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Transposon-mediated mutagenesis of somatic cells in the mouse for cancer gene identification

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Abstract

To successfully treat cancer we will likely need a much more detailed understanding of the genes and pathways meaningfully altered in individual cancer cases. One method for achieving this goal is to derive cancers in model organisms using unbiased forward genetic screens that allow cancer gene candidate discovery. We have developed a method using a “cut-and-paste” DNA transposon system called Sleeping Beauty (SB) to perform forward genetic screens for cancer genes in mice. Although the approach is conceptually similar to the use of replication competent retroviruses for cancer gene identification, the SB system promises to allow such screens in tissues previously not amenable to forward genetic screens such as the gastrointestinal tract, brain, and liver. This article describes the strains useful for SB-based screens for cancer genes in mice and how they are deployed in an experiment.

Keywords

Sleeping Beauty; Transposon; Cancer genetics; Mouse transgenesis; Somatic mutagenesis

1. Introduction

Sleeping Beauty (SB) is a member of the Tc1/Mariner family of DNA transposable elements that transpose in a “cut-and-paste” manner and integrate at “TA” dinucleotides. SB was originally identified as a long-dormant transposable element and its relevant transposase in the genomes of salmonid fish. Directed mutagenesis was used to correct mutations that silenced the activity of the transposase [1]. SB is a two-component system, composed of the transposase transgene and a transposon vector, flanked by special inverted terminal repeats or IRs. When the transposon vector is present in a cell expressing the transposase transgene, the “cut-and-paste” transposition reaction occurs. The SB transposase enzyme recognizes specific binding sites within the IRs excises and then integrates the transposon elsewhere at a TA dinucleotide. The excision site is repaired by the host cell machinery leaving behind a 3 bp footprint, either CAG or CTG [1]. The transposition reaction can occur from a transfected plasmid to another plasmid or to a chromosome in a transfected cell [2]. Similarly, an SB transposon vector can jump from one place on a chromosome to another in transgenic mouse germline and somatic cells [3–5]. SB has been shown to be transpositionally active in zebrafish, vertebrate cell culture, the mouse germline and in mouse somatic tissues when delivered on plasmid DNA [1,3,6–14].

Based on our studies using SB transposons for germline mutagenesis [15,16], we have modified SB for use as a somatic insertional mutagen to identify cancer genes in multiple types of cancer including sarcoma, leukemia, gastrointestinal tract tumors, and hepatocellular carcinoma [4, 5,17,18]. We hypothesized that an SB transposon could be used to “tag” cancer genes in mouse somatic cells. We created a transposon, T2/Onc, which contains splice acceptor (SA) signals in both orientations (Fig. 1). Upon intronic insertion, these elements are designed to intercept upstream splice donors and elicit premature transcript truncation by virtue of a bidirectional polyadenylation (PA) signal also included in T2/Onc and lying between the two splice acceptors. Also between the two SA's are sequences from the 5' long terminal repeat (LTR) of the murine stem cell virus (MSCV), which contain strong promoter and enhancer elements that are methylation-resistant and active in stem cells [19–21]. Immediately downstream of the LTR is a splice donor (SD) for splicing of a transcript initiated from the LTR into a neighboring gene. The T2/Onc transposon is thus specialized to identify both tumor suppressors and oncogenes. However, in the case of tumor suppressor genes, typically both alleles must be inactivated for cancer to occur. So, we expected that T2/Onc mobilization could identify tumor suppressor genes if they showed haplo-insufficiency for tumor suppression, if both alleles were inactivated by two independent insertions, or if the second allele were inactivated by another means after the first allele was inactivated by T2/ onc insertion. Indeed, we have shown that bone fide tumor suppressor genes are recovered at recurrent sites of transposon insertion in tumors induced by SB transposition [17].

In published work, we have shown that SB transposase-induced mobilization of T2/Onc in somatic cells of *p19Arf*^{-/-} mice accelerated sarcoma development and, in wild type mice, could induce T cell leukemia and medulloblastoma [4,5]. In both the accelerated sarcomas and the T cell leukemia T2/Onc insertions were found to cluster near or within specific genes in multiple independent tumors. These clusters are called common insertion sites (CIS) and reflect *selection* for these rare events among the many, many random somatic insertion mutations that are occurring in SB mice. Some CISs were near or within previously known or suspected tumor suppressors and oncogenes.

We have now made modifications to the system that improve the utility of SB for cancer genetics studies in a wide variety of tissue types [17,18]. We refer readers to several reviews that discuss how the system could be modified to be most useful [22–24]. A modification that has been especially useful involves creating a transgenic line that expresses the catalytically improved SB transposase, SB11 [25], from the endogenous *Rosa26* locus, but only after the Cre recombinase has excised a stop cassette separating the SB11 cDNA from the ROSA26 promoter (designated RosaSB^{LSL} or R26-*lsl*-SB11). Using this tool we can restrict SB mutagenesis to just tissues expressing the Cre recombinase and avoid the highly penetrant, rapid lymphomagenesis that would otherwise occur if the transposase is expressed ubiquitously [4]. We have verified that the RosaSB^{LSL} transgene responds to the Cre recombinase *in vivo* in mice, resulting in tissue-specific expression of SB11 via IHC, excision of T2/Onc vectors from a donor concatemers with reinsertion elsewhere, and tumor development in that tissue. We used a hepatocyte-specific *albumin*-Cre (*Alb*-Cre) transgene to activate transposase expression, specifically in the liver, from the RosaSB^{LSL} transgene. As mutations in *p53* are one of the most common mutations in human HCC, a conditional dominant negative *p53* transgene, the *LSL-p53*^{R270H} allele [26], was included. When Cre is present, the dominant negative *p53*^{R270H} protein is produced. Triple transgenic (RosaSB^{LSL}; T2Onc; *Alb*-Cre) and quadruple transgenic (RosaSB^{LSL}; T2Onc; *Alb*-Cre; *LSL-p53*^{R270H}) mice and controls were generated. Multiple hepatic adenomas, some of which progressed to HCC with metastases, occurred in both the triple and quadruple transgenic mice but, were more frequent and were accelerated in mice that also carried the *LSL-p53*^{R270H} allele. HCC genes already implicated in the development of this disease as well as novel candidates were identified at CIS [18]. We also published a similar study in which SB transposition was restricted to epithelial cells of the

gastrointestinal (GI) tract using the Villin-Cre transgene [17]. In this study, triple transgenic mice carried the Villin-Cre; T2/Onc; RosaSB^{LSL} transgenes. The great majority of 47 experimental triple transgenic mice developed GI tumors by 10 months of age. Analysis of over 16,000 non-redundant insertions from 135 GI tumors identified 77 CIS candidate GI cancer genes. The *Adenomatous polyposis coli (Apc)* gene, a tumor suppressor and member of the Wnt/ β -catenin signaling pathway, whose loss is rate-limiting in ~80% of human CRC, was by far the most common site of SB-mediated insertional mutagenesis in this screen. Other major known GI tumor suppressor genes on the list of 77 CIS candidate genes were *Smad4* and *Pten*. Notably, the list of top 10 genes by insertional frequency contained many novel GI cancer genes such as *Rspo2*, *Kcnq1*, *Wac*, *Tcf12*, *Cugbp1*, *Ptpkr*, *Nr6a1*, and *Zcchc7*. Sixty of the 77 CIS GI cancer candidate genes have been reported to be dysregulated in one or more human cancers, either mutant at the sequence level or aberrantly expressed [17]. However, only a minor subset of the CIS-associated genes, ~10%, were well known GI tract cancer genes from mouse or human. A similar frequency of known cancer genes can be expected from typical SB screens at present.

In this article we describe methods to perform a cross to generate experimental class mice in which SB-initiated or accelerated cancer could occur. Specific concerns regarding this approach are discussed. One important issue is to verify the expression of the SB transposase in appropriate (and enough) cells of the correct tissue. Therefore, also included is a protocol for detection of SB transposase using immunohistochemistry on mouse tissues.

2. Materials

2.1. PCRs

1. Platinum taq supplied with 10× PCR buffer and 50 mM MgCl₂ (Invitrogen, Carlsbad, CA).
2. dNTPs—2.5 μ M each for a total concentration of 10 μ M in PCR grade water. Aliquot and store at –20°.
3. PCR primers should be dissolved in Millipore purified or PCR grade water at 25 μ M.
4. Agarose.
5. Ethidium bromide.
6. 50× TAE is 242.2 g Tris base, 100 ml .5 M EDTA pH 8, 57.1 ml glacial acetic acid (caution—hazardous, use in fume hood) brought up to 1 l with Millipore water.
7. 100 bp DNA ladder.

2.2. Immunohistochemistry for SB transposase protein

1. Citrosolv (Fisher Scientific, Fairlawn, NJ).
2. 70%, 80%, 95% and 100% Ethanol.
3. Phosphate buffered saline (pH 7.5).
4. VectaStain Elite ABC reagent (Vector Laboratories, Burlingame, CA).
5. Permount (Fisher Scientific, Fairlawn, NJ).
6. Diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA).
7. 3% Hydrogen peroxide in water.
8. Phoenix blue nuclear stain (Thermo Scientific, Waltham, MA).

9. M.O.M. Mouse Ig Blocking Reagent (Vector Laboratories, Burlingame, CA).
10. Acid alcohol (1% HCl in 70% ethanol = 1 ml concentrated HCl (12 M) in 100 ml 70% ethanol).
11. ImmunoEdge Pen (Vector Laboratories, Burlingame, CA).
12. Ammonia water (1% ammonium hydroxide in water (1 g ammonium hydroxide in 100 ml H₂O)).
13. Unmasking solution (Vector Laboratories, Burlingame, CA).

3. Description of method

3.1. Overview

The basic method is to create mice that express the SB transposase and harbor a multi-copy concatemer of the T2/Onc. There are several choices for the source of the transposase and the T2/Onc transgenes. Two SB transposase transgenic lines are described in the literature: A CAG-SB10 transgenic line produced by pronuclear injection [15] and a Rosa26-SB11 “knock-in” line produced by gene targeting in ES cells [4]. Although designed to be ubiquitous, the CAG-SB10 line expresses transposase best in the male germline and in mesenchymal or connective tissue cells and is mosaic in most tissues (unpublished observations). The Rosa26-SB11 line expresses ubiquitously, but when combined with T2/Onc lines produces leukemia within 4–6 months in nearly all recipients ([4]; and unpublished observations). Therefore, in most applications it is desirable to use some method for tissue-specific expression of SB transposase. This can be done using a tissue-specific promoter to drive transposase or a Cre/loxP regulated *Rosa26* “knock-in” allele, RosaSB^{LSL}, as described above (unpublished observations). We have had good success inducing tumors with the second method, using the conditional RosaSB^{LSL} allele combined with a tissue-specific Cre transgenic line and a T2/Onc transgenic line (unpublished observations). Many tissue-specific Cre transgenic lines are available now [<http://www.mshri.on.ca/nagy/Cre-pub.html>]. Several T2/Onc transgenic mice have been described in the literature on various donor chromosomes and of varying copy numbers [4,5]. It is important to use a multi-copy concatemer line for T2/Onc since presumably multiple independent insertion mutations must accumulate in the same clone of cells for a tumor to develop. Although the lower limit of copies present in a concatemer for efficient tumorigenesis is not known at present, good results are obtained with concatemers of ~25 copies [17,18]. In practice, we simply generate multiple T2/Onc transgenic lines by standard pro-nuclear injection and screen for lines with a high enough copy number by Southern blotting since copy number cannot be controlled in this process. Custom donor concatemers in specific regions of the genome could conceivably be created by homologous recombination in mouse embryonic cells. In this context, it is important also to note that SB displays a “local hopping” tendency similar to other cut-and-paste transposons [15]. The local hopping trend means a mobile SB transposon is more likely to integrate into a TA site physically linked to its donor site. Any bias due to local hopping can be overcome by performing mutagenesis screens twice, using at least two transposon donor concatemers that map to different chromosomes, an approach we routinely take and recommended for all screens. By doing this, one can discard all same-chromosome T2/Onc insertions from the results prior to the analysis to determine the CIS. Any CIS on the same chromosome as one of the T2/Onc donor lines would have to be defined only by insertions having originated from transposition from another chromosome. It is possible that by placing the T2/Onc concatemer in a specific region of the genome thought to harbor cancer genes they could be discovered more easily than by transposition from a donor on another chromosome.

3.2. Maintenance of transgenic lines

It is important to maintain the RosaSB^{LSL} mice separately from the Cre transgenic lines, since the Cre transgene may cause inadvertent activation of the SB11 transposase transgene in the germline. The T2/Onc transgenic lines can be maintained separately or together with the RosaSB^{LSL} transgene. Following is the PCR genotyping protocol the T2/Onc transposon lines that we use to generate experimental class mice.

3.2.1. PCR genotyping for T2/Onc

1. Isolate tail biopsy DNA using standard methods.
2. It is advisable to prepare DNA samples using a phenol:chloroform extraction method and diluted to a final concentration of 100 ng/μl in TE.
3. In practice we have used CLP Taq polymerase from CLP, San Diego, CA.
4. CLP Taq PCR Setup (T2/Onc).

a.	10× CLP buffer	5µl
b.	50 mM MgCl ₂	3 µl
c.	25 mM dNTP	0.4 µl
d.	10 µM sense primer	1 µl
e.	10 µM anti-sense primer	1 µl
f.	Double-distilled water	28.35 µl
g.	5× Sucrose red dye	10 µl
h.	CLP Taq (5U/µl)	0.25 µl
i.	DNA sample (100 ng/µl)	1 µl
		50 µl

5. PCR primer sets

a. T2/Onc sense: CGCTTCTCGCTTCTGTTGC.

b. T2/Onc anti-sense: CCACCCCCAGCATTCTAGTT.

6. PCR reaction conditions

a.	94 °C	5 min
b.	94 °C	1 min
c.	55 °C	1 min (×35 cycles of b–d)
d.	72 °C	1 min
e.	72 °C	7 min
f.	10 °C	Hold

7. Agarose gel electrophoresis of PCR products

- a. Pour a 1–1.5% Agarose gel containing 6 µl ethidium bromide (10 mg/ml) per 100 ml of TAE buffer.
- b. T2/Onc product size: 250 bp

3.3. Mouse cross

In order to produce experimental class mice we generally cross a tissue-specific Cre transgenic line (TSP-Cre) to mice that are doubly transgenic for both T2/Onc and the RosaSB^{LSL} transgene (Fig. 2A). In this way, the Cre transgene and the RosaSB^{LSL} transgene come together for the first time in the experimental class mice. Thus, inadvertent activation of the SB transposase in the germline does not occur. This is a big concern since many Cre transgenes are also active in the male germline. Alternatively, it is possible to include a mutation that predisposes mice to cancer from the same parent carrying the TSP-Cre transgene (Fig. 2B). If this approach is taken, the TSP-Cre transgene would be first crossed with a cancer predisposing mutation to make a double transgenic mouse, heterozygous for both the TSP-Cre transgene and a cancer predisposing mutation. This double heterozygous mouse is crossed to the double homozygous T2/Onc and RosaSB^{LSL} transgene mice to produce experimental class mice. All offspring from this cross will be heterozygous for T2/Onc and the RosaSB^{LSL} transgenes. One half of the offspring will carry the TSP-Cre transgene. If a cancer predisposing mutation or transgene is included in the cross (Fig. 2B), then a quarter of the offspring would carry both the TSP-Cre and cancer predisposing mutation. The crosses described here do not include many variations that can be conceived. For example, it may be necessary for cancer predisposing mutations to be homozygous in experimental class mice. Some tumor suppressor gene mutations do not result in significant cancer predisposition unless in a homozygous state, but are lethal when homozygous in mice. Therefore, conditional “knockout” mice could be useful in this regard.

3.4. Analysis of experimental class mice

Experimental class mice carry a tissue-specific Cre transgene, a T2/Onc transgene array, and the conditional Cre-regulated RosaSB^{LSL} transgene. These mice may also carry a fourth transgene that predisposes mice to cancer development. It is useful to sacrifice one or a few experimental class mice to verify that SB transposase protein is expressed in the appropriate target cell and in a high percentage of target cells. We find that immunohistochemical detection of the SB protein in the nuclei of cells is useful in this regard. An example is shown in Fig. 3 of SB11 expression in CNPase-Cre positive cells in the brain. We perform this assay on tissue sections, on glass slides, from formalin-fixed paraffin embedded organs.

3.4.1. Immunohistochemical detection of SB transposase protein—This protocol starts with paraffin-embedded tissue sections on slides. Other published protocols describe generation of such slides from mouse tissues. Good positive controls for all tissues are obtained from Rosa26-SB11 mice [4].

To deparaffinize place slides in:

- 1 Citrisolv—5 min.
- 2 Citrisolv—5 min.

To re-hydrate place slides in:

- 3 100% Ethanol—2 min.
- 4 100% Ethanol—2 min.
- 5 95% Ethanol—2 min.
- 6 90% Ethanol—2 min.
- 7 70% Ethanol—2 min.
- 8 Water—2 min.
- 9 1× PBS—2 min (can pause here).

Antigen retrieval step to remove methyl-bonds between proteins that were initiated during formaldehyde fixation, in order to open up epitopes.

- 10 Boil slides for 30 min in unmasking solution (purchased from Vector Laboratories, Burlingame, CA) using microwave or heating plate (make sure special care is taken so the unmasking solution stays above the slide level as some of the solution will boil off). Let cool to room temperature by placing beaker in ice. Slides must not be allowed to dry after this step.
- 11 Rinse 1× PBS—5 min.

Block all endogenous peroxidases in the tissue by placing slides in:

- 12 3% Hydrogen peroxide in water—10 min.
- 13 1× PBS—5 min.
- 14 1× PBS—5 min.

Block non-specific binding of antibody

- 15 Place slides in humidity chamber. (A humidity chamber = any enclosed box with wet paper towels, for example, a plastic box in which 4 cut-off pipets have been placed, which are used to raise the slides off of the floor of the chamber. Both below and on top of the pipets includes a layer of paper towels. Water is poured into the chamber so some small puddles form on top of the paper towels.)
- 16 Blocking step—use an ImmunoEdge Pen (Vector Laboratories, Burlingame, CA) and draw a box around the tissues of each of the slides being stained (the smaller the box, the better). Add around 100 µl of 2.5% sheep serum in PBS to each slide. The box drawn by the pen will keep the liquid from dispersing across the entire slide. Incubate at room temperature in the humidity chamber for 1 h. Alternatively, place ~100 µl of diluted (2 drops or 90 µl into 2.5 ml PBS) M.O.M. Mouse Ig Blocking Reagent (Vector Laboratories, Burlingame, CA) on the slide and incubate 1 h at Room temp. Note: Vector Labs recommends using the exact timing indicated below for optimal staining and avoidance of background signal.
- 17 Wash slides 2× in PBS for 2 min.
- 18 Place ~100 µl (enough to cover tissue) of M.O.M. diluent (600 µl stock into 7.5 ml PBS) on each tissue section, incubate 5 min at room temp.

Primary antibody staining

- 19 Tip off excess M.O.M. diluent. Dilute primary Ab in M.O.M. diluent. (For SB, dilute 1:100 or 2.5 µg/ml). Place 100 µl of 1° Ab solution on tissue sections and incubate overnight at 4 °C in humidity chamber (Note: this step is variable and can be adjusted for better staining depending upon the primary antibody being used). While Ab is incubating, prepare the VectaStain Elite ABC reagent, so it can stand for 30 min before using. Use anti-SB11 antibody (Mouse monoclonal clone 324622) from R&D Systems (Minneapolis, MN) diluted to 2.5 µg/ml.
- 20 Wash 2× in PBS for 2 min.

Secondary antibody staining

- 21 Place 100 µl of diluted M.O.M. Biotinylated anti-mouse IgG (10 µl stock into 2.5 ml of MOM diluent) onto tissue sections and incubate 10 min RT.
- 22 Wash 2× in PBS for 2 min.

Horse radish peroxidase (HRP) avidin–biotin addition

- 23 Place 100 µl VectaStain Elite ABC (avidin/biotin-HRP complex) reagent and incubate for 5 min. (2 drops of reagent A to 2.5 ml of PBS, mix, then add 2 drops of Reagent B and mix, let stand 30 min before using).
- 24 Wash 2× in PBS for 5 min.

Apply diaminobenzidine (DAB) substrate to visualize antibody staining

- 25 Prepare fresh substrate diaminobenzidine (DAB from Vector Laboratories, Burlingame, CA) by adding 2 drops of buffer stock solution to 5 ml distilled H₂O, mix well. Add 4 drops of DAB stock solution and mix well. Add 2 drops of H₂O₂ solution and mix well.
- 26 Place 100 µl on tissue sections and incubate for 2–10 min.
- 27 Wash in H₂O 5 min.

Stain with phoenix blue nuclear stain

- 28 70% Ethanol—5 min.
- 29 Add phoenix blue nuclear stain (Thermo Scientific, Waltham, MA)—2 min.
- 30 Add Water (rinse under tap about 1 min or until the blue stain no longer runs off the slides).
- 31 Add acid alcohol (1% HCl in 70% ethanol = 1 ml concentrated HCl (12 M) in 100 ml 70% ethanol)—5 s.
- 32 Water—10 s dip.
- 33 Ammonia water (1% ammonium hydroxide in water (1 g ammonium hydroxide in 100 ml H₂O)—10 s.
- 34 Water (rinse under tap 1 min).

Dehydrate tissue sections

- 35 70% Ethanol—5 min.
- 36 85% Ethanol—5 min.
- 37 95% Ethanol—5 min.

- 38 100% Ethanol—5 min.
- 39 100% Ethanol—5 min.
- 40 CitriSolv—5 min.
- 41 CitriSolv—5 min.
- 42 Mount in permount, place coverslip and get rid of bubbles.
- 43 View under microscope. Positive staining is brown and nuclear as SB transposase is a nuclear protein.

3.4.2. Aging mice for tumor development—In practice we age cohorts of 40 or more mice per experimental class. This number helps ensure that enough tumors will be recovered to identify genes recurrently mutated by T2/Onc insertion. It is useful to sacrifice small numbers of mice at intervals, such as every three months, to determine if cancer is developing in experimental class mice faster than in controls. In our experience, the time it takes for tumor development varies from less than 3 or 4 months to more than a year and a half, depending on the tissue type and whether the mice carry a cancer predisposing mutation(s). Indeed, for some tissues no tumors develop after transposition without a pre-disposed background [5] (and unpublished data). In any case, once SB-induced tumors have been obtained, a variety of methods can be used to characterize them. A key part of the process is to use polymerase chain reaction (PCR) to amplify T2/Onc/genomic DNA junction fragments, which can be sequenced to determine the site of insertion. In this way, genes recurrently mutated by T2/Onc insertion, which are strong candidate cancer genes, can be identified. A protocol for this procedure has been described in detail elsewhere [17,27].

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Fig. 1.

The T2/Onc transposon vector. This vector contains elements designed to elicit either transcriptional activation (MSCV 5' LTR and splice donor [SD]) or inactivation (splice acceptors [SA] and polyadenylation signals [pA]). The position of primers used to detect T2/Onc in transgenic mice is shown.

Fig. 2.

Typical crosses to generate tissue-specific mobilization of T2/Onc in mice. (A) Tissue-specific Cre transgenic mice (TSP-Cre) are crossed to mice doubly homozygous for a T2/Onc multi-copy transgene (T2/Onc) and the Cre/LoxP—regulated, or conditional, Rosa26 SB11 “knock-in” allele designated RosaSB^{LSL}. The desired experimental class offspring are triply transgenic. (B) The same cross could also be performed using a parent transgenic for a TSP-Cre transgene and a cancer predisposing mutation or transgene, here referred to generically as Tg.

Immunohistochemistry: anti-Sleeping Beauty

Fig. 3.

Photomicrographs of immunohistochemical detection of SB transposase protein. Images from formalin fixed paraffin embedded sections from the cerebrum and cerebellum are shown in the top and bottom images respectively. Sections were stained with anti-SB transposase antibody (brown stain) and counterstained with Phoenix blue nuclear stain. Mice carrying Cnp-Cre; RosaSB^{LSL}; T2/Onc (the chromosome 15 concatemer from [5]) transgenes were the source of the tissues in the two right-hand images and control mice which do not carry a Cre transgene, but carry the other two transgenes, are shown in the two left-hand images. Strong nuclear staining is observed in a subset of cells in both tissues in triple transgenic mice, but not Cre-negative control tissues.