Chapter 3. Slingshot: A *piggyBac* based transposon system for tamoxifen-inducible 'self-inactivating' insertional mutagenesis

3.1 Introduction

The complexity of the genes, pathways and networks that dictate many cellular phenotypes rarely makes it possible to employ a candidate gene (reverse genetic) approach to identify potential mediators of biological processes. In contrast genome-wide forward genetic screens which may be performed without making *a priori* assumptions about the candidature of individual genes in a process, represents a powerful approach for gene discovery. Classically forward genetic screens in higher order organisms have been performed using ionizing radiation, chemical mutagens, or viruses each of which are likely to target a different repertoire of genes. While these approaches can be extremely efficient at generating mutants with a phenotype of interest the subsequent identification of causal mutations is often cumbersome. This is particularly the case for traditional chemical mutagens such as ENU and EMS, which generate genome-wide point mutations and in so doing significant background noise from which a candidate gene carrying a mutation must be identified and then validated. Similarly, while ionizing radiation is a powerful tool for mutagenesis that generates sufficiently small chromosomal rearrangements so that a candidate gene can be identified

using approaches such as comparative genomic hybridization (CGH), it requires high doses of radiation to be used which generates a significant number of rearrangements, the majority of which represent background. Lower doses produce rearrangements of large chromosomal regions, in some cases containing hundreds of genes, complicating follow-up analysis. Even viruses, which can be introduced as single copy integrants into the genome, may function many megabases distal to their integration site. Viruses also exhibit strong insertional biases preferring to insert into active promoters and open chromatin, which effectively reduces the compendium of genes that they can mutate.

Transposons are mobile genetic elements that constitute a major part of the repetitive sequence of eukaryotes genomes (1). Transposons may be classified into two groups; DNA transposons and retrotransposons. DNA transposons consist of two terminal repeats flanking a transposable element, which allows them to be mobilized and relocated to other locations in the genome by a *'*cut-and-paste*'* mechanism. Retrotransposons function by a *'*copy-and-paste*'* mechanism. In lower organisms (worms, bacteria, *Drosophila*), DNA transposons have been used extensively for genetic manipulation and for mutagenesis (59,112,113). In recent years, DNA transposons have also been used for insertional mutagenesis screens in vertebrates, for example in zebrafish, *Tol2* has gained popularity, but has limited activity in mammalian cell cultures and *in vivo* (114). Other transposons including *Minos* have also been trialled in mammalian cells but with mixed success (115). In contrast the *Tc1*-family transposon, *Sleeping Beauty* (SB), has been shown to be effective for cancer gene discovery screens *in vivo* (88,89), and is an active mutagen in the germ line (116-118). However, while *Sleeping Beauty* is active in some cell lines such as 293T and HeLa cells, it appears to be weakly active in embryonic stem cells (108). *Sleeping Beauty* also exhibits significant 'local hopping', a phenomenon whereby a mobilised transposon re-integrates close to the donor locus (108,116,118). It also has a limited cargo capacity with mobilisation being significantly reduced when elements of more than 3kb are cloned between the inverted repeats/direct repeats (IR/DR) of the transposon (119). These factors, coupled with overexpression inhibition (where overexpression of the transposase inhibits transposition and optimal transposition is only obtainable within a narrow window of transposase expression) limits the utility of *Sleeping Beauty* as a universal mutagen. Despite these factors *Sleeping Beauty*-mediated screens have been successfully performed in cell culture systems (73).

More recently considerable effort has been invested in developing the transposon *piggyBac* (PB) from the moth *Trichoplusia ni*, as a tool for insertional mutagenesis (75,76,109,120-

122). Mobilisation of *piggyBac* from donor loci results in a more random distribution of transposon insertion events around the genome than is obtainable with *Sleeping Beauty* or *Tol2*. *piggyBac* can mobilise large cargo containing transposons of up to 50 kb (unpublished results; Bradley laboratory, Wellcome Trust Sanger Institute, Cambridge, UK) and unlike *Sleeping Beauty,* expression of the *piggyBac* transposase at high levels does not appear to result in overexpression inhibition (109). Another advantage of *piggyBac* is that the *piggyBac* transposase is still active when fused with other proteins such as the estrogen receptor ligand-binding domain (ERt2), which opens up a range of possibilities for temporally controlled mutagenesis (122). In addition to these factors, *piggyBac* appears to be highly active in mammalian cells and generates multiple independent insertions in cells into which the transposon is introduced. Collectively these factors suggest that *piggyBac* is a powerful mutagen that complements tools that are currently available for genetic screens in mammals.

The most common method for introducing *piggyBac*, or indeed other transposons, into mammalian cells, is to co-transfect a plasmid expressing the transposase ('helper' plasmid) and another plasmid carrying the transposon ('donor' plasmid) (**Figure 3-1 A**) (123). Once transfected into the host cells the transposase enzyme mobilizes the transposon from the donor plasmid and integrates it into the host genome. This plasmid based transposon system in cell culture has several drawbacks. Obviously, if only the 'helper' or 'donor' plasmid presented in a cell, transposition would not take place (**Figure 3-1 B and C**). Although the number of transposon integration events can be controlled to some extent by titering the amount of plasmid and the ratio of the helper and donor plasmids, integration patterns are frequently complex, thus increasing the difficult in isolating the useful insertion sites (**Figure 3-1 D**) (121). Similarly once integrated the continued expression of the transposase can re-mobilize transposon integration events, generating a complex pattern of integrations in subsequent cell divisions. Furthermore, efficient transfection of both *Sleeping Beauty* and *piggyBac* has proven difficult in some somatic cell lines which have limited the use of transposon systems for insertional mutagenesis screens in these cell lines. Therefore, although the transposon systems including *Sleeping Beauty* and *piggyBac* represent novel and powerful tools for mutagenesis study in mammalian cells, there is a lack of efficient methods to introduce these transposon systems into cell cultures for high efficiency insertional mutagenesis screening.

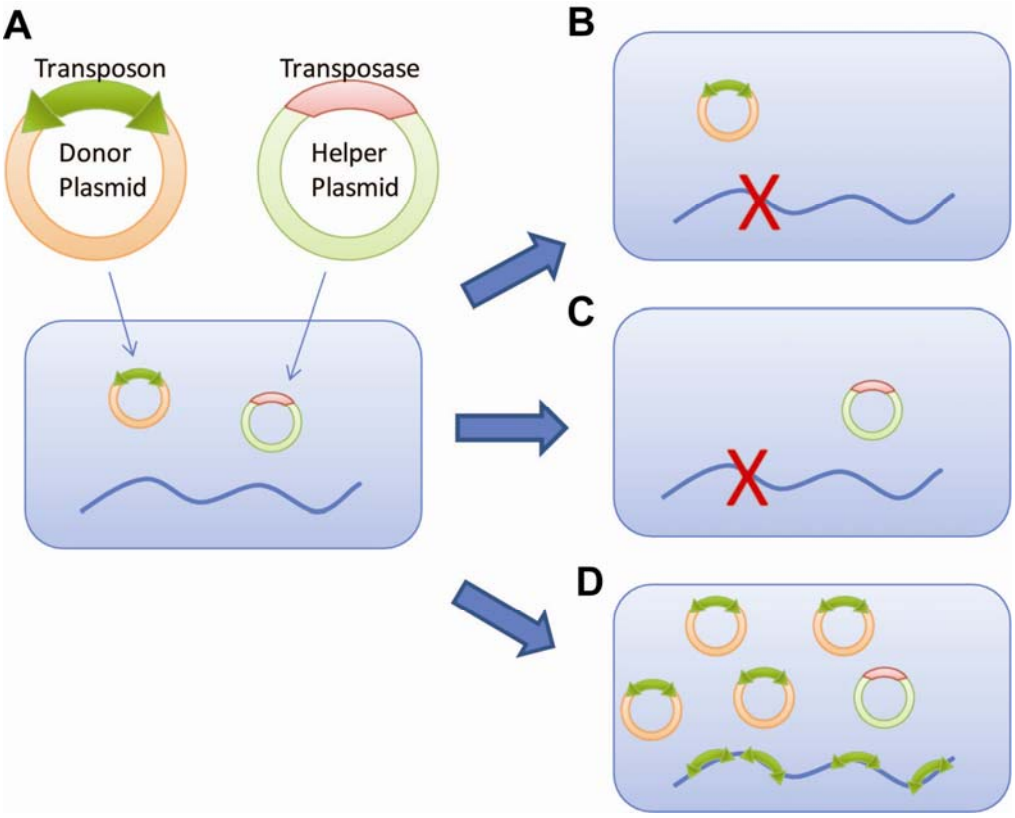


Figure 3-1. Plasmid based PB Transposon system in cell culture

(A) The Plasmid based PB Transposon system requires both the ‘donor’ and ‘helper’ plasmids to be introduced into one cell for transpostion taking place. If only ‘donor’ plasmid or ‘helper’ plasmid were present in a cell no transposition would result (B and C). Multiple copies of ‘donor’ plasmids in a cell would result in multiple jumping in the genome (D), therefore complicating downstream analysis.

3.2 Aim and summary of the project

To overcome the above-mentioned limitations, I set out to develop a novel, transposon-based insertional mutagenesis system with high efficiency for cell culture applications. The *piggyBac* (PB) transposon system was chosen for this purpose since it shows much higher activity than *Sleeping Beauty* and other transposon systems in different types of mammalian cells (123). In addition, the PB transposase shows favourable properties for molecular engineering. In a recent application, an estrogen receptor ligand-binding domain (ERt2) was added to the C-terminal of the PB transposase (mPB-L3-ERt2) to provide conditional activation of the transposase while retaining the efficiency of this system (122). Mouse ES cells were used for developing and testing this system because of their superior colony forming ability, and because the system has many potential applications in ES culture systems. For this project I will aim to increase the efficiency of PB transposition by combining both the transposon and inducible ERt2 transposase (mPB-L3-ERt2) in a single plasmid and use it to identify candidate genes in an insertional mutagenesis screen in ES cells.

The novel transposon system should have following advantages for cell culture applications:

1. The system should be easily introduced into cells, including ES cells and other somatic cell lines, for insertional mutagenesis screens.

2. The system should have superior transposition activity, and subsequent screening should be easy to carry out on a large scale.

3. When considering the difficulty in isolating the common insertion sites from a complicated insertion site pattern, it is preferable that the system introduces a low copy number of integrations per cell and the copy number should be controllable during the screen.

4. Ideally, the new transposon system should avoid re-mobilisation, which is caused by constitutive expression of the transposase enzyme. This should be accomplished by switching-off transposase expression after the first integration.

Based on these criteria, I have designed a self-inactivating *piggyBac* transposon system for tamoxifen inducible insertional mutagenesis. This system, which we have named *'*Slingshot*'* (referring to a single shot handheld weapon), contains a PB transposon upstream of a transposase sequence in one cassette as shown in **Figure 3-2 A**. The transposase (mPB-L3-ERt2), driven by the CAGGS promoter is tamoxifen inducible and has been described

previously (122). After stable integration of the Slingshot cassette into the genome and Blasticidin (BSD) selection (**Figure 3-2 B**), transposition can be initiated by the administration of 4-Hydroxytamoxifen (4-OHT). The Slingshot transposon contains a ubiquitous CAGGS promoter and a splicing donor (SD) (124) for gain of function mutagenesis (**Figure 3-2 D**), and elements for gene trapping to generate loss of function mutations upon integration into the genome (**Figure 3-2 C and E**). Mobilization, however, translocates the CAGGS promoter away from the Slingshot donor site, eliminating further expression of the transposase and effectively prevents remobilization (**Figure 3-2 F**).

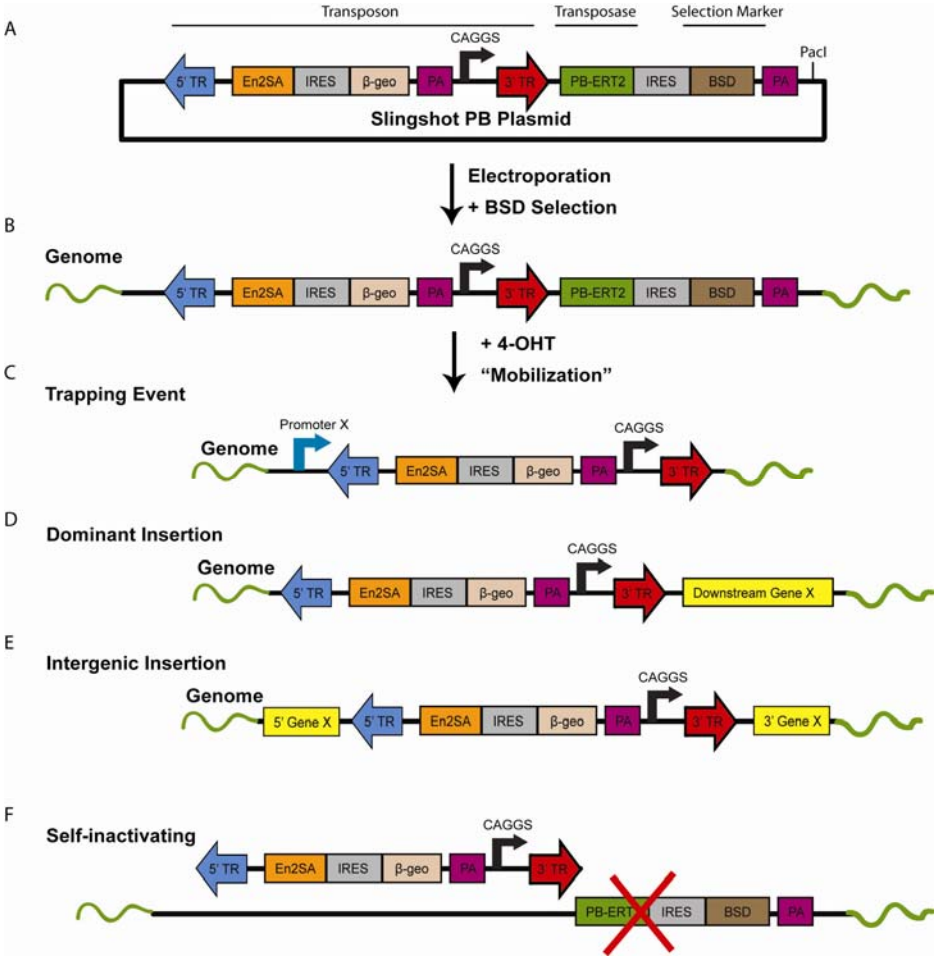


Figure 3-2. Schematic diagram of the Slingshot plasmid and the mutagenesis scenarios possible with this system

(A) The Slingshot system was constructed using a pBluescript II SK(+) vector as backbone The construct can be linearized using the restriction enzyme *Pac*I and stably integrated into a host cell genome using Blasticidin (BSD) selection. (B) The Slingshot transposon can be mobilized from the stable integrated Slingshot plasmid by treating cells with 4-OHT which translocates the PB-ERt2 fusion protein to the nucleus and mobilizes the transposon. (C) If the Slingshot transposon re-integrates downstream of an endogenous promoter 'X' it can hijack the promoter resulting in G418 resistance. (D) If the Slingshot transposon re-integrates upstream of a gene it can overexpress the gene by expression from the CAGGS promoter. (E) It is also possible for the Slingshot transposon to generate neomorphic alleles such as dominant negative alleles and loss of function events by generating intergenic insertions. (F) Mobilization of the transposon translocates the CAGGS promoter away from the PB-ERt2 preventing further re-mobilization events.

In this chapter I have develop and further characterize the Slingshot transposon system described above. My results show that the Slingshot transposon can be efficiently mobilized from a range of chromosomal loci with high inducibility and low background generating insertions that are randomly dispersed throughout the genome. Transposition and trapping efficiency is extremely high such that as many as 30,000 clonal insertion events can be generated from a single 10 cm plate of ES cells, making it theoretically feasible to screen all permissive genes and regions of the genome in just a few plates of cells in culture. To illustrate the efficacy of Slingshot as a screening tool experiments were set out to identify mediators of resistance to puromycin and the chemotherapeutic drug vincristine by performing a gain-of-function screen in mouse ES cells. From these genome-wide screens multiple independent insertions were identified in the multidrug resistance transporter genes *Abcb1a/b* and *Abcg2* and these insertions were shown to up-regulate the expression of these genes conferring resistance to drug treatment. Importantly the Slingshot transposon system was also been shown to be functional in other human somatic cell lines, suggesting that it may be used in a range of cell culture systems for genetic screens. From above results, Slingshot represents a flexible and potent system for genome-wide transposon-mediated mutagenesis with many potential applications.

3.3 Materials and Methods

3.3.1 Plasmids construction

The PB transposon element was constructed with 5' and 3' PB terminal repeats flanking a promoterless *β-geo* and a CAGGS promoter using elements derived from the plasmids 5'-PTK-3', pGTo1xr and pcDNA3.1. The *piggyBac* transposase estrogen receptor fusion (mPB-L3-ERt2) cDNA was obtained from Cadinanos *et al*. (122). The final plasmid was constructed on a pBluescript II SK(+) vector backbone using standard molecular cloning approaches and was sequenced in full. Genbank Accession: GU937109.

3.3.2 Cell culture media

Mouse embryonic stem (ES) cells were cultured in KNOCKOUT^{TM} DMEM (Invitrogen Cat. No.: 10829018) containing 2 0% Foetal Bovine Serum (Invitrogen Cat. No.: 16000044), 1× GPS, 1× BME and Leukemia inhibitory factor (LIF, Activity Varies). The human embryonic kidney cell line HEK293, and the ovarian carcinoma cell lines OVCAR-3 and PE01 were

cultured in KNOCKOUT^{TM} DMEM medium containing 8 % Foetal Bovine Serum (Invitrogen Cat. No.: 16000044) and 1× GPS.

3.3.3 Generation of an cell lines with stable integration of the Slingshot plasmid

To generate stable integrants carrying the Slingshot plasmid, 10^7 E14TG2a ES cells were electroporated at 230 V, 500 mF (BIO-RAD Gene Pulser II Electroporator) with 40 μg of Slingshot plasmid DNA following linearisationed with *Pac* I. Cells were cultured in 10 cm culture dishes for two days after transfection and subsequently selected with 15 μg/ml Blasticidin (BSD) for 10 days. Individual clones were picked into 96-well plates for downstream analysis. For transfection of HEK293, OVCAR-3 and PE01, 40 μg of *Pac* I linearised Slingshot plasmid was introduced into 10^7 cells by electroporation (300V, 800 μF) and stable integrants were selected with Blasticidin, (8 μg/ml for HEK293, 4 μg/ml for OVCAR-3 and 3 μg/ml for PE01). Round monolayer colonies formed after 2-4 weeks of selection. Cells were treated for two days with 4-OHT and G418 selection was carried out for 2-3 weeks at 500 μg/ml (HEK293), 400 μg/ml (OVCAR-3) and 200 μg/ml (PE01) to generate data on the efficiency test in these cells lines.

3.3.4 Trapping efficiency test and mobilisation assay

Mobilisation activity was tested by plating cell lines in duplicate into 6-well plates and treating with 1 μM 4-OHT (Sigma: H7904) or vehicle control (95 % EtOH) for two days. Cells were then cultured under G418 selection (175 μg/ml) for 8 days to select for gene trap events. The number of colonies was counted and the ratio of these events calculated to determine the transposon mobilisation/trapping activity. To further assess the trapping efficiency for each cell line, cells were treated with 1 μM 4-OHT for two days before a defined numbers of cells were plated on 10 cm culture dishes and treated with or without 175 μg/ml G418. Colony numbers were counted and the trapping efficiency was calculated by dividing the colony number from the plate treated with G418 (G418 resistant colonies)/by the colony number from the plate without G418 treatment (total colonies).

3.3.5 Excision PCR on DNA for 4-OHT treated cells

Excision PCR was performed to determine whether the transposon had been excised from the donor site. PCR was performed using Thermo start *Taq* Polymerase (Abgene) with primers

(forward: 5'-AAGTGTAGCGGTCACGCTGC-3' and reverse: 5'-CTCGATCACGTTCTGCTCGT-3') and the following reaction conditions: 95 °C 15 min/ 95 °C 0.5 min, 62 °C 0.5 min, 72 °C 1.5 min: 40 cycles/ 72 °C 5 min. The forward and reverse PCR primers flanked the transposon sequence in the Slingshot cassette, generating a PCR band of 526 bp if the transposon had been excised from the donor site. If the transposon remained intact, a much larger band (> 6 kb) or no band was generated.

3.3.6 Drug resistance screen using puromycin

To screen for genes responsible for puromycin resistance, 10^6 cells from clone 'PB/PB-1', which showed stable integration of Slingshot PB, were plated on 10 cm dishes. Cells were first treated with 1 μM 4-OHT or vehicle control (95 % EtOH) for two days and then cultured in normal M-15 media for another two days. Cells were then selected with 1 μg/ml puromycin for 10 days. Colonies from 4-OHT treated plates were then picked and genomic DNA was extracted to perform splinkerette PCRs to identify the insertion sites.

3.3.7 Drug resistance screen using vincristine

To screen for genes responsible for vincristine resistance, a titration test was performed to determine the lowest vincristine (Sigma Cat. no. V8388) concentration required to kill all ES cells. 10^6 ES cells from clone 'PB/PB-1' were plated on 10 cm dishes and treated with 1 μM 4-OHT or vehicle control (95 % EtOH) for two days before culturing in normal M-15 media for another two days. Cells were then selected with 10 pg/ml vincristine for 10 days. Colonies from 4-OHT treated plates were then picked and genomic DNA was extracted to perform splinkerette PCRs to identify the insertion sites.

3.3.8 Splinkerette PCR and insertion sites analysis

For splinkerette PCR 4 μg genomic DNA was digested overnight with 20 units of *Sau*3AI (NEB) in a 50 μl reaction volume. After heat inactivation at 65 °C for 20 min, 2.5 μl of the digestion mix was ligated with 1.5 μl of annealed linker oligonucleotides and 1 μl DNA ligase (NEB: M0202L) overnight. To prepare the linker oligos, 1.5 μM forward and 1.5 μM reverse linker oligonucleotides (in water) were boiled at 100 °C for 10 minutes and cooled to room temperature. One-microlitre of the ligation mixture was used for the first PCR using the 1st run splinkerette PCR primers. One-microlitre of the first PCR reaction was used as

template for the second PCR using 2nd run splinkerette primers (for all primers and oligo sequences used for splinkerette PCR see **Appendix A).** All PCRs were performed using Thermo-Start *Taq* DNA Polymerase (ABgene: AB-0908/B) with standard formula on the user's manuscript. PCR conditions were: 95 °C 15 min/ 95 °C 0.5 min, 62 °C 0.5 min, 72 °C 1.5 min: 40 cycles/ 72 °C 5 min. The PCR products were separated by agarose gel electrophoresis. Bands containing the junction site genomic DNA sequences were cut from the gel and sequenced using linker and PB sequencing primers. Sequencing reads were analyzed using the *iMapper* online web tool for identification of insertion sites (104).

3.3.9 Western blotting

Cells were lysed in NP-40 lysis buffer with 1 mM phenylmethanesulfonyl fluoride (PMSF). The protein concentration of each lysate was determined using the BCA (bicinchoninic acid) Protein Assay (Pierce). Approximately 50 μg of each lysate was fractionated on an SDS-PAGE gel. The protein was transferred to an Immobilon-P PVDF membrane (Millipore). The membrane was blocked with 5 % milk in TBST for 1 hour at room temperature and then incubated with primary antibodies [anti-Estrogen receptor (ER) (Santa Cruz Technology) and β-Actin (Cell signaling)] and then HRP-conjugated secondary antibody. The protein bands were visualized using the ECL Western blotting system (GE Healthcare).

3.4 Results

3.4.1 Generation of the Slingshot PB system and Slingshot ES cell lines

The Slingshot PB system was constructed as shown in **Figure 3-2**. To test whether the Slingshot PB cassette could introduce tamoxifen inducible transposition activity, 40 μg of Slingshot PB plasmid was transfected into ES cells. After transfection, half of the cells were plated on 10 cm dishes with 4-OHT treatment and half were plated without treatment. Two days later medium was changed to G418 selection at 175 μg/ml for another 10 days. If transposons re-integrated into an endogenous promoter (**Figure 3-2 C**), the β-geo gene in the transposon will be expressed to provide G418 resistance to the cell. After selection around 1000 G418 resistant colonies were generated on the 4-OHT treated plate while the non-treated plate had very few colonies formed (**Figure 3-3**). A similar result was obtained by

electroporating the PB transposon (neomycin + CAGGS version) and mPB-L3-ERt2 inducible transposase plasmids separately into cells (20 μg each), indicating that the Slingshot PB plasmid alone could generate comparable inducible transposition activity by combining the PB transposon and transposase on one cassette.

Since the PB transposon and transposase are located on one cassette in the Slingshot plasmid, in theory this design could increase the jumping efficiency when compared to a method which delivers the transposon and transposase via separate plasmids. However, the efficiency of the slingshot system could also be compromised by the large size of the plasmid itself (over 15 kb in length). To further improve the efficiency of the Slingshot system, I investigated the performance of Slingshot in cells with a stable integration (**Figure 3-2 B**). The Slingshot plasmids were electroporated into ES cells and selected with BSD (15 μg/ml) to generate stable integrated cell lines (see Materials and Methods). 53±5 (mean ± SD) BSD resistant colonies were formed from three independent electroporations. These colonies should all contain at least one stable Slingshot PB integration to enable the CAGGS promoter in the transposon region to drive the expression of the BSD selection marker in the cassette and provide BSD resistance. In total 18 colonies were picked and derived into cell lines for further characterisation.

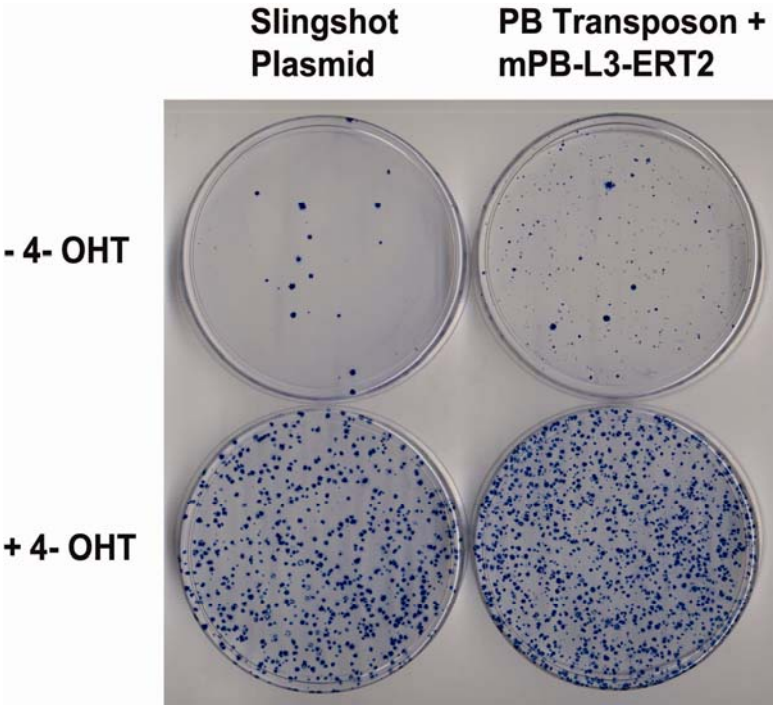


Figure 3-3. Functional test for the Slingshot PB plasmid

To test the activity of the Slingshot transposon system, the Slingshot plasmid (40 μg) was transfected into 10^7 mouse ES cells followed by 4-OHT treatment to activate transposition. After selection with G418 colonies were formed in the plate due to mobilization and re-integration of the PB transposon. As a positive control for this experiment, ES cells were transfected with two separate plasmids containing the PB transposon (neomycin + CAGGS version) and mPB-L3-ERt2 transposase (20 μg each).

3.4.2 Evaluating the jumping efficiency of the Slingshot transposon from a stable donor

To test the transposition activity from the 18 Slingshot ES cell lines showing stable integration of the Slingshot transposon system, each cell line was amplified and plated on a six-well plate and treated with 4-OHT to activate transposition events or vehicle control (95 % EtOH). After G418 selection, colonies were counted and colony numbers from 4-OHT treated and non-treated wells were compared. The cell lines with large numbers of colonies following 4-OHT treated but few colonies in non-treated wells represent cell lines with a high jumping activity that is tightly controlled for tamoxifen inducibility. To simplify the quantification, we defined a ratio of 2 (colony number in 4-OHT well/ colony number in non-treated well) as the threshold for a cell line having transposition activity.

As shown in **Figure 3-4 A**, 10 cell lines out of 18 showed jumping activity after 4-OHT treatment (colony number in 4-OHT well/ colony number in non-treated well > 2). Some cell lines were BSD resistant but showed no obvious jumping activity, which might suggest that the Slingshot PB array has been damaged during integration or that the plasmid has integrated into a site in the genome where expression from the CAGGS promoter is not permissive. Several cell lines formed colonies in 4-OHT treated wells (clones 3, 12, 18), but also formed colonies in vehicle treated wells suggesting that Slingshot has integrated into a site where β-geo is promiscuously expressed therefore resulting in G418 resistance. We selected clone 1 (PB/PB-1) for further analysis because it showed low background and high inducibility which was maintained over 12 passages (**Figure 3-4 B**). The transposition activity in PB/PB-1 clones after 4-OHT treatment and G418 selection was demonstrated by excision PCR; DNA were extracted from eight randomly picked colonies and PCR was performed to prove that transposon has been excised from the original donor site (**Figure 3-4 C**). The Slingshot plasmid integration sites for this clone were characterised by Southern Blotting and Splinkerette PCR; only one donor site was identified which was subsequently mapped to chromosome 18 (See **Figure 3-4 D**, red arrow). To assess the transposon re-integration we picked 48 G418 resistant PB/PB-1 clones and amplified their transposon insertion sites using splinkerette PCR. *iMapper* (104) was used to generate a *KaryoView* picture of the insertion sites and showed that there does not appear to be any local hopping from the original donor site on chromosome 18 for this clone, and that insertions appeared to be widely distributed throughout the genome (**Figure 3-4 D**).

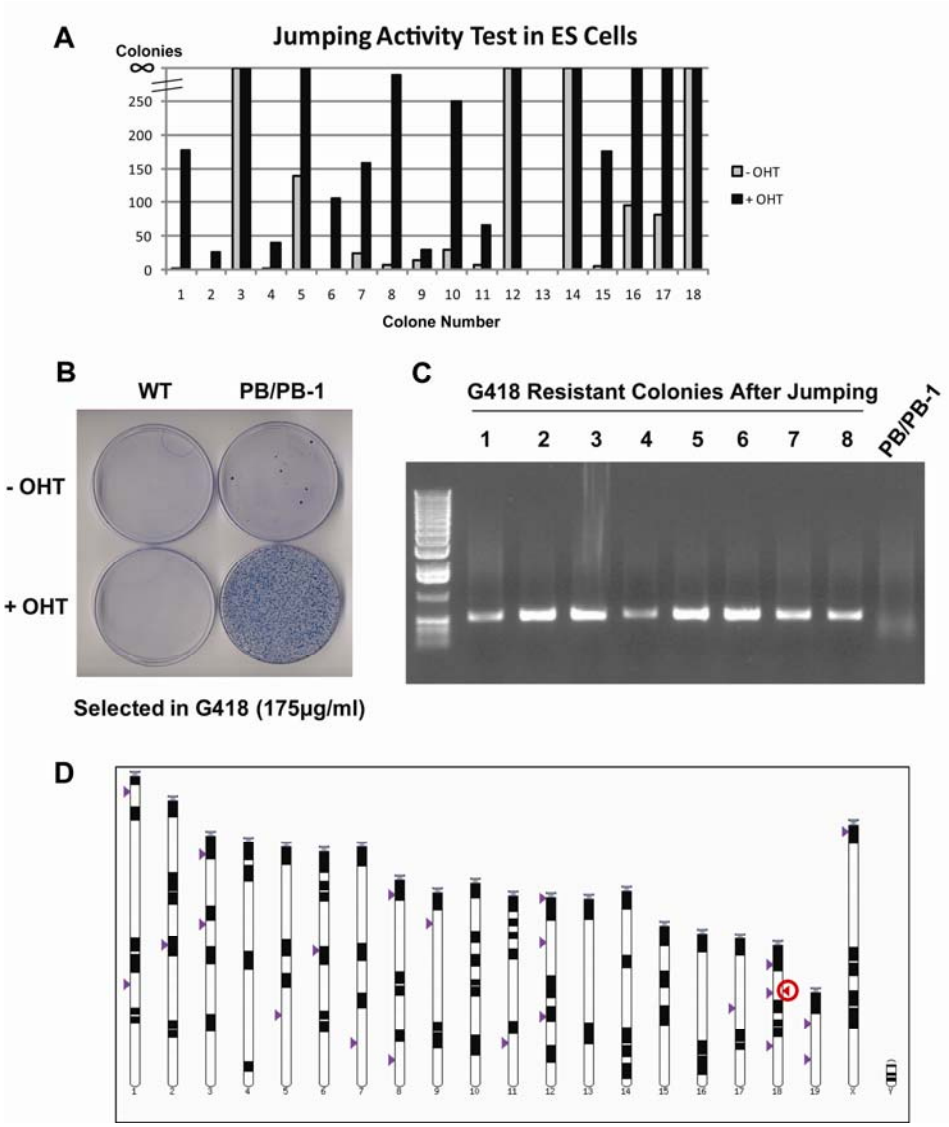


Figure 3-4. Testing of transposition activity and re-integration sites in Slingshot PB integrated ES cell lines

(A) The transposition activity in 18 ES cells lines showing stable integration of Slingshot PB was tested. The x-axis shows the individual ES colony number and the y-axis shows the colony counts for 4-OHT treated and non-treated cells cultured in six-well plates after G418 selection. (B) Colony one (PB/PB-1) ES cells treated with 4-OHT had high transposition activity and formed G418 resistant colonies in 10 cm culture dishes. (C) Excision PCR of genomic DNA for 8 PB/PB-1 G418 resistant colonies after 4-OHT treatment resulted in a band of 526 bp. The last lane is the PB/PB-1 control DNA without 4-OHT treatment. (D) *KaryoView* picture of the insertion sites isolated from 48 PB/PB-1 G418 resistant colonies generated by *iMapper*. The red arrow indicates the original Slingshot transposon donor site.

To further characterise the transposition efficiency from the Slingshot donor we performed a time series of mobilisation tests to quantify jumping efficiency with 4-OHT treatment (**Table 3-1**). From these tests we obtained mobilisation efficiencies ranging from 0.2-0.4 %, with longer 4-OHT treatment resulting in slightly higher jumping efficiency. This means that a confluent 10 cm dish with 10^7 ES cells would theoretically be sufficient for whole genome coverage (about 3×10^4 insertions). Although the Slingshot donor uses the mPB-L3-ERt2 transposase which has lower activity compared with the wild type version, Slingshot transposition is considerably higher when compared with other studies using plasmid based delivery methods (76,109,122). It is worth noting in these experiments that we are equating trapping efficiency with mobilisation efficiency. It is likely that the mobilisation efficiency of Slingshot is orders of magnitude higher than the systems' trapping efficiency.

Table 3-1. Evaluation of PB/PB-1 transposition efficiency using a time Series of 4-OHT treatment

4-OHT treatment	Cell number plated	Colony number - G418 plate *	Colony number + G418 plate **	Jumping Efficiency (Trapping) ***
1-Day	1 X 10^4	~ 3000	5 ± 2	> 0.17 %
2-Day	1 X 10^4	~ 3000	7 ± 2	> 0.23 %
4-Day	1 X 10^4	~ 3000	11 ± 2	> 0.37 %

* The colony numbers in non-G418 treated plates were estimated

** The colony numbers in G418 treated plates were calculated by mean ± SD in three parallel plates

*** The jumping efficiency is represented as trapping efficiency therefore the actual transposon jumping efficiency should be much higher

3.4.3 Drug resistance screens using the Slingshot system

The transposon used in the Slingshot system contains a CAGGS promoter and splicing acceptor- polyA 'trapping' element to generate both gain and loss of function transposition events. The trapping experiments described above essentially validate the loss-of-function elements of the transposon. Here the gain of function capabilities of Slingshot were tested by performing drug resistance screens *in vitro* using two agents, the aminonucleoside antibiotic puromycin and vincristine, an antimicrotubule spindle poison.

3.4.3.1 Puromycin resistance screen

For the puromycin screen, 18 and 26 colonies from two independent experiments (using 8 and 20 plates each) were derived after puromycin selection (**Figure 3-5 A and B**). Insertion sites were isolated from these clones by splinkerette PCR (**Figure 3-5 C**) and mapped to the genome with *iMapper*. From a total of 44 colonies, the majority of the insertion sites mapped independently to two genomic loci . Twenty-one of the insertions mapped to chromosome 5 between 8.5 - 8.8 MB (**Figure 3-5 D**), where genes encoding the ABC Transporters *Abcb1a* and *Abcb1b* are localised. Sixteen of these insertion sites were located upstream of the gene *Abcb1a,* ranging from 0.2 Mb away from the first exon to the first intron. Five were located upstream of *Abcb1b* before the first exon. The transposons all inserted in a sense orientation at this locus and the CAGGS promoters were all facing the gene orientation. Since the genes *Abcb1a* and *Abcb1b* are paralogous, having evolved as a result of a gene duplication, their drug transporter activity is essentially identical. The other locus that had multiple insertion sites was located on chromosome 6 between 58.54 - 58.59 MB (**Figure 3-5 E**), where another five transposon insertion sites were located. These transposition events are predicted to drive overexpression of *Abcg2*. *Abcb1a/b* and *Abcg2* are ABC drug transporters and these results indicate that that puromycin is a substrate for both of these drug efflux pumps (125). Six clones derived from control plates were also analyzed by splinkerette PCR and no insertion sites could be identified.

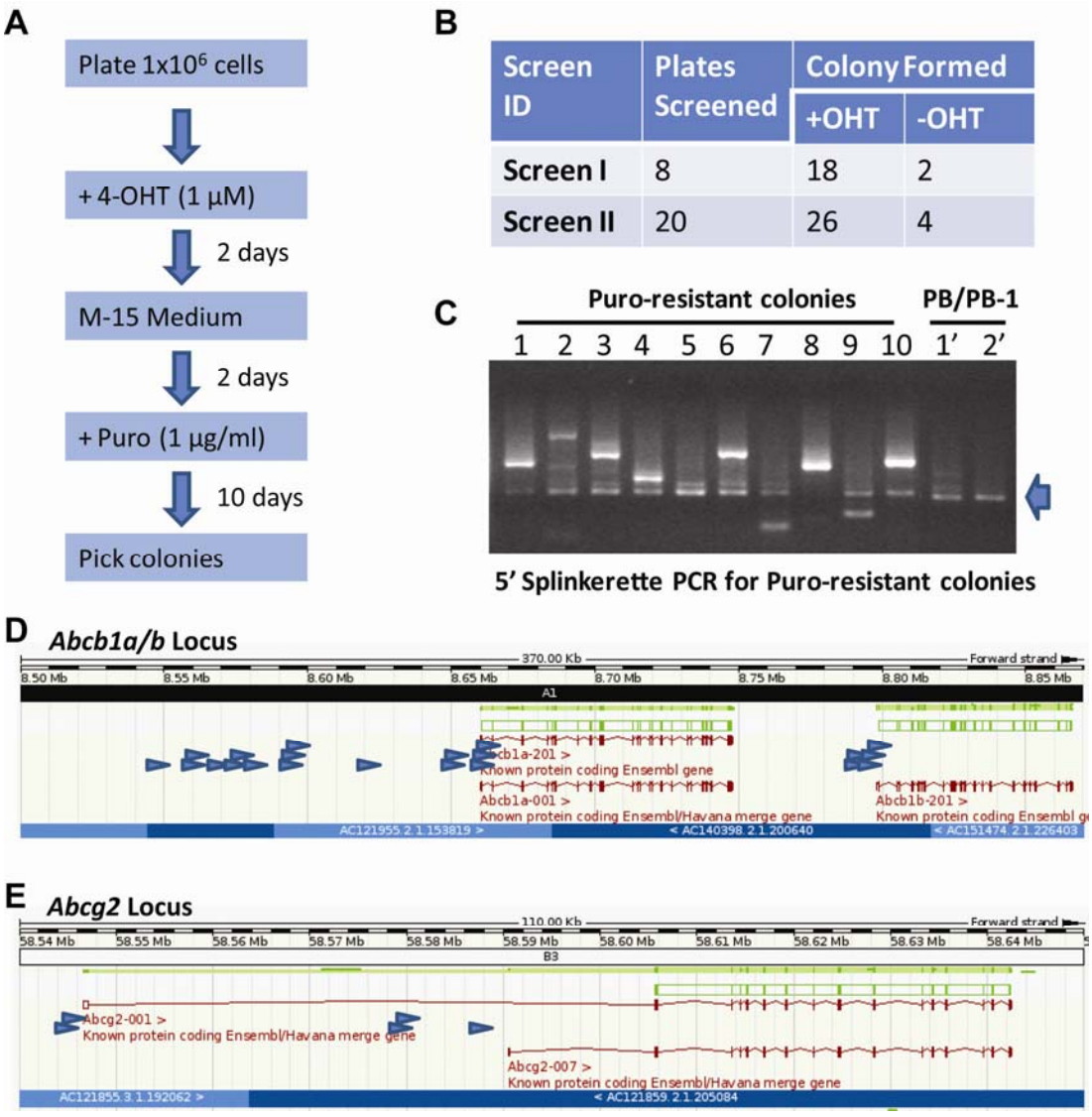
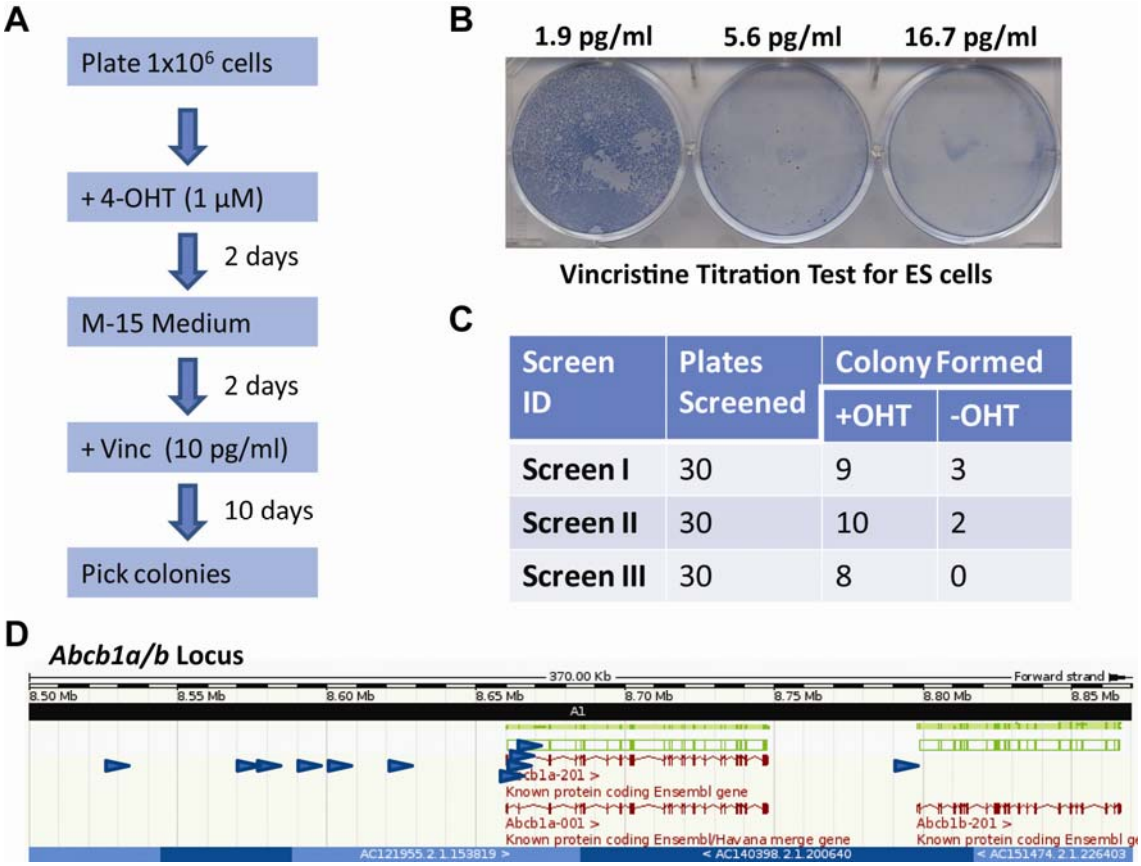


Figure 3-5. Drug resistance screen in Slingshot PB cell line PB/PB-1 using puromycin

(A) Flow chat showing overview of the drug resistance screen in PB/PB-1 using puromycin. (B) Information and results of two independent experiments. (C) Splinkerette PCR gel picture using 5 prime PCR primers as shown in Appendix A. Colonies 1-8 are puromycin resistant colonies isolated from the screen. Lane 1' and 2' are control DNA from PB/PB-1. The blue arrow indicates a background PCR band cloned from the Slingshot plasmid sequence. (D) Twenty-one independent insertion sites (indicated in blue arrowhead) were mapped to *Abcb1a/b* Locus on chromosome 5 between 8.5-8.8 MB. (E) Five independent insertion sites (indicated by blue arrowhead) were mapped to the *Abcg2* Locus on chromosome 6 between 58.54 - 58.59 MB. The orientation of the arrows indicates the CAGGS promoter orientation after transposon integration.

3.4.3.2 Vincristine resistance screen

For the vincristine resistance screen using a titration test was first performed to establish the optimal concentration of drug required to kill all cells (**Figure 3-6 B**). From this test we determined that a drug concentration of 10 pg/ml is sufficient to kill all the ES cells seeded at a density of 10^7 cells in 10 cm plates within 4 days and this concentration was used in subsequent experiments. In total three independent experiments were carried out using 30 plates each time and in total 27 colonies were obtained (**Figure 3-6 C**). The insertion sites were cloned by splinkerette PCR and 11 independent insertions were mapped to the *Abcb1a/b* locus on chromosome 5 between 8.5 - 8.8 MB (**Figure 3-6 D**). Therefore this screen has independently identified *Abcb1a/b* as a gene for anti-vincristine resistance. In both the puromycin screen and also in the vincristine screen insertions from several clones could not be mapped. This was generally due to the short sequence length of the splinkerette product or repetitive sequences that could not be mapped.



Screen ID	Plates Screened	Colony Formed	
		+OHT	-OHT
Screen I	30	9	3
Screen II	30	10	2
Screen III	30	8	0

Figure 3-6. Screen for vincristine resistance in the Slingshot PB cell line PB/PB-1

(A) Overview of the screen. (B) Titration test to determine the optimal vincristine concentration in ES cells. (C) Results of three independent screening experiments. (D) 11 independent insertion sites (indicated by blue arrowhead) were mapped to the *Abcb1a/b* Locus on chromosome 5 between 8.5-8.8 MB. The orientation of the arrows indicates the CAGGS promoter orientation after transposon integration.

3.4.4 Self-inactivation of the transposon after transposition

Transposon remobilisation caused by constitutive expression of a transposase is a major problem in insertional mutagenesis studies. In mouse tumour studies the *Sleeping Beauty* transposon may 'jump' multiple times before landing in the identified insertion sites. At each site of integration, *Sleeping Beauty* leaves a TA footprint which could cause a frame shift in the coding region. *piggyBac* has an advantage over *Sleeping Beauty* in that it is faithfully excised and leaves no footprint in the donor site. However remobilisation of *piggyBac* during transposition could still generate a complex insertion site profile and cause difficulty in isolating the common insertion sites.

During transposition the Slingshot transposon and its CAGGS promoter sequence are excised from the original donor site and re-integrate elsewhere in the genome. In theory this translocates the CAGGS promoter away from the transposase shutting down further expression to prevent re-mobilization of the transposon. To prove this theory I used puromycin resistant colonies generated using PB/PB-1 that had integrated upstream of *Abcb1a* but were G418 sensitive (puromycin resistant clones 1, 8 and 14). The presence of the transposon in these three clones was reconfirmed by splinkerette PCR and further verified by genomic PCR (data not shown). These cells were treated with 4-OHT and then selected in G418 to identify re-mobilization events, however none were detected. These data illustrate that transposase activity is completely shut down following mobilisation of the Slingshot transposon (**Figure 3-7 A**). To prove that re-mobilisation from the *Abcb1a* locus is possible, puromycin resistant clones 1, 8 and14 were transfected with the mPB-L3-ERt2 plasmid by electroporation, cells were treated with 4-OHT for 2 days, and then selected in G418. All three cell lines treated in this way generated hundreds of colonies per plate (**Figure 3-7 A**), indicating that the lack of transposition in these puromycin resistant clones was due to lack of PB transposase expression in these cells. Eight colonies were picked from each of the three puromycin resistant cultures and mobilisation of the Slingshot transposon from the *Abcb1a* locus was confirmed by excision PCR (data not shown). Finally, while PB-ERt2 expression was easily detected in the PB/PB-1 clone using an anti-ER antibody (see Materials and Methods), PB-ERt2 transposase expression was absent in whole cell lysates from puromycin resistant clones 1, 8 and 14 further confirming that transposition from the Slingshot donor represses further transposase expression (**Figure 3-7 B**).

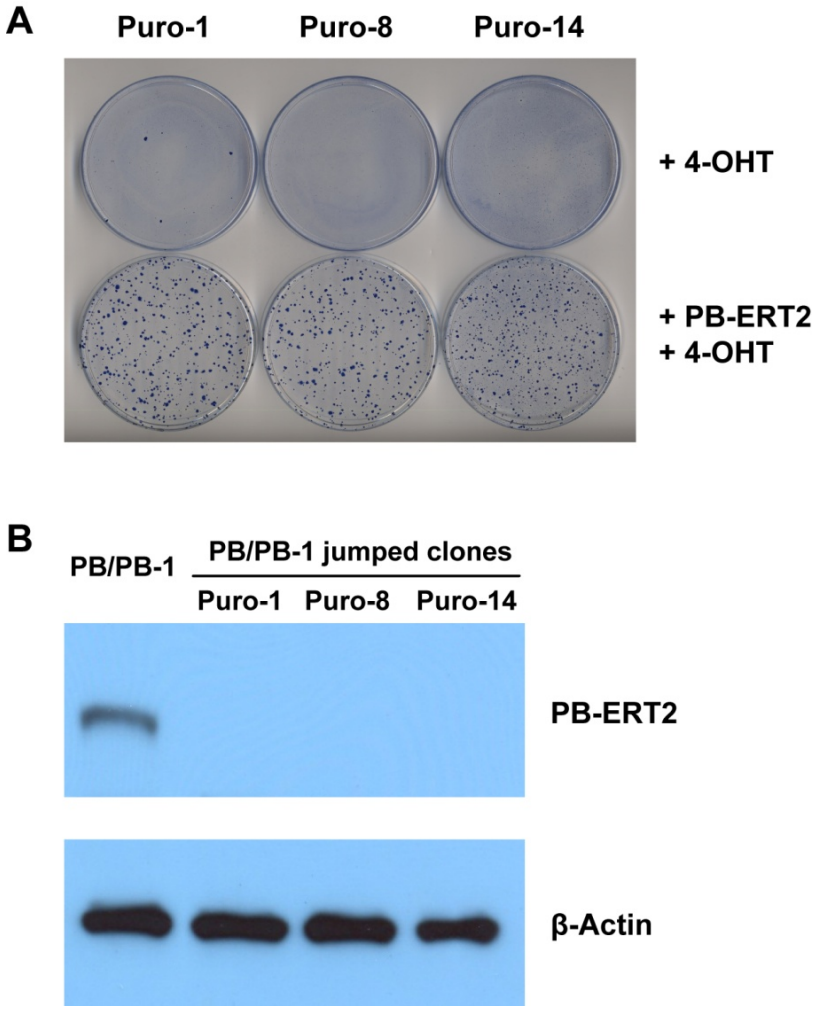


Figure 3-7. Slingshot is a self-inactivating transposon system

(A) Colony forming assay to test remobilization in three puromycin resistant clones, Puro-1, -8, -14. These three clones are derived from PB/PB-1 and all contain a PB transposon copy inserted upstream of the ABC transporter gene *Abcb1a* but are G418 sensitive. While there was no transposition after 4-OHT treatment of Puro-1, -8, -14 (top plates) transfection of a PB-ERt2 plasmid into these cell lines followed by 4-OHT treatment reveals that the PB transposon can be remobilised from the *Abcb1a* locus (bottom plates) (B) Western Blot for PB-ERt2 transposase expression using an anti-ER antibody. Expression of PB-ERt2 is readily detectable in the PB/PB-1 control but completely absent from Puro-1, -8, -14.

3.4.5 The Slingshot transposon system is active in somatic cell lines

To expand the application of the Slingshot system to somatic cell lines, in which many cell culture screening systems have been established, the Slingshot donor plasmid was also introduced into three commonly used human experimental somatic cell lines to test their transposition activities: the human embryonic kidney cell line HEK293, and the ovarian carcinoma cell lines OVCAR-3 and PE01. All three cell lines were transfected with 40 μg of linearized Slingshot plasmid which was introduced into 10^7 cells by electroporation (300V, 800 μF) and stable integrants were selected with Blasticidin, treated with 4-OHT for two days and selected with G418 for 2-3 weeks.

All three human somatic cell lines showed considerable trapping activity after 4-OHT treatment (**Figure 3-8**): 6 colonies out of 19 for HEK293, 2 colonies out of 16 for OVCAR-3 and 3 colonies out of 11 for PE01 cells had obvious transposition activity (colony number in 4-OHT well/ colony number in non-treated well > 2). The excision of the transposon from the Slingshot cassette in these three cell lines was confirmed by excision PCR (**Figure 3-8 C, F, I**). The constitutive expression of the PB-ERt2 transposase in OVCAR-3 cell lines was detectable by Western blot even after 12 passages (**Figure 3-8 J**). These results indicate that the Slingshot transposon system could be an efficient tool for mutagenesis studies in these somatic cell lines.

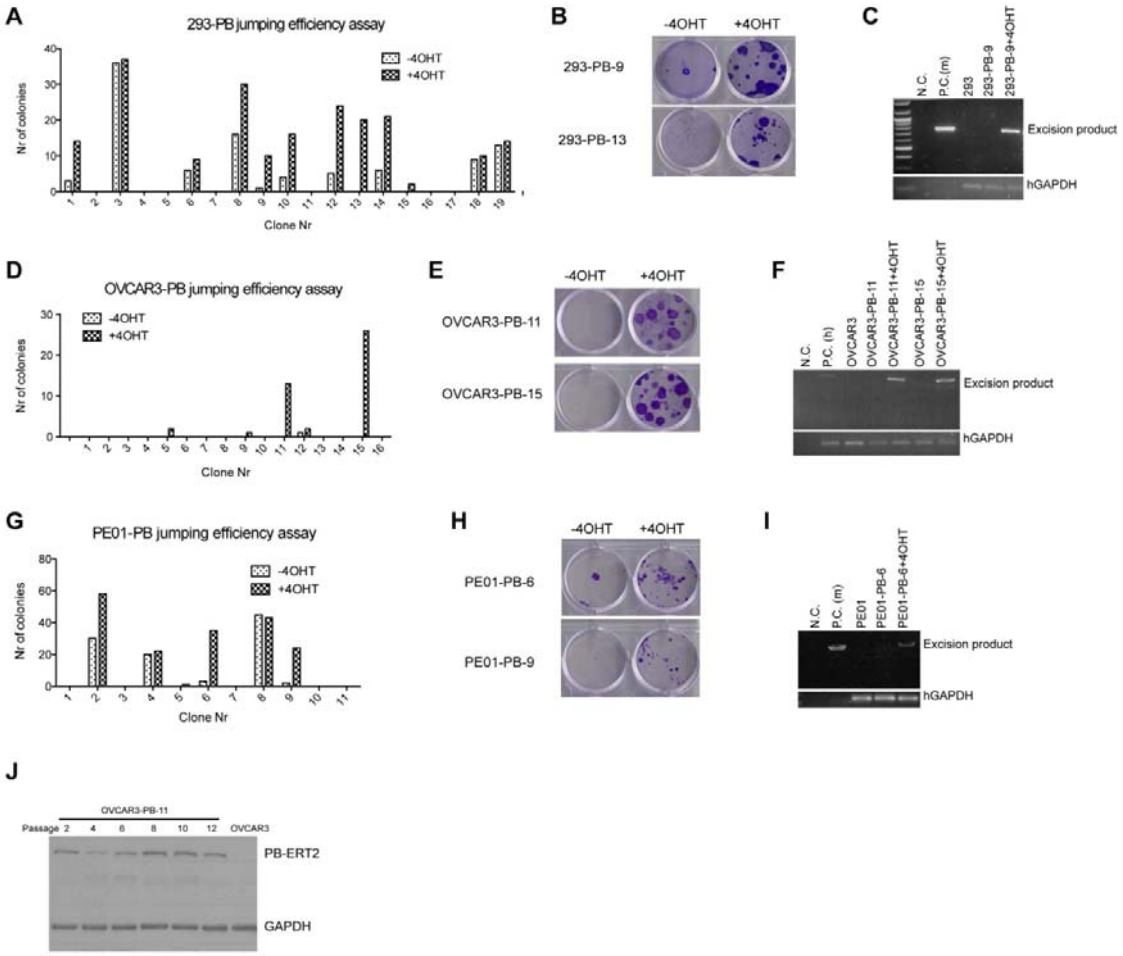


Figure 3-8. Slingshot is functional in three human somatic cell lines

HEK293, OVCAR3 and PE01 cells were stably transfected with the Slingshot plasmid and trapping efficiency assays and excision PCRs were performed. The Slingshot transposon was mobilised in HEK293 (**A, B, C**), OVCAR3 (**D, E, F**) and also in PE01 (**G, H, I**) following analysis of 19, 18 and 11 clones from these cell lines, respectively. Excision PCR was performed on DNA extracted from clones from each cell line pre- and post- 4-OHT treatments. N.C., negative control; P.C., positive control. (DNA from the Puro-1 cell line) Human GAPDH was used as a gDNA loading control. **(J)** Western blot analysis shows expression of the fusion protein PB-ERt2 in a highly active PB-carrying OVCAR3 clone following serial passage. The fusion protein was detected using an antibody recognising the ERt2 domain. Parental OVCAR3 cells were used as a negative control. GAPDH was used as a loading control.

3.4.6 Increasing the integration efficiency using chicken insulator sequence cHS4

In most transgenic experiments, the expression of integrated transgenic elements is subject to the influence of surrounding chromatin structure, a phenotype called chromosomal position effects (126). Chicken hypersensitive site 4 (cHS4), a well characterised insulator sequence was used to flank and protect the Slingshot PB cassette from chromosomal position effects (**Figure 3-9 A**). The cHS4 sequence is identified from the 5' element of the chicken β-globin domain and has been shown to improve the expression of integrating gene transfer vectors by reducing the position effects (127-129). To test the efficiency of cHS4 sequence, identical amounts of cHS4 flanked Slingshot or non-flanked Slingshot were electroporated into ES cells (40 μg DNA per electroporation) and selected with BSD (15 μg/ml). In three independent electroporations, 885 ± 48 (Means ± SD) colonies formed on plates seeded with ES cells transfected with cHS4 flanked Slingshot PB plasmid, a 17-fold increase when compared to the number of colonies formed on plates transfected with the non-flanked Slingshot (53 ± 5) (**Figure 3-9 B and C)**. To compare the transposition activity for the cHS4-flanked and non-flanked Slingshot clones, 24 clones were picked from each for mobilization activity test. 16 of non-flanked and 18 of cHS4 flanked Slingshot colonies showed obvious jumping activity after 4-OHT induction followed by G418 selection and there was no obvious increase in colony numbers for the cHS4 flanked Slingshot colonies. From the above results the chicken insulator sequence cHS4 significantly increased the colony number after electroporation, indicating that it could protect the Slingshot cassette from position effects while integrating into cells. Among cell lines with stable Slingshot integration, however, there seems little difference in transposition efficiency between colonies transfected with the cHS4-flanked and non-flanked Slingshot cassette. Of course, it is possible that the cHS4-flanked Slingshot could improve mobilisation efficiency in somatic cell lines, in which the transgenic elements are more likely to be silenced by position effects.

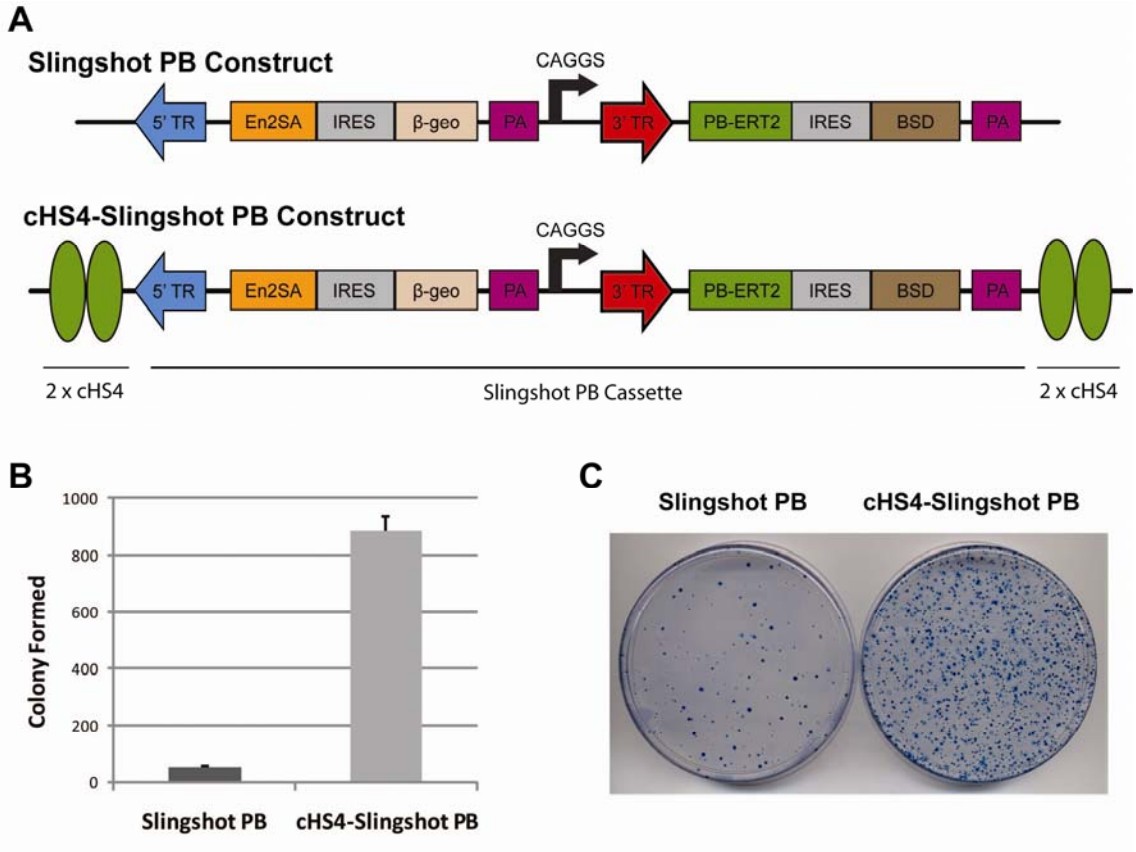


Figure 3-9. Improvement of the Slingshot PB donor colony formation efficiency using chicken insulator sequence cHS4.

(A) Slingshot PB and cHS4-Slingshot PB constructs. The cHS4-Slingshot PB is flanked with two cHS4 sequences. (B) 10^7 ES cells were electroporated at 230 V, 500 mF with Slingshot PB and cHS4-Slingshot PB constructs and the number of colonies counted following BSD selection (mean ± SD from three experiments) (C) Representative example of colony plates.

3.5 Discussion

The recent discovery of *piggyBac* (PB), a transposon derived from the cabbage looper moth *Trichoplusia ni*, which is active in mammalian cells has opened up new opportunities for insertional mutagenesis in mammalian (75). When compared to other insertional mutagens *piggyBac* has been shown to exhibit much higher rates of transposition, less local hopping and can carry large cargo sequences. In addition the *piggyBac* transposase can be fused to other sequences which has made it possible to control the transposition temporally (75,76,109,123). These advantages have made the PB system an ideal insertional mutagen for genetic screens.

I have developed a system called Slingshot, which can be used for self-inactivating insertional mutagenesis in mouse embryonic stem cells and also in a range of human somatic cell lines. The Slingshot PB cassette showed stable integration into host cell genomes and the transposition activity of each Slingshot donor cell line was easily evaluated using a colony formation assay. This 4-OHT inducible Slingshot PB system has many advantages over the PB system carried by donor and helper plasmids for cell culture applications. By stable integration into the host cell genome, the Slingshot PB cassette provides transposition activity in almost every cell, which significantly increased the transposition efficiency compared with plasmid-based transposition experiments. Because the transposon is mobilized from the integrated Slingshot PB cassette, the transposon copy number is determined by the actual copy of integrated Slingshot PB cassette per cell and this could be determined by splinkerette or Southern blot. In addition, since transposition is controlled by 4-OHT, transposition could be terminated by withdrawing 4-OHT to avoid multiple re-integration events. I have also shown that once the transposon has jumped out of the Slingshot cassette, transposase activity is shut down to prevent re-mobilisation.

To further characterise the transposition ability of the Slingshot PB system, a cell line with stable Slingshot PB integration (clone ‘PB/PB-1’) was used to characterise the jumping efficiency by colony formation assay. A single copy of the Slingshot PB cassette was identified by splinkerette PCR and mapped to chromosome 18. We identified the integration sites in 48 clones and found that the insertion sites were randomly distributed throughout the genome with no obvious local hopping. This is a particularly important feature of *piggyBac* that has previously been described and makes it ideal for PB to transpose from a defined genomic locus. The Slingshot PB cell line showed a trapping efficiency of around 0.3%.

Since the assay only detects trapping rather than mobilisation events, the actual jumping efficiency of the Slingshot system could be much higher. Nevertheless, this efficiency is much higher than that reported for studies using the plasmid transformation method and a PB trapping cassette (121,122), though it is slightly lower than the efficiency reported for mobilisation of PB from the HPRT locus, which measures the jumping efficiency rather than trapping efficiency (76). In addition, the Slingshot PB system showed promising activity in human somatic cell lines such as the human embryonic kidney HEK293 and human ovarian carcinoma cell lines OVCAR-3 and PE01. Although the actual jumping efficiency is difficult to quantify in these somatic cell lines as they can only form mono-layer colonies which are unfavourable for the colony formation assay, these experiments demonstrated that the stable integration of the Slingshot PB system is an ideal tool for high-efficient mutagenesis studies in ES cells as well as other somatic cell lines.

Here I have shown that Slingshot can be used to identify candidate genes in genome-wide mutagenesis studies by performing two screens to identify mediators of resistance to the compounds puromycin and vincristine. In the screen for puromycin resistance three ABC drug transporter genes *Abcb1a*, *Abcb1b* and *Abcg2* were hit multiple times by independent transposon insertions. *Abcb1a*, *Abcb1b* encode the same drug transporter protein ABCB1 and are located next to each other in the genome. *Abcg2* encodes the drug transporter protein ABCG2. The orientation of the CAGGS promoter in all of the insertions faced the gene orientation, indicating that the transposon activates these drug transporter genes via its CAG promoter sequence. In the screen for vincristine resistance, the drug transporter genes *Abcb1a*, *Abcb1b* were also hit multiple times suggesting that activation of these genes also results in vincristine resistance. We did not identify all of the genes that have previously been shown to result in vincristine resistance, such as the Multi-Drug Resistance Protein coding genes *MRP-1* and *MRP-2*. However, this may be because the pilot screen was not large enough or the *MPR* gene loci are not accessible for PB integration. Nevertheless, these screens have shown that the Slingshot PB system is an efficient tool for identifying activating mutations. In theory the Slingshot PB system could also be used to identify recessive mutations via the disruption of gene transcription. Since the Slingshot PB cell line we used for these drug resistance screens contained only a single copy of Slingshot, this cell line is more suited to the identification of dominant mutations.

We have found that the insertion sites generated by low copy numbers of transposons are easier to isolate and characterise by standard splinkerette protocols, while the insertions

generated by high copy numbers of transposons require more complicated, deep sequencing technology. Due to its large size, the Slingshot PB system is more likely to introduce a low copy number of integrations per cell. In fact, of all the cell lines we analysed, we did not identify any with more than one copy of Slingshot. Therefore the Slingshot PB system would be an ideal tool for the identification of phenotypes caused by single-cell events under defined cell culture conditions, such as the identification of drug resistant genes or cooperating gene mutations in a defined cellular background. More complicated cellular processes that are caused by cooperative genetic events, however, will only be identified using mutagenesis tools such as retroviruses or multi-copy transposon systems.

Although the identification of a candidate gene from insertional mutagenesis screens largely depends on the accessibility of the genomic locus and DNA modifications, methylation, acetylation status etc., the efficiency of the insertional system plays a very important role in mutagenesis screens. Therefore it is worthwhile investigating strategies that would further improve the transposition efficiency of the Slingshot system. The most obvious approach would be to reduce the size of transposon itself by, for example, removing the LacZ sequence in the transposon region, since mobilisation of the transposon is exponentially dependent on the size of the transposition molecule. Furthermore, it would also be possible to add a GFP tag to indicate any mobilisation events within a cell. This would enable the isolation and enrichment of cells by flow-assisted cell sorting. Last but not least, most of the Slingshot donor cell lines identified during these screens had only a single copy Slingshot integration. It is worth considering an alternative method of transfection such as Lipofectamine to increase copy numbers which could potentially improve the transposition efficiency several fold.

In summary, Slingshot is a stable, self-inactivating mutagenesis system that has several potential applications. Firstly, as I have shown here, Slingshot represents a useful tool for genome-wide screens. Secondly, since the transposon is mobilised from a stable chromosomal donor by adding tamoxifen to the culture medium, Slingshot can be used in heterogeneous populations of cells, in three dimensional culture systems, or where it is impractical to transiently transfect donor and helper plasmids into cells. Thirdly, since the transposon is mobilised only once following the administration of tamoxifen the Slingshot system can be used to *'barcode'* populations of cells making it possible to track the dynamics of growth of the population over time. When combined with high-throughput sequencing which could read the barcodes or insertion sites from a population of cells, it would be possible to use Slingshot in synthetic genetic screens such as those performed in yeast.