construction

The pCMV-*piggyBac* transposase plasmid and the transposon plasmids pTpB, pIRII-eGFP, and pT3-Nori have been described previously (Wilson *et al.*, 2007; Nakazawa *et al.*, 2009). i7*piggyBac* and m7*piggyBac* were synthesized (GenScript USA Inc., Piscataway, NJ) and cloned into pCMV-*piggyBac* by standard molecular biology techniques. All enzymes used were purchased from New England BioLabs (Ipswitch, MA). SB100X (kindly provided by Dr. Zoltán Ivics) (Mates *et al.*, 2009) was excised from the donor plasmid by *Apa*I/*Spe*I digest and bluntly cloned into pCMV-*piggyBac* to swap out the cDNAs. pCMV-HA-*piggyBac* was created by adding a hemagglutinin (HA) tag with flanking 5′ *Sac*II and 3′ *Spe*I restriction sites to the *piggyBac* transposase by PCR, and the resulting fragment was cloned into pCMV-*piggyBac* *Sac*II/*Kpn*I. i7*piggyBac* and m7*piggyBac* were amplified with 5′ *Spe*I and 3′ *Kpn*I sites and cloned into pCMV-HA-*piggyBac*. SB100X was amplified with 5′ *Spe*I and 3′ *Psi*I sites and cloned into pCMV-HA-*piggyBac*. The zinc finger DNA-binding domain (Tan *et al.*, 2003; Kettlun *et al.*, 2011) (Sangamo Biosciences, Richmond, CA) was amplified with 5′ *Sac*II and 3′ *Spe*I sites and cloned into pCMV-HA-*piggyBac*, pCMV-HA-m7*piggyBac*, and pCMV-HA-SB100X to create N-terminal fusions. To create the *Sleeping Beauty* enhanced green fluorescent protein (eGFP) plasmid pT2-eGFP, the contents of the transposon pIRII-eGFP were excised and cloned into the transposon vector pT2/HB (Cui *et al.*, 2002) *Bgl*II/blunt. All constructs were confirmed by restriction digest and sequencing.

Colony-count assay

Plasmids were transiently transfected into 1×10^6 HEK293 or HeLa cells using FuGENE 6 (Roche Applied Science, Indianapolis, IN). Tranfections consisted of the indicated amount of transposon and transposase plasmid plus pUC19 plasmid to a constant DNA amount of 2 μg. Two days after transfection, cells were split 1:400 unless otherwise indicated and plated in medium with 1 mg/ml G418. After 2 weeks of selection, cells were fixed with 10% formalin and stained with 500 mg/L methylene blue in PBS.

Western blot

HEK293 cells were transfected as for colony-count assays. Two days after transfection, cells were harvested in hypotonic buffer (10 m*M* Tris, pH 7.4, 1 m*M* EDTA, 0.01% SDS) plus protease inhibitors (Complete Mini; Roche Applied Science) and lysed by repeated passage through a 27-gauge needle. The lysate was clarified by centrifugation and protein quantified by BCA assay (Thermo Scientific, Waltham MA). Twenty micrograms of protein per lane was loaded onto precast NuPAGE 10% polyacrylamide gels (Invitrogen, Carlsbad, CA) and subjected to electrophoresis. Gels were transferred to nitrocellulose and probed with monoclonal antibodies to HA (Covance, Princeton, NJ) and β-actin (Novus Biologicals, Littleton, CO), both diluted 1:2,000, followed by CW800-conjugated goat anti-mouse secondary antibody (LI-COR Biosciences, Lincoln, NE) diluted 1:10,000 and imaged with an Odyssey infrared imager (LI-COR).

Gene delivery in human T cells

T cells were isolated from previously frozen peripheral blood mononuclear cells using a Pan T Cell Isolation Kit (Miltenyi Biotech, Auburn, CA) and rested overnight in T-cell medium [Advanced RPMI (Invitrogen) supplemented with GlutaMAX-I (Invitrogen), 5 m*M* *N*-acetylcysteine (Sigma, St. Louis, MO), and 5% fetal bovine serum (FBS; Hyclone, Logan, UT)] containing 5 ng/ml interleukin-15 (IL-15; R&D Systems, Minneapolis, MN). The following day, 1×10^7 T cells were transfected with 5 μg of pIRII-eGFP and 5 μg of pCMV-pB or pCMB-m7pB or 5 μg of pT2-eGFP and 5 μg of pCMV-SB100X using the P3 Primary Cell 4D-Nucleofector Kit (Lonza, Basel, Switzerland) and program FI-115 (high-efficiency, unstimulated T cells) on a 4D-Nucleofector. Immediately following transfection, the cells were placed in 2 ml of prewarmed T-cell medium containing 5 ng/ml IL-15 in a 24-well plate and incubated overnight at 37°C. The next day, transfected cells were counted by trypan blue exclusion, analyzed for GFP expression by flow cytometry, and stimulated on OKT3/anti-CD28–coated plates (1 μg/ml each) in T-cell medium and fed three times per week with 5 ng/ml IL-15. Cells were analyzed for GFP expression, counted, and restimulated weekly in a 1:1 ratio with artificial antigen-presenting cells (aK652) with 50 ng/ml OKT3 and 5 ng/ml IL-15 (Nakazawa *et al.*, 2009).

Plasmid rescue of genomic integration sites

HEK293 cells were transfected with 1 μg of pTpB and 1 μg of pCMV-m7*piggyBac* using FuGENE 6. Two days post transfection, the cells were split into G418 selection medium and grown to confluence. Genomic DNA was harvested using DNAzol (Invitrogen). Integration sites in human cells were rescued as previously described (Wilson *et al.*, 2007). DNA was digested with *Nde*I, which cuts the transposon vector outside the transposon, and treated with Antarctic Phosphatase. DNA was then digested with *Nhe*I, *Spe*I, and *Xba*I (New England BioLabs), which create compatible cohesive ends and do not cut inside the transposon. The DNA was then self-ligated using T4 DNA ligase and transformed into DH10B cells by electroporation. Colonies were screened for sensitivity to kanamycin and ampicillin (pTpB contains a β-lactamase cassette on the plasmid backbone), and kanamycin-resistant, ampicillin-sensitive colonies were sent for colony sequencing (GeneWiz, South Plainfield, NJ) with a primer that reads through the 5′ inverted repeat (5′IR) of the *piggyBac* transposon (5′-TTCCACACCCTAACTGACAC-3′).

Analysis of genomic integration sites

Genomic integration sites were determined by query of the BLAT genome browser as described previously (human February 2009 assembly) (Wilson *et al.*, 2007). The location of genomic integrations was determined in reference to RefSeq genes, transcriptional start sites, and CpG islands. The frequency of m7*piggyBac* integration into these genomic elements was compared with our previously published determination of *piggyBac* integration (Wilson *et al.*, 2007) by χ^2 analysis.

Real-time PCR of transposon copy number

HEK293 cells were transfected as for colony-count assays, split 1:4 into G418 selection medium at day 2, and grown for 2–3 weeks until confluent. Genomic DNA was harvested using DNAzol reagent. To purify the DNA and remove any unintegrated transposon plasmid, DNA was subjected to agarose gel electrophoresis. The genomic DNA band was visualized under ultraviolet light and excised, and the gel fragments containing the genomic DNA were solubilized in QG buffer (QIAGEN Inc., Valencia, CA). The DNA was loaded on QIAGEN DNeasy Mini Spin columns and purified using QIAGEN gel extraction reagents, and recovery of genomic DNA was confirmed by agarose gel electrophoresis. Kan/Neo cassette, *piggyBac* transposon 5′IR, and *RNaseP* copy number were determined from 20 μg and 4 μg of DNA as described previously (Kettlun *et al.*, 2011). For determination of the Kan/Neo cassette and 5′IR copy number, the DNA amount was kept constant at 20 ng by addition of genomic DNA from untransfected HEK293 cells. Real-time PCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Life Science, Hercules, CA) and SYBR Green Supermix (Quanta BioSciences, Inc., Gaithersburg MD) reagents. For determination of Kan/Neo copy number, the primer set 5′-CGGATGGAAGCCGGTCTTGTC-3′ and 5′-AGAAGGCGATAGAAGGCGATG-3′ was used; for determination of 5′IR copy number, the primer set 5′-CTAAA TAGCGCGAATCCGTC-3′ and 5′-TCATTTTGACTCACGC GG-3′ was used. For determination of the *RNaseP* copy number control, the primer set 5′-AGATTTGGACCTGC GAGCG-3′ and 5′-GAGCGGCTGTCTCCACAAGT-3′ was used. Copy number was determined using a standard curve consisting of 6 log order dilutions of plasmid containing the respective target genes (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline/hum).

In vivo *gene delivery*

All animal procedures were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. Plasmid DNA was delivered to 8–10-week-old female FVB mice (Charles River Labs, Wilmington, MA) via hydrodynamic tail-vein injection as previously described (Liu *et al.*, 1999; Saridey *et al.*, 2009). Mice were injected with the indicated amounts of transposase and luciferase transposon plasmids in 100-μl volume per gram of body weight of TransIT-QR hydrodynamic injection solution (Mirus Bio, Madison, WI). Mice were imaged at the indicated time points after injection using a Xenogen IVIS imaging system (Caliper Life Sciences, Hopkinton, MA) following intraperitoneal injection of luciferin substrate 5 min prior to injection according to the manufacturer's instructions.

Results

Hyperactive piggyBac *expression and activity in human cells*

To enable objective comparison, all *piggyBac* transposase open reading frames were initially cloned into a vector with an N-terminal HA tag to create expression plasmids that are identical except for the transposase cDNA. Prior to the recent development of 7pB, the highest reported levels of *piggyBac* transposition were obtained using a transposase expression vector codon-optimized for expression in mouse cells (Cadinanos and Bradley, 2007). We therefore compared the expression and activity of the nonoptimized open reading

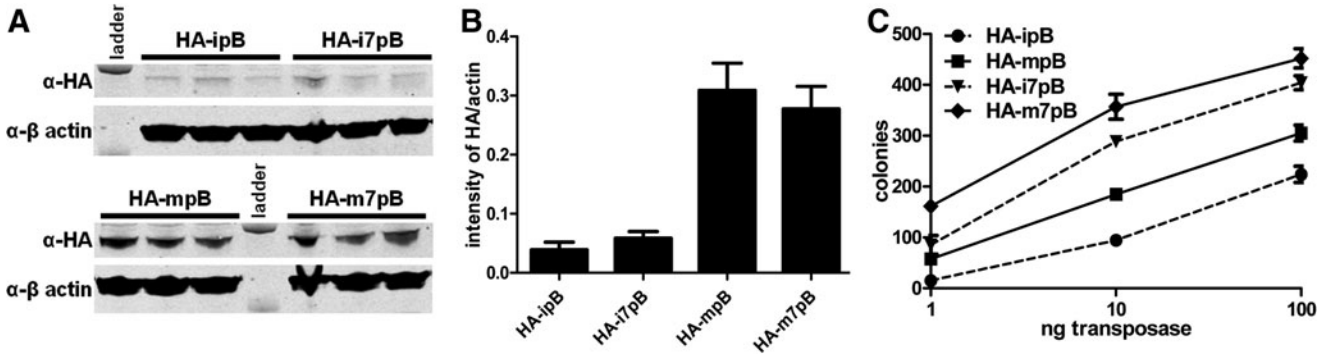

FIG. 1. 7pB expression in human cells. **(A)** Western-blot analysis demonstrating similar expression of *piggyBac* and 7pB transposases. HEK293 cells were transfected with 1 μg of transposase plasmid and lysed 2 days after transfection. Protein was loaded at 20 μg of protein per lane. **(B)** Densitometric analysis of blot reveals no significant difference between native and mutant *piggyBac* transposase expression. **(C)** Colony-count assay demonstrating increased transposition with 7pB. HEK 293 cells were transfected with 1 μg of a *piggyBac* neomycin resistance transposon (pTpB) and the indicated amount of transposase plasmid, then split 1:400 at day 2, and selected with 1 mg/ml G418 for 2 weeks. Codon optimization of both transposases (HA-mpB, HA-m7pB) increased transposition, especially at low transposase plasmid doses ($n = 3$; means ± SEM).

frame as originally isolated from insect cells, termed ipB (Fraser *et al.*, 1996) (pCMV-HA-ipB), the insect transposase open reading frame with the seven mutations as described (Yusa *et al.*, 2011) (pCMV-HA-i7pB), and mouse codon-optimized versions of both transposase sequences (pCMV-HA-mpB and pCMV-HA-m7pB). Western-blot analysis revealed a five- to 10-fold increase in transposase expression in human cells with codon optimization; however, there was no significant difference in expression between wild-type and hyperactive versions of *piggyBac* (Fig. 1A and B).

We next compared the transposition activity of native and hyperactive ipB and mpB transposase constructs in human cells. To quantify transposition, we used a colony-count assay following cotransfection of transposase plasmid and a plasmid with a *piggyBac* transposon containing a neomycin resistance cassette. Colony-count assays were performed in both HEK293 and HeLa human cell lines after selection in medium containing G418. We found that 7pB mediated formation of more G418-resistant colonies at a wide range of transposase plasmid doses, an effect that was amplified at low doses (Fig. 1C). For example, whereas pCMV-HA-i7pB led to the formation of five to six times as many colonies as pCMV-HA-ipB with 1 ng of transposase plasmid, at the 100-ng dose it was only approximately twofold better. A similar relationship was observed between the codon-optimized transposase plasmids, pCMV-HA-mpB and pCMV-HA-m7pB. Although increasing *piggyBac* expression by codon optimization of the transposase open reading frame does increase transposition, as has been reported previously (Cadinanos and Bradley, 2007), the greatest increase in activity is obtained through use of the mutated transposase, as evidenced by the increase in activity obtained by use of codon-optimized native *piggyBac* (mpB) and non–codon-optimized hyperactive *piggyBac* (i7pB) as compared with wild-type *piggyBac* (ipB) (Fig. 1).

7pB compared with SB100X

The recently described SB100X transposase is the most active variant of the *Sleeping Beauty* transposase, and reportedly catalyzes transposition at a rate 100 times greater than that of the first-generation SB transposase (Mates *et al.*, 2009). However, reports of its activity when directly compared with native *piggyBac* vary (Liang *et al.*, 2009; Mates *et al.*, 2009; Grabundzija *et al.*, 2010). We therefore decided to directly compare *piggyBac* and 7pB with SB100X at a wide range of transposon and transposase plasmid doses in human cells. As the *Sleeping Beauty* family of transposases is known to have decreased activity when a protein tag is added (Wilson *et al.*, 2005; Ivics *et al.*, 2007; Yant *et al.*, 2007), we cloned all transposase open reading frames into an identical plasmid expression vector without a tag. The transposons used for comparison were the *piggyBac* transposon pTpB and the hyperactive *Sleeping Beauty* transposon pT3-Nori (Yant *et al.*, 2004), which vary only in the inverted repeat elements required for transposase recognition (Wilson *et al.*, 2007). Transposition activity was compared among wild-type *piggyBac* (pCMV-ipB), non–codon-optimized and codon-optimized hyperactive *piggyBac* (pCMV-i7pB and pCMV-m7pB), and SB100X in the human HEK293 (Fig. 2A–C) and HeLa (Fig. 2D–F) cell lines. Comparisons were performed using 4 log orders of transposase (1–1,000 ng of transposase plasmid) at transposon doses of 10 ng (Fig. 2A and D), 100 ng (Fig. 2B and E), and 1,000 ng (Fig. 2C and F).

Hyperactive codon-optimized m7pB resulted in the highest number of colonies among the transposases in all conditions tested except one (1,000 ng of transposase plasmid with 10 ng of transposon), and in that case hyperactive but not codon-optimized i7pB was most active (Fig. 2). This observation at 1,000 ng of transposase plasmid with 10 ng of transposon may be due to the expression level difference in *piggyBac* transposase observed at high codon-optimized transposase dosage (Fig. 1A) with a limiting dose of transposon. Overproduction inhibition is a phenomenon where excessive expression of transposase leads to decreasing transposition, and is a widely recognized characteristic of Tc1/*mariner* transposons, including *Sleeping Beauty* (Geurts *et al.*, 2003). Whether or not *piggyBac* demonstrates overproduction inhibition is debated, as reports vary depending on the cell type studied and the transfection methodology used (Wu *et al.*, 2006; Cadinanos and Bradley, 2007; Wilson *et al.*, 2007; Grabundzija *et al.*, 2010).

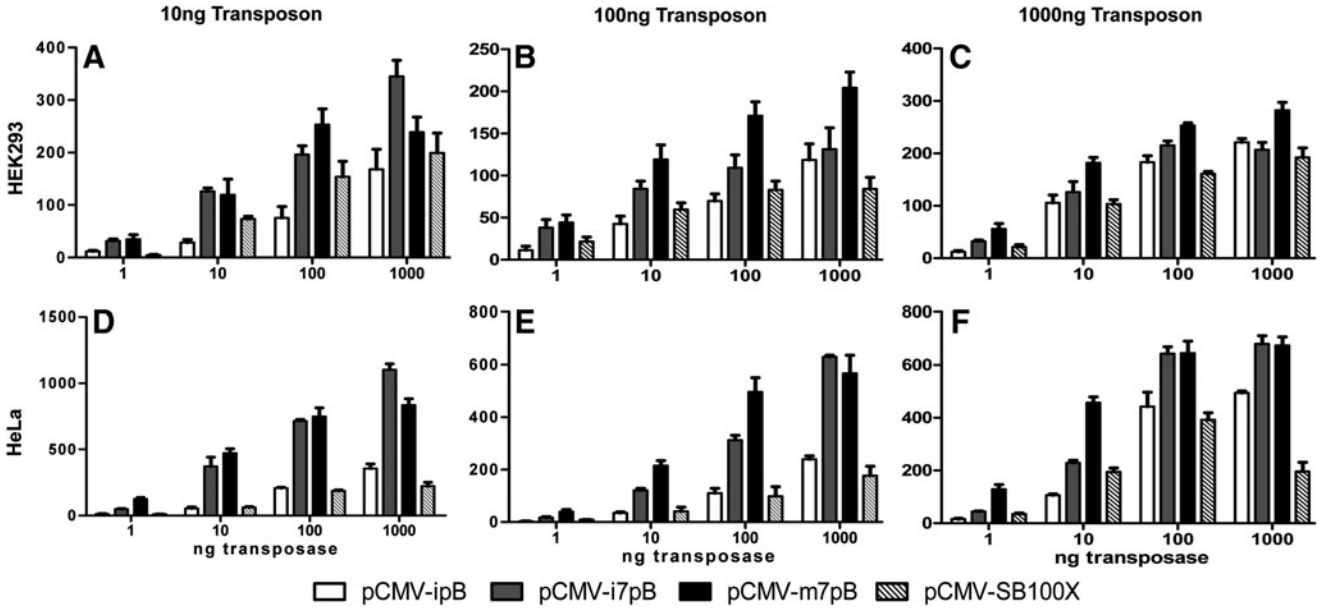

FIG. 2. *PiggyBac* and SB100X transposition in human cells. Native and hyperactive *piggyBac* transposases were compared with SB100X at a range of doses of transposon and transposase plasmids in both HEK293 **(A-C)** and HeLa **(D-F)** cells by colony-count assay, quantifying transposition activity by transfer of a neomycin resistance cassette; the number of colonies formed (indicated on the *y*-axis) was used as a proxy for transposition activity. Cells were split at day 2 after transfection (10-ng transposon transfections split 1:40; 100-ng and 1,000-ng transfections split 1:400) and selected for 10–14 days in 1 mg/ml G418. Whereas native *piggyBac* and SB100X resulted in similar numbers of resistant colonies at a range of conditions, 7pB resulted in more colonies, especially under more limiting conditions, such as lower transposon and transposase doses ($n = 3$; means ± SEM).

Overall, hyperactive m7pB was 1.5–12-fold more active than SB100X, depending on the DNA dosage, and this difference in activity was more pronounced in HeLa cells when compared with HEK293 cells.

7pB increases gene delivery in human T cells

Recently, the *SB* transposon system has been approved for a human trial for clinical application in the modification of human T cells for cancer immunotherapy of $CD19^+$ malignancies (Hackett *et al.*, 2010). We and others have previously reported the use of *piggyBac* to modify primary human T cells (Nakazawa *et al.*, 2009; Huang *et al.*, 2010), and the use of cells modified using *piggyBac* for cancer immunotherapy *in vitro* and *in vivo* (Raja Manuri *et al.*, 2009; Nakazawa *et al.*, 2011). We therefore evaluated the ability of hyperactive *piggyBac* to improve the efficiency of the modification of human primary T cells.

Human T lymphocytes were purified from three separate donors and then nucleofected with transposase plasmid and a corresponding transposon plasmid encoding eGFP. Following transduction, gene expression was monitored out to 28 days using flow cytometry. Although the proportion of stably transduced cells was approximately equal when pCMV-ipB and pCMV-SB100X were used, pCMV-m7pB increased the proportion of $eGFP^+$ lymphocytes two- to threefold (Fig. 3) ($p < 0.01$ by ANOVA). Cell recovery and expansion did not vary significantly among the transposases (Supplementary Fig. S2). These results suggest that 7pB should enable more efficient generation of clinically relevant human cell types for potential cell-therapy applications.

7pB's activity is unaffected by N-terminal tags

The ability to maintain transposase activity in the setting of protein domain or tag fusion significantly increases the flexibility of a transposon system, for example, for creating inducible transposases (Cadinanos and Bradley, 2007) or adding domains capable of targeting the transposase to

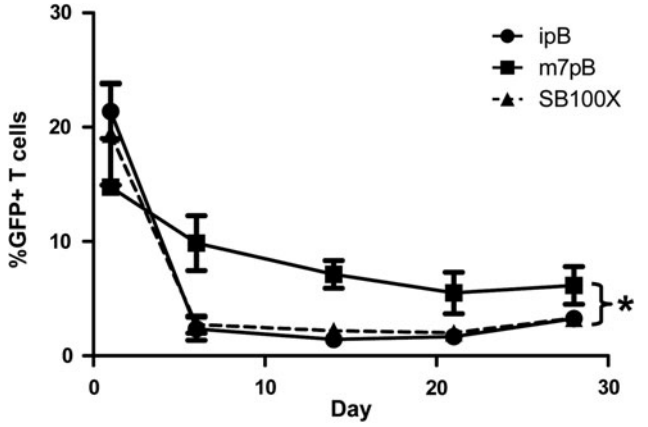

FIG. 3. Modification of human T cells by hyperactive *piggyBac*. Human peripheral blood mononuclear cells from three separate donors were nucleofected with pCMV-ipB, pCMV-m7pB, and pCMV-SB100X and corresponding *piggyBac* and *Sleeping Beauty* transposons containing eGFP, and were followed in culture by FACS to day 28. The fraction of CD3 and eGFP double-positive cells is indicated. Cells with m7pB exhibited two- to threefold greater stable gene expression than cells with either *piggyBac* or SB100X. *$p < 0.01$ by ANOVA.

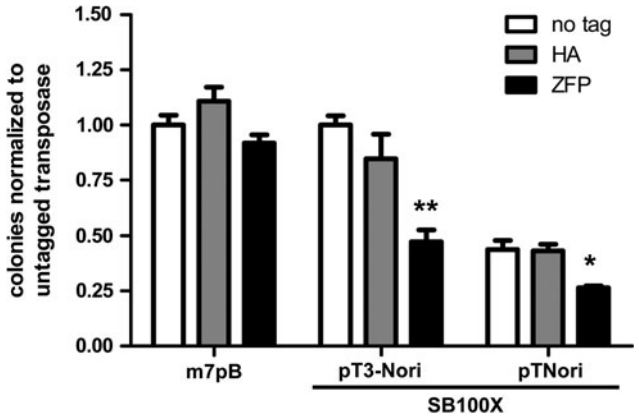

FIG. 4. Effect of N-terminal tags on transposition. The effect of addition of a 1.5-kDa HA or 24-kDa ZFP to the N terminus of the transposase was assayed by colony count in HEK293 cells. Although neither tag affected transposition by codon-optimized 7pB, the addition of the ZFP tag decreased SB100X transposition significantly, using both a hyperactive (pT3-Nori) and a first-generation (pTNori) *Sleeping Beauty* transposon. $*p < 0.01$; $**p < 0.001$ versus untagged transposase by Student's t test ($n = 3$; means ± SEM).

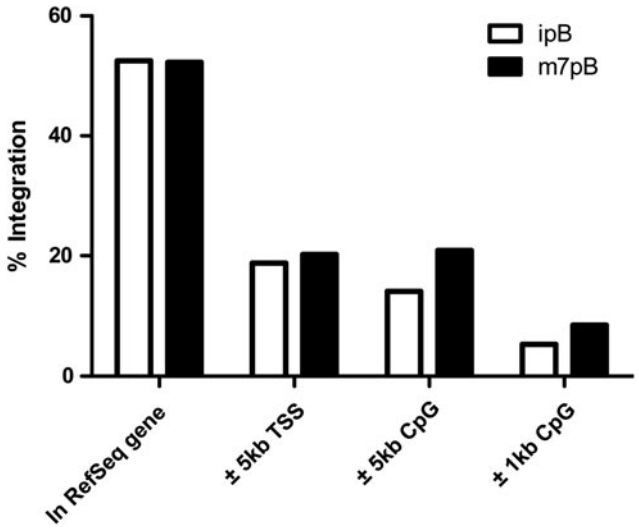

FIG. 5. Integration frequency of hyperactive *piggyBac* in intragenic regions of HEK293 cells. Two hundred eighty-one integrations using m7-*piggyBac* were recovered from HEK293 cells and compared with 318 native *piggyBac* integrations in HEK293 cells previously reported by us (Wilson *et al.* 2007). χ^2 analysis revealed no significant difference between ipB and m7pB.

specific sites in the genome (Wilson *et al.*, 2005; Kettlun *et al.*, 2011). We and others have previously reported that adding a tag or protein domain to the *Sleeping Beauty* transposase significantly attenuates transposition (Wilson *et al.*, 2005; Ivics *et al.*, 2007; Yant *et al.*, 2007), whereas doing the same to *piggyBac* has no apparent effect on its activity (Wilson *et al.*, 2007; Meir *et al.*, 2011); however, the effect of a tag on the activity of the 7pB and SB100X transposases has not been demonstrated.

To examine the effect of a tag on 7pB and SB100X, we added either a 1.5-kDa HA tag or a 24-kDa zinc finger protein (ZFP) domain to the N-terminus of the transposases. Transposition was compared with untagged transposase by colony-count assay using 1,000 ng of transposon and 100 ng of transposase plasmid. Consistent with previous results describing modifications of the *piggyBac* transposase, m7pB maintained its activity with both tags. Although the activity of SB100X was not changed by addition of the 1.5-kDa HA tag, the addition of the ZFP domain decreased its activity approximately 50% as compared with the untagged transposase; this was observed with both a first-generation (pTNori) and a hyperactive (pT3-Nori) SB transposon (Fig. 4).

Characteristics of 7pB integration in HEK293 cells

A number of previous reports have examined the integration preferences of *piggyBac* in a variety of cell types (Wilson *et al.*, 2007; Galvan *et al.*, 2009; Huang *et al.*, 2010; Meir *et al.*, 2011). Our previous results have indicated that native *piggyBac* is more prone to integrate in or near genes than *Sleeping Beauty*, but is significantly less likely than retroviral vectors to integrate in or near proto-oncogenes.

We examined the integration preferences of hyperactive m7pB by plasmid rescue and sequencing of integration sites from HEK293 cells, and compared them with the *piggyBac* integrations we have previously described using the exact same methods in the identical cell type (Fig. 5) (Wilson *et al.*, 2007). Integration sites were determined by BLAT search and were analyzed for their location in reference to genes and proximity to transcriptional start sites and CpG islands. We compared 281 m7pB integrations (available in Supplementary Table S1) to our 318 previously published *piggyBac* integrations and found a similar pattern of integration of the two transposases ($p = 0.58$ by χ^2). Approximately half of the recovered integrations were in intragenic regions and, as we have observed previously with *piggyBac*, there was a bias shown toward a 10-kb region around transcriptional start sites. We did not observe any integration into exons, although we did observe two each into 5′ and 3′ untranslated regions (Supplementary Table S1). Therefore, there does not appear to be a dramatic difference in integration preference when comparing native *piggyBac* with hyperactive *piggyBac.*

We determined transposon copy number in populations of cells transfected with varying amounts of a transposon plasmid containing a neomycin resistance cassette and 1,000 ng of transposase plasmid, the maximal dose of transposase used for our *in vitro* experiments. After transfection, cells were selected with G418 for 2–3 weeks, and genomic DNA was isolated and gel-purified to remove any remaining unintegrated plasmid. Transposon copy number was determined by real-time quantitative PCR for the integrated neomycin resistance cassette and for the 5′IR of the *piggyBac* transposon (Supplementary Fig. S3). Genome number per reaction was normalized to genomic copy number of *RNaseP*, which is well-characterized in human cells as present at one copy per haploid genome. Under restrictive conditions (10 ng of transposon, 1,000 ng of transposase), ipB catalyzed 1.2 ± 0.1 transposon integrations per haploid genome as compared with 2.3 ± 0.1 with m7pB and 1.3 ± 0.4 with SB100X. Under conditions where we observed maximal transposition by colony count (1,000 ng each of transposon and transposase), integration copy number increased to

14.6 ± 1.8 with ipB, 7.6 ± 1.0 with m7pB, and 10.4 ± 1.9 with SB100X.

Although copy number increased with increasing transposon dose, there was no significant difference found in copy number among the transposases [$p = 0.50$ and 0.41 by ANOVA, respectively, for neomycin cassette (Supplementary Fig. S3A) and 5′IR copy number (Supplementary Fig. S3B)].

7pB increases gene delivery in vivo

We and others have previously reported that *piggyBac* can mediate long-term gene expression *in vivo* following hydrodynamic tail-vein injection (Saridey *et al.*, 2009; Nakanishi *et al.*, 2010). Although SB100X has also been used for gene delivery by hydrodynamic injection (Mates *et al.*, 2009), there has been no direct comparison of the various systems for *in vivo* gene delivery. We therefore compared the ability of ipB, m7pB, and SB100X to mediate long-term gene expression following hydrodynamic tail-vein injection of plasmid DNA. We chose to use a low dose of transposase plasmid (1 μg) to objectively compare the various systems at a limiting dosage to examine possible differences in efficiency in gene transfer to liver *in vivo*.

Luciferase gene expression was monitored out to 6 months post injection by *in vivo* imaging. After gene expression stabilized around day 21 post injection, no significant difference was found between pCMV-ipB and pCMV-SB100X, whereas mice injected with pCMV-m7pB had approximately threefold higher luciferase activity than either of the other transposase groups (Fig. 6A) ($p < 0.01$). When luciferase activity was measured 6 months after injection (Fig. 6B), m7pB had approximately 10-fold greater signal than mice that received either ipB or SB100X ($p < 0.01$ by Dunnett's multiple comparison test).

Discussion

Although the *piggyBac* transposon system has been studied for several years for potential use in gene therapy and other applications, it is only recently that a hyperactive transposase has been developed (Yusa *et al.*, 2011). This is in contrast to the *Sleeping Beauty* transposon system, which has been extensively modified since its introduction in 1997 (Yant *et al.*, 2004; Baus *et al.*, 2005; Mates *et al.*, 2009). Development of the first hyperactive *piggyBac* transposase and the promise of future improvements to the system enhance its potential therapeutic and research applications.

We undertook this study to evaluate a hyperactive *piggyBac* transposase for use in potential gene-therapy applications by studying its activity in human cells and *in vivo*. We have shown that mutations of the *piggyBac* transposase identified in yeast and characterized in cultured mouse embryonic stem cells can increase gene delivery in human cells and *in vivo* in mice. This hyperactive transposase displays similar characteristics to wild-type *piggyBac*, including similar integration site preferences and integration copy number *in vitro* and the ability to efficiently catalyze transposition when the transposase is expressed as a fusion protein. Recent reports (Liang *et al.*, 2009; Mates *et al.*, 2009; Grabundzija *et al.*, 2010) have varied on how native *piggyBac* compares in activity with SB100X, the most active transposase of the *Sleeping Beauty* transposon family, and a comparison of *piggyBac* and SB100X for *in vivo* gene delivery has not yet been published. We therefore conducted a comparison of *piggyBac*, hyperactive *piggyBac*, and SB100X in cultured human cell lines, primary human cells, and *in vivo* in mice. We found that SB100X enables gene delivery at a rate similar to that of wild-type *piggyBac in vitro* and *in vivo*. These results imply that the recently developed hyperactive *piggyBac* transposase is the most active transposon system in human cells and *in vivo* in mice.

Grabundzija *et al.* (2010) reported that SB100X was more efficient in gene transfer in human cells when compared with native *piggyBac* in human HeLa and $CD34^+$ cells. This is in contrast to what we observed in HeLa cells (as well as human T cells and mice) when comparing both native and hyperactive *piggyBac* with SB100X. Possible reasons for these differing observations include the use of different promoters, different transfection reagents, different numbers of cells

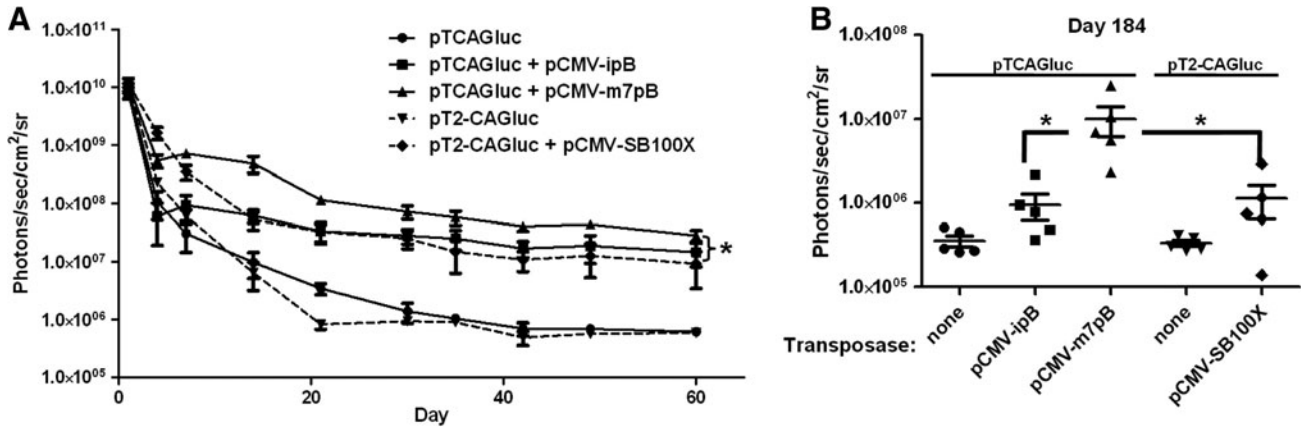

FIG. 6. Delivery of a luciferase transposon by hydrodynamic tail-vein injection. Twenty-five micrograms of luciferase transposon (pTCAGluc and pT2-CAGluc) was codelivered with 1 μg of transposase plasmid via hydrodynamic tail-vein injection to FVB mice ($n = 5$; means ± SEM). After injection, luciferase expression was monitored by *in vivo* imaging following injection of luciferin substrate. **(A)** From day 21 to day 60, luciferase expression in mice that received m7pB was, on average, 2.5–3.5× higher than that in mice that received either ipB or SB100X. **(B)** Six months after injection, mice injected with m7pB had ~10× greater luciferase expression than those injected with ipB or SB100X. *$p < 0.01$ by ANOVA.

transfected with the given reagents, and experiments performed in different laboratories. SB100X, native *piggyBac*, and hyperactive *piggyBac* are all very efficient nonviral vectors capable of stably transfecting cells.

Using identical expression vectors and plasmid backbones, we compared hyperactive *piggyBac* transposition with wild-type *piggyBac* and SB100X in two human cell lines (HEK293 and HeLa) over a wide range of transposon and transposase conditions, and found hyperactive *piggyBac* to consistently be the most active transposase. As *piggyBac* is capable of catalyzing transposition in a range of clinically relevant human cell types (Nakazawa *et al.*, 2009; Woltjen *et al.*, 2009; Chen *et al.*, 2010), the use of a hyperactive transposase should enable more efficient production of sufficient numbers of cells for therapies. Also, hyperactive *piggyBac* maintained full activity with the addition of a 24-kDa DNA-binding domain, whereas SB100X was reduced in activity by approximately 50%, consistent with previously described results of adding a tag to *piggyBac* (Wilson *et al.*, 2007; Meir *et al.*, 2011) and earlier versions of *Sleeping Beauty* (Wilson *et al.*, 2005; Ivics *et al.*, 2007; Yant *et al.*, 2007). The ability to be modified without loss of activity increases potential applications of the *piggyBac* transposon system, such as targeting the transposase to specific genomic locations by the addition of a site-specific DNA-binding domain, as we have recently described (Kettlun *et al.*, 2011). However, the ability of transposases to function normally with a tag may vary depending on the sequence added, and the transposition activity should be evaluated for the conditions desired.

We have previously used *piggyBac* to modify human T cells (Nakazawa *et al.*, 2009) and demonstrated the potential of these cells for cancer immunotherapy (Raja Manuri *et al.*, 2009; Nakazawa *et al.*, 2011). In this study, we show that the use of hyperactive *piggyBac* can be used to increase the proportion of cells expressing the transgene. Increasing the efficiency of T-cell modification can decrease the time required to produce sufficient cells for therapy, and delivering greater numbers of tumor-specific cells should improve clinical response.

We have previously demonstrated long-term gene expression in mice using *piggyBac* (Saridey *et al.*, 2009). In this study, we have shown that hyperactive *piggyBac* can increase gene delivery *in vivo* following somatic cell gene transfer. Following hydrodynamic tail-vein injection in immunocompetent mice with limiting doses of transposase plasmid, we observed 10-fold higher luciferase expression at 6 months post injection in mice that received hyperactive *piggyBac* as compared with those that received either wild-type *piggyBac* or SB100X. The ability to more efficiently deliver genes *in vivo* should aid in the use of the *piggyBac* system as a vector for gene delivery in research models of disease and, ultimately, in clinical trials. Additionally, the use of hyperactive transposases should allow for the administration of lower doses for the same therapeutic effect and lower the risk of toxicity. In cell therapy applications, hyperactive *piggyBac* should permit the generation of more cells stably expressing transgenes. In addition, recent studies have described alterations of the *piggyBac* inverted repeat elements to increase transposition, but these have not yet found their way into common use (Lacoste *et al.*, 2009; Meir *et al.*, 2011). The use of these elements with the hyperactive transposase could increase gene delivery to levels greater than we have observed in our experiments.

Transposon systems are nonviral- and plasmid-based. Therefore, their ability to achieve long-term transgene expression is constrained by gene delivery, which is a known limitation of nonviral-based systems. Although hydrodynamic delivery is an effective tool for *in vivo* gene transfer in small animal models, its potential for clinical application remains uncertain (Sawyer *et al.*, 2009). Recent advances, such as the use of nanocapsules, may improve gene delivery *in vivo*, which will be needed for therapeutic application (Kren *et al.*, 2009). The *Sleeping Beauty* transposon has recently been approved for a clinical trial for *ex vivo* modification of human T lymphocytes for adoptive immunotherapy (Hackett *et al.*, 2010).

As with any integrating gene-delivery system, transposons pose a risk of genotoxicity when used for gene-therapy purposes. Although *piggyBac* and *Sleeping Beauty* both appear less likely than retroviral vectors to cause malignant transformation (Yant *et al.*, 2005; Wilson *et al.*, 2007; Galvan *et al.*, 2009), the theoretical risk of insertional mutagenesis leading to oncogene activation remains with any untargeted integrating vector; this risk naturally rises as the number of integrations per genome increases. Strategies to mitigate this risk are currently in development; these include introducing an inducible suicide gene along with the therapeutic transgene, allowing modified cells to be selectively killed if they proliferate inappropriately (Nakazawa *et al.*, 2009). Another strategy to which *piggyBac* may be well-suited is modifying the transposase to target integration to defined safe sites in the genome (Kettlun *et al.*, 2011).

The development of a hyperactive *piggyBac* transposase increases its potential for *in vitro* and *in vivo* therapeutic and research applications. Further refinement of the transposase could lead to a wider range of uses for the system. Much about the transposase remains to be studied. The mutations in the hyperactive *piggyBac* transposase used in this study were identified through random mutagenesis and screening of a large number of transposase mutants. Better understanding of the structure and function of the transposase could lead to a more rational design of mutants with desired characteristics.

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Author Disclosure Statement

No competing financial interests exist.

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