Published in final edited form as:

Prostate. 2014 May ; 74(7): 781–791. doi:10.1002/pros.22797.

A novel method for somatic transgenesis of the mouse prostate using the Sleeping Beauty transposon system

Kimberly D.P. Hammer, Jim Alsop, Rita A. Buresh-Stiemke, Katsiaryna Frantskevich, Rita Malinowski, Laura Roethe, Ginny L Powers, and Paul C. Marker

Division of Pharmaceutical Sciences, School of Pharmacy and University of Wisconsin Carbone Cancer Center; University of Wisconsin-Madison, Madison, Wisconsin

Abstract

BACKGROUND—*In vivo* ectopic gene expression is a common approach for prostate research through the use of transgenes in germline transgenic mice. For some other organs, somatic transgenesis with the Sleeping Beauty transposon system has allowed *in vivo* ectopic gene expression with higher throughput and lower cost than germline transgenic approaches.

METHODS—Mouse e16 urogenital sinuses (UGSs) were co-injected with plasmids expressing the Sleeping Beauty transposase and plasmids with control or activated *BRAF* expressing transposons. Following electroporation, the transduced UGSs were grown as allografts in mouse hosts for 8 weeks, and the resulting allografts were evaluated for several endpoints.

RESULTS—Transposon-transduced UGS allografts developed into prostatic tissue with normal tissue structure and cellular differentiation. Integration of transposon vectors into the genomes of transduced allografts was confirmed using linker-mediated PCR, sequencing, and *in situ* PCR. Transduction of UGS allografts with transposons expressing activated *BRAF* resulted in ectopic *BRAF* expression that was detectable at both the mRNA and protein levels. Prostatic ducts over-expressing activated *BRAF* also had ectopic activation of the ERK1/2 mitogen activated kinases and increased epithelial cell proliferation.

CONCLUSIONS—The Sleeping Beauty transposon system can be used to achieve somatic transgenesis of prostatic allografts. This new method for achieving ectopic gene expression in the prostate will complement other existing approaches such as ectopic gene expression in cell lines and in germline transgenic mice. Advantages of this new approach include preservation of stromal-epithelial interactions not possible with cell lines, and higher throughput and lower cost than traditional germline transgenic approaches.

Keywords

Sleeping Beauty transposon; transgenic; mouse prostate; BRAF; ERK1; ERK2

Introduction

There is considerable interest in understanding the molecular and genetic pathways that regulate the biology of the prostate gland during normal development, during normal reproductive function, and during the progression of prostatic diseases including prostate cancer and benign prostatic hyperplasia. Although a number of experimental model systems are used to study the prostate, recent years have seen an increasing reliance upon mice due to the relative ease of genetic manipulations that can alter gene expression in the mouse prostate. One common type of genetic manipulation is ectopic gene expression in the mouse prostate using transgenic techniques to modify the germline (1). Such studies have been facilitated by the identification of promoters such as the ARR2PB version of the rat probasin promoter that can drive robust and prostate-specific expression of genes in transgenic mice (2). While extremely useful, germline transgenic approaches are very time and labor intensive. Single cell mouse embryos must be injected with the transgene and implanted into pseudopregnant females. The resulting progeny must be screened for transgenic founders and then bred, often for multiple generations, before the desired experiments can be conducted.

Other common approaches for investigating the mouse prostate include the study of prostatic organ rudiments grown using *in vitro* culture techniques and the study of prostate allografts grown under the renal capsules of nude mice (3). In the context of prostate organ cultures, transient ectopic gene expression has been achieved using electroporation to introduce plasmid expression constructs into the urogenital sinus (UGS), the embryonic precursor of the prostate gland (4). Prostate allografts have also been conducted with genetically modified prostates. However, these have typically been allografts of prostates from mice with germline genetic modifications such as prostate organ rescue experiments using embryos with germline deletions of *Fgf10* or *Shh* that cause embryonic or perinatal lethality before the prostate fully develops (5,6). One exception is the mouse prostate reconstitution (MPR) model in which retroviruses have been used to achieve ectopic gene expression in prostate allografts (7).

This study explores the potential utility of the Sleeping Beauty (SB) transposon system for genetically modifying prostate allografts. The SB system consists of two parts. The SB transposase enzyme and a DNA transposon that consists of two inverted repeat, direct repeat (IRDR) elements that define the left and right boundaries of the transposon (8). The SB transposase binds to the IRDR elements and mobilizes the transposon by a cut-and-paste mechanism. The DNA cargo between the two IRDR elements is also mobilized and can be of variable size and sequence composition. SB can mediate transposition from one location in the genome to another, or it can mediate transposition from an extra-genomic plasmid into the genome. The capacity to integrate transposons from a plasmid donor into a host cell's genome has previously been used to achieve somatic transgenesis in several mouse tissues including the liver, lung, and components of the hematopoietic system (9-11).

For the present study, we investigated the utility of the SB system for achieving transgenesis in the mouse prostate. Both empty vector control transposons and experimental transposons expressing a truncated and constitutively activated form of BRAF were successfully

introduced into the epithelium of the mouse UGS. When transduced UGSs were grown as prostate allografts under the renal capsules of nude mice, a subset of prostatic ducts retained the transgenes. Furthermore, in the case of activated BRAF, functional expression of the transgene was demonstrated by the activation of the ERK1/2 mitogen activated kinases and increased epithelial proliferation for transgenic prostatic ducts.

Materials and Methods

UGS transduction and allografting

Part of the male embryonic day 16 (e16) lower urinary tract including the bladder, urogenital sinus (UGS), and a segment of urethra distal to the UGS was dissected and co-transfected with two plasmids, one containing a transposon vector and the other an SB11 transposase expression construct. Transposon plasmids included T2-*BRAF*, containing a cDNA encoding a C-terminal truncated kinase domain (12) and pT2/HB as an empty vector transposon control plasmid (13). The transposase expressing plasmid was pCMV-SB11 (14). For the transfections, plasmid DNA at 10 µg/µl (10:1 ratio of transposon vector plasmid to SB11 expressing plasmid) was mixed with 2 mg/ml BSA and 0.1% (w/v) Fast Green FCF dye (Sigma-Aldrich) immediately before use and approximately 0.5 µl injected into the lumen of the urethra and UGS using a 36-gauge needle and 10-µl syringe (World Precision Instruments, Sarasota, Florida). Tissues were immediately electroporated (Gene Pulser Xcell, BioRad Instruments, Hercules, California) in a 5mm cuvette with 10 mM Tri-HCl, pH 7.5, 1 mM EDTA buffer using a square wave profile (100 volts, 20 msec pulse/1 sec interval, 7 pulses). Following electroporation, the UGS was dissected away from the bladder and urethra and cultured overnight. For the cultures, UGSs were placed on Millicell-CM Culture Plate Inserts (30 mm, 0.4 µm pore size; Millipore Corp) in 4-well plates (Nunc A/S, Roskilde, Denmark) at the air/medium interface and floated over 0.5 ml of medium consisting of DMEM/F12 50/50 mix supplemented with 0.37 g/L L-glutamine, 10 U/ml penicillin, 10 µg/ml streptomycin, 1X insulin, transferin, and selenium (ITS), and 0.5% DMSO, and 10⁻⁸ M testosterone. Following an overnight culture in a 5% CO₂, 37°C incubator, UGSs were surgically grafted under the kidney capsules of male CD-1 nu/nu mice (Charles River). Allografts were grown *in vivo* for 8 weeks at which time the mice were sacrificed and allografts were excised and photographed. Half of the allograft was fixed in 10% buffered formalin and embedded in paraffin blocks for histology and immunohistochemistry while the other half was snap-frozen and later used for the isolation of RNA and/or DNA. All animal experimentation in this study was conducted in accord with accepted standards of humane animal care as outlined in the NIH Guide for the Care and Use of Laboratory Animals, and the experimentation was approved by the Animal Care and Use Committee of the University of Wisconsin-Madison.

Immunohistochemistry

Tissue sections were cut at 6-µm thickness, deparaffinized and rehydrated through a series of graded ethanols. Slides underwent antigen retrieval by boiling for 30 minutes in antigen unmasking solution (Vector Laboratories). Endogenous peroxidases were quenched with 3% (v/v) hydrogen peroxide solution for 10 minutes. For antibodies against E-cadherin (Cell Signaling Technology; 1:400 dilution), wide spectrum cytokeratin (Dako; 1:250 dilution),

and BRAF (Santa Cruz Biotechnology; 1:500 dilution), slides were blocked with 2.5%(v/v) sheep serum at room temperature for four hours before incubating overnight with primary antibody. Following a series of washes, slides were incubated with secondary biotinylated anti-rabbit antibody (Vector Laboratories; 1:500 dilution) for one hour at room temperature. For antibodies against Ki67 (Vector Laboratories; 1:100 dilution), p63 (Santa Cruz Biotechnology; 1:400 dilution), and smooth muscle actin (Sigma-Aldrich; 1:500 dilution), slides were blocked with M.O.M. blocking reagents (Vector Laboratories) at room temperature for four hours before incubating overnight with primary antibody. Following a series of washes, slides were incubated with secondary biotinylated anti-mouse antibody (Vector Laboratories; 1:200 dilution) for one hour at room temperature. For the antibody against phospho-ERK1/2 (Cell Signaling Technology; 1:200 dilution) slides were blocked with 5%(v/v) goat serum at room temperature for four hours before incubating overnight with primary antibody. Following a series of washes, slides were incubated with secondary biotinylated anti-rabbit antibody (Vector Laboratories; 1:500 dilution) for one hour at room temperature. After incubation with secondary antibodies, slides were washed and incubated with the Vectastain ABC reagent (Vector Laboratories) for 30 minutes at room temperature, washed again, and stained using peroxidase substrate kit DAB (Vector Laboratories). Finally slides were counterstained with hematoxylin, dehydrated and mounted.

Transposon-genome junction fragment isolation and analysis

Transposon-genome junction fragments were amplified from transduced allograft DNA using linker-mediated PCR (LM-PCR) as previously described (15). LM-PCR products were cloned using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Cloned LM-PCR products were subjected to Sanger sequencing by the University of Wisconsin-Madison Biotechnology Center. Sequences were examined for the presence of transposon-genome junctions, and the genomic sequences were aligned to assembly GRCm38 of the mouse genome using the BLAT search tool available at ensembl.org.

RT-PCR

RNA was extracted from ducts dissected from 4 allograft samples for each treatment (T2/HB empty vector control, T2-*Braf*) and was reverse transcribed according to previously published methods (16). For quantitative studies of total *Braf* transcripts, RNA was subjected to RT-PCR on a StepOnePlus system (Applied Biosystems). Relative *Braf* mRNA expression levels were normalized to an *18S ribosomal RNA* and calculated as described previously (17). *Braf* primers were forward 5' GGA GGA CAG AA GTC GGA TGA 3' and reverse 5' GTG TGG GTG CTG TCA CAT TC 3' (amplicon 179 bp) and 18S rRNA primers were forward 5' GAA TGG GAT GGT CTT AGT A 3' and reverse 5' TGG ACT GAA CAG TGA ATT 3'. For transgene-specific RT-PCR analysis, primers were designed to amplify the junction of the vector and truncated form of *Braf*. Outer (forward 5' GGA CTG GCT ACT TGA AGG CT 3' and reverse 5' GTG TGG GTG CTG TCA CAT TC 3') and inner (forward 5' CGC CCA TCA AGC TTG CTA CT 3' and reverse 5' ATC CGA CTT CTG TCC TCC GA 3') primers were designed to anneal to the vector and truncated *Braf* insert. 1 µg of RNA from T2-*Braf* treated UGSs were reverse transcribed. Outer primers amplified a 379bp product. The amplicon was isolated using a PCR purification kit

(QIAquick, Qiagen). A second round of PCR was performed on this amplicon using the inner primers, which resulted in a 195bp band. DNA sequencing confirmed the junction sequence of the transcript derived from the T2-*Braf* vector. 18S primers were used for a control.

In situ PCR

Sections were cut at 6 μ M thickness, and rehydrated through a series of graded ethanols. Slides underwent proteinase K (5 μ g/mL) digestion in 1X PBS at 37°C for 30 min and then heated in the microwave in 1X PBS for 60 sec. For primary PCR, 150 μ l of PCR master mix (1X Amplitaq PCR buffer (Invitrogen), 200 μ M each of dATP, dCTP, dGTP, and dTTP (Invitrogen), 4 mM MgCl₂, 6.4% BSA, 5 U/ μ l Amplitaq DNA polymerase (Invitrogen), and 20 pM both sense and anti-sense primers) was added, a coverslip was applied, and the slide was sealed with clear nail polish. Primary PCR primers were 5' CAG TCC TCC GAT AGA CTG CG 3' and 5' GGA GGA CAG AAG TCG GAT GA 3'. Slides were placed in a glass slide adaptor (MJ Research) and cycled as follows: 94°C 8 min; 30 cycles of (94°C 1 min, 56°C 0.5 min, 72°C 2 min); and a final hold at 72°C for 15 min. Following the primary PCR reaction, slides were washed with 100% EtOH 3 x 5 min each to remove coverslips and further washed with 1x PBS with 1% Tween. Secondary PCR conditions were identical to the primary PCR except 5 mM biotin-16-dUTP (Roche) was added to the Master mix recipe and primers for secondary PCR were 5' GGA GGA CAG AGT CGG ATG A 3' and 5' GTG TGG GTG CTG TCA CAT TC 3'. Following the secondary PCR, cover slips were removed, slides were washed and then incubated with the vectastain ABC reagent (Vector Laboratories) for 30 minutes at room temperature, washed again, and stained using the peroxidase substrate kit DAB (Vector Laboratories). Slides were counterstained with hematoxylin, dehydrated, and mounted with permount.

Results

The mouse UGS will develop into a prostate when grafted under the renal capsule of a male host mouse (3). We have also previously shown that short-term ectopic gene expression could be achieved in the UGS when expression plasmids were introduced by electroporation (4). This led us to explore the possibility of achieving durable ectopic gene expression in the prostate by using the Sleeping Beauty transposon system to integrate expression vectors into prostate progenitor cells present in the UGS followed by renal capsule grafting (Fig. 1A, overview of strategy). Although the UGS could be successfully transduced as an isolated tissue, we achieved most consistent results when a segment of the lower urinary tract including the bladder, UGS, and a segment of urethra distal to the UGS was dissected and kept intact through the electroporation step. At e16, the lumens of the urogenital sinus (Fig 1B, arrow) and urethra (Fig 1B, arrowhead) were clearly visible under a dissecting microscope. At this stage, a 36 gauge needle was inserted into the lumen of the UGS (Fig 1C) and approximately 0.5 μ l of a plasmid DNA solution (10 μ g/ μ l, 10:1 ratio of transposon vector plasmid to SB11 expressing plasmid with 2 mg/ml BSA and 0.1% Fast Green FCF dye) was injected. The presence of the Fast Green dye in the DNA solution allowed visual confirmation of successful injections (Fig. 1D). Immediately following DNA injection, the bladder/UGS/urethra was immediately subjected to electroporation (square wave profile,

100 volts, 20 msec pulse, 1 sec interval, 7 pulses). Following electroporation, the UGS was dissected away from the bladder and urethra and cultured overnight in a serum-free medium. The next day, UGSs were surgically grafted under the kidney capsules of male CD-1 nu/nu mice and grown for 8 weeks *in vivo*. Allografts included a group transduced with the T2/HB empty vector control transposon, a group transduced with the T2-*Braf* transposon expressing a truncated and constitutively activated form of BRAF, and a control group of UGSs not subjected to DNA injection and electroporation. For all groups, the resulting allografts grew on the surface of the kidney into ductal structures resembling normal mouse prostate (Fig 1E and data not shown). Although the mean weight of allografts transduced with T2-*Braf* was higher than empty vector controls (Fig. 1F), there was substantial variability in weights for both the experimental and control groups such that observed differences were not statistically significant. The tissue organization and cellular differentiation of allografts was evaluated using histology and immunohistochemistry. In the case of T2/HB empty vector transposon transduced allografts, hematoxylin and eosin stained sections confirmed the presence of prostatic ducts that resembled normal mouse prostate (Fig. 2A, B). The ducts contained a continuous layer of luminal cells that stained positive for epithelial cytokeratins (Fig 2C) and E-cadherin (Fig. 2D). The ducts also contained a discontinuous layer of basal epithelial cells that stained positive for p63 (Fig. 2E), and they contained a thin stromal layer that surrounded the epithelium and stained positive for smooth muscle actin (Fig. 2F). In the case of T2-*Braf* transposon transduced allografts, hematoxylin and eosin stained sections also confirmed the presence of prostatic ducts that resembled normal mouse prostate (Fig. 3A, B). These ducts also had normal tissue organization as revealed by staining for epithelial cytokeratins, E-cadherin, p63, and smooth muscle actin (data not shown).

Prior to embedding for histology, a portion of some allografts was dissected away and used for the isolation of RNA and DNA. *Braf* transcript levels were analyzed for T2/HB empty vector transduced and T2-*Braf* transduced allografts using real-time RT-PCR. An overall increase in *Braf* transcripts was observed in the T2-*Braf* transduced allografts (Fig. 3C) suggesting at least some cells in the allografts expressed the transgene. RT-PCR was also conducted using a primer pair directed against sequences present in *Braf* transcripts expressed from the transposon expression vector but not present in endogenous *Braf*. This amplified bands of the predicted size for transgene-derived *Braf* in 3 of 4 T2-*Braf* transduced allografts (Fig. 3D). The identity of these bands as transposon-derived truncated *Braf* transcripts was confirmed by sequencing (data not shown). DNA from T2-*Braf* transduced allografts was also subjected to linker-mediated PCR to amplify transposon-genome junction fragments. A smear of different sized linker-mediated PCR products was observed in individual allografts (Fig. 3E). This suggested that allografts contained multiple independent transposon insertions leading to varying sized linker-mediated PCR products derived from multiple transposon-genome junction fragments. This was confirmed by cloning and sequencing of linker-mediated PCR products from a representative T2-*Braf* transduced allograft and identifying the presence of multiple independent transposon insertions at different locations in the genome (Table 1).

To further understand the pattern of transgenesis within T2-*Braf* transduced allografts, they were evaluated along with empty vector controls using immunohistochemistry for the

carboxy-terminal domain of BRAF expressed by the transposon vector. Empty vector allografts had little BRAF staining (Fig. 4A) while T2-*Braf* transduced allografts contained groups of ducts that stained strongly for BRAF (example indicated by arrow in Fig. 4B) and other groups of ducts that stained weakly for BRAF (example indicated by arrowhead in Fig. 4B). At higher magnification, it was clear that the strong BRAF expression occurred as continuous epithelial staining in some ducts (Fig. 4E, epi) with little staining in the associated stroma (Fig. 4E, str). It was anticipated that expression of the truncated and constitutively activated form of BRAF would activate the ERK1 and ERK2 mitogen activated kinases (ERK1/2) so near-adjacent tissue sections to those stained for BRAF were also stained with an antibody against the phosphorylated and activated form of ERK1/2. Empty vector allografts had little activated ERK1/2 staining (Fig. 4C) while the same groups of ducts in T2-*Braf* transduced allografts that had strong BRAF staining also stained strongly for activated ERK1/2 (Fig. 4D arrow). Other groups of ducts with little staining for BRAF also had weak staining for activated ERK1/2 (Fig. 4D arrowhead). These data suggested that the allografts had a mosaic pattern of expression for the BRAF transgene with some groups of ducts strongly expressing the transgene throughout the epithelium and other ducts not expressing the transgene. To further clarify whether this pattern reflected the presence/absence of an integrated transposon vector in some ducts but not others, we developed an *in situ* PCR assay to directly detect the T2-*Braf* transposon vector in tissue sections. Staining of near-adjacent T2-*Braf* transduced allograft tissue sections with anti-BRAF (Fig. 4 E,H), anti-phospho-ERK1/2 (Fig. 4 F,I) and *in situ* PCR for the T2-*Braf* transposon vector (Fig. 4 G,J) suggested that ducts with high expression of BRAF and activated ERK1/2 had also incorporated the transposon vector (compare E-G) while ducts with little BRAF or activated ERK1/2 had not incorporated the transposon vector (compare H-J). We further quantified ducts with ectopic expression of BRAF expression and ectopic activation of ERK1/2 in 6 allografts to estimate the extent of transgenesis achieved using electroporation of the UGS with transposon vectors. Transgene expressing ducts were present in 6/6 allografts, and the percentage of ducts with transgene-expressing cells within a particular allograft ranged from 35% to 54% (overall 778 transgene-expressing ducts were observed among 1753 counted ducts). We also stained near-adjacent tissue sections by *in situ* PCR for T2-*Braf* and for the proliferation associated marker Ki67 (18) and showed that epithelial proliferation was increased for ducts that had integrated the BRAF transgene compared to ducts transduced with the empty vector transposon (Fig. 5).

Discussion

Ectopic gene expression is a powerful approach for interrogating gene function in the prostate gland. The most common method for achieving ectopic gene expression is through the use of germline transgenesis in the mouse. This has been most extensively and successfully used for studies on the molecular mechanisms driving prostate cancer initiation and/or progression (19). Relatively few alternatives to germline transgenesis have been available for achieving ectopic gene expression in the prostate. One successful approach was the mouse prostate reconstitution (MPR) model that was initially used to investigate the roles of ras and myc in prostate cancer (20,21). In this model, dissociated UGSs were infected with replication deficient retroviral vectors that carried ras and/or myc transgenes,

and then the UGSs were reconstituted and grafted into male hosts. This gave expression of transgenes in approximately 0.1% of cells and was sufficient to induce multistage carcinogenesis in response to ras + myc. A variant of the MPR model has also been described that utilizes UGS mesenchyme combined with adult mouse prostate epithelial cells and uses lentiviral vectors for gene transfer (22). Comparing our results to the MPR model, there were both similarities and differences. In both cases, gene transfer was achieved by introducing expression constructs into cells of the UGS followed by grafting under the renal capsules of male mouse hosts, and both approaches achieved durable somatic transgenesis and long-term gene expression. In the case of the MPR model, the retroviral vectors infected both stromal and epithelial cells with the majority of infected cells present in the stroma. In their study, Thompson et al. estimated that epithelial cells represented only about 1% of infected cells (20). In contrast, electroporation of the UGS with transposon vectors resulted in transgene expression predominantly in the epithelium of the subsequent allografts. This occurred even though the transposon expression vector used the CAG promoter (23) that is active in most cell types. The most likely explanation for the epithelial-specific expression of the transgene was transposon mediated gene transfer that was largely restricted to the epithelium. This is consistent with our *in situ* PCR results for the T2-*Braf* vector (Figs. 4 and 5) that detected the presence of the transposon sequences predominantly in the epithelium. It is also consistent with our previous observation that UGSs electroporated with expression plasmids yielded epithelial and not mesenchymal gene expression (4) likely due to epithelial-restricted uptake of the expression plasmids. We also observed a higher rate of transgenesis than has been reported for the MPR model with, on average, about 40% of prostatic ducts containing transgene-expressing cells in the case of the BRAF transposon vector. However, the potential differences in efficiency of gene transfer in the transposon model relative to the MPR should be interpreted with caution as the T2-*Braf* transgene clearly gave cells a growth advantage as indicated by the higher proliferation rate for BRAF expressing ducts (Fig. 5). Future studies with additional transgenes that don't confer a growth advantage are needed to further clarify the general efficiency of gene transfer by transposon vectors. In parallel to allografts transduced with T2-*Braf* + transposase expression vector, we also electroporated and allografted UGSs with T2-*Braf* in the absence of the transposase vector. In these allografts, we attempted to detect retention the T2-*Braf* vector after 8 weeks using nested PCR for transposon DNA, RT-PCR for transposon-derived transcripts, and immunohistochemistry for BRAF. None of these methods detected retention of T2-*Braf* in the absence of transposase (data not shown).

For this proof-of-principle study, the experimental transgene was a truncated form of BRAF that retained the carboxy-terminal kinase domain but lacked the amino-terminal RAS-binding domain and as a result had constitutive signaling activity. This is similar to the truncated forms of BRAF that are expressed from fusion genes that have been observed in human prostate cancers (24). Constitutive BRAF activation can also result from point mutations. Although *BRAF* activating point mutations are prevalent in other types of cancers, only select cohorts of prostate cancer cases in the literature harbor the V600E activating mutation. Cho et al. described an Asian cohort with 10% prevalence of V600E mutations in prostate cancer samples (25). However, other groups have not been able to detect V600E mutations in different prostate cancer samples (26-31). Transgenic mouse

models have also been used to investigate BRAF in prostate cancer. Jeong et al. used a doxycycline-inducible *BRAF* V600E activating mutation targeted to the prostate epithelium of mice on an *Ink4a/Arf* null background and found that the *BRAF* activating mutation was capable of initiating the development of invasive prostate adenocarcinoma (32). Genetic extinction of *BRAF* V600E in established tumors did not lead to tumor regression. Therefore, the activating *BRAF* mutation was sufficient to initiate development of prostate adenocarcinoma, but was not required for its maintenance. Because these studies were performed on an *Ink4a/Arf* null background, which is not commonly altered in prostate cancer, it is difficult to determine if these results can be extended to other models of prostate cancer. In the current study, ectopic expression of a carboxy-terminal truncated form of *Braf* on a normal background dramatically increased epithelial cell proliferation without invasion (Fig. 5), contrasting to the increased proliferation and invasion with the V600E *Braf* activating mutation on an *Ink4a/Arf* null background (32). In spite of the increased proliferation, the average weight of T2-*Braf* transduced allografts was not significantly higher than empty vector controls (Fig. 1). Based on histology of the allografts (Fig. 2, Fig. 3), most of the allograft weight was contributed by prostatic secretions rather than cellular content, limiting the utility of allograft weight as a measure of cellular outcomes in the model.

One potential concern for both the transposon-based and the MPR model is the possibility that transposon or viral insertions may act as mutagens by disrupting normal genes that could in some cases induce phenotypes that would mask effects of the intended transgene. Because each transduced UGS would have unique genomic insertions, phenotypes that occur in most or all transduced prostates are very likely to result from the intended transgene. However, it is important to be mindful that less commonly observed phenotypes that are restricted to one or a few transduced prostates may be the result of vector-induced mutational events. An appealing feature of using transposons to introduce genetic elements into the mouse UGS is the possibility of creating transgenic allografts in syngeneic immunocompetent mice and/or strains of genetically modified mice. This would represent an advantage over other grafting-based models that must rely on using immune-compromised mice.

Conclusions

The Sleeping Beauty transposon system can be used to achieve somatic transgenesis of prostatic allografts. This new method for achieving ectopic gene expression in the prostate will complement other existing approaches such as ectopic gene expression in cell lines, the MPR model, and germline transgenic mice. Advantages of this new approach include preservation of stromal-epithelial interactions not possible with cell lines, and higher throughput and lower cost than traditional germline transgenic approaches. Use of this approach to model the effects of truncated forms of BRAF that are expressed from fusion genes in human prostate cancers demonstrated that activated BRAF can drive prostate epithelial proliferation in the absence of other cooperating mutations.

Acknowledgments

We thank members of the Marker and Ricke laboratories for their assistance throughout this project, and we thank consulting veterinary pathologist Ruth Sullivan for her insights in interpreting the outcomes of allografting experiments.

Grant Sponsor: National Institutes of Health; Grant Numbers: AG024278, CA140217, CA141798, DK091193, CA157322; Grant Sponsor: Department of Defense; Grant Numbers: W81XWH-05-1-053, W81XWH-10-1-0571

References

1. Gordon JW, Ruddle FH. Integration and stable germ line transmission of genes injected into mouse pronuclei. *Science*. 1981; 214(4526):1244–1246. [PubMed: 6272397]
2. Zhang J, Thomas TZ, Kasper S, Matusik RJ. A small composite probasin promoter confers high levels of prostate-specific gene expression through regulation by androgens and glucocorticoids in vitro and in vivo. *Endocrinology*. 2000; 141(12):4698–4710. [PubMed: 11108285]
3. Doles JD, Vezina CM, Lipinski RJ, Peterson RE, Bushman W. Growth, morphogenesis, and differentiation during mouse prostate development in situ, in renal grafts, and in vitro. *Prostate*. 2005; 65(4):390–399. [PubMed: 16114054]
4. Buresh RA, Kuslak SL, Rusch MA, Vezina CM, Selleck SB, Marker PC. Sulfatase 1 is an inhibitor of ductal morphogenesis with sexually dimorphic expression in the urogenital sinus. *Endocrinology*. 2010; 151(7):3420–3431. [PubMed: 20410206]
5. Donjacour AA, Thomson AA, Cunha GR. FGF-10 plays an essential role in the growth of the fetal prostate. *Dev Biol*. 2003; 261(1):39–54. [PubMed: 12941620]
6. Doles J, Cook C, Shi X, Valosky J, Lipinski R, Bushman W. Functional compensation in Hedgehog signaling during mouse prostate development. *Dev Biol*. 2006; 295(1):13–25. [PubMed: 16707121]
7. Thompson TC, Timme TL, Park SH, Yang G, Ren C. Mouse prostate reconstitution model system: A series of in vivo and in vitro models for benign and malignant prostatic disease. *Prostate*. 2000; 43(4):248–254. [PubMed: 10861743]
8. Ivics Z, Hackett PB, Plasterk RH, Izsvak Z. Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. *Cell*. 1997; 91(4):501–510. [PubMed: 9390559]
9. Montini E, Held PK, Noll M, Morcinek N, Al-Dhalimy M, Finegold M, Yant SR, Kay MA, Grompe M. In vivo correction of murine tyrosinemia type I by DNA-mediated transposition. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2002; 6(6):759–769. [PubMed: 12498772]
10. Belur LR, Frandsen JL, Dupuy AJ, Ingbar DH, Largaespada DA, Hackett PB, Scott McIvor R. Gene insertion and long-term expression in lung mediated by the Sleeping Beauty transposon system. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2003; 8(3):501–507. [PubMed: 12946324]
11. Xue X, Huang X, Nodland SE, Mates L, Ma L, Izsvak Z, Ivics Z, LeBien TW, McIvor RS, Wagner JE, Zhou X. Stable gene transfer and expression in cord blood-derived CD34+ hematopoietic stem and progenitor cells by a hyperactive Sleeping Beauty transposon system. *Blood*. 2009; 114(7):1319–1330. [PubMed: 19414858]
12. Collier LS, Carlson CM, Ravimohan S, Dupuy AJ, Largaespada DA. Cancer gene discovery in solid tumours using transposon-based somatic mutagenesis in the mouse. *Nature*. 2005; 436(7048):272–276. [PubMed: 16015333]
13. Cui Z, Geurts AM, Liu G, Kaufman CD, Hackett PB. Structure-function analysis of the inverted terminal repeats of the sleeping beauty transposon. *Journal of molecular biology*. 2002; 318(5):1221–1235. [PubMed: 12083513]
14. Geurts AM, Yang Y, Clark KJ, Liu G, Cui Z, Dupuy AJ, Bell JB, Largaespada DA, Hackett PB. Gene transfer into genomes of human cells by the sleeping beauty transposon system. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2003; 8(1):108–117. [PubMed: 12842434]

15. Largaespada DA, Collier LS. Transposon-mediated mutagenesis in somatic cells: identification of transposon-genomic DNA junctions. *Methods in molecular biology* (Clifton, NJ. 2008; 435:95–108.
16. Buresh RA, Kuslak SL, Rusch MA, Vezina CM, Selleck SB, Marker PC. Sulfatase 1 Is an Inhibitor of Ductal Morphogenesis with Sexually Dimorphic Expression in the Urogenital Sinus. *Endocrinology*.
17. Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. *BioTechniques*. 2005; 39(1):75–85. [PubMed: 16060372]
18. Bubendorf L, Sauter G, Moch H, Schmid HP, Gasser TC, Jordan P, Mihatsch MJ. Ki67 labelling index: an independent predictor of progression in prostate cancer treated by radical prostatectomy. *The Journal of pathology*. 1996; 178(4):437–441. [PubMed: 8691323]
19. Jeet V, Russell PJ, Khatri A. Modeling prostate cancer: a perspective on transgenic mouse models. *Cancer Metastasis Rev*. 2010; 29(1):123–142. [PubMed: 20143131]
20. Thompson TC, Southgate J, Kitchener G, Land H. Multistage carcinogenesis induced by ras and myc oncogenes in a reconstituted organ. *Cell*. 1989; 56(6):917–930. [PubMed: 2538247]
21. Thompson TC, Timme TL, Kadmon D, Park SH, Egawa S, Yoshida K. Genetic predisposition and mesenchymal-epithelial interactions in ras+myc-induced carcinogenesis in reconstituted mouse prostate. *Mol Carcinog*. 1993; 7(3):165–179. [PubMed: 8489712]
22. Xin L, Ide H, Kim Y, Dubey P, Witte ON. In vivo regeneration of murine prostate from dissociated cell populations of postnatal epithelia and urogenital sinus mesenchyme. *Proc Natl Acad Sci U S A*. 2003; 100(Suppl 1):11896–11903. [PubMed: 12909713]
23. Miyazaki J, Takaki S, Araki K, Tashiro F, Tominaga A, Takatsu K, Yamamura K. Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. *Gene*. 1989; 79(2):269–277. [PubMed: 2551778]
24. Palanisamy N, Ateeq B, Kalyana-Sundaram S, Pflueger D, Ramnarayanan K, Shankar S, Han B, Cao Q, Cao X, Suleman K, Kumar-Sinha C, Dhanasekaran SM, Chen YB, Esgueva R, Banerjee S, LaFargue CJ, Siddiqui J, Demichelis F, Moeller P, Bismar TA, Kuefer R, Fullen DR, Johnson TM, Greenson JK, Giordano TJ, Tan P, Tomlins SA, Varambally S, Rubin MA, Maher CA, Chinnaiyan AM. Rearrangements of the RAF kinase pathway in prostate cancer, gastric cancer and melanoma. *Nat Med*. 16(7):793–798. [PubMed: 20526349]
25. Cho NY, Choi M, Kim BH, Cho YM, Moon KC, Kang GH. BRAF and KRAS mutations in prostatic adenocarcinoma. *Int J Cancer*. 2006; 119(8):1858–1862. [PubMed: 16721785]
26. Burger M, Denzinger S, Hammerschmied C, Tannapfel A, Maderstorfer A, Wieland WF, Hartmann A, Stoehr R. Mitogen-activated protein kinase signaling is activated in prostate tumors but not mediated by B-RAF mutations. *Eur Urol*. 2006; 50(5):1102–1109. discussion 1109–1110. [PubMed: 16413100]
27. Liu T, Willmore-Payne C, Layfield LJ, Holden JA. Lack of BRAF activating mutations in prostate adenocarcinoma: a study of 93 cases. *Appl Immunohistochem Mol Morphol*. 2009; 17(2):121–125. [PubMed: 18987552]
28. Carter BS, Epstein JI, Isaacs WB. ras gene mutations in human prostate cancer. *Cancer Res*. 1990; 50(21):6830–6832. [PubMed: 2208148]
29. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the BRAF gene in human cancer. *Nature*. 2002; 417(6892):949–954. [PubMed: 12068308]
30. Moul JW, Friedrichs PA, Lance RS, Theune SM, Chang EH. Infrequent RAS oncogene mutations in human prostate cancer. *Prostate*. 1992; 20(4):327–338. [PubMed: 1608859]
31. Shen Y, Lu Y, Yin X, Zhu G, Zhu J. KRAS and BRAF mutations in prostate carcinomas of Chinese patients. *Cancer genetics and cytogenetics*. 2010; 198(1):35–39. [PubMed: 20303012]

32. Jeong JH, Wang Z, Guimaraes AS, Ouyang X, Figueiredo JL, Ding Z, Jiang S, Guney I, Kang GH, Shin E, Hahn WC, Loda MF, Abate-Shen C, Weissleder R, Chin L. BRAF activation initiates but does not maintain invasive prostate adenocarcinoma. PloS one. 2008; 3(12):e3949. [PubMed: 19079609]

Figure 1. Somatic transgenesis of the prostate using the Sleeping Beauty transposon system (A) Overview of the procedure used to create transgenic prostatic allografts using the Sleeping Beauty transposon system. (B) A portion of the mouse embryonic day 16 (e16) lower urinary tract is shown including the bladder (BL), the urogenital sinus (UGS), and the segment of the urethra distal to the urogenital sinus. The continuous lumen of the UGS (arrow) and urethra (arrowhead) is easily visible at this stage. (C) For the transgenesis procedure, a 36 gauge needle was inserted into the e16 UGS lumen via the urethra and used to inject approximately 0.5 μ L of DNA solution. (D) To aid in the injection process, the DNA solution also contained 0.1% Fast Green dye that is clearly visible within the UGS lumen (arrow) following a successful injection. (E) A prostatic allograft (arrowheads) for a UGS that had undergone DNA injection (transposase expression plasmid + T2/HB empty vector plasmid) and electroporation is shown on the surface of a mouse kidney following 8 weeks of growth under the renal capsule of a CD1 nu/nu mouse host. (F) Wet weights for transposase expression plasmid + T2/HB empty vector plasmid (SB + T2-EV; n = 10) and transposase expression plasmid + T2-*Braf* expression vector plasmid (SB + T2-BRAF; n = 14) are shown. Although the mean graft weight was higher for the SB + T2-BRAF group, the observed difference was not statistically significant by T test.

Figure 2. Normal prostate morphogenesis and differentiation in transposon-transduced urogenital sinus allografts

UGSs that had been transduced with a pCMV-SB11 expression plasmid + an empty vector pT2/HB transposon plasmid were grown as allografts in CD-1 nu/nu mice for 8 weeks and then processed for histology and immunohistochemistry. (A) Low magnification and (B) high magnification views of hematoxylin and eosin (H & E) stained allografts showed ductal tissue architecture similar to the normal mouse prostate. Immunohistochemistry with antibodies directed against cell type specific proteins including (C) wide spectrum cytokeratin (Cyto), (D) E-cadherin (Ecad), (E) p63, and (F) smooth muscle actin (SMA) further confirmed normal prostate tissue architecture with a continuous layer of cytokeratin and E-cadherin positive luminal cells, a discontinuous layer of p63 positive basal cells (examples indicated by arrows in E), and a thin layer of smooth muscle actin positive stroma. A magnification scale for panel A is shown in A. A magnification scale for panels B-F is shown B.

Figure 3. Somatic transgenesis with activated BRAF

UGSs that had been transduced with a pCMV-SB11 expression plasmid + a pT2-*Braf* transposon expression vector were grown as allografts in CD-1 nu/nu mice for 8 weeks and then processed for histology, RNA analysis, and DNA analysis. (A) Low magnification and (B) high magnification views of hematoxylin and eosin stained allografts showed predominantly normal ductal tissue architecture similar to empty vector allografts. (C) Real-time RT-PCR for *Braf* transcripts showed an overall increase in *Braf* expression in allografts that had been transduced with pT2-*Braf* transposon relative to allografts transduced with an empty vector transposon. The observed increase in *Braf* expression was statistically significant ($P < 0.001$ by Student's T test). (D) Reverse transcription followed by PCR using a primer pair present in *Braf* transcripts expressed from the transposon expression vector but not present in endogenous *Braf* transcripts detected transgene expression in 3 of 4 T2-*Braf* transduced allografts (lanes 1-4, NT = no template control). (E) Agarose gel of linker-mediated PCR products containing transposon-genome junction fragments revealed a smear of many different product sizes for individual allografts transduced with a pCMV-SB11

expression plasmid + a pT2-*Braf* transposon expression vector (SB + T2-BRAF) suggesting multiple independent transposon insertions are present in individual allografts.

Figure 4. Pattern of BRAF expression and ERK activation in transgenic allografts
UGSs that had been transduced with a pCMV-SB11 expression plasmid + a T2 empty control vector (A, C), or transduced with a pCMV-SB11 expression plasmid + T2-*Braf* transposon expression vector (B, D-J) were grown as allografts in CD-1 nu/nu mice for 8 weeks and then processed for immunohistochemistry. (A) Low magnification view of an empty vector control allograft tissue section stained with anti-BRAF antibodies showed minimal staining. (B) Low magnification view of a T2-*Braf* transduced allograft tissue section stained with anti-BRAF antibodies showed groups of ducts that strongly expressed BRAF (example indicated by arrow) and other groups of ducts with little or no BRAF expression (example indicated by arrowhead). (C) Low magnification view of an empty vector control allograft tissue section stained with anti-phospho-ERK1/2 (pERK) showed minimal staining. (D) Low magnification view of a T2-*Braf* transduced allograft tissue section (near-adjacent to the tissue section shown in B) stained with anti-pERK antibodies showed groups of ducts that were stained strongly with anti-BRAF also stained strongly for activated ERK1/2 (example indicated by arrow) while groups of ducts that stained weakly with anti-BRAF also stained weakly for activated ERK1/2 (example indicated by arrowhead). Analysis of near-adjacent T2-*Braf* transduced allograft tissue sections with anti-

BRAF (E, H), anti-pERK (F, I) and *in situ* PCR for the T2-*Braf* transposon vector (G, J) suggested that ducts with high expression of BRAF and pERK had also incorporated the transposon vector (compare E-G) while ducts with little BRAF and pERK expression had not incorporated the transposon vector (compare H-J). A magnification scale for panels A-D is shown in B. A magnification scale for panels E-J is shown in G.

Figure 5. Increased epithelial proliferation induced by activated BRAF

Tissue sections from empty vector (T2-EV) transduced (A, B) and T2-*Braf* transduced (C, D) allografts were analyzed using an *in situ* PCR assay for the BRAF-expressing transposon vector (A, C) and for labeling by the Ki67 proliferation-associated antigen (B, D). (A) T2-EV transduced allografts had no staining using the *in situ* PCR assay, but (C) groups of ducts had positive staining (brown stained nuclei) for T2-*Braf* transduced allografts. (B) Ki67 positive cells were infrequent in T2-EV transduced allografts, but (D) were more common in prostatic ducts positive for T2-*Braf* by *in situ* PCR. (E) Quantification of the epithelial Ki67 labeling index confirmed that it was increased for prostatic ducts that were transgenic for the T2-*Braf* vector when compared to prostatic ducts in the T2-EV control allografts. The observed increase in Ki67 labeling index for prostatic ducts with activated BRAF expression was statistically significant ($P < 0.001$ by Student's T test). A magnification scale for panels A-D is shown B.