

Somatic integration of an oncogene-harboring *Sleeping Beauty* transposon models liver tumor development in the mouse

Corey M. Carlson*[†], Joel L. Frandsen*, Nicole Kirchoff[†], R. Scott McIvor*, and David A. Largaespada*^{††}

*The Arnold and Mabel Beckman Center for Transposon Research, Institute of Human Genetics, Department of Genetics, Cell Biology, and Development, and [†]University of Minnesota Cancer Center, Minneapolis, MN 55455

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The *Sleeping Beauty* (SB) transposon system can integrate foreign sequences of DNA in the genome of mouse somatic cells eliciting long-term expression *in vivo*. This technology holds great promise for human gene therapy as a nonviral technology to deliver therapeutic genes. SB also provides a means to study the effects of defined genetic elements, such as oncogenes, on somatic cells in mice. Here, we test the ability of the SB transposon system to facilitate somatic integration of a transposon containing an activated *NRAS* oncogene in mouse hepatocytes to elicit tumor formation. *NRAS* oncogene-driven tumors developed when such vectors were delivered to the livers of p19^{Arf}-null or heterozygous mice. Delivery of the *NRAS* transposon cooperates with *Arf* loss to cause carcinomas of hepatocellular or biliary origin. These tumors allowed characterization of transposon integration and expression at the single-cell level, revealing robust *NRAS* expression and both transposase-mediated and random insertion of delivered vectors. Random integration and expression of the SB transposase plasmid was also observed in one instance. In addition, studies using effector loop mutants of activated *NRAS* provide evidence that mitogen-activated protein kinase activation alone cannot efficiently induce liver carcinomas. This system can be used to rapidly model tumors caused by defined genetic changes.

cancer | gene therapy | Nras | p19^{Arf}

The genetic modification of mice through transgenesis or gene targeting allows researchers to model tumorigenesis caused by defined genetic changes (1). Specifically, transgenic overexpression of an oncogene or disruption of a tumor suppressor gene predisposes animals to tumors whose growth depends on a known genetic modification. Although these technologies have proven useful to the study of many cancer genes, the continuous expression of germline transgenes in a field of somatic cells does not accurately mimic the spontaneous and sporadic activation of a protooncogene by mutation in an adult somatic cell (2, 3). Tissue-specific and conditional expression of transgenes has helped narrow the spectrum of cell types susceptible to tumorigenesis in transgenic and knockout models (1, 4) but ultimately necessitates the production of a unique transgenic animal for each gene of interest.

Retroviruses have been used to deliver activated oncogenes to mouse somatic cells and recapitulate defined somatic mutations that occur in human cancer. Transgenic mice engineered for cell-specific expression of an avian retrovirus receptor (TVA) have been generated and infected with an avian retrovirus that is otherwise incapable of infecting normal cells (5). Studies have shown the utility of this system to model brain tumors (6) and ovarian carcinoma (7). Replication-defective retroviral vectors have also been used to deliver and integrate genes in mouse somatic cells without the need for transgenesis. This technique has been used to model carcinomas of the rat mammary gland (8) as well as mouse leukemias (9). **However, this technology is not of great utility for the delivery of genetic elements to nondividing cells.**

Sleeping Beauty (SB) is a synthetic transposon system resurrected by site-directed mutagenesis of inactive salmonid transposable

elements (10). The SB transposase recognizes and binds inverted/direct repeats flanking a given sequence of DNA and excises it from its original location, subsequently inserting it at a new location within a TA dinucleotide. SB is active in human cells (10–12), mouse embryonic stem cells (13), the one-cell mouse embryo (14), the mouse germline (15–19), and mouse somatic tissues (17, 20). The SB system has been successfully used as a nonviral method to integrate reporter or therapeutic genes within the lung, liver, and brain of the mouse (20–22). **The ability to achieve long-term expression *in vivo* by using SB suggests that somatic integration of oncogenes using SB is a feasible approach to model molecularly defined tumorigenesis in mouse. Here, we assess the ability of the SB transposon system to integrate activated oncogenes in mouse somatic cells to promote tumorigenesis.**

Materials and Methods

Vector Construction. pT/CMV-GFP (14) was digested with EcoRV and religated to remove GFP. It was then linearized with BclI and ligated to a \approx 750-bp BamHI fragment containing human G12V *NRAS* from pNras12 (23) to form pT/CMV-V12Nras. The Transformer Site-Directed Mutagenesis Kit (Clontech) was used to generate the D38A, T35S, and E37G mutations in the effector-loop domain of *NRAS*. The selection primer is: 5'-Phos.-GCT GGA ATT CTG CAG TTA TCA AGC TTA TCG-3'. The mutagenic primers are: 5'-Phos-CCC ACC ATA GAG GCT TCT TAC AG-3' (D38A), 5'-Phos-GAA TAT GAT CCC TCC ATA GAG G-3' (T35S), and 5'-Phos-CCC ACC ATA GGG GAT TCT TAC-3' (E37G). All vectors were digested with SpeI to excise the cytomegalovirus promoter and religated. Resultant plasmids were digested with XhoI and ligated to a SalI/XhoI fragment of pCaggs-SB10 (16). Resultant plasmids are pT/Caggs-V12Nras, pT/Caggs-V12A38Nras, pT/Caggs-V12S35Nras, and pT/Caggs-V12G37Nras. pPGK-SB13 contains a version of the SB10 transposase with two hyperactive mutations reported by Yant *et al.* (24) (T83A, K33A) (J. R. Ohlfest, personal communication) regulated by the human phosphoglycerate kinase (PGK) promoter taken from pKO Select-Puro (Stratagene).

Mice and Hydrodynamic Injection. C57BL/6J p19^{Arf}-null mice were kindly provided by Martine Roussel of St. Jude Children's Research Hospital (Memphis, TN). Plasmids were prepared by using the Qiagen (Valencia, CA) EndoFree Maxi Kit. Animals received a 2:1 molar ratio of transposon to transposase-encoding plasmid. Twen-

Conflict of interest statement: D.A.L. and R.S.M. are cofounders of, and consultants to, Discovery Genomics, Inc. (DGI), a biotechnology company that has licensed SB technology from the University of Minnesota. DGI is pursuing human gene therapy using SB. The work reported in this manuscript is entirely independent of DGI personnel or resources.

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Abbreviations: SB, *Sleeping Beauty*; PGK, phosphoglycerate kinase; RFLP, restriction fragment length polymorphism;

[†]To whom correspondence should be addressed. E-mail: larga002@umn.edu.

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Table 1. Experimental groups

Group	Genotype	Constructs injected	No. harboring tumors (tumor multiplicity)
1	<i>Arf</i> $-/-$	pT/Caggs-V12Nras + pPGK-SB13	8/8 (TNTC)
2	<i>Arf</i> $-/-$	pT/Caggs-V12Nras + pPGK	4/7 (0-1)
3	<i>Arf</i> $-/-$	pT/Caggs-V12A38Nras + pPGK-SB13	1/7* (NA)
4	<i>Arf</i> $-/-$	pT2/Caggs-Luciferase + pPGK-SB13	0/5 (NA)
5	<i>Arf</i> $+/-$	pT/Caggs-V12Nras + pPGK-SB13	7/8 (2-10)
6	<i>Arf</i> $-/-$	pT/Caggs-V12S35Nras + pPGK-SB13	2/3 [†] (1)
7	<i>Arf</i> $-/-$	pT/Caggs-V12G37Nras + pPGK-SB13	0/3 (NA)

Each group received a different combination of transposon and transposase and was monitored for sickness. When moribund, liver tumor burden was assessed, and approximate numbers are indicated. TNTC, too numerous to count; NA, not applicable.

*This mouse developed a different tumor type than the others.

[†]The third mouse died during the study and was not recovered.

ty-five micrograms of pT/Caggs-V12Nras was used for Groups 1 and 5 (Table 1), and this plasmid was used as the molar standard, i.e., each animal received the same molar amount of transposon and transposase-encoding plasmid. DNA was suspended in lactated ringers at a final volume 10% the weight of the animal and injected via tail vein in ≤ 10 sec (25, 26).

Histopathology. Animals were killed when moribund. Tissues were fixed in 10% formalin and paraffin-embedded sections were stained with hematoxylin/eosin. Select sections were also immunostained with pan-cytokeratin (Dako AE1/AE3 clone, DakoCytomation, DAKO) or assayed with myeloperoxidase and chloroacetate esterase.

Cell Line Production. Individual *Arf* $-/-$ liver tumors were placed in ASM (DMEM-high glucose + glutamine, nonessential amino acids, pen/strep, 10 mM Hepes/1 mM sodium pyruvate/10% FBS/0.2 units/ml insulin/ 10^{-5} M 2-mercaptoethanol/1 mM oxaloacetic acid/10% medium NCTC-109), dissociated, and passed through a 70- μ m cell strainer. Cells were subcultured at least once before DNA and protein extraction.

Southern Hybridization. DNA extracted from tumors was analyzed for transposon integrants by Southern hybridization, as described (27). Five micrograms of genomic DNA was digested with SacI, electrophoresed on a 1% agarose gel, and transferred to a nylon membrane. pT/Caggs-V12Nras was digested with EcoRI to generate a 1,755-bp probe, consisting of the Caggs promoter. An 896-bp probe, consisting of sequences immediately flanking the left inverted/direct repeat within the plasmid backbone, was generated by digesting pT/Caggs-V12Nras with PvuI.

Linker-Mediated PCR. Transposon-chromosome junctions were recovered by linker-mediated PCR, as described (28-30).

Western Blotting. Cells or primary tumors were lysed in IPWB buffer [50 mM Tris, pH 7.4/14.6 mg/ml NaCl/2 mM EDTA/2.1 mg/ml NaF/1% Nonidet P-40/1 mM NaVO₄/1 mM Na₂PO₄ with protease inhibitors (Roche Diagnostics, Mannheim, Germany)]. For Fig. 6B, 293T cells were transiently transfected (CellPfect Transfection Kit, Amersham Pharmacia Biosciences) with the indicated forms of NRAS and serum-starved. Samples were electrophoresed on a 12% acrylamide (29:1) gel and transferred to nitrocellulose (Bio-Rad) by using the NuPAGE system (Invitrogen). Appropriate antibodies were used to detect the EE (Glu-Glu) epitope tag (Covance/Babco, Richmond, CA) on N ras, SB transposase (custom polyclonal), phosphorylated Erk (Cell Signaling Technology, Beverly, MA), and total Erk-1 (Santa Cruz Biotechnology).

Results

Modeling Spontaneous Molecularly Defined Tumors in Mouse Liver Using Transposons.

To test whether the SB transposon system can be used to model tumors caused by defined genetic elements, p19*Arf*-null or heterozygous mice were coadministered two plasmids (Fig. 1A). The first contains a transposon that expresses either a constitutively active human *NRAS* oncogene (pT/Caggs-V12Nras), an effector loop mutant of activated human *NRAS* (pT/Caggs-V12A38Nras), or the firefly luciferase gene (pT2/Caggs-Luciferase), each regulated by the ubiquitous Caggs promoter. The second plasmid encodes the SB transposase via the human PGK promoter (pPGK-SB13) or harbors the PGK promoter alone (pPGK) (Fig. 1B). A hydrodynamics-based delivery technique was used to deliver naked plasmid DNA to mouse liver (25, 26). Ten-week-old animals received the combinations of plasmids indicated in Table 1 in a 2:1 molar ratio of transposon to transposase. Animals were aged, monitored for sickness, and killed when moribund. All mice were killed at 181 days (6 mos) postinjection if they had not succumbed to illness.

All *Arf* $-/-$ recipients (8/8) of a transposon encoding a constitutively active human *NRAS* gene (G12V) and the SB transposase-encoding plasmid (Group 1) developed multiple, nodular, hepatic neoplasms (Fig. 2A), becoming moribund at an average latency of 43 days postinjection (Fig. 2B). A percentage of these animals (3/8) also developed tumors at the base of the tail. This may be attributed to the pooling of injected material here during injection. Most (7/8)

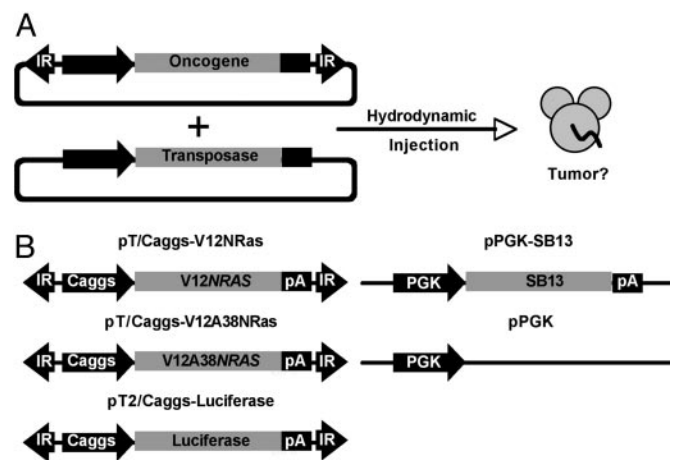


Fig. 1. Experimental design and constructs. (A) Transposition-mediated somatic integration of an oncogene could be used to elicit tumors of defined genetic origin. (B) Transposon and transposase constructs used to test this possibility (IR, inverted/direct repeats; pA, polyadenylation signal).

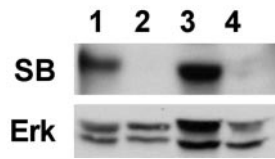


Fig. 5. Stable somatic expression of the transposase plasmid. Expression of SB transposase protein was detected by Western blot in one tumor-derived cell line (lane 1, HeLa cells transiently transfected with pCMV-SB11; lane 2, 293T cells transfected with pT/Caggs-V12Nras; lane 3, tumor-derived cell line; lane 4, uninjected *Arf*^{-/-} liver).

sion, Western blot analysis using a polyclonal antibody specific for the transposase was performed. SB transposase protein was detected in one of the tumor-derived cell lines (Fig. 5) but was absent in all others tested (data not shown).

Testing of Mutant *NRAS* Constructs. This technology can feasibly be used to assess the oncogenic potential of mutant or candidate oncogenes. We thus tested two effector loop mutants of *NRAS* predicted to signal exclusively to a particular downstream effector pathway of ras. To determine whether *NRAS* signaling to either Raf or Ral-GDS alone is sufficient to promote liver tumorigenesis, versions of the constitutively active *NRAS* containing mutations at sites that have been characterized in *HRAS* to limit signaling to either Raf (V12S35) or Ral (V12G37) were generated (Fig. 6A) (31). Human *NRAS* and *HRAS* are 85% identical, including 100% identity of the first 85 amino acids, which contain the effector loop domain of ras. Transient transfection experiments demonstrated that the mitogen-activated protein kinase signaling of these two mutants was consistent with their predicted activities (Fig. 6B). Both effector loop mutants of the activated *NRAS* were coinjected with the transposase-encoding plasmid (Groups 6 and 7, Table 1). After 6 mo, two of three *Arf*^{-/-} recipients of the V12S35 double mutant harbored at least one tumor (Table 1, Fig. 6C and D). These mice did not, however, develop the multifocal liver tumors observed in recipients of the activated *NRAS* with an intact effector loop domain. The third mouse from Group 6 died during the study, and the liver was not recovered. None of three V12G37 recipients developed tumors within 6 mo.

Discussion

We demonstrate effective use of the SB transposon system to integrate activated oncogenes in mouse somatic cells to model

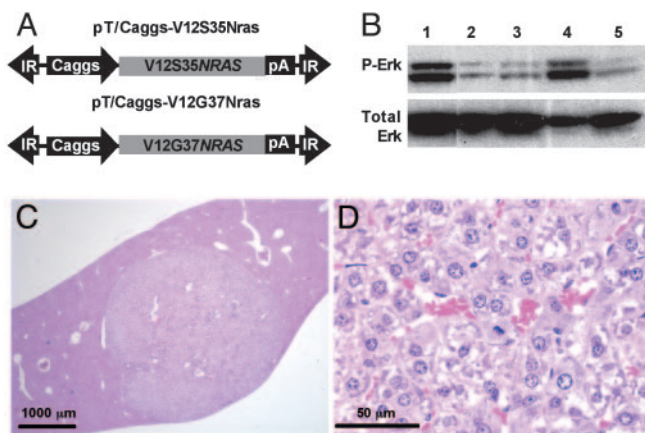


Fig. 6. Testing effector loop mutants of activated *NRAS*. (A) Activated *NRAS* transposons were constructed harboring either the T35S or E37G mutations. (B) Erk phosphorylation assay indicates mitogen-activated protein kinase signaling of the S35 but not G37 mutant in serum-starved 293T cells (lane 1, V12S35; lane 2, V12G37; lane 3, V12A38; lane 4, V12; 5, untransfected). (C and D) Hepatocellular carcinomas from two distinct recipients of the V12S35 effector loop mutant.

tumor formation. An activated human *NRAS* transposon plasmid was codelivered with a plasmid expressing the SB transposase to mouse liver via hydrodynamic injection (25, 26). Both p19*Arf*-null and heterozygous recipients of these plasmids developed multifocal liver cancer. Resulting tumors are carcinomas of hepatocellular and biliary (cholangiocarcinomas) origin. Both transposase-mediated and random integration events were observed, and N-ras protein was expressed in all tumors. A plasmid containing the SB expression cassette was also integrated randomly in a percentage of cases and sometimes expressed. We have used this system to test mutant versions of the *NRAS* oncogene, encoding proteins with altered signaling potential, for their tumor-inducing properties.

Similar initial experiments using wild-type mice failed to yield any liver tumors, presumably due to the number of genetic insults necessary for tumorigenesis (data not shown). A constitutively active *NRAS* oncogene is insufficient to promote efficient liver tumorigenesis on an otherwise wild-type background, consistent with previous work demonstrating that activated *NRAS* does not transform primary cells but instead promotes cell senescence (32). p19*Arf* is a positive regulator of the p53 tumor suppressor, and loss of *Arf* predisposes to a wide spectrum of tumors (33). *NRAS* appears to cooperate with *Arf* deficiency to promote tumorigenesis in the liver. This is consistent with previous work demonstrating cooperation of the *Arf* and *ras* pathways in cellular transformation (34) and the ability of *NRAS* to transform a nontumorigenic liver epithelial cell line (35). Interestingly, *NRAS* combined with a heterozygous *Arf* mutation was sufficient to promote tumorigenesis in this model without observable loss of heterozygosity, although at a much-reduced penetrance. However, as noted, the possibility remains that the remaining wild-type *Arf* allele may be silenced by an alternative mechanism (e.g., epigenetic).

Liver tumors generated in this model included both hepatocellular carcinomas or biliary tract tumors known as cholangiocarcinomas. Both human cholangiocarcinomas (36, 37) and hepatocellular carcinomas (38) are known to harbor defects in the p53 pathway and *RAS* oncogene activation, suggesting that this model may recapitulate a disease reminiscent of that which occurs in humans. Cellular transformation was also noted in tissues besides the liver using the hydrodynamic delivery method. We observed partially cytokeratin-positive anaplastic soft-tissue tumors at the base of the tail and myeloid hyperplasia with an expansion of granulocytes and their precursors in the spleen. The masses at the base of the tail appear to represent genuine tumors, as evidenced by the presence of clonal integrated transposons and expression of N ras. Although the spleen myeloid hyperplasia depends on coadministration of both *NRAS* and transposase plasmids in *Arf*^{-/-} mice, it is unclear whether this manifestation is a clonal process.

The applications of this technology to tumor biology are not limited to simply generating liver tumors caused by known genetic insults. We have shown that the analysis of mutant or candidate oncogenes can be facilitated by this technology. A mutant version of *NRAS* predicted to signal exclusively to the Raf-mitogen-activated protein kinase (MAPK) pathway was sufficient to promote hepatocellular transformation on the *Arf*^{-/-} background but with greatly reduced efficiency, whereas the Ral-specific *NRAS* did not elicit any observable tumors. This is consistent with previous work demonstrating the importance of the Raf effector of Ras in the transformation of mouse cells (39, 40). Signaling to Raf, however, was insufficient to produce the multifocal liver disease observed in recipients of the activated *NRAS* oncogene with a wild-type effector loop. Also, the tumors from recipients of this mutant harbored hepatocellular carcinomas but not cholangiocarcinomas. Additional constitutive signaling to alternative downstream pathways (e.g., Ral and PI3K) likely dramatically increases the efficiency of transformation elicited by the Raf-MAPK pathway to produce the multifocal pathology.

Other modes of delivery could be used to initiate tumorigenesis in alternative tissue types. For example, polyethylenimine has been

shown to be an effective method to deliver naked SB transposon DNA to the lung (21). Tissue-specific promoters could also be incorporated to limit expression to a cell type of interest. One could also feasibly use a transposon expressing a siRNA specific for a tumor suppressor gene to model tumors (41). In addition, cDNA libraries might be screened for cancer-promoting genes using this system. A library could be cloned downstream of a strong promoter and delivered to somatic cells of the mouse. Transposon insertions from any tumors that develop could subsequently be sequenced and putative responsible genes identified.

These experiments enabled characterization of somatic cell gene delivery of transposon and transposase at the single-cell level. Because tumors generally initiate from single transformed cells, the outgrowth of such cells produces a clonal population of cells allowing analysis of transposon insertion and expression. An average of 4.6 RFLPs that may represent transposase-mediated insertions was observed in primary tumors and tumor-derived cell lines. In addition, bands corresponding to random integration of the entire plasmid were also observed. In fact, half of the mice that received the *NRAS* transposon plasmid without transposase developed one to two liver tumors each. Because random integration may account for approximately one to two RFLPs on a Southern blot, the insertion rate observed here is roughly equivalent to that observed in experiments performed in HeLa cells (approximately three integration events per stable clone) (11). However, random integrants are rarely observed in transposition assays performed in HeLa cells (10, 11). Also, a very low frequency of random integration (<2%) was estimated in studies using the hydrodynamic delivery method and the SB transposon system to treat mice suffering from tyrosinemia I (42). Random integration of episomal plasmid DNA may be unique to rapidly dividing transformed cells.

The transposase-encoding plasmid was also integrated in a percentage of cases, resulting in expression in one instance. This underscores the need for increased safety testing if DNA molecules are to be used to deliver transposase in gene therapy clinical trials using SB. Stable expression of the transposase could cause genomic instability and insertional mutagenesis attributed to continuous transposition in the cell. In fact, in recent work, we demonstrated that SB can be used to induce cancer in wild-type or tumor-prone mice via random insertional mutagenesis (28, 29). To reduce this potential risk, transposase RNA or protein could be delivered with transposon DNA to provide a transient burst of transposase enzyme expression. This will ultimately be the ideal way to deliver the transposase if this system is to be successfully used as a vehicle for human gene therapy.

This system provides researchers with an effective way to model molecularly defined somatic mutations and tumors in the mouse. This method has significant advantages over other technologies used to model tumorigenesis. It has the capability of modeling spontaneous sporadic tumors of known molecular origin without the complications and expense associated with mouse germline transgenesis or knockout approaches. In addition, unlike retroviruses, this system is less limited by cell type. This technology also affords other unique applications to the study of tumor biology.

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