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***Sleeping Beauty* Transposon Mutagenesis of the Rat Genome in Spermatogonial Stem Cells**

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Abstract

Since several aspects of physiology in rats has evolved to be more similar to humans than that of mice, it is highly desirable to link the rat into the process of annotating the human genome with function. However, the lack of technology for generating defined mutants in the rat genome has hindered the identification of causative relationships between genes and disease phenotypes. As an important step towards this goal, an approach of establishing transposon-mediated insertional mutagenesis in rat spermatogonial stem cells was recently developed. Transposons can be viewed as natural DNA transfer vehicles that, similar to integrating viruses, are capable of efficient genomic insertion. The mobility of transposons can be controlled by conditionally providing the transposase component of the transposition reaction. Thus, a DNA of interest such as a mutagenic gene trap cassette cloned between the inverted repeat sequences of a transposon-based vector can be utilized for stable genomic insertion in a regulated and highly efficient manner. Gene trap transposons integrate into the genome in a random fashion, and those mutagenic insertions that occurred in expressed genes can be selected *in vitro* based on activation of a reporter. Selected monoclonal as well as polyclonal libraries of gene trap clones are transplanted into the testes of recipient/founder male rats allowing passage of the mutation through the germline to F1 progeny after only a single cross with wild-type females. This paradigm enables a powerful methodological pipeline for forward genetic screens for functional gene annotation in the rat, as well as other vertebrate models. This article provides a detailed description on how to culture rat spermatogonial stem cell lines, their transfection with transposon plasmids, selection of gene trap insertions with antibiotics, transplantation of genetically modified stem cells and genotyping of knockout animals.

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1. Introduction

DNA transposons are discrete pieces of DNA with the ability to change their positions within the genome *via* a “cut and paste” mechanism called transposition. In nature, these elements exist as single units containing the transposase gene flanked by terminal inverted repeats (TIRs) that carry transposase binding sites (Fig. 1A). However, under laboratory conditions, it is possible to use transposons as bi-component systems, in which virtually any DNA sequence of interest can be placed between the transposon TIRs and mobilized by *trans*-supplementing the transposase in form of an expression plasmid (Fig. 1B) or mRNA synthesized *in vitro*. In the transposition process, the transposase enzyme mediates the excision of the element from its donor plasmid, followed by reintegration of the transposon into a chromosomal locus (Fig. 1C). This feature makes transposons natural and easily controllable DNA delivery vehicles that can be used as tools for versatile applications ranging from somatic and germline transgenesis to functional genomics and gene therapy.

Transposons have been successfully used in plants as well as in invertebrate animal models, including *Arabidopsis*, rice, *C. elegans* (1-3) and *Drosophila* (4-6) for transgenesis and insertional mutagenesis, but there was no known transposon that was active enough to be tailored as a tool for such purposes in vertebrates. In 1997, Ivics *et al.* succeeded to engineer the *Sleeping Beauty* (*SB*) transposon system by molecular reconstruction of an ancient, inactive Tc1/*mariner*-type transposon found in several fish genomes (7). This newly reactivated element allowed highly efficient transposition-mediated gene transfer in major vertebrate model species without the potential risk of cross-mobilization of endogenous transposon copies in host genomes. Indeed, *SB* has been successfully used as a tool for genetic modifications of a wide variety of vertebrate cell lines and species including humans [reviewed in (8-10)].

In evolutionary terms, the *SB* transposon represents a successful element that was able to colonize several fish genomes millions of years ago (7). However, even successful transposons have not been selected for the highest possible activity in nature, since, unlike viruses, they have to coexist with their hosts and, consequently, there is strong selective pressure to avoid insertional mutagenesis of essential genes. Indeed, although the resurrected *SB* element was active enough to be mobilized in vertebrate cells, its transpositional activity still presented a bottleneck for some applications. For example, requirements for transfection of primary cells and other hard-to-transfect cell types or for remobilization of transposons from chromosomally resident single-copy donor sites demanded an enzyme with more robust activity. Thus, enhancing transpositional activity has been one of the main targets for transposon vector development. A high-throughput, PCR-based, DNA-shuffling strategy and screening in mammalian cells produced a variant of *SB* that was hundred-fold more potent in chromosomal insertion of a transgene than the originally reconstructed protein (11). The use of *SB100X* demonstrated that it is possible to establish a transposon-based, non-viral vector system, that is capable of stable gene transfer at an efficiency comparable to that of viral strategies (11). Thus, the hyperactive *SB100X* transposase holds great promise by offering a broad utility in genetic applications including functional genomics.

The post-genomic era presented the scientific community with the new challenge of functional annotation of every gene and identification of elaborate genetic networks. Diverse methods have been employed to address this task, including mutational analysis that proved to be one of the most direct ways to decipher gene functions. There are versatile strategies for creating mutations, including insertional mutagenesis by discrete pieces of foreign DNA. Insertional mutagenesis has the advantage that the inserted DNA fragment can serve as a molecular tag that allows rapid identification of the mutated allele (usually by PCR). Since the function of the gene in which the insertion has occurred is often disturbed, such loss-of-

function insertional mutagenesis is frequently followed by functional analysis of mutant phenotypes. In many instances, retroviral vectors were utilized to introduce mutagenic cassettes into genomes, but their chromosomal insertion bias does not allow full coverage of genes (12). The random integration pattern of the *SB* transposon combined with its ability to efficiently integrate versatile transgene cassettes into chromosomes established this system as a highly useful tool for insertional mutagenesis in both embryonic stem cells (ESCs) (13, 14) as well as in somatic (15, 16) and germline tissues (17-25) in animal models.

2. Materials

2.1. Culturing and preserving rat spermatogonial stem cell lines

1. DMEM-high glucose: Dulbecco's modified Eagles Medium-high glucose (cat. no. D5648, Sigma, Inc.)
2. Sodium Bicarbonate (cat. no. S5761, Sigma, Inc.)
3. PBS: Dulbecco's phosphate-buffered saline (PBS; cat. no. D8537, Sigma, Inc.) 200 mg/l KCl (w/v), 200 mg/L KH₂PO₄ (w/v), 8 g/l NaCl (w/v), 1.15 g/l Na₂HPO₄ (w/v)
4. Heat Inactivated Fetal Bovine Serum: FBS (cat. no. 104, Tissue Culture Biologicals)
5. Gelatin from Porcine Skin- Type A (cat. No. G1890, Sigma, Inc.)
6. DR4 mouse embryonic fibroblasts (MEFs; cat. no. ASF 1001P Applied Stemcell, Inc)
7. Recovery Cell Culture Freezing Medium (cat. no. 12648-010, Invitrogen, Inc.)
8. T225 Flasks Angled Neck (cat. no. 431081, Corning, Inc.)
9. Costar Clear TC-Treated Microplates, Individually Wrapped, Sterile (cat. no. 3516, 3513, 3524, 3548, 3596, Corning, Inc.)
10. DHF12 Medium (Dulbecco's modified Eagle's medium: Ham's F12 medium 1:1) (cat. no. D8437, Sigma, Inc.)
11. B-27 Supplement minus vitamin A (cat. no. 12587-010, Invitrogen, Inc.)
12. L-glutamine (cat. no. 25030-149, Invitrogen, Inc.)
13. Antibiotic-antimycotic solution: 10,000 U/ml penicillin G sodium (U/v), 10,000 µg/ml streptomycin sulfate (w/v), and 25 µg/ml amphotericin B (w/v) (cat. no. 15240-062, Invitrogen Inc.).
14. 2-Mercaptoethanol (cat. no. M3148, Sigma, Inc.)
15. Recombinant human FGF2 (cat. no. F0291, Sigma, Inc.)
16. Recombinant GDNF (cat. no. 512-GF, R and D Systems, Inc)
17. Dimethyl Sulfoxide: DMSO (cat no D2650, Sigma, Inc.)
18. 5100 Cryo 1°C Freezing Container "Mr. Frosty" (cat. no. 15-350-50, Thermo Fisher Scientific Nalgene, Inc.)
19. Cryogenic vials (cat. no. 03-337-50, Thermo Fisher Scientific Nalgene, Inc.)

2.2. Transfection of rat spermatogonia by nucleofection

1. Pre-warmed, SG Medium (Sec 3.3.1)

2. Highly purified plasmid DNA in TE buffer at 1-2 µg/µl.
3. Gelatin-coated plates (**Sec 3.3.2**)
4. Plates with fresh MEF feeder layers (**Sec 3.3.2**)
5. Nucleofector® device (cat. no. AAD-1001, Lonza)
6. Cell Line Nucleofector Kit L (cat. no. VCA-1005, Lonza)

2.3. Transfection of rat spermatogonia by lipofection

1. Pre-warmed, SG Medium (**Sec 3.3.1**)
2. Opti-MEM (cat. no. 31985062; Invitrogen, Inc.)
3. Lipofectamine 2000 (cat. no. 11668019; Invitrogen)
4. Highly purified plasmid DNA in TE buffer at 1-2 µg/µl
5. Gelatin-coated plates (**Sec 3.3.2**)
6. Plates with fresh MEF feeder layers (**Sec 3.3.2**).

2.4. Clonal selection for gene-trap mutations in rat spermatogonia

1. Geneticin Selective Antibiotic: G418 (cat no 11811-031, Invitrogen Inc.).
2. *Sleeping Beauty* gene-trap construct (Fig. 2A) expressing a resistance gene that selects for survival in G418-containing medium (i.e. neomycin phosphotransferase gene).
3. MEF feeder cell line expressing a resistance gene for survival in G418-containing medium (DR4 MEFs; cat. no. ASF 1001P, Applied Stemcell, Inc.).

2.5. Germline transmission from genetically modified spermatogonia

1. Disposable Pasteur Pipettes (cat. no. 13-678-20C, Thermo Fisher Scientific Nalgene Inc.)
2. Busulfan (cat. no. 154906, ICN Biomedicals)
3. Dimethyl Sulfoxide (DMSO) (cat. no. 317275, Calbiochem)
4. Trypan Blue (cat. no. T6146-25G, Sigma Inc.)
5. Triadine (cat. no. 10-8208, Triad Disposables)
6. Ethanol (cat. no. 111ACS200, Pharmco-AAPER)
7. PBS: Dulbecco's phosphate-buffered saline (PBS; cat. no.D8537, Sigma Inc.) 200 mg/l KCl (w/v), 200 mg/l KH₂PO₄ (w/v), 8 g/l NaCl (w/v), 1.15 g/l Na₂HPO₄ (w/v).
8. Kimwipes (cat. no. 34155, Kimberly-Clark)
9. Bead Sterilizer; Germinator 500 (Cellpoint Scientific Inc)
10. Flaming/Brown Micropipette Puller; Model P-97 (Sutter Instruments Co.)
11. Glass Capillaries for needles; 100 µl micropipette (Drumond Scientific Co.)
12. Heat Therapy Pump Pad (cat. no. P/N 0799-000, Gaymar)
13. Acepromazine (cat. no. 038ZJ03, Vedco)
14. Rompin (cat. no. LA33806A, Lloyd Laboratories)

15. Ketaset (cat. no. 440761, Fort Dodge Animal Health)
16. Buprenex Injectable (cat. no. 12496-0757-1, Reckitt Benckiser)
17. Shaving Razors – Stainless Steel Surgical Prep Blades (cat. no. 74-0001, Personna)
18. Suture Thread; Spool Suture (cat. no. SUT-15-2, Roboz Surgical Inc.)
19. Suture Needles; Eye 3/8 circle (cat. no. RS-7981-4, Roboz Surgical Inc.)
20. Michel Wound Clips (cat. no. RS-9272, Roboz Surgical Inc.)
21. Michel Wound Clip Forceps (cat. no. RS-9294, Roboz Surgical Inc.)
22. Ear Puncher – 2 mm diameter (cat. no. RS-9902, Roboz Surgical Inc.)
23. Hemostat (cat. no. RS-7110, Roboz Surgical Inc.)
24. Straight Sharp Microdissecting Scissors (cat. no. RS-5882, Roboz Surgical Inc.)
25. Curved, Sharp Microdissecting Scissors (cat. no. RS-5883, Roboz Surgical Inc.)
26. Full-Serve Microdissecting Forceps (cat. no. RS-5137, Roboz Surgical Inc.)
27. Straight Tip, Dumostar Tweezers (cat. no. RS-4978, Roboz Surgical Inc.)
28. 5/45 INOX Tweezers (cat. no. RS-5005, Roboz Surgical Inc.)
29. Polyethylene capillary tubing (cat. No. 19-0040-01, Pharmacia, Inc.)
30. 24-day old, busulfan-treated, male, Sprague Dawley rats (**Section 3.6.1.1.**)

3. Description of method

3.1. Overview

Insertional mutagenesis can be applied in cultured, germline-competent - rat spermatogonial stem cells (SSCs) (26, 27). One advantage of this approach lies in the possibility to perform preselection of modified cell clones before generating mutant animals. It is possible to perform large-scale, transposon-based, insertional mutagenesis screens in SSCs by simply transfecting or electroporating transposon donor and transposase expression plasmids into the cells. The amounts of the delivered plasmids can be adjusted for obtaining the desired insertion frequencies per cell.

Most intronic integrations of foreign DNA (also applicable to *SB*, as discussed above) are expected to end up spliced out without having a mutagenic effect on endogenous gene expression. Thus, considerable effort has been put into improving mutagenicity as well as reporting capabilities of the internal components of mutagenic vectors (28, 29). There are several types of mutagenic cassettes that can be efficiently combined with transposon-based gene delivery for insertional mutagenesis. Gene-trap cassettes include splice acceptors and polyadenylation sequences so that transcription of genes can be disrupted upon vector insertion into introns (Fig. 2A) (12). Frequently, such cassettes are also equipped with a reporter gene (usually, a fluorescent protein, β -galactosidase or antibiotic resistance) whose expression is dependent on the correct splicing between exons of the trapped gene and the splice acceptor site carried by the transposon vector (**for more details refer to Chapter 1**) (29, 30). Commonly, such gene traps have been used for ESC mutagenesis (31, 32), and were also employed in combination with the *SB* delivery system in several studies for mutagenizing ESCs (14) as well as the germlines of experimental animals (**see Chapter 1**) (17-25).

SB transposition can be used to tag and simultaneously mutate thousands of genes in culture, by taking advantage of gene-trap cassettes. Importantly, culture conditions maintain the potential of genetically manipulated rat SSCs to produce viable sperm cells (26, 27). The spermatogonial clones are transplanted to repopulate the testes of sterilized, wild-type recipient male rats. The stem cell genome is then passed on to transgenic offspring upon crossing the recipient males with wild-type females (Fig. 3). Although transposition events in a given target gene occur by chance, the tissue culture conditions allow screening for a large number of events. Transposition-mediated gene insertion and cell culture conditions thus allow generation of libraries of gene knockouts in rat SSCs (Fig. 3). This technology has the potential to develop powerful genomic tools for the rat, offering the opportunity to create a bridge between physiology and genomics.

3.2. Functional testing of gene-trap transposition in cultured human cells

The *SB* transposon has a close-to-random genomic insertion profile with a ~35% frequency of landing in a gene. Since most hits in genes are localized in introns, these transposon insertions are not expected to be mutagenic, because they are likely spliced out from the primary transcript. Thus, the *SB* transposon was equipped with a β -geo gene trap cassette containing an adenoviral splice acceptor (SA) sequence followed by the *lacZ-neo* (β -geo) fusion (Fig. 2A) widely used to trap genes in mouse ESCs. Gene trapping is based on the activation of a promoterless reporter gene that is dependent on splicing between the exons of the trapped gene and a splice acceptor site carried by the transposon (Fig. 2B). Thus, gene trap vectors both report the insertion of the transposon into an expressed gene, and have a mutagenic effect by truncating the transcript through imposed splicing and premature termination of translation (**more details in Chapter 1**).

In order to assess gene trapping in human HeLa cells, a two-component transposition assay (Fig. 1B) based on co-transfection of a donor plasmid carrying a non-autonomous transposon marked with the β -geo gene trap cassette, with or without a helper plasmid expressing the *SB* transposase is applied. Transposition events into transcriptionally active genes give rise to G418-resistant colonies, if transcriptional orientation of the β -geo marker coincides with that of the trapped gene and if splicing preserves the reading frame of the β -geo coding region (this is not an issue if transposon insertion occurs downstream of a nontranslated exon, in which case translation initiates from the ATG of the β -geo coding sequence). Following antibiotic selection, transposition efficiency is calculated from the numbers of G418-resistant cell colonies in the presence vs. absence of the transposase. These transposition assays established that the most efficient gene trapping was obtained at the 10 : 1 transposon : transposase plasmid ratio (by mass) (Figs. 2C and 2D).

3.3. Culturing and preserving rat spermatogonial stem cell lines

In this section, protocols are presented for routine culture of rat spermatogonial lines. Established spermatogonial lines are sub-cultured on feeder layers of MEFs in *spermatogonial medium* (SG medium) (33, 34) and then preserved in *spermatogonial freezing medium* (SG freezing medium) (33, 34). The generated spermatogonial stocks can be thawed after >1 year in cryostorage, re-expanded over multiple passages in culture, and then used to produce fully functional spermatozoa in recipient rat testes (33, 34). Donor derived spermatozoa produced in recipient rat testes yield high rates of germline transmission through natural mating (33, 34).

3.3.1. Formulating spermatogonial culture medium

1. Spermatogonial Culture Medium (SG Medium) is prepared by supplementing DHF12 medium with 20 ng/ml GDNF, 25 ng/ml FGF2, 100 μ M 2-

mercaptoethanol, an additional 4 mM L-glutamine (final concentration = 6 mM), and a 1X concentration of B27 Supplement Minus Vitamin A.

3.3.2. Preparing fibroblast feeder cell lines

1. Primary stocks of DR4 MEFs are purchased from Applied StemCell, Inc. at passage 2, and expanded after plating into Dulbecco's modified Eagle's medium supplemented with 1.5 g/l sodium bicarbonate, and 15% heat-inactivated FBS (MEF medium) at 37°C/5% CO₂ for up to 4 passages following their thawing and initial plating from the vial received from the manufacturer.
2. Following expansion to passages 5 and 6, secondary stocks of MEFs are irradiated (120 Gy) and then cryo-preserved in liquid nitrogen for future use in Recovery Cell Culture Freezing Medium (Invitrogen) according to the manufacturer's protocol.
3. Culture dishes are pre-coated with a solution of sterile 0.1% gelatin for 1 hr at room temperature, and then rinsed 1 × with sterile PBS before plating the MEFs. To prepare 0.1% gelatin solution, dissolve gelatin in MQ water (1 g gelatin/liter) and autoclave on liquid cycle.
4. Prior to use for culture with spermatogonia, MEFs are thawed and plated into gelatin-coated dishes (4.5×10^4 cells/cm²) in MEF medium for 16-48 hr, rinsed 1 × with PBS and then pre-incubated in SG medium for an additional 16-48 hr. The SG medium used for pre-incubation is then discarded and spermatogonia are plated onto the MEFs in fresh SG medium.

3.3.3. Culturing rat spermatogonial lines—Starting Notes: *All culture incubation steps with spermatogonia are performed using SG medium at 37°C, 5% CO₂.*

1. After an initial thaw from cryostorage, spermatogonia from a single cryostorage vial are suspended to 10 ml with fresh SG medium and then pelleted at $400 \times g$ for 4 min. Supernatant is discarded to remove the freezing medium. The pellet is suspended to 3.5 ml with fresh SG medium and then the total cell suspension is plated into a 9.5 cm² well of a 6-well culture dish containing feeder layers of irradiated MEFs (see **Section 3.3.2.** for preparing MEF feeder layers).
2. Initial passaging of spermatogonial cultures post-thaw often requires a 1:1 to 1:2 split into the same size wells 10-12 days after first seeding onto the MEFs. After the second or third passage on MEFs, cultures of rat spermatogonial lines are passaged at ~1:3 dilutions onto fresh monolayers of MEFs every 10-14 days at $\sim 3 \times 10^4$ cells/cm².
3. To harvest spermatogonia for passaging, cultures are removed from the MEFs by repeated pipetting over the surface area of the culture using a 5 ml pipette. After harvesting, "clusters" of spermatogonia are dissociated by gentle trituration with 20-30 strokes through a P1000 pipet tip in the SG Medium.

Note: *Protease treatment is not required for sub-culturing in SG medium.*

4. The dissociated cells are pelleted at $400 \times g$ for 4 minutes and the number of cells recovered during each passage is determined by counting on a hemocytometer.
5. Spermatogonia are easily distinguished during counting as the predominant population of smaller, round cells with smooth surfaces, as compared to occasionally observed, larger and often irregular shaped irradiated MEFs. Typically, once expanded in numbers, $2-4 \times 10^6$ spermatogonia can be harvested from a single, 10 cm dish.

3.3.4. Cryo-preserving stocks of spermatogonial lines

1. Prepare Spermatogonial Freezing Medium (SG Freezing Medium) (33) by adding DMSO at a concentration of 10% (v/v) in SG Medium. Filter-sterilize and cool the prepared freezing medium on ice prior to use.
Note: Spermatogonial Freezing Medium can be stored frozen at -20°C for up to 1 month prior to use.
2. Prepare a “Mr. Frosty” freezing container by adding 200 ml fresh isopropanol to the outer chamber. Chill the container by equilibrating it to ~4°C in a refrigerator prior to use.
3. Harvest spermatogonia as described previously in **Section 3.3.3**.
4. Suspend the harvested spermatogonial pellet in ice-cold, SG Freezing Medium at 2×10^5 to 2×10^6 cells/ml and then aliquot stocks into cryovials at 1 ml/vial. Work quickly and place filled cryovials on ice while finishing aliquots.
5. Place cryovials of spermatogonial stocks into the pre-chilled “Mr Frosty” and close container firmly.
6. Store the freezing container of spermatogonial stocks at -80°C for 24 hours, then transfer vials into a liquid nitrogen cryostorage unit.

Note: Record each use of the “Mr. Frosty” container so that fresh isopropanol can be replenished after the 5th use (see Manufacturer’s instructions).

3.4. Transfection of rat spermatogonia

In this section, we present gene delivery protocols for rat spermatogonia, which are based on electroporation and lipofection. Electroporation consistently results in 20-40% transfection efficiency of circular plasmid DNA into undifferentiated rat spermatogonia (27). This approach is most convenient from a user standpoint, but requires a specialized “Nucleofector” apparatus from AMAXA, Inc. Thus, a second protocol is also presented for transfecting rat spermatogonial stem cells with plasmid DNA. This lipofection-based protocol also yields up to 40% transfected rat spermatogonia (35), and therefore can be performed without special electroporation equipment.

3.4.1. Transfection of rat spermatogonia by nucleofection

1. Set up the Nucleofector® device according to the manufacture’s instructions and enter program A-020.
2. Harvest spermatogonia by pipetting in SG medium as described in **Section 3.3.3**.
Note: If transfecting spermatogonia from an established line maintained on MEFs, first pre-incubate the harvested cells on a gelatin-coated plate in SG medium for 30-45 min at 37°C, 5% CO₂ to deplete contaminating MEF feeder cells from the spermatogonial suspension. Then re-harvest spermatogonia for transfection.
3. After counting harvested cells, split the suspension into 15 ml tubes to obtain $2-3 \times 10^6$ cells/transfection, pellet them by centrifugation for 5 min at $400 \times g$.
4. Carefully remove the supernatant as completely as possible from the tube.
5. Suspend the spermatogonial pellet with 100 µl of Nucleofector® Solution L, add 10 µg total plasmid DNA [1 µg transposase expression plasmid plus 9 µg transposon plasmid (27)], and transfer the spermatogonial suspension into a transfection cuvette provided in the kit for Solution L.

6. Insert the cuvette containing cells and DNA into the Nucleofector® holder and press the “X” button to execute program A-020.
Note: *Do not keep the cells in Nucleofector Solution longer than 15 min.*
7. Promptly remove the cuvette from the Nucleofector® device and add 500 µl of pre-warmed SG Medium directly to the transfected cells in the cuvette.
8. Carefully remove the cell suspension with a plastic pipette provided with the kit by avoiding visibly coagulated DNA, and plate the suspension drop-wise directly onto freshly prepared feeder layers of MEFs in SG Medium for further expansion and selection of transgenic lines (**Section 3.5**).

3.4.2. Transfection of rat spermatogonia by lipofection

1. Prepare a Transfection Mixture containing Lipofectamine 2000 (Invitrogen) and plasmid DNA in Opti-MEM, as follows:
 - a. In a 1.5 ml microfuge tube, dilute 1 µg total plasmid DNA (0.1 µg transposase expression plasmid plus 0.9 µg transposon plasmid) in 100 µl Opti-MEM.
 - b. In a separate 1.5 ml microfuge tube, dilute 2 µl Lipofectamine 2000/100 µl Opti-MEM.
 - c. Incubate tubes separately for 5-10 min.
 - d. Combine contents of each tube together and incubate at room temperature for at least 20 minutes (but no longer than 6 hr) to obtain the Transfection Mixture. During this incubation step, proceed to harvesting cells for transfection.
2. Harvest spermatogonia by pipetting in SG medium as described in **Section 3.3.3**.
Note: *If using proliferating cultures of spermatogonia maintained on MEF feeder layers, first plate the cells onto a fresh gelatin-coated plate and incubate for 30 min (37°C, 5% CO₂) to deplete the number of residual MEFs present in the cell suspension.*
3. Suspend spermatogonia to ~10⁶ cells/ml in SG Medium.
4. Add Transfection Mixture to the cell suspension at a ratio 20% vol Transfection Mixture : 80% vol spermatogonial suspension, and incubate at 37°C, 5% CO₂ for 40-120 min (routinely 80 min) in a vented tube.
Note: *As a typical example, 40 µl volume of the Transfection Mixture prepared in Step 1 is used to transfect ~2×10⁵ spermatogonia in a total transfection volume of 200 µl.*
5. During transfections lasting longer than 1 h, mix the transfection by gently pipetting cells up and down two times midway through the incubation period.
6. After the transfection incubation period, wash spermatogonia by first suspending the transfection suspension to 20 times its volume using fresh culture medium (i.e. 4 ml medium/200 µl transfection reaction), and then pellet the cells for 5 min at 400 × g.
7. Discard the supernatant, and wash the pellet(s) two additional times using fresh culture medium at an equivalent of the 20× volume/wash used in Step 6.

8. After the third wash, suspend the cell pellet in fresh medium and then plate transfected cells onto fresh MEF feeder layers for selection of transgenic spermatogonial lines (**Section 3.5**).

Note: Take care to completely remove all supernatant after each wash in Steps 6-8 to minimize toxic effects of the transfection reaction.

3.5. Clonal selection for gene-trap mutations in cultures of rat spermatogonia

In this section, we describe methods for monoclonal and polyclonal expansion of *SB* gene-trap-modified rat spermatogonial cells.

1. After transfecting spermatogonia with transposon and transposase DNA plasmids, the treated spermatogonia are plated directly into SG Medium at densities of $\sim 3 \times 10^5$ spermatogonia/well (9.5 cm^2) in a 6-well plate containing freshly prepared MEFs (see **Section 3.3.3**).
2. Transfected spermatogonia are then allowed to proliferate for 7-10 days.
3. Spermatogonia are then passaged onto fresh MEFs and allowed to incubate for ~2 days prior to initiating selection in SG medium supplemented with $\sim 75 \text{ } \mu\text{g/ml}$ G418 (Invitrogen, Inc.).

Note: G418 selection of gene-trapped spermatogonia is dependent on the relative expression levels of the neomycin-resistance gene in spermatogonia, which is dependent on the trapped cellular promoters. Therefore, the lowest G418 concentration still capable of killing wild-type spermatogonia should be used to maximize the number of gene trap events.

4. After initiating selection, cultures are fed fresh SG medium containing G418 every two days during an 8-10 day selection period. Thereafter, cells are fed every two days using SG medium alone to expand clonally enriched lines of rat spermatogonia that can be used to produce rats harboring *SB* gene-trap mutations, as described in the following sections.

3.5.1. Clonal expansion of spermatogonial cells containing gene-trap

transposon insertions—After genetic modification of spermatogonial lines using *SB* transposition, mixtures of genetically modified spermatogonial colonies harboring distinct gene-trap mutations can be expanded collectively and then pooled to form a library of mutated rat germlines for transplantation into testes of recipient-founders (see **Section 3.1**). This type of polyclonal expansion of mutant spermatogonial lines using randomly integrating constructs will mobilize the production of numerous distinct transgenic rat lines following transplantation into testes of a single recipient/founder (26, 27). The newly generated rat lines can then be screened to identify genomic sites of transposon integration within animals using PCR techniques (27).

Individual G418-resistant spermatogonial colonies harboring gene-trap mutations can also be picked from a 6-well plate using a p200 Eppendorf tip. For monoclonal expansion, dilute G418-resistant spermatogonial libraries to $3\text{--}4 \times 10^3$ cells/ cm^2 on fresh MEFs in SG medium. After 25-35 days, and working under a stereomicroscope, transfer individual colonies into wells of a 96-well plate to facilitate clonal expansion of mutant germlines to larger numbers upon subsequent passages into larger culture dishes. A single 10 cm dish typically yields enough spermatogonia from a given line for both preservation of frozen stocks (**Section 3.3.4**), and for the production of multiple recipient-founders (**Section 3.6.1**).

Note: During the initial 25-35 day period of clonal expansion, fresh MEFs should be supplemented into spermatogonial cultures (i.e. “spiked” into ongoing cultures) every 11-12 days.

3.5.2. Characterization of SB gene-trap insertion sites—Transposon insertion sites are recovered from genomic DNA samples prepared from spermatogonial clones carrying gene-trap insertions. A linker-mediated splinkerette-PCR is applied that specifically amplifies the genomic flanks of transposons by primers that are designed for the TIR sequences of SB and for a linker (or splinkerette) that is ligated to the ends of restriction enzyme-digested genomic DNA (Fig. 4).

1. Digest 5 µg genomic DNA isolated from spermatogonial cells with 15 units of *Mbo*I (New England Biolabs) in a 200 µl reaction volume for 5 hours.
2. Chloroform-extract and ethanol-precipitate DNA. Dissolve pellet in 10 µl TE.

Note: Run a 1 µl aliquot of the digested DNA on a 0.7% agarose gel to check if the average size of the DNA fragments is between 0.5 and 1.0 kbp. If this is not the case, do another round of digest.

3. Ligate 2 µl of digested DNA solution with 10 pmol of splinkerette linker and 5 U T4 DNA ligase in a 10 µl reaction overnight at room temperature.
4. Heat-inactivate reaction at 65°C for 10 min.
5. Set up the first round PCR in a 50-µl reaction containing:

34.5 µl H₂O
 5 µl 10X PCR buffer
 3 µl MgCl₂ (25 mM)
 1 µl dNTP (10 mM)
 2 µl (10 pmol/µl) splinkerette-specific primer Link3
 2 µl (10 pmol/µl) transposon-specific primer Bal Rev3
 2 µl of ligated DNA
 0.5 µl Taq polymerase

Touchdown PCR program:

94°C 3 min
 94°C 30``
 70°C 30``
 72°C 30`` (15x)
 94°C 30``
 63°C 30``
 72°C 2`` + 2``/cycle (5x)
 94°C 30``
 62°C 30``
 72°C 12`` + 2``/cycle (5x)

94°C 30''
 61°C 30''
 72°C 22'' + 2''/cycle (5x)
 94°C 30''
 60°C 30''
 72°C 30'' (5x)
 72°C 5 min
 4°C until next step

6. Set up the second, nested round PCR in a 50-μl reaction containing:

35.5 μl H₂O
 5 μl 10X PCR buffer
 3 μl MgCl₂ (25 mM)
 1 μl dNTP (10 mM)
 2 μl (10 pmol/μl) nested splinkerette-specific primer Link4
 2 μl (10 pmol/μl) nested transposon-specific primer Bal Rev
 1 μl of 100X-diluted PCR-1
 0.5 μl Taq polymerase

PCR program:

94°C 3 min
 94°C 30''
 65°C 30''
 72°C 30'' (10x)
 94°C 30''
 58°C 30''
 72°C 30'' (20x)
 72°C 5 min
 4°C until next step

Note: *The expected size of the PCR products is between 400 bp and 700 bp.*

- 7.** In most cases, a single dominant product is seen, which can later be sequenced directly after gel isolation. Multiple bands often represent multiple insertions, which need to be isolated from gel or cloned into a plasmid vector (e.g. pGEM-T Vector Systems, Promega) followed by sequencing.
- 8.** Map the insertion site by doing a BLAST search with the DNA sequence directly flanking the transposon at the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

3.6. Germline transmission from genetically modified spermatogonia

A major advantage of genetically modified spermatogonia is their ability to colonize the germline after direct transplantation into the testes of sterile males. This approach ensures the transmission of the transgene to F1 progeny by simple breeding with an efficiency of up to 100 percent (27, 33, 34, 36). We have found male-sterile, *DAZL*-deficient rats (37) to be most effective for germline transmission of mutant alleles created in spermatogonial cells (34). High efficiency of germline transmission by *DAZL*-deficient rats is due to the lack of endogenous sperm production (37), caused by an early, post-meiotic block (34). Thus, this mode of sterile-testis complementation with genetically modified spermatogonial stem cells in rats can be used to directly generate germline founders in that it bypasses the generation of a genetically mosaic animal.

3.6.1. Generation of recipient-founders by testicular transplantation—Busulfan-treated wildtype Sprague Dawley rats (Harlan, Inc), or male-sterile *DAZL*-deficient, Sprague Dawley rats at 24 days of age can be used as recipients for spermatogonial lines (34).

3.6.1.1. Recipient preparation: *Note: All reagents (solutions and tools) used for transplantation should be sterilized prior to use to ensure sterile surgical technique.*

- 1 Rats need to be received from the supplier at 8-10 days of age.
- 2 At 12 days of age recipient rats receive a single dose of busulfan (12.5 mg/kg, i.p. for wildtype Sprague Dawley rats; 12.0 mg/kg for *DAZL*-deficient Sprague Dawley rats), and subsequently housed in a quiet, clean and well ventilated location of an approved animal facility. *Under guidelines of an approved safety plan**, a 4 mg/ml working stock of busulfan in 50% DMSO is prepared by dissolving busulfan in 100% DMSO at 8 mg/ml to which an equal volume of filter-sterilized, deionizer water is added.

***Safety Information:** *Busulfan is considered a biohazardous compound. Its use for studies in animals warrants an approved safety plan certified by both the parent research institution's Environmental Health and Safety program, and the Institutional Animal Care and Use Committee (IACUC). Personal protection equipment (doubled gloves; lab coat; safety glasses; ventilation mask) should be worn when working with busulfan to prevent any type of exposure. The preparation, use and disposal of busulfan should be restricted to certified chemical and/or biosafety cabinets to prevent exposure.*

Note: *Recipients are pre-treated with busulfan in order to deplete their endogenous spermatogonial populations prior to transplantation; this enhances engraftment by the transplanted spermatogonia.*

3.6.1.2. Transplantation procedure

1. On the day of transplantation, rat spermatogonia are harvested from culture and suspended in ice cold medium at concentrations of $2-6 \times 10^5$ spermatogonia/100 μ l. The cellular suspension is transferred to a sterile microfuge tube and kept on ice until transplantation.
2. Just prior to transplantation, the cell suspension is supplemented with a 20% volume of a filter-sterilized, 0.04% trypan blue solution freshly made in PBS. We typically add 100 μ l of pre-chilled 0.04% trypan blue solution directly to 400 μ l of a cell suspension containing 1-3 million cells, which is then gently mixed by pipetting up and down ~5 times.
3. Once spermatogonia are harvested, the first busulfan-treated recipient rat is anesthetized by intraperitoneal (i.p.) injection of a cocktail containing 100 mg/ml

ketaset, 20 mg/ml rompin plus 10 mg/ml acepromazine at 0.1 ml/100 g body weight to achieve a surgical plane (as monitored by the lack of response to a toe pinch).

4. The abdominal skin is opened just rostral to the pelvis, and the testis is exposed. The efferent ductules leading into the rete testis are then accessed by blunt dissection using micro-dissection forceps, after which an absorbent wick made of a small, *tightly* twisted corner of a Kimwipe is inserted directly under the efferent ductules to provide support during injection (Fig. 5).
5. The ductules and wick are soaked with several drops of PBS and the ductules are further dissected up to the base of their respective testis to yield visible access to the rete, which is the injection site (Fig. 5).
6. Once the rete is exposed, the spermatogonial cell suspension is mixed gently by pipetting up and down ~5 times with a p200 tip. About ~70-80 μ l of the suspension is loaded into a 100 μ l glass capillary injection needle (~50 μ m opening) using a flame pulled, transfer pipette (i.e. made from Pasteur pipettes) and rubber squeeze bulb.
7. The injection needle containing spermatogonia is manually inserted into the rete testis, and the cells are transferred into the testis using a stationary 10 ml syringe connected to the injection needle by flexible plastic tube (Fig. 5).
8. The injected testis is then carefully placed back into the abdominal cavity and the same procedure can be performed on the contra-lateral testis to increase efficiency.
9. Once injected and placed back in the abdominal cavity, the abdominal wall (sutured) and skin (wound clips) are surgically closed.

Note: Care should be taken not to inadvertently suture the fat pad of the epididymus to the abdominal wall, which would prevent proper descending of the testis and subsequent spermatogenesis.

10. The procedure can be applied to multiple recipients using the same spermatogonial suspension, which can be kept on ice for up to 5 hours.

3.6.1.3. Post-operative steps

1. After surgically closing the abdominal cavity and skin, all animals are maintained on a warming pad set to 34°C and receive post-operative care to assure their safe recovery from anesthesia and to alleviate pain and distress.
2. For recovery from anesthesia, each animal is observed with respect to its breathing rate, muscle control and external stimuli, prior to housing in a quiet, well ventilated location of the animal facility.
3. As a post-operative analgesic to alleviate pain, each rat receives a single dose of buprenorphine hydrochloride (25 μ g/kg) (Buprenex Injectable, Reckitt Benckiser). If necessary, additional doses can be administered every 6-12 hours for the next 48 hours upon signs of discomfort or pain.
4. Wound clips are removed after 12-14 days.
5. Breeding is usually started about 65 days post-transplantation.

3.6.2. Germline transmission from recipient-founders—Recipient males transplanted with spermatogonial lines are crossed to wild-type female Sprague Dawley rats of similar age. Typically, the first F1 progeny are born between 100 and 150 days post-

transplantation, and recipients can continue to sire litters over 300 days post-transplantation due to the long-term spermatogenesis colony forming potential of cultured rat spermatogonia (26, 33). Mutant rat progeny from recipient-founders and wild-type females are identified by genomic PCR and/or Southern Blot analysis of tail DNA using probes specific to the mutation of interest.

4. Conclusions

As a rapidly emerging alternative to embryonic stem cells, spermatogonial stem cells combined with *SB* gene-trap mutagenesis provide a relatively simple and fast procedure for creating thousands of mutations in the rat. Genetic manipulation in spermatogonial rather than embryonic stem cells bypasses the need to produce and breed chimeric rats. Thus, F1 generation rats derived from injected spermatogonial cells harboring gene-trap mutations in individual genes can be intercrossed immediately to obtain F2 generation knockout rats (27, 33, 34). Finally, knockout rats will provide animal models of human disease, particularly for diseases that are difficult to replicate in mice.

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Figure 1. General organization and use of class II transposable elements as gene vectors
(a) Autonomous transposable elements consist of terminal inverted repeats (TIR; black arrows) that flank the transposase gene. **(b)** Bicomponent transposon vector system for delivering transgenes that are maintained in plasmids. One component contains a DNA of interest between the transposon TIRs carried by a plasmid vector, while the other component is a transposase expression plasmid, in which the black arrow represents the promoter driving expression of the transposase. **(c)** The transposon carrying a DNA of interest is excised from the donor plasmid and is integrated at a chromosomal site by the transposase.

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Figure 3. Generation of knock-out rats by insertional mutagenesis with gene trap transposons in spermatogonial stem cells

Cultured stem cells are transfected with gene trap transposon and transposase constructs that will lead to thousands of transposon insertions covering all chromosomes. Those cells in which insertions occurred in expressed genes can be selected based on activation of the gene trap marker, and the insertion sites can be mapped. Cell clones or polyclonal insertion libraries can be transplanted into the testes of sterile males, in which the spermatogonial stem cells will undergo spermatogenesis. These transplanted males are crossed with wild-type females to pass the insertions through the germline and generate transgenic/knock-out animals.

Figure 4. Identification of transposon insertion sites

After transposition, the genomically integrated transposon (red) is flanked by chromosomal DNA sequences (gray); for simplicity, only one flank is illustrated. Genomic DNA isolated from gene-trap spermatogonial colonies is first digested with a restriction enzyme (blue arrow), followed by ligation of a linker (splinkerette, green) molecule to the ends of the digested DNA. The DNA sequences directly flanking the integrated transposon are PCR-amplified in two, nested PCR reactions by primers that are specific for the splinkerette (green arrows) and for the ends of the transposon (red arrows). PCR products can be directly sequenced after gel purification, or can be first cloned into a plasmid vector followed by sequencing.

Figure 5. Transplanting spermatogonial cultures into rat testes

(a-d) Images illustrate transplantation of rat spermatogonial suspension into the right testis of a busulfan-treated rat. Spermatogonia within a 100 μ l glass injection needle (N) are manually injected into the rete testis (R) located at the base of the efferent ductules (E). Injecting cells into the rete testis will result in retrograde filling of the seminiferous tubules (ST) composing the testis, which is tracked by the addition of trypan blue to the donor cell suspension. V = testicular vasculature; KW = Kimwipe wick. Scale bars: a = 2 mm; b = 2 mm; c = 4 mm; d = 1.5 mm

Table 1

Primer sequences

Primer designation	Sequence
Link1SAU	CGAATCGTAACCGTTCGTACGAGAATCGCTGCCTCTCCAACGAGCCAAGG
Link2SAU	GATCCCTTGGCTCGTTTTTTTTTGCAAAAA
Link3	CGAATCGTAACCGTTCGTACGAGAA
Link4	TCGTACGAGAATCGCTGCCTCTCC
SB LEFT TIR (BAL REV3)	AAAGCCATGACATCATTTTCTGGAATT
SB LEFT TIR (BAL REV)	CTTGTCATGAATTGTGATACAGTGAATTATAAGTG

Primers Link1SAU and Link2SAU are to be annealed to form the splinkerette linker (10 pmol/μl final). Link3 and Link4 are the splinkerette-specific primers, whereas SB LEFT TIR (BAL REV3) and SB LEFT TIR (BAL REV) are the transposon-specific primers to be used in the first and second rounds of PCR, respectively.