

Evaluating the potential for undesired genomic effects of the *piggyBac* transposon system in human cells

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ABSTRACT

Non-viral transposons have been used successfully for genetic modification of clinically relevant cells including embryonic stem, induced pluripotent stem, hematopoietic stem and primary human T cell types. However, there has been limited evaluation of undesired genomic effects when using transposons for human genome modification. The prevalence of *piggyBac*(PB)-like terminal repeat (TR) elements in the human genome raises concerns. We evaluated if there were undesired genomic effects of the PB transposon system to modify human cells. Expression of the transposase alone revealed no mobilization of endogenous PB-like sequences in the human genome and no increase in DNA double-strand breaks. The use of PB in a plasmid containing both transposase and transposon greatly increased the probability of transposase integration; however, using transposon and transposase from separate vectors circumvented this. Placing a eGFP transgene within transposon vector backbone allowed isolation of cells free from vector backbone DNA. We confirmed observable directional promoter activity within the 5'TR element of PB but found no significant enhancer effects from the transposon DNA sequence. Long-term culture of primary human cells modified with eGFP-transposons revealed no selective growth advantage of transposon-harboring cells. PB represents a promising vector system for genetic modification of human cells with limited undesired genomic effects.

INTRODUCTION

Class II DNA cut-and-paste transposon systems have recently been explored for delivery of genetic cargo to human cells. Transposons are being developed as gene delivery systems for the production clinical grade cells for gene and cell therapy (1). Transposons have multiple advantages over viral vectors including lower cost, less innate immunogenicity and the ability to co-deliver multiple therapeutic genes. Of the current transposon systems in use, *Sleeping Beauty* (SB) (2) and *piggyBac* (PB) (3) have been studied as gene delivery vehicles in human cells. T cells modified with SB are currently in a clinical trial for the treatment of B-cell malignancies (4). The PB transposon system has the added benefits of a large cargo capacity (>200 kb) (5), low frequency of foot print mutations (6) and is perhaps the most active transposon system in human cells (7). It also retains its activity after the addition of a DNA binding domain allowing further modification of its activity for possible human genomic targeting (8–10). PB has successfully been used to reprogram cells for the generation of induced pluripotent stem cells (11–13) and to modify antigen-specific T cells (14–16).

The presence of ~2000 PB-like elements in the genome raises the question of whether there is a risk of genomic mobilization or re-arrangement upon expression of the exogenously delivered transposase (17), which would be a concern for the clinical application of PB (18). An additional concern is whether endogenous PB-like transposase proteins can mobilize integrated transposons, resulting in genomic instability (1). Finally, transposition from plasmid DNA leads to linearization of the plasmid backbone and the fate of this DNA segment has not been studied. To further consider PB for genome modification of human cells, it is necessary to study the integrity of PB-mediated transposition within the human genome. Within the context of this manuscript, we are defining PB-mediated transposition in-

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tegrity as transposon integration without undesired genome alteration, such as mobilization of endogenous genomic elements, residual transposase expression or promotion or enhancement of neighboring genes.

In the current study, we determined whether PB could mobilize endogenous PB transposon-like DNA elements within the human genome, whether transfected transposase increases the frequency of double-stranded DNA breaks in human cells, and we determined the frequency of backbone DNA integration during transposition both when the transposase is expressed from the transposon plasmid backbone DNA and from separate DNA plasmid. We compared the stability of transgene expression in mice after gene transfer using transposase supplied on the same or separate from the transposon plasmid. We also evaluated promoter and enhancer activity within the transposon terminal repeats (TRs) in human cells and tested whether the PB transposon provides a selective growth advantage to primary human cells. In summary, we analyzed in detail the potential for undesired genomic effects when using the PB transposon to gene-modify human cells, a necessary evaluation for future clinical application.

MATERIALS AND METHODS

Double-strand break assay

Human embryonic kidney (HEK-293) cells were transfected with 1 μ g of pT-CMV-enhanced green fluorescent protein (eGFP) (19) or pCMV-PB (3) with FuGENE 6 (Promega, Madison, WI, USA). pUC19 transfected cells were used as negative controls and cells treated for 2 h with 2.5 μ M camptothecin were used as positive controls (20). Histones were extracted as described previously (20). Samples were resolved on a 10% bis-tris gel in 2-(N-morpholino)ethanesulfonic acid (MES) buffer and probed with mouse anti-phospho H2A.X (Cell Signaling Technologies, Danvers, MA, USA) and mouse anti-histone H1.0 antibody (Abcam, Cambridge, MA, USA) followed by anti-mouse secondary antibody conjugated to IR-800 dye and imaged on an Odyssey infrared imager (LICOR Biosciences, Lincoln, NE, USA). Fold change in H2A.X phosphorylation was calculated by normalizing the band intensity of phospho-H2A.X with H1.0 band intensity using ImageJ.

Identification of *piggyBac*-like genomic sequences

The 17 bp *piggyBac* TR sequence (TRS) was used as the query sequence to search for *piggyBac* TR-like sequences in the human genome using the Basic Local Alignment Search Tool (BLAST) at NCBI (<http://blast.ncbi.nlm.nih.gov>). Possible TRS-like sequences (Supplementary Information) were polymerase chain reaction (PCR) amplified starting with the terminal TTAA sequence and an adjoining 300–400 bp region with the expand high fidelity PCR kit to add flanking NdeI, EcoRI restriction sites (Roche Applied Science, Indianapolis, IN, USA). The PCR products were cloned into pTpB (3) replacing the native 5'TR or 3'TR with the genomic sequences. All plasmid sequences were verified with DNA sequencing.

Colony count assay

The plasmids carrying TRS-like sequences (or the non-splice version of pTpB) were co-transfected with pCMV-PB (1 μ g each) in HEK-293 cells with FuGENE 6 (Promega). Forty-eight hours after transfection cells were trypsinized and plated at a ratio of 1:10 000 in G418 or puromycin containing media. After 10 days, the cells were fixed in 10% neutral buffered formalin and stained with methylene blue for the colony count assay as described previously (3).

Excision assay

The plasmids carrying TRS-like sequences were co-transfected with pCMV-PB in HEK-293 cells with FuGENE 6. Forty-eight hours after transfection cells were trypsinized and washed with phosphate buffered saline. Plasmid DNA was prepared using a plasmid miniprep kit (Qiagen, Valencia, CA, USA) and nested PCR was performed with primer sets nestF and nestR (round 1) and exF and exR (round 2) (Supplementary Information) and TaqPro Red PCR mastermix (Denville Scientific, Metuchen, NJ, USA) as described previously (3).

piggyBac expression time course

HEK-293 cells were transfected with pCMV-HA.*piggyBac* (3) using FuGENE 6. Whole cell protein lysates were prepared every 24 h and frozen at -80°C . Lysates were run on a 10% bis-tris gel in MOPS buffer (Life technologies, Grand Island, NY, USA) and probed with mouse anti-HA antibody (Covance, Emeryville, CA, USA) and anti-mouse secondary antibody conjugated to IR-800 dye and imaged on an Odyssey infrared imager (LICOR Biosciences).

Transposase expression in 'cis' and 'trans' clones

HEK-293 cells were transfected with HA.PB-pT-Nori, pT3SB12 or pTpB and pCMV-HA.*piggyBac* (described in (3)) using FuGENE 6 and 48 h after transfection plated in G418 selection media. Single cell clones were expanded for at least 8 weeks and whole cell protein lysates were prepared for western blots and analyzed as described above and in (3).

In vivo gene delivery

All animal procedures were approved by the Institutional Animal Care and Use Committee at the Baylor College of Medicine. Plasmid DNA was delivered to 8- to 10-week-old female FVB mice (Charles River Labs, Wilmington, MA, USA) via hydrodynamic tail vein injection as previously described (21). Mice were injected with the indicated amounts of transposase and luciferase transposon plasmids in 100 μ l volume per gram body weight of TransIT QR hydrodynamic injection solution (Mirus Bio, Madison, WI, USA). Mice were imaged at the indicated time points after injection using a Xenogen IVIS imaging system (Caliper Life Sciences, Hopkinton, MA, USA) following intraperitoneal injection of luciferin substrate 5 min prior to injection according to the manufacturer's instructions.

Luciferase reporter assay

A set of test plasmids with the firefly luciferase reporter and the *piggyBac* TRs in different orientations were constructed using standard molecular biology techniques. HEK-293 cells were co-transfected with 0.25 pMoles each of the test plasmids and pRL-TK (Promega) carrying the renilla luciferase. pUC19 was used to normalize for the differences in the amount of the test plasmids transfected. Luciferase activity was measured after 48 h using Dual-Glo[®] Luciferase Assay System (Cat# E2920; Promega). Relative ratio was calculated by normalizing the fLUC/rLUC values to the fLUC/rLUC value of pCAG-LUC or pGL3 where appropriate.

Backbone integration

Standard molecular biology techniques were used to place a CMV-eGFP cassette in the backbone of pTpB (3) to create pCMV-eGFP-pT-Nori. HEK-293 cells were then transfected with pCMV-eGFP-pT-Nori and pCMV-PB in ratios of 1 μ g:1 μ g, 1 μ g:100 ng, 1 μ g:10 ng and 1 μ g:0 ng. The total amount of plasmid DNA was normalized to 2 μ g with pUC19. Forty-eight hours post-transfection cells were harvested and replated in media containing 1 mg/ml G418. Two weeks later colonies were fixed with 10% neutral buffered formalin, stained with methylene blue and counted. Cells were also analyzed to determine the percent of cells that were eGFP-positive and the genomic integration site of the transposon backbone (CMV-eGFP cassette) after 8 weeks of G418 selection. eGFP expression was analyzed using a BD CantoII flow cytometer (BD Biosciences, San Jose, CA, USA). Backbone integration sites were identified by nested inverse PCR using primers bb1F, bb1R for round 1 and bb2F-bb2R for round 2 (Supplementary Information), genome walking using the APA Gold Genome Walking kit (Bio S&T, Quebec) according to the manufacturer's instructions, or plasmid rescue as described previously (3).

Cell sorting and quantitative PCR (qPCR)

HEK-293 cells transfected with pCMV-eGFP-pT-Nori were pooled for sorting on a fluorescence-activated cell sorting (FACS) Aria (BD, Franklin Lakes, NJ, USA). Cells were sorted into 0.5 ml fetal bovine serum (FBS) to improve viability. The sorted cells were pooled, separated into three equal vials of 1–2 million cells each, centrifuged and cell pellets were frozen. Genomic DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen). RNaseP and neomycin primers (10) and PCR conditions (22) have been previously described. eGFP primers have been previously described: forward primer eGFP-QF (23) and reverse primer eGFP-Rev (24). Each well contained 6 ng of genomic DNA. We did gradient PCR to optimize the eGFP copy number amplification program to produce only the desired product. The program was as follows: 95°C 3 min; 40 cycles of 95°C 10 s then 68°C 30 s; 95°C 10 s. The neomycin and eGFP standard curves contained a constant 6 ng of naïve HEK-293 genomic DNA with a serial dilution of a known number of copies of the pCMV-eGFP-pTNori plasmid, similar to the standard curve previously described (22).

Melting curves confirmed amplification of only the desired product.

Clonality of human foreskin fibroblasts

Human telomerase reverse transcriptase (hTERT) coding sequence was PCR amplified from pBABE-Neo-hTERT (Addgene, Cambridge, MA, USA) with primers that add a 2A self-cleaving peptide sequence. The hTERT.2A sequence was then cloned into pIRII-IRES-eGFP (25) replacing the IRES to obtain pIRII-hTERT.2A.eGFP. Human foreskin fibroblasts (HFF-1) were obtained from ATCC (Manassas, VA, USA). Cells were transfected with either pT-CMV-eGFP or pIRII-hTERT.2A.eGFP and pCMV-*piggyBac* with the NEON transfection system (Life Technologies, Grand Island, NY, USA). The percentage of eGFP-positive cells was determined by flow cytometry on a BD FACS CantoII (BD Biosciences, San Jose, CA, USA).

RESULTS

Lack of PB-mediated endogenous human genome mobilization

PB creates double-strand breaks at genomic target sites in order to insert the transposon carrying the gene(s) of interest. If PB transposase can recognize endogenous PB-TR-like sequences within the human genome, then an increase in double-stranded DNA breaks should be observed in the presence of PB transposase and the expression of the transposase alone could cause genomic instability. To ascertain whether the transposase can increase the frequency of double-strand breaks, we transfected HEK-293 cells with the transposase (pCMV-PB), the transposon (pT-CMV-eGFP) or the combination of the two and measured the levels of phosphorylated H2A.X, a marker for DNA damage (26). We observed no significant increase in the levels of phosphorylated H2A.X in cells transfected with the transposase alone as compared to cells transfected with the pUC19 negative control (Figure 1). By contrast, we observed a 1.9-fold increase in phospho-H2A.X levels when cells were treated for 2 h with 2.5 mM camptothecin (positive control). Elevated levels of phosphorylated H2A.X were observed in the presence of transposase with transposon but not with transposase alone.

In order for PB to excise a transposon segment from a plasmid and integrate it into the genome, TR sequences are required (27,28). The PB TRs are made up of identical 17 bp TRSs including the TTAA sequence and 19 bp internal repeat sequence (IR) separated by asymmetrical spacers (29). Feschotte estimates that there are ~2000 PB-like elements throughout the human genome (17). This has recently been reiterated by Vandendriessche *et al.* as a potential drawback to using PB to gene modify human cells (1). Although comparative genomic hybridization analysis after the use of PB in mouse cells revealed no genomic alterations (30), and karyotype analysis of human T cells modified with PB revealed no detectable alteration (16), a more direct analysis of the possibility of PB mobilizing endogenous PB-like elements in human cells has not been undertaken.

To identify sequences in the human genome that could potentially be recognized by the transposase, we performed

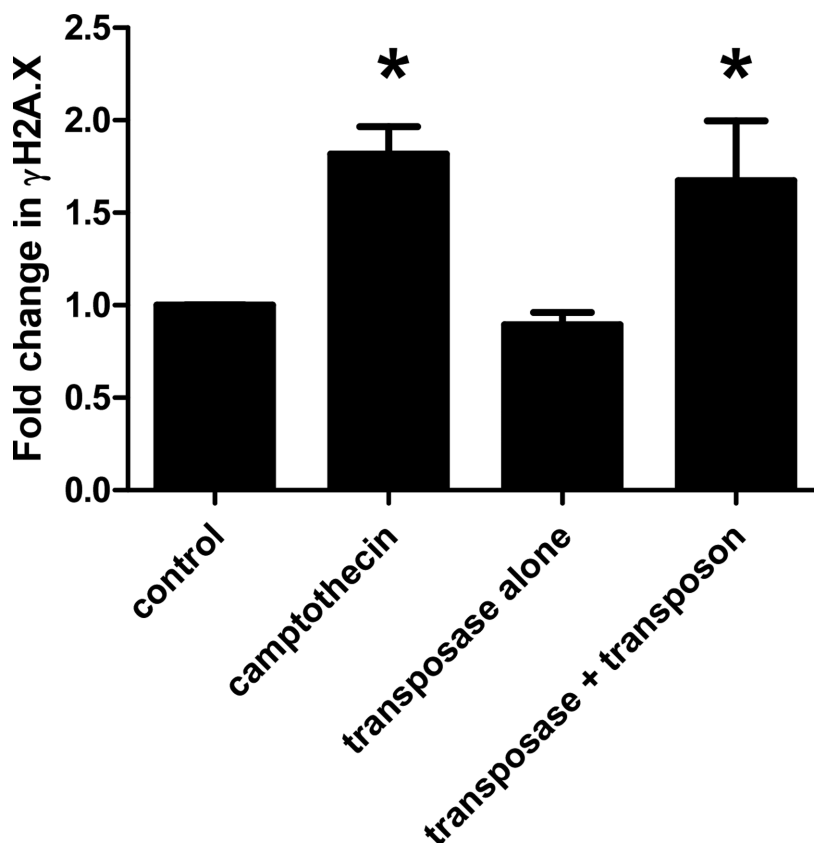


Figure 1. *piggyBac* transposase requires the presence of the transposon to induce DNA double stranded breaks (DSBs) in human cells. Formation of DSBs by *piggyBac* transposase was quantified by determining the level of phosphorylated H2A.X at serine 139. HEK293 cells were transfected with either a vector carrying the transposase (pCMV-PB), a transposon (pT-CMV-eGFP) or both. Cells transfected with pUC19 were used as negative control and cells treated with 2.5 μ M camptothecin served as positive control. Approximately 24 h after transfection histones were extracted and probed with anti-phospho-H2A.X. H1.0 was used as loading control. Quantitative analysis using ImageJ demonstrated a statistically significant increase (*) in pH2A.X levels after 2.5 μ M camptothecin or transposase + transposon compared to control ($N = 3-5$ independent experiments, ANOVA with Bonferroni post-test).

a BLAST search with the 17-bp TR as the query sequence. We chose this sequence as PB has been shown to directly bind this sequence in gel shift and footprint assays (31). There were no sequences in the human genome identical to the 17-bp PB TR with a terminal TTAA. Recently, Li *et al.* demonstrated that 97.9% of PB integration sites are TTAA. The next most common integration site is CTAA (1.4%) with other possibilities (6). They demonstrated that full length IRs ending in CTAA could be mobilized from plasmid vectors. Our analysis revealed no TR-like sequences in the human genome corresponding to the 17-bp TR ending in CTAA. There were, however, sequences identical to the first 16, 15 or 14 bps of a TR ending in TTAA. We selected the top 14 sequences with 16 and 15 bp similarities (Supplementary Information) and replaced the native 5'TR or 3'TR of the pTpB plasmid (3) containing an antibiotic resistance gene, with the human sequences including flanking DNA such that a 300-bp DNA fragment was inserted. Transposons lacking either the 5' or 3'TR or the intact transposon transfected without transposase were used as negative controls. We evaluated the ability of the transposase to recognize and transpose plasmids containing these sequences by both colony count assay and excision assay. The colony count assay serves as a proxy for transposition by quantitating the number of selected cell colonies resultant from

integration of an antibiotic resistance gene cassette. When the genomic sequences were used in place of native PB TR elements, we observed no more colonies than we observed with the negative controls (Figure 2A and B) indicating that the PB-TR-like endogenous DNA sequences are not sufficient to mediate transposition and cannot be mobilized.

The excision assay uses PCR amplification of the excision product to provide a more sensitive means to detect transposition compared to the traditional colony count assay (32). We therefore evaluated the possibility of mobilization of these genomic fragments using excision PCR and found no such mobilization (Figure 2C and D). We observed no evidence of PB-mediated mobilization of endogenous PB-TR-like DNA fragments when they were placed into a cognate transposon plasmid in either the 5' or 3' TR. Therefore, we observed no mobilization of endogenous human genomic DNA by the PB transposase using colony count assays, excision assays and double-stranded DNA break assays. Further refinement of PB vectors has demonstrated that shortening the 5'TR to 35 bp and the 3'TR to 63 bp may still mediate gene transfer (33). It is likely that a high degree of sequence specificity is required to mediate transposition from both TRs. Therefore, even though we directly evaluated only a few of possible PB TR-like elements, it seems unlikely that expression of the PB transposase can rearrange

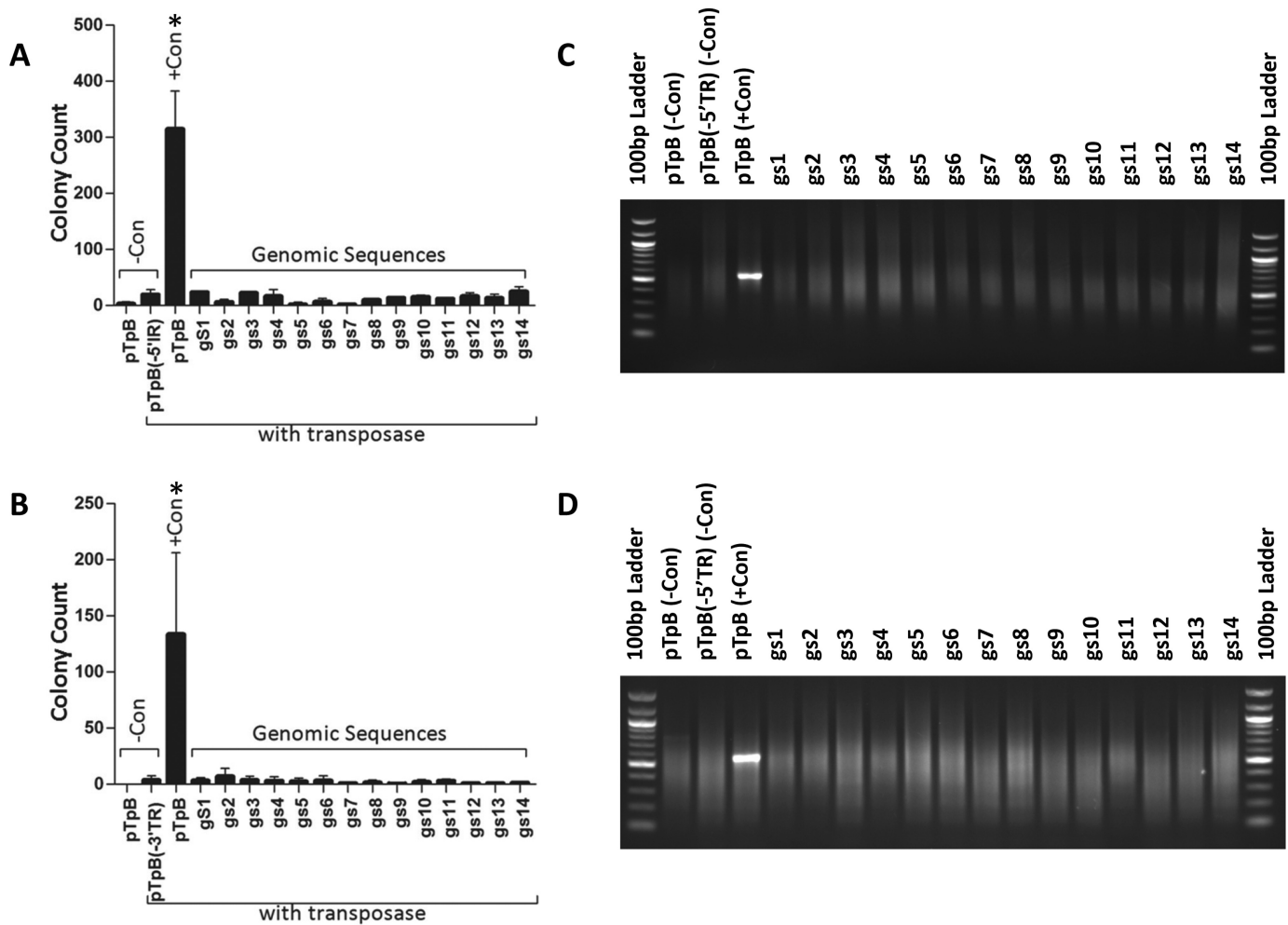


Figure 2. *piggyBac* does not mobilize endogenous human TR-like sequences. 5' or 3' native TRs were replaced with PCR amplified genomic sequences. (A) and (B) The colony count was used as a measure of stable transposition. Only the native 5' (A) or 3' (B) in presence of the transposase leads to efficient and stable transposition in HEK293 cells. Elimination of the TRs or the transposase led to significantly reduced transposition and serve as negative controls. Genomic sequences similar to the *piggyBac* TRS did not lead to efficient and stable transposition and were comparable to the negative controls ($N = 3$ independent experiments, ANOVA with Bonferroni post-test; *, statistically different from negative control). An excision assay was used to determine whether a transposon is recognized and excised out of the vector by the transposase. A transposon with native TR sequences was efficiently recognized and excised by the *piggyBac* transposase, so a PCR product was visible on the agarose gels shown (pTpB lanes). Transposons carrying the Genomic TR-like sequences were not recognized and excised from the transposon either in the 5' (C) or 3' (D) position.

the human genome given the specific sequence requirements of PB for transposition. Our conclusion is strengthened by previous evidence using CGH arrays and karyotype analysis after using PB to gene modify cells (16,30).

PB transposase or backbone DNA integration

Integration and long-term expression of the transposase could result in genome instability via transposon remobilization. The transposase can be delivered with transposon DNA in either in a 'cis' or in 'trans' configuration. In the 'cis' configuration, the transposase and transposon are on the same DNA vector (Figure 3A), whereas in the 'trans' configuration the transposase is expressed from a separate non-integrating helper plasmid (Figure 4A). We have previously reported increased gene delivery when using the 'cis' configuration, presumably because all transfected cells received both transposase and transposon as both are

encoded on the same DNA vector (3). However, the 'cis' configuration raises the possibility of transposase integration (34). Therefore, we sought to compare the degree of transposase integration between the 'cis' and 'trans' configurations.

In order to evaluate long-term transposase expression, we transfected HEK-293 cells with a transposon harboring the neomycin resistance gene and the transposase in either the 'cis' or 'trans' configuration. Cells were then selected with G418 and transposase expression was assessed after 8 weeks of selection by western blot. All clones derived from 'cis' transfection contained residual PB transposase expression, even after 8 weeks of antibiotic selection (Figure 3B). Additionally, a population of selected 'cis' cells expressed residual transposase on day 31 (Figure 3C). Transposase integration in the 'cis' configuration was not unique to the PB system as 5 out of 6 clones raised with SB when using a

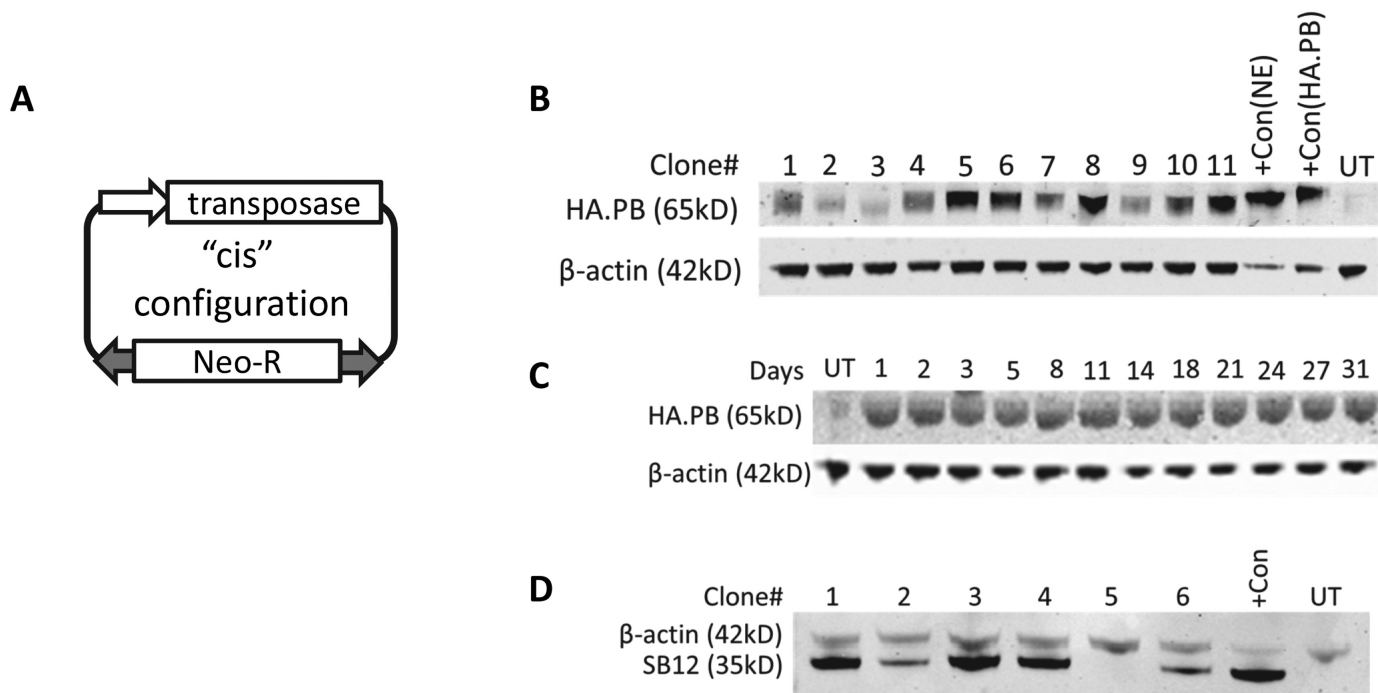


Figure 3. Residual transposase expression after ‘cis’ delivery. (A) In the ‘cis’ configuration, the transposase and transposon are on the same plasmid DNA vector. (B) HEK293 cells were transfected with a vector carrying both the transposon and the transposase. The transposon had a Kan/Neo selection gene and cells were passaged in G418 containing media to select for cells with stable transposon integrations. After 8 weeks of selection in G418 media, protein purified from single cell clones was analyzed by western blot for residual transposase expression. +CON(NE), nuclear extract from HEK293 cells transfected with PB transposase harvested at 48 h. +CON(HA.PB), whole cell extract from HEK293 cells transfected with HA tagged PB transposase harvested at 48 h. (C) A mixed population of polyclonal HEK293 cells had residual transposase expression at day 31. (D) Residual transposase expression after ‘cis’ delivery is also noted for the *Sleeping Beauty* (SB12) transposon system. Positive controls were whole cell extracts as described above.

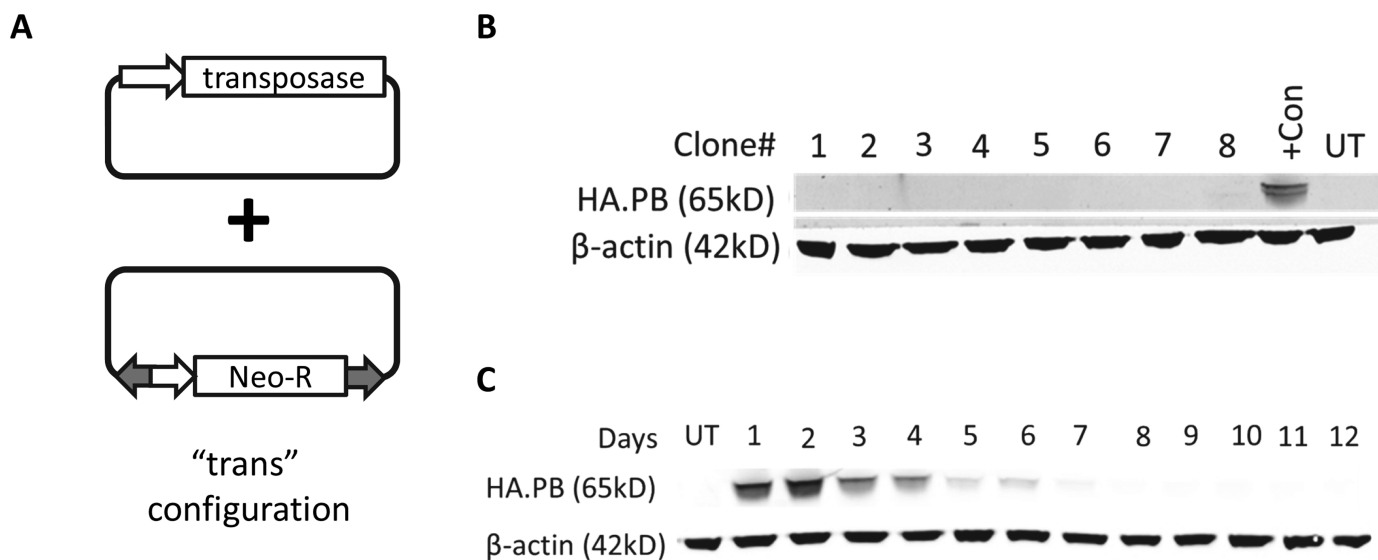


Figure 4. Lack of residual transposase expression after ‘trans’ delivery. (A) In the ‘trans’ configuration the transposase and transposon are supplied on separate DNA plasmid. (B) HEK293 cells were transfected with two separate vectors individually carrying the transposon and the transposase. The transposon had a Kan/Neo selection gene and cells were passaged in G418 containing media to select for cells with stable transposon integrations. Single cell clones had no residual transposase expression after 8 weeks of selection in G418 media. (C) A mixed population of HEK-293 cells had no residual transposase expression after approximately day 7.

'cis' configuration also expressed transposase (Figure 3D). In contrast, clones generated after transfecting cells with a PB transposase in 'trans' did not show any residual transposase expression (Figure 4B). Nor did we observe significant transposase expression after day 7 in a population of 'trans' transfected and antibiotic selected cells (Figure 4C). Therefore, the 'trans' configuration reduces the possibility of transposase integration; however, backbone DNA integration does occur at a high frequency as demonstrated when evaluating the 'cis' configuration. We tested whether the use of the 'cis' configuration would lead to transgene instability after gene delivery in mice. A 'cis' vector containing luciferase was delivered using hydrodynamic tail vein injection in adult mice and it was compared to supplying the transposon and transposase separately. We observed no difference in transgene stability and luciferase expression whether the transposase was delivered in 'cis' or 'trans' (Supplementary Figure S1).

Due to the high frequency of integration of the transposase backbone in the 'cis' model, we investigated whether the transposon:transposase ratio could be optimized to eliminate the integration of the plasmid backbone in target cells. HEK-293 cells were transfected with 1000 ng of transposon and a wide range of transposase concentrations/amounts. The transposon vector pCMV-eGFP-pT-Kan/Neo carried a Kan/Neo selection cassette within the transposon and an eGFP reporter within the plasmid backbone (Figure 5A, top). Cells with stable integration of the transposon were selected with G418 and analyzed for integration of the backbone by evaluating for the expression of the eGFP reporter. We observed a decrease in the frequency of backbone integrations in clones that were transfected with lower amounts of transposase (Figure 5A, bottom).

We hypothesized that the DSBs induced by PB could lead to integration of the linearized backbone DNA. Therefore, we used colony counts to determine if PB-mediated transposition would increase the integration rate of a linearized puromycin-resistance cassette (Figure 5B, top). We used a puromycin-resistant transposon as a control. We observed no significant increase in the integration rate of a linearized puromycin-resistant cassette when PB was used to integrate a neomycin-resistant transposon (Figure 5B, bottom).

Given the high frequency of plasmid backbone integration, we sought to determine if this was a PB-mediated integration event. We mapped plasmid backbone genomic integration sites by incorporating two steps to reduce the recovery of non-integrated plasmid DNA. First, stably transfected cells were cultured for 4–6 weeks to reduce recovery of non-integrated plasmid DNA. Second, genomic DNA samples from the cells were treated with the restriction enzyme *DpnI* which cuts bacterially methylated DNA to eliminate recovery of non-integrated plasmid DNA segments. We hypothesized that the linearized plasmid backbone DNA segment was integrating into the genome. If our hypothesis was correct, then most if not all of the integration sites recovered would demonstrate a plasmid-genome junction in the backbone precisely where the transposon was excised from the plasmid.

Genome walking recovered two sequences of repaired (i.e. re-ligated) plasmid-backbone DNA present in genomic

DNA. We also used inverse PCR of genomic DNA isolated from stably transfected cells as above to recover backbone DNA integration sites. Twenty-six inverse PCR products were analyzed and 18 revealed repaired plasmid backbone. The remainder inverse PCR products mapped to three different genomic sites; however, none of these occurred at a genomic TTAA which occurs >95% of the time with a piggyBac-mediated integration event (6,30). As the transposon plasmid backbone contains ampicillin resistance and a bacterial origin of replication, we also used plasmid rescue to recover backbone DNA integration events. We recovered 11 events of which 10 revealed repaired backbone and one was not mappable to a unique sequence in the human genome. We sequenced each rescued plasmid completely and recovered one genomic integration that could be mapped. In this plasmid, the plasmid-genome junction occurred 30 bp away from the repaired transposon excision site. Therefore, the majority of the time, once the transposon is excised from its parent plasmid, the plasmid backbone is repaired by re-ligation and integrated into the genome. Alternatively, a rarer event may occur in which the linearized plasmid backbone can be integrated without excision repair (Supplementary Information).

Isolation of PB-transposed cells lacking plasmid backbone DNA

The integration of the plasmid backbone at relatively high rates may be undesirable for certain applications of the PB transposon system. Therefore, we devised a strategy to measure and remove the backbones. The plasmid pCMV-EGFP-pTNori (Figure 6, top) contains a neomycin-resistance gene in the transposon portion of the plasmid and the eGFP marker in the backbone. This allowed us to track backbone plasmid marker expression. To quantify the number of copies of transposon and backbone per cell, we purified genomic DNA and performed qPCR with probes to the neomycin resistance gene, the eGFP gene and the human RNaseP gene. We transfected HEK-293 cells with the pCMV-EGFP-pTNori and PB transposase expression plasmids and selected and propagated the transfected cells to eliminate residual unintegrated plasmids. We performed a FACS sort of the cells to separate the eGFP-positive and eGFP-negative cell populations. Of the unsorted population, 55% were eGFP-positive, 34% were eGFP-negative and the middle 11% were discarded. We obtained 6.7 million eGFP-positive cells and 3.9 million eGFP-negative cells that were separated post-sorting into three vials per group for extracting the genomic DNA. Of note, we found during post-sorting analysis that ~2% of the cells were not sorted correctly, i.e. there were 2% eGFP-positive cells in the eGFP-negative sorted population and vice versa. We performed qPCR assays on the samples to detect three separate targets: (i) RNaseP, a unique gene found once per haploid genome in human cells; (ii) the neomycin-resistance gene present on the transposon; and (iii) the eGFP gene present on the plasmid backbone (Figure 6). While the selected cells all contained ~10 copies of the transposon per diploid cell, the sort was successful in removing cells containing the plasmid backbone to levels ~40× lower than those of unsorted cells. While unsorted cells typically had 1–2 copies of plas-

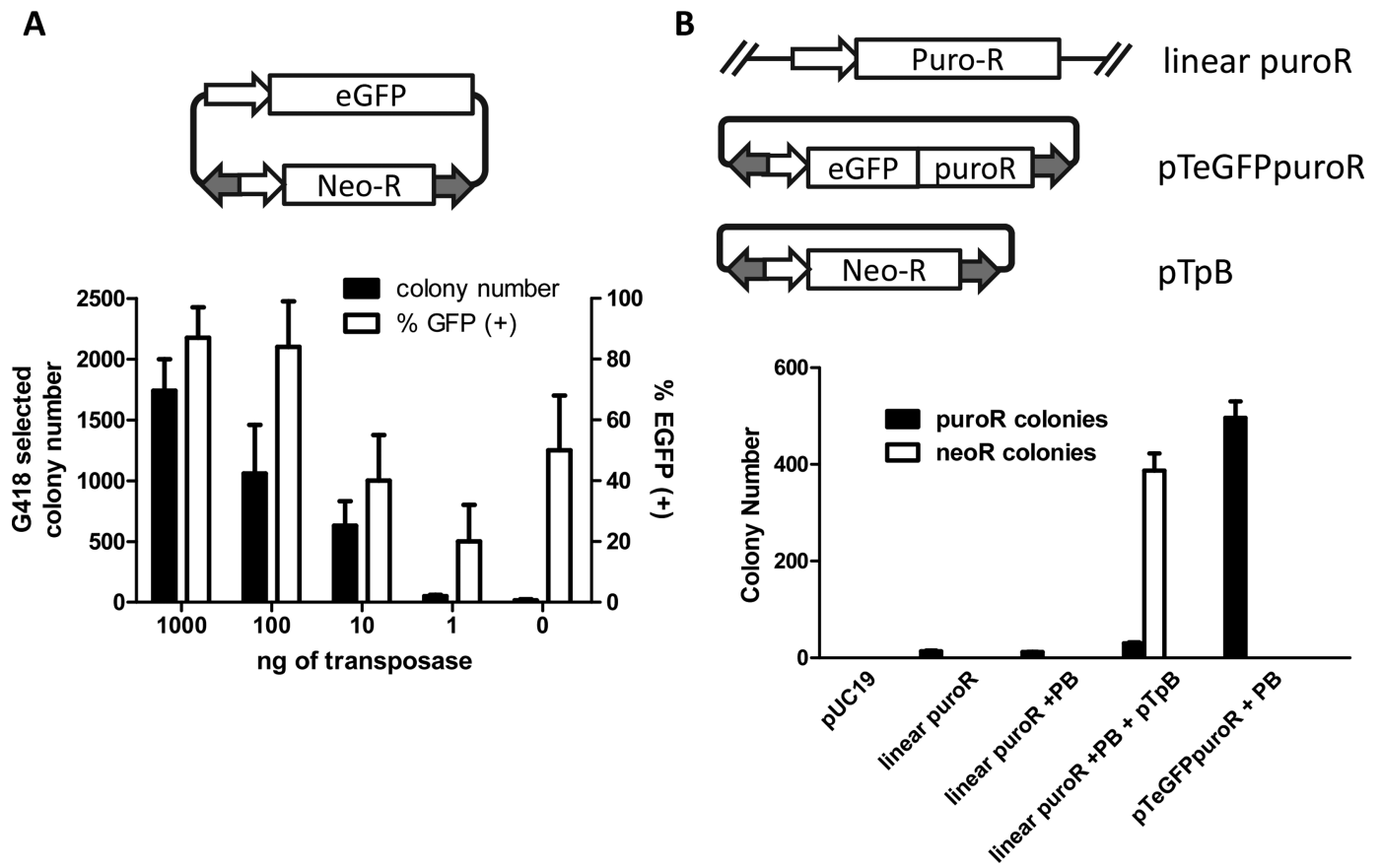


Figure 5. The effect of varying the transposon-to-transposase DNA ratio on backbone-free transposon integration. (A, top) Schematic of pCMV-eGFP-pT-Nori vector carrying an eGFP reporter on the backbone and a Kan/Neo selection gene in the transposon. (A, bottom) Flow cytometry for eGFP expression was used to evaluate for backbone-free transposon integration at transposon-to-transposase ratios of 1000 ng:1000 ng and 1000 ng:100 ng and 1000 ng:10 ng. (B, top) Schematics of vectors used. A linearized puromycin-resistance cassette was transfected with a neomycin resistant transposon and integration was measured evaluating for puromycin-resistant colonies after 2 weeks of selection in puromycin (B, bottom). A puromycin-resistant transposon was used as a positive control. ANOVA revealed pT-eGFP-puroR + PB was different from linear puroR. Linear puroR + PB + pTpB was not statistically different from linear puroR alone ($N = 3$).

mid backbone per diploid genome, the eGFP-negative cells had 0.026 copies of plasmid backbone per haploid genome. The eGFP-negative population has a backbone copy number that is precisely accounted for by the expected contribution from the contaminating eGFP-positive cells (2% sorted incorrectly $\times 1.28$ backbone copies per haploid genome = 0.026 copies expected per haploid genome from the contaminating population). If desired, cells could be sorted a second time to remove nearly all of the cells with an integrated plasmid backbone.

PB-TR mediated effects within human cells

Previous reports have demonstrated promoter activity within the PB 5' TR in cultured cells (35). To further test for and quantify possible PB-TR-mediated promoter activity in human cells, we created a set of plasmids carrying the luciferase reporter and the TRs either singly or together and in various orientations and tested their ability to mediate luciferase gene expression in HEK-293 cells (Figure 7). We used the luciferase reporter without a promoter or the TRs as the negative control. A cytomegalovirus (CMV) early enhancer/chicken β actin (CAG) promoter-driven lu-

ciferase was used as the positive and normalization control. We observed that the 5' TR exhibited some promoter activity though weak when compared to the strong CAG promoter. This activity was directional as flipping the 5' TR in the opposite orientation eliminated the promoter activity. Therefore, the 5' TR exhibits weak promoter activity in human cells; however, it is directional to promote gene expression within the transposon and not outside of the transposon. The 3' TR exhibited no promoter activity in either orientation.

To test whether PB TRs can act as enhancers we investigated the effect of the TRs on the activity of a SV40 promoter-driven firefly luciferase reporter in HEK-293 cells. TRs were cloned upstream of the SV40 promoter in the pGL3-basic vector. A promoter-less firefly luciferase sequence was used as a negative control. PB TRs did not significantly enhance the SV40 promoter (Supplementary Information). Although there was an apparent increase comparing 5' TR-SV40-LUC to SV40-LUC alone, this was not statistically significant using analysis of variance and the increase may be due to the directional promoter activity of the 5' TR observed in Figure 7. Enhancer activity was not

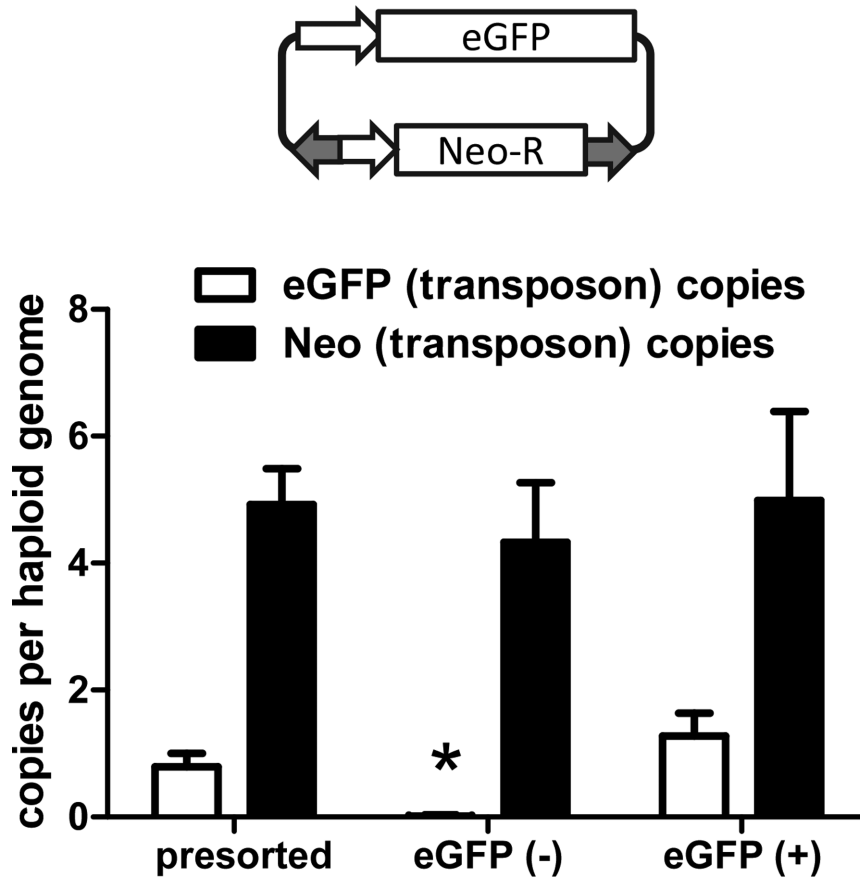


Figure 6. Quantitation of transposon and backbone copy number and isolation of backbone-free *piggyBac*-modified human cells. pCMV-eGFP-pTNori and transposase plasmids were transfected into HEK-293 cells that were cultured in selection media to produce a population of cells carrying the PB transposon. The plasmid backbone contained eGFP and cells were either unsorted, eGFP-negative (-), or eGFP-positive (+). The number of copies of the transposon (black bars) and plasmid backbone (white bars) per haploid genome (copy of RNaseP) was determined by qPCR. There was no significant difference between groups with regard to the transposon copy number by ANOVA. There was a significant difference between the copies of plasmid backbone per haploid genome by ANOVA ($P = 0.001$). We found that both unsorted versus eGFP-negative ($P < 0.05$) and eGFP-negative versus eGFP-positive ($P < 0.01$) were significantly different while there was no significant difference between unsorted and eGFP-positive cells (Tukey HSD test).

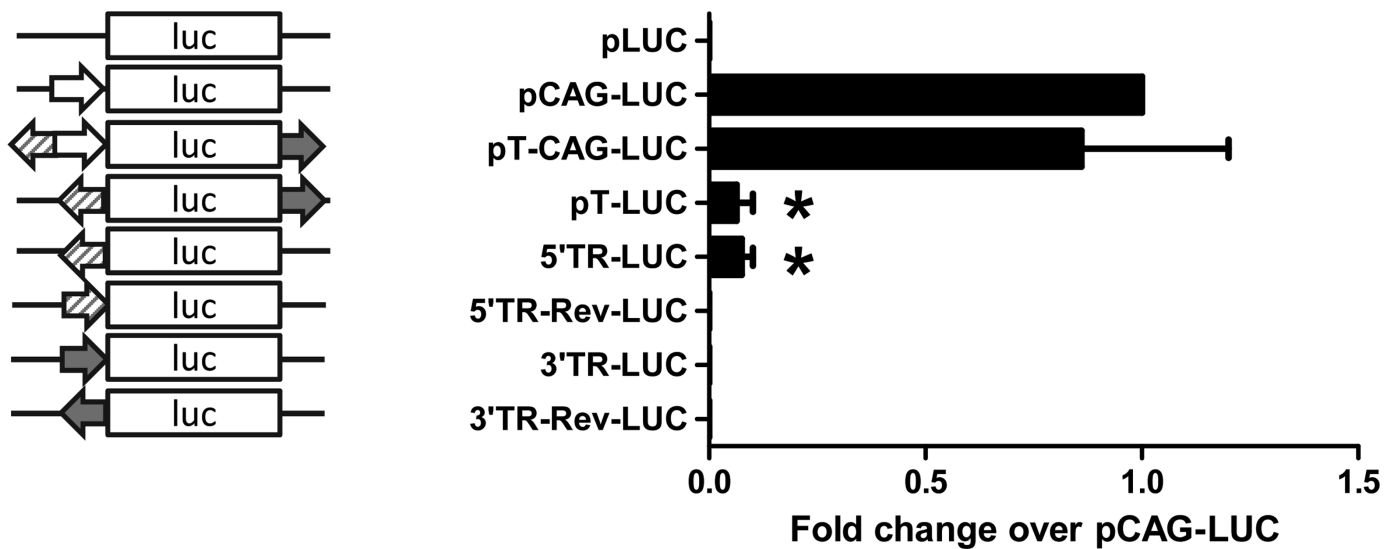


Figure 7. Evaluation of promoter activity and enhancer effects of PB TRs in human cells. The PB 5'TR has weak directional promoter activity only inside the transposon. The 3'TR has no detectable promoter activity in either orientation. There is no significant change in the activity of a CAG-driven luciferase reporter activity when placed inside the transposon ($N = 3$). Rev, the TR is flipped to the reverse orientation. The dashed arrow indicates the 5'TR, whereas the solid gray arrow indicates the 3'TR.

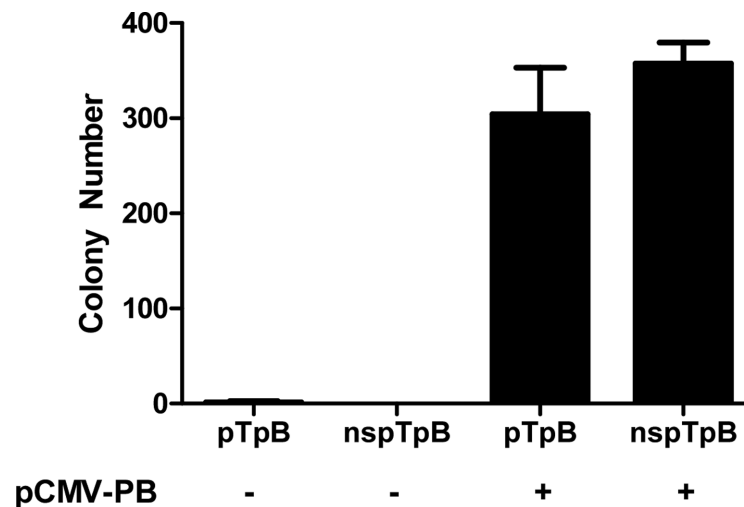


Figure 8. Mutation of the cryptic splice site in the PB 5' TR has no effect on transposition. The 5' TR is 311 bp. The mutated non-splice version (nspTpB) contains a G to C mutation at position 73, a G to C mutation at position 149 and a T to A mutation at position 184. These mutations eliminate predicted splice sites derived from NetGene2 (<http://www.cbs.dtu.dk/services/NetGene2/>) and those proven in Wang *et al.* (36). ($N = 3$).

observed with either TR when using a strong promoter like CAG (Supplementary Information). Therefore, the PB TRs exhibit a 5' TR directional promoter activity pointed inside the transposon. Although we observed no statistically significant enhancer activity within the PB TRs, this may warrant further evaluation in specific human cell types if considering using PB for genomic modification.

Wang *et al.* previously reported a cryptic splice site within the 5' TR of the PB transposon when evaluating PB as a mutagen attempting to target the mouse *HPRT* locus (36). We mutated this cryptic splice site and determined if it affected PB-mediated gene transfer in human cells. Using a colony count assay, we observed no statistical change in PB activity when using the 5' TR no-splice variant when compared to the native 5' TR (Figure 8). Therefore, the 5' TR of PB can be engineered to remove the cryptic splice site if desired for its use in human cells.

To determine whether PB-modified cells could have a selective growth advantage, we transfected HFF-1 with either eGFP transposon or a transposon with both eGFP and hTERT dual expression. hTERT imparts immortalization by maintaining telomere lengths (37). We saw no selective advantage in HFF-1 cells modified with eGFP alone. In contrast, the cells modified with the dual expression transposon enriched the population and constituted 57% of the population at day 140 (Figure 9A and B).

DISCUSSION

In this study we assessed the potential for undesired genomic effects when using the PB transposon system for genetic modification of human cells. In order to consider using PB for human use, we sought to determine if PB could mobilize endogenous human genomic PB-like sequences and to what extent promoter/enhancer activity exists within PB TRs. We observed no mobilization of endogenous PB-like elements when an active PB transposase was expressed as measured in double-stranded DNA break, colony count and excision assays. Although we observed a high rate of

transposase integration when using a 'cis' configuration of transposase and transposon, we did not observe transgene instability after gene transfer in mice when using the 'cis' configuration and transposase integration was eliminated when the transposase was expressed from a separate plasmid. The stability of transgene expression *in vivo*, even following delivery of our 'cis' vector, could be due to silencing of the CMV promoter used to drive transposase expression and/or because the rate of 're-excision' of PB transposons has been reported to be low in mammalian cells (36). The rate of transposon plasmid backbone integration could be somewhat reduced by altering the ratio of transposase to transposon. Importantly, cell sorting could be utilized to obtain plasmid-backbone-free PB modified human cells. We confirmed directional promoter activity in human cells from the 5' TR element, but its activity was directionally located within the transposon.

The lack of mobilization of PB-like human genomic elements when PB transposase is expressed is encouraging. These observations fit well with the use of PB in mouse cells as comparative genomic hybridization analysis revealed no genomic alterations with the presence of transposase (30). Karyotype analysis of human T cells modified with PB revealed no detectable alteration after transposition (16). Evaluation of PB-modified human T cells has revealed no clonal outgrowth in culture (15).

Integration of the PB transposase has previously been observed when using PB in a 'cis' configuration (34). Urschitz *et al.* attempted to overcome this issue by having the transposase span the 3' TR using an intron (34). In this case, transposition splits the transposase cDNA so that even if the backbone is integrated it will lack a full transposase cDNA. However, this configuration also creates a promoter directionally placed to promote genes outside of the transposon segment which could result in undesired genomic effects.

The degree of transposon plasmid backbone DNA integration we observed may be due to the high degree of ac-

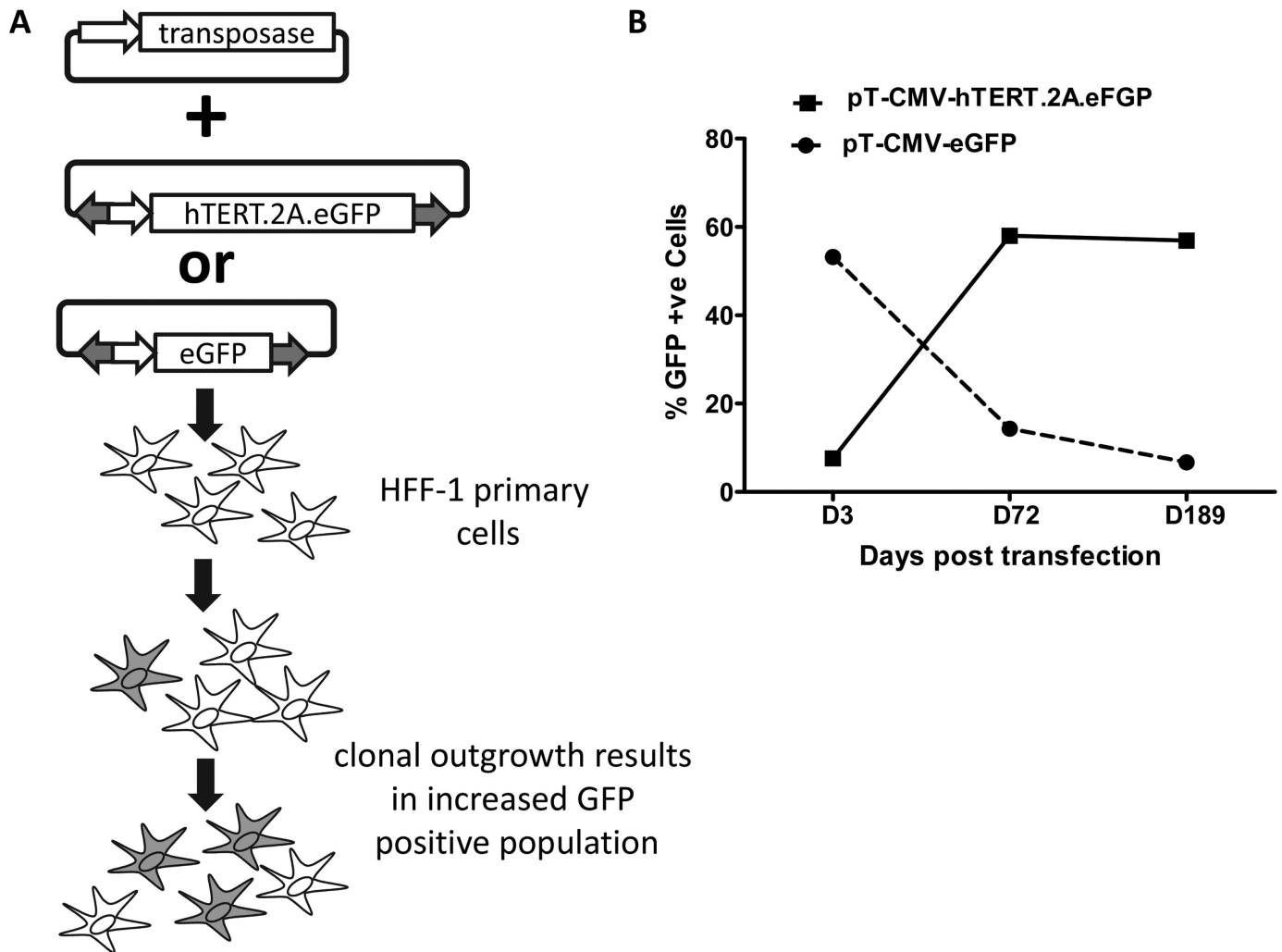


Figure 9. Lack of observable selective advantage of primary cells modified with the PB gene delivery system. (A) Human foreskin fibroblast-1 (HFF-1) cells were transfected with either pT-CMV-eGFP or pT-CMV-eGFP.2A.eGFP. If PB transposon integration led to any selective advantage the population would be expected to become increasingly eGFP positive. A dual expression vector expression both an eGFP reporter and the human telomerase reverse transcriptase (hTERT) was used as a positive control. (B) HFF-1 cells transfected with only an eGFP reporter transposon had no selective advantage, whereas cells transfected with the positive control exhibited selective advantage. Shown is a representative of 3 independent experiments.

tivity of PB in HEK-293 cells. We previously observed a high number of integrations per cell when using PB in a population of HEK-293 cells (7,10). The use of a weaker promoter driving PB transposase expression may alleviate this issue. Recently, Cai *et al.* packaged PB into integrase-defective lentiviral particles and observed an average of one transposon integration per cell (38). Given the high degree of plasmid backbone DNA integration when using transposon plasmids, the ‘trans’ configuration seems best to eliminate transposase integration when using plasmids. Alternatively, transposase can be supplied as RNA (39). However, plasmid backbone integration from the transposon plasmid remains a possibility. To reduce the risk of this occurrence, the plasmid backbone DNA size can be reduced (40) or a suicide gene (14) placed within the plasmid backbone could be used to eliminate cells with backbone DNA integration and enrich for cells with only transposon integration. Our results suggest that this should be achievable as cell sorting substantially enriched for cells with genomes that con-

tained only the drug-resistance transposon and not the accompanying eGFP-expressing backbone. It may be possible to deliver transposase RNA with linearized transposon DNA segment; however, this has not yet been tested in the context of a mammalian cell. Nonetheless, our findings of a high rate of transposase integration when using a ‘cis’ configuration are important as the use of PB for genome modification increases and ‘cis’ plasmid vectors are available. A recent investigation reported integration of ‘minimal’ PB components when full-length TRs are provided elsewhere in the plasmid; however, these results can be explained via our observation of transposon plasmid backbone DNA integration (33).

Promoter activity has been previously observed when using PB in cultured cells (35,41). We sought to further evaluate the potential for both directional promoter and enhancer activity within human cells. The 5’TR promoter activity is directional and acts only within the transposon. This activity, as well as any possible minimal enhancer ac-

tivity, could be isolated by using insulator elements within the transposon segment (42). The transposon orientation can be flipped such that the 5' TR acts in the opposite direction to the transgene promoter if needed (35). Ultimately, it may be possible to mutate the TR elements to eliminate promoter activity while retaining transposition. Herein, we demonstrated that transposon IRs can be mutated to eliminate a cryptic splice site while retaining transposition.

To determine if the PB transposon conferred a survival advantage, we used a reporter transposon to modify primary human cells. We found that there was no observable growth advantage of the transposon-modified cells during our 140 day experiment. These results are consistent with a lack of clonality after PB-mediated gene modification of human T cells to express a chimeric antigen receptor (15). Longer term experiments in mice may be needed to further evaluate genotoxicity occurring after PB-mediated gene transfer. Deep sequencing analysis of thousands of PB integrations in human cells has confirmed a weak bias toward genes and the 5' end of genes (43). However, recent investigations are directed at targeting transposition so as to deliver therapeutic transgenes to low-risk chromosomal regions (8–10).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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