De novo Induction of Genetically Engineered Brain Tumors in Mice Using Plasmid DNA

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Abstract

Spontaneous mouse models of cancer show promise to more accurately recapitulate human disease and predict clinical efficacy. Transgenic mice or viral vectors have been required to generate spontaneous models of glioma, a lethal brain tumor, because nonviral gene transfer is typically transient. To overcome this constraint, we used the Sleeping Beauty transposable element to achieve chromosomal integration of human oncogenes into endogenous brain cells of immunocompetent mice. Genetically engineered, spontaneous brain tumors were induced with plasmid DNA in a matter of weeks in three separate mouse strains. The phenotype of tumors was influenced by the combination of oncogenes delivered, resembling human astrocytoma or glioblastoma in the majority of cases. At least five different genes can be cotransfected simultaneously including reporters, allowing measurement of tumor viability by in vivo imaging. This model can accelerate brain tumor research in a variety of ways such as generation of “humanized” models for high throughput drug screening and candidate gene validation with exceptional speed and flexibility. [Cancer Res 2009;69(2):431–9]

Introduction

Malignant glioma (MG) is a devastating primary brain tumor. Gliomas are classified according to the WHO criteria ranging from grade I, a typically treatable tumor, to grade IV, a glioblastoma multiforme (GBM; ref. 1). GBM is a lethal brain tumor claiming over 12,000 lives each year in the United States (2). Prognosis for patients with high-grade MG is poor and has remained relatively unchanged for decades. MG often displays marked genetic and phenotypic heterogeneity (3). For example, as many as three different alleles of the Trp53 tumor suppressor gene are detectable in a single GBM; such tumors contain mixed regions appearing as high- or low-grade histologically and exhibit heterogeneous expression of the epidermal growth factor receptor (EGFR) immunohistochemically (4, 5). The heterogeneity of MG may account for the failure of therapies with a single mechanism of action. Animal models that recapitulate the complexity of human MG would be useful to better understand glioma biology and predict therapeutic response.

The most widely used rodent model of MG involves intracerebral transplantation of cultured glioma cells, a very different scenario from the human disease. There has been great interest in spontaneous models of MG in which the tumor evolves with the host immune system with the expectation that they will more faithfully mimic human disease progression and better predict clinical efficacy. A number of genetically engineered mice (GEM) are available, harboring constitutive or conditional alleles of genes associated with MG development (6). Some models combine various GEM to test cooperativity of particular mutations in tumor development (7–9). Spontaneous MG can also be induced by intracerebral injection of a retroviral vector encoding platelet-derived growth factor (10, 11). Hybrid models have also been created by using GEM that express a receptor for a replication-competent ALV splice acceptor (RCAS) avian retrovirus in a tissue specific pattern, leading to MG after oncogene transfer (12, 13). This GEM has been bred to a second GEM expressing firefly luciferase (FLuc) in mitotic cells to allow cell division to be noninvasively monitored using bioluminescent imaging (14). Bioluminescent imaging provides unparalleled convenience and speed for determining the efficacy of therapeutic agents in living mice (15). To date, all spontaneous mouse models of MG have required the use of GEM or viral vectors. Production of new GEM or viral vectors can take months to years to develop and characterize. In some models, mice develop tumors with incomplete penetrance and exhibit relatively long survival times making them inconvenient for preclinical trials. We sought to develop a more flexible, rapid, spontaneous MG model that was independent of strain background while retaining the ability to monitor tumor viability with bioluminescence.

Several investigators have previously achieved nonviral transfection of the murine brain with polyethyleneimine/plasmid DNA (PEI/DNA) complexes (16–19). Unfortunately, gene expression after plasmid DNA transfection is typically transient. To overcome this constraint, we used the Sleeping Beauty (SB) transposable element delivered as plasmid DNA to achieve chromosomal integration and long-term expression (17). SB is a synthetic transposable element that was created by genetically engineering inactive transposase gene sequences isolated from salmonid fish (20). SB is a two-part system composed of a transposon DNA substrate and a transposase enzyme. SB transposase mediates “cut and paste” excision and insertion of transposon DNA into a TA dinucleotide of the host genomic DNA (21). The gene encoding the SB transposase enzyme can be provided on the plasmid DNA backbone or on a separate plasmid relative to transposon DNA.

Here, we show that injection of PEI/DNA complexes into the lateral cerebral ventricle of neonatal mice leads to SB-dependant...
long-term gene expression. Using this method, we describe the development of a series of spontaneous MG models that express several combinations of reporter genes, human oncogenes, and inhibitors of tumor suppressor function. This model is readily adaptable for rapid high throughput drug screening, candidate gene validation, and basic biology studies that could accelerate brain tumor research in a variety of ways.

Materials and Methods

Animal care. Mice were purchased from The Jackson Laboratory or Charles River Corporation for all experiments. Mating pairs were setup and carefully monitored each day until they gave birth. All animals were maintained in a specific pathogen–free facility. Experiments were conducted according to the guidelines of the University of Minnesota Animal Care and Use Committee. Neonatal mice that were ages <2 d were used for the studies with three exceptions (see Table 1).

Plasmid vectors. PT2/C-FLuc, pT/CMV-SV40-LgT, pT/CAGGS-NRASV12, and PGK-SB13 were created as previously described (22). PT2/C-Luc//PGK-SB13 was created by excising the PGK-SB transposase expression cassette from pPGK-SB13 as a XmnI fragment and ligating into pT2/C-Luc as a XmnI fragment. PT3.5/CMV-GFP as a PvuII fragment. MSCV-LTRmiR30-SV40 (23) contained a microRNA containing fragment, followed by ligation into pT3.5/CMV-GFP as a XhoI/AGEI fragment. MSCV-LTRmiR830-SV40 (23) contained a microRNA short hairpin against Trp53 and a second expression cassette encoding green fluorescent protein (GFP); it was a kind gift from Dr. Scott Lowe (Cold Spring Harbor, NY, USA). The shp53 and GFP expression cassette–containing fragment was released from MSCV-LTRmiR830-SV40 as a PvuII fragment and ligated into PT2/HC (24) as an EcoRI V fragment to generate pt2/shp53/GFP4. The MSCV-AKT vector containing a human AKT cDNA with a myristylation site was a kind gift from Dr. Scott Lowe. MSCV-AKT was cut with EcoRI/UNeo 1 to release the AKT cDNA and ligated into Litmus 29, followed by final ligation into pKT2/CLP as a Neo 1/Bgl II fragment to generate pKT2/CLP-AKT. Plasmids were purified using a maxiprep kit (Invitrogen) and stored in 0.1X TE buffer (pH 8.0).

PEI/DNA administration. In all oncogene experiments, 25% mannitol (20 μL) was injected i.p. immediately prior hypothermia anesthesia and PEI/DNA injection. Neonatal mice were then placed on ice for 5 min to induce anesthesia before being secured in a cooled, “neonatal rat” stereotactic frame (Stoelting) maintained at 4°C to 6°C by a dry ice/ethanol reservoir. A 10 μL syringe (Hamilton Company) fitted with a 30 gauge hypodermic needle (12.5″ bevel; Hamilton Company) attached to a micropump (Stoelting) was used to inject plasmids at a flow rate of 0.7 μL/min into the right lateral cerebral ventricle. Coordinates for injection were +1.5AP, 0.7ML, and −1.5DV from lambda. PEI/DNA complexes were prepared according to the manufacturer’s instructions to achieve N/P ratio of 7 (Polyplus Transfection; ref. 25). One-half to 2 μL of PEI/DNA solution was administered at a maximum concentration of 0.5 μg/μL. No incision was made for injection. The skull of a neonate was penetrated with the needle for all injections.

Luciferase assays and immunohistochemical staining. Luciferase expression was assessed in vivo as previously described (17). For in vitro luciferase assays, animals were deeply anesthetized before transcardial perfusion with PBS (pH 7.0–7.4). The brains were removed and homogenized in 400 μL of lysis buffer (Promega). Luminescence was determined by mixing 100 μL of lysate with 20 μL of substrate solution provided in the luciferase assay kit (Promega) and immediately measured on a luminometer with a 15 second exposure time. Relative light units were normalized to milligrams of protein as determined by Bradford assay.

For histologic analysis, animals were perfused with PBS followed with 2% paraformaldehyde. For immunofluorescence, the brains were dissected and placed in 30% sucrose for 48 h for cryoprotection. Brains were sectioned and immunofluorescence stained was carried out using rabbit anti-GFP (Molecular Probes). Images were acquired and processed as described (26). Immunohistochemistry (IHC) and H&E staining was carried out using standard clinical techniques on formalin-fixed, paraffin-embedded tissue as described (27). The EGFRVIII antibody was a kind gift from Dr. Darrel Bigner (Duke University, Durham, North Carolina).

Table 1. Effect of vector, DNA dose, volume, mouse strain, and age on tumor formation

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Vector 1</th>
<th>Vector 2</th>
<th>Vector 3</th>
<th>Vector 4</th>
<th>Ratio*</th>
<th>Total DNA (μg)</th>
<th>Volume (μL)</th>
<th>Age (h)</th>
<th># w/ tumor</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SB13/FLuc</td>
<td>SV40-LgT</td>
<td>NRAS G12V</td>
<td>—</td>
<td>1:2</td>
<td>1.0</td>
<td>2.0</td>
<td>≤48</td>
<td>13/15 (87%)</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>2</td>
<td>SB13/FLuc</td>
<td>SV40-LgT</td>
<td>NRAS G12V</td>
<td>—</td>
<td>1:2</td>
<td>0.5</td>
<td>2.0</td>
<td>≤48</td>
<td>4/4 (100%)</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>3</td>
<td>SB13/FLuc</td>
<td>SV40-LgT</td>
<td>NRAS G12V</td>
<td>—</td>
<td>1:2</td>
<td>0.5</td>
<td>1.0</td>
<td>≤48</td>
<td>3/4 (75%)</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>4</td>
<td>SB13/FLuc</td>
<td>SV40-LgT</td>
<td>NRAS G12V</td>
<td>—</td>
<td>1:2</td>
<td>0.25</td>
<td>1.0</td>
<td>≤48</td>
<td>4/5 (80%)</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>5</td>
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<td>SV40-LgT</td>
<td>NRAS G12V</td>
<td>—</td>
<td>1:2</td>
<td>0.25</td>
<td>0.5</td>
<td>≤48</td>
<td>3/6 (50%)</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>6</td>
<td>SB13/FLuc</td>
<td>SV40-LgT</td>
<td>Empty Vector</td>
<td>—</td>
<td>1:2</td>
<td>1.0</td>
<td>2.0</td>
<td>≤48</td>
<td>0/4 (0%)</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>7</td>
<td>SB13/FLuc</td>
<td>SV40-LgT</td>
<td>Empty Vector</td>
<td>—</td>
<td>1:2</td>
<td>0.5</td>
<td>1.0</td>
<td>24–48</td>
<td>11/11 (99%)</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>8</td>
<td>SB13/FLuc</td>
<td>Empty Vector</td>
<td>NRAS G12V</td>
<td>—</td>
<td>1:2</td>
<td>1.0</td>
<td>2.0</td>
<td>≤48</td>
<td>0/4 (0%)</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>9</td>
<td>SB13/FLuc</td>
<td>Empty Vector</td>
<td>NRAS G12V</td>
<td>—</td>
<td>1:2</td>
<td>0.5</td>
<td>1.0</td>
<td>24–48</td>
<td>8/8 (0%)</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>10</td>
<td>SB13/FLuc</td>
<td>SV40-LgT</td>
<td>NRAS G12V</td>
<td>—</td>
<td>1:2</td>
<td>1.0</td>
<td>2.0</td>
<td>≤48</td>
<td>7/7 (100%)</td>
<td>FVB/n</td>
</tr>
<tr>
<td>11</td>
<td>SB13/FLuc</td>
<td>Akt</td>
<td>NRAS G12V</td>
<td>—</td>
<td>1:2</td>
<td>1.0</td>
<td>2.0</td>
<td>≤48</td>
<td>5/10 (50%)</td>
<td>FVB/n</td>
</tr>
<tr>
<td>12</td>
<td>SB13/FLuc</td>
<td>SV40-LgT</td>
<td>EGFRVIII</td>
<td>—</td>
<td>1:2</td>
<td>1.0</td>
<td>2.0</td>
<td>≤48</td>
<td>2/10 (20%)</td>
<td>FVB/n</td>
</tr>
<tr>
<td>13</td>
<td>SB13/FLuc</td>
<td>Akt</td>
<td>NRAS G12V</td>
<td>shP53/GFP</td>
<td>1:1:1</td>
<td>1.0</td>
<td>2.0</td>
<td>≤48</td>
<td>9/12 (75%)</td>
<td>FVB/n</td>
</tr>
<tr>
<td>14</td>
<td>SB13/FLuc</td>
<td>EGFRVIII</td>
<td>NRAS G12V</td>
<td>shP53/GFP</td>
<td>1:1:1</td>
<td>1.0</td>
<td>2.0</td>
<td>48–72</td>
<td>9/9 (100%)</td>
<td>FVB/n</td>
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<tr>
<td>15</td>
<td>SB13/FLuc</td>
<td>EGFRVIII</td>
<td>NRAS G12V</td>
<td>Empty Vector</td>
<td>1:1:1</td>
<td>1.0</td>
<td>2.0</td>
<td>24–48</td>
<td>0/6 (0%)</td>
<td>FVB/n</td>
</tr>
<tr>
<td>16</td>
<td>SB13/FLuc</td>
<td>EGFRVIII</td>
<td>NRAS G12V</td>
<td>Empty Vector</td>
<td>1:1:1</td>
<td>1.0</td>
<td>2.0</td>
<td>24–48</td>
<td>0/6 (0%)</td>
<td>FVB/n</td>
</tr>
<tr>
<td>17</td>
<td>SB13/FLuc</td>
<td>Empty Vector</td>
<td>NRAS G12V</td>
<td>shP53/GFP</td>
<td>1:1:1</td>
<td>1.0</td>
<td>2.0</td>
<td>24–48</td>
<td>3/5 (60%)</td>
<td>FVB/n</td>
</tr>
<tr>
<td>18</td>
<td>SB13/FLuc</td>
<td>SV40-LgT</td>
<td>Empty Vector</td>
<td>shP53/GFP</td>
<td>1:1:1</td>
<td>1.0</td>
<td>2.0</td>
<td>24–48</td>
<td>2,160 (0%)</td>
<td>FVB/n</td>
</tr>
<tr>
<td>19</td>
<td>SB13/FLuc</td>
<td>SV40-LgT</td>
<td>NRAS G12V</td>
<td>—</td>
<td>1:2</td>
<td>1.0</td>
<td>2.0</td>
<td>≤96</td>
<td>3/6 (50%)</td>
<td>FVB/n</td>
</tr>
<tr>
<td>20</td>
<td>SB13/FLuc</td>
<td>SV40-LgT</td>
<td>NRAS G12V</td>
<td>—</td>
<td>1:2</td>
<td>0.25</td>
<td>1.0</td>
<td>24–48</td>
<td>5/5 (100%)</td>
<td>BALB/c</td>
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<tr>
<td>21</td>
<td>SB13/FLuc</td>
<td>SV40-LgT</td>
<td>NRAS G12V</td>
<td>—</td>
<td>1:2</td>
<td>0.125</td>
<td>1.0</td>
<td>24–48</td>
<td>4/4 (100%)</td>
<td>BALB/c</td>
</tr>
</tbody>
</table>

*With respect to vectors listed left to right.
Tissue culture and Western blot. Western blot was conducted as described (22) using the following antibodies: mouse anti-LgT (Calbiochem), rabbit anti-NRAS, rabbit anti-p53, mouse anti-ha actin, and rabbit anti–extracellular signal-regulated kinase (all from Santa Cruz). Cell lines were derived from mice as previously described using TrypLE enzymatic digestion (28). All cells derived from spontaneous tumors were cultured in media consisting of DMEM/F12, N2, and B27 supplements (1/C2), 1% penicillin/streptomycin (Invitrogen), and supplemented with 20 ng/ml EGF and fibroblast growth factor (Peprotech). Cytokines were added every 2 to 3 d, and cells were passaged once to twice each week depending on density. GL261 glioma cells were cultured as described (29).

Southern blot and insertion cloning. Southern blot hybridization for NRAS was performed as described (22). Transposon insertion sites were identified by a combination of linker-mediated PCR (30), using the pCR4-TOPO cloning vector and One-Shot TOP10 competent cells (Invitrogen) for shot-gun cloning, and pyrosequencing (454 Life Sciences; Roche) as previously described (31). Genomic sequences directly flanking the transposon were mapped using Ensemble.

Statistical analysis. Survival was analyzed by log-rank test as described (32). Graphing and statistical analysis was performed using Prism4 software (Graph Pad Software) as described (32).

Results

Characterization and optimization of SB-mediated gene transfer. To determine the localization of transfected cells, PEI/DNA complexes encoding simian virus 40 large T antigen (SV40-LgT) were injected into the lateral cerebral ventricle of neonatal mice. IHC staining for SV40-LgT was conducted on brains harvested 48 h after gene transfer. A representative sagittal section is shown. Gray boxes labeled with roman numerals represent >20 magnification of the corresponding black boxes shown at low power. Black arrows, positive cells. The lateral ventricle (LV) and inferior horn (IH) of the lateral ventricle are labeled. B, similar experiment as in A except that a GFP vector was injected into mice that were or were not given an i.p. injection of mannitol 1 h before gene delivery (n = 4 per group). Coronal sections were processed 48 h later for GFP immunofluorescence. Red arrows, cells reacting with anti-GFP antibody. C, quantification of the cells counted from B. Columns, mean of GFP-positive cells counted per section (n = 4 sections per mouse and 4 mice were used); bars, SD. D, neonatal mice were injected with PT2/C-FLuc alone or in combination with PGK-SB13 at 1:30 ratio (n = 4 per group; 1 µg total dose). Thirty days later, brain lysates were assayed for luciferase activity (graph indicates average ± SD).
in serial sections to quantify the extent of transfection. Mannitol more than doubled the number of GFP-positive cells within 1 mm of the lateral ventricle and also increased transfection deeper into the brain parenchyma (Fig. 1C). To determine if SB could enhance transgene expression in the brain, neonatal mice were intracere- brally injected with a FLuc transposon with or without transposase-encoding DNA. One month later, brain lysates were assayed for FLuc activity as a measure of long-term expression. Luciferase activity was over seven times higher in brains injected with a plasmid encoding SB transposase relative to brains injected without SB-encoding DNA (Fig. 1D). I.p. administration of mannitol before cotransfection with SB-encoding DNA further increased FLuc activity 3-fold (Fig. 1D).

**Oncogene transfection resulted in development of spontaneous glioma.** To promote spontaneous tumor formation, we delivered transposons encoding SV40-LgT, a hyper-active human NRAS (22), and a transposon carrying a FLuc expression cassette on the same DNA molecule as the SB transposase gene into C57BL/6 mice (Fig. 2A). In experiment 1 (Table 1), tumors arose rapidly, visualized by increasing bioluminescence within 3 to 4 weeks (Fig. 2B). In general, well-demarcated tumors arose in the right (injected) cerebral hemisphere that exhibited infiltrative clusters of tumor cells frequently surrounding blood capillaries (Fig. 3A). In some mice, apparent multifocal tumors arose near both lateral ventricles and macroscopic hydrocephalus was observed with tumors occupying the ventricular space (Supplementary Fig. S1). All tumors exhibited a high cell density with two to five mitotic cells often visible in a single microscopic field (Fig. 3A). Areas of necrosis were apparent in some of the tumors (Fig. 3B).

These tumors exhibited relatively homogenous expression of nestin (Fig. 3B), a marker for neural progenitor cells that is expressed in several types of brain tumors including gliomas. Immunopositivity for glial fibrillary acid protein (GFAP), a marker for astrocytes, was heterogeneous within individual tumors (Supplementary Fig. S2). To determine whether the GFAP-positive cells were reactive astrocytes or tumor cells, two-color IHC was conducted staining for SV40-LgT and GFAP (Fig. 3C). Numerous tumor cells were immunopositive for GFAP and SV40-LgT (Fig. 3C), ruling out reactive astrocytes as the only source of GFAP immunoreactivity. Consistent with an astrocytic phenotype, tumor cells also stained positive for S100. These tumors were negative for neuronal markers including NeuN and synaptophysin, but the surrounding normal brain was positive as an internal control (Fig. 3C). The histologic features of these murine tumors are consistent with the diagnosis of an astrocytoma corresponding to WHO grade III and IV, although human tumors are typically more diffusively invasive. We refer to tumors induced in this murine mode as “GEM” glioma of various grades hereafter to acknowledge that such models are only an approximation of the human disease. To determine the frequency of each tumor grade, 13 tumors that had been induced by NRAS/SV40-LgT were graded based on the presence of necrosis; 77% of exhibited minimal or microscopic necrosis (grade III) and 23% exhibited large regions of necrosis (grade IV; Supplementary Table S1).

The injection volume and concentration of PEI/DNA affected penetrance and lethality of oncogene transfection. The highest injection volume (2 μL) tended to result in the development of hydrocephalus that was apparent by an enlarged skull, particularly in C57BL/6 mice, which are prone to this developmental

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**Figure 2.** Oncogene transfection scheme, imaging, and survival. A. schematic of cotransfection technique used to induce tumors by oncogene transfer. SB transposase expression is regulated by the phosphoglycerate kinase (PGK) promoter on the plasmid DNA backbone of the same vector harboring the FLuc transposon regulated by the CAG promoter. Double gray arrows, inverted repeats of the transposon, marking the termini of the integrating DNA after transposition. B. neonatal mice were injected with SV40-LgT, NRAS, and FLuc vector loaded with SB-encoding DNA as detailed in Table 1. Survival after each injection condition is shown above with representative bioluminescence images from experiment 1 below. C. survival and bioluminescent imaging of FVB/n mice from experiment 14 detailed in Table 1.
In experiment 1, mice developed rapidly lethal tumors with 87% penetrance and median survival of 39 days (2 of 15 mice died from complications of hydrocephalus with no apparent tumor; Fig. 2A). By reducing volume and PEI/DNA dose, hydrocephalus development was attenuated in C57BL/6 mice (experiments 2–5; Table 1). Tumor initiation required the combination of NRAS and SV40-LgT. With the exception of one SV40LgT-treated mouse in experiment 7, tumors did not arise when the single genes were injected in combination with empty vector to control for total PEI/DNA dose (Table 1). In C57BL/6 mice, the lowest injection volumes tended to decrease penetrance (experiments 4 and 5; Table 1). In the BALB/c strain, as little as 0.125 μg of DNA in a 1 μL volume initiated tumors with 100% penetrance without hydrocephalus (experiment 21; Table 1).

Characterization of cell lines derived from NRAS/SV40-LgT/Fluc tumors. Four cell lines were established from the brains of C57BL/6 mice treated with NRAS/SV40-LgT that we designated M2, M4, M7, and M10. These mice were littermates sacrificed after in vivo imaging at 29 days after injection. Cells were cultured in serum-free conditions shown to enrich for nonadherent neurospheres with an undifferentiated phenotype (34, 35). Each cell line grew as nonadherent neurospheres (Fig. 4A). Three of the four cell lines expressed SV40-LgT and every line expressed NRAS at levels detectable by Western blot (Fig. 4B). Transposase-mediated insertions were cloned and mapped to the mouse genome. We cloned a total of 46 insertions from M2, M7, and M10 cells (Supplementary Table S2). Twenty-eight separate insertions were recovered from M7 and fewer were isolated from M2 and M10 (Supplementary Table S2). Four of nine insertions cloned from M10 mapped to chromosome 2. M7 and M2 had a more random pattern. Twenty-eight percent of the integrants mapped within introns of known or predicted genes (Supplementary Table S2). To investigate the clonogenicity of these tumors, individual subclones were isolated from M2, M7, and M10 cell lines that had been established from the entire tumor mass. Subclones were expanded into independent cell lines. Southern blots were conducted using a probe specific for NRAS vector sequence. Each of the four subclones derived from the parental cell lines had identical banding patterns (data not shown).

Tumor initiation was tested in transplantation experiments in severe combined immunodeficient (SCID) recipient mice. All mice

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Figure 3. Characterization of NRAS/SV40-LgT–induced tumors. A, representative images of histology including clustering of tumor cells around capillaries and mitotic figures (black arrows). B, representative images of necrosis, nestin, and S100 IHC are shown. C, two-color IHC staining for GFAP in brown and SV40-LgT in pink (arrows, some dual positive cells; left). Other panels show that the bulk tumor (T) is negative for neuronal markers NeuN and synaptophysin, whereas normal brain (NB) is strongly positive by IHC.
injected with 10,000 M7 cells developed tumors, whereas only 66% of mice injected with M10 cells did. As few as 1,000 cells initiated tumors in 75% of mice (Supplementary Fig. S3). Tumors that arose were traceable by bioluminescent imaging similarly to the primary tumor from which M7 was derived. The histologic phenotype of the primary tumors shown in Fig. 3 was indistinguishable from the tumors that arose upon transplantation of M7 including areas of necrosis (Supplementary Fig. S3).

Development of alternative MG models with human oncogenes. We next sought to determine how flexible the requirements for tumor initiation via SB-mediated transfection could be. Some of the most commonly dysregulated pathways in human MG are high-level RAS and AKT activation via signaling from receptor tyrosine kinases (RTK), Trp53 loss, and mutation of EGFR, rendering it constitutively active (EGFRvIII; ref. 36). We delivered a microRNA-based short hairpin RNA vector that reduces p53 expression (shp53; ref. 23) in combination with NRAS (22), AKT, and EGFRvIII in seven different combinations (Table 1). The combination of NRAS, EGFRvIII, and shp53 was the most robust, inducing MG with 100% penetrance in experiment 14 (Table 1).

All mice treated with NRAS/shp53/EGFRvIII exhibited increasing bioluminescence that correlated with survival (median survival, 83 days; Fig. 2C). These tumors were mitotically active and highly invasive, exhibiting extensive infiltration of normal brain (Fig. 5A). Hemorrhaging and pseudopalisading necrosis was apparent in some tumors (Fig. 5A–B). Nearly the entire tumor mass expressed nestin. However, tumor cells were heterogeneous in appearance including the presence of giant cells and mixed immunoreactivity for GFAP (Supplementary Fig. S4). Similar to the human disease, expression of EGFRvIII was also patchy (Fig. 5B). Despite this heterogeneity, a significant fraction of tumor cells expressed glial markers but not neuronal markers (Supplementary Fig. S4; Fig. 5). The expression of astrocytic markers, hemorrhaging, whole brain infiltration, and pseudopalisading necrosis closely resembles the most common forms of human GBM (1). However, ~60% of these tumors lacked appreciable necrosis and were thus considered grade III GEM astrocytoma (Supplementary Table S1). The penetrance of tumor initiation was attenuated when one of the three oncogenes was removed from the cocktail. Mice injected with shp53/EGFRvIII and NRAS/EGFRvIII did not develop tumors, whereas 60% of mice injected with NRAS/shp53 developed GEM grade III or IV gliomas (Supplementary Table S1; experiments 15–17 in Table 1).

Cell lines were established from mice injected with NRAS/shp53/EGFRvIII when they developed neurologic symptoms. We designated the lines derived from mice in experiment 14 as 14-1 and 14-2, and 2 additional lines not shown in Table 1 as M11 and M12. Because the shp53 vector contains a GFP expression cassette, the expression of GFP was investigated using fluorescent microscopy. The vast majority tumor spheres in any microscopic field exhibited green fluorescence (Fig. 4A), confirming presence of shp53/GFP vector. Both 14-1 and 14-2 cells expressed NRAS by Western blot (M11 and M12 were not tested; Fig. 4B). All cell lines derived from tumors induced with NRAS/shp53/EGFRvIII lacked detectable p53 protein (Fig. 4C).

Discussion

Despite decades of research, the prognosis for MG patients has only improved marginally (37). Arguably, unsatisfactory progress may be attributable to the paucity of animal models that are biologically accurate yet convenient enough to use in preclinical trials. A model that uses mutations relevant to human MG and can be flexibly modified to mimic the biology of human MG would be valuable. This SB-induced somatic model seems to meet these criteria, producing transplantable tumor initiating cells, histologic, and genetic heterogeneity similarly to human MG. Whether this model will be better at predicting clinical responses relative to established models remains to be determined.

There are several key advantages of the SB-induced model that make it novel and potentially useful for a variety of applications. The SB system can integrate transposons up to 10 kb in size (24), and 80% of human cDNAs are <7 kb (38). Therefore, after including a promoter and polyadenylation signal for gene expression, SB is suitable to deliver 70% to 80% of all human cDNAs. In contrast, the RCAS model is limited to 2.6 kb (12) and most retroviruses >7.0 kb in size are difficult to produce. The SB-induced model could be used to rapidly validate MG candidate genes discovered by comparative genomic approaches or forward genetic screens. Straightforward approaches to measure glioma viability using bioluminescence in spontaneous models have been lacking. In the RCAS model, FLuc was expressed only in dividing cells (14) or bioluminescence in spontaneous models have been lacking. In the RCAS model, FLuc was expressed only in dividing cells (14) or
where FLuc expression is regulated by a ubiquitous CAG promoter, making it more flexible and convenient for measuring viability of the bulk tumor mass. When the appropriate combinations of oncogenes are used 100% of animals develop SB-induced tumors. This rate of tumor formation is equivalent or better than existing GEM models and is therefore for very appealing for preclinical trials. The SB-induced model offers unprecedented speed and flexibility because it can be used in existing GEM rather than requiring creation of new GEM to interrogate new biological questions. Although our initial studies have focused on mice, we speculate that the SB-induced model could be applied to rats and perhaps larger animals. Molecular analyses showed that all but one cell line derived from the tumor expressed the genes delivered by cotransfection. Therefore, the SB-induced model could be useful to produce genetically engineered cell lines in several species.

It is possible that insertional mutagenesis played a role in the heterogeneity of SB-induced tumors, as 28% of insertions mapped to introns (1 in an exon) and others could activate transcription of nearby genes (Supplementary Table S2). The observation that no common gene was hit twice argues against this possibility. In addition, NRAS or SV40-LgT gene transfer alone did not result in detectable tumor (Table 1). However, more insertions will have to cloned and characterized to determine what extent insertional mutagenesis may play in this model.

The clonality and cell of origin of these tumors are not entirely clear. Southern blot revealed that single clones derived from the cell lines exhibited identical banding patterns (data now shown), indicating tumors were monoclonal or that a single clone overtook the culture via selective outgrowth. In experiment 10, we analyzed tumors histologically before overt symptoms developed at 28 days. This experiment revealed that tumors arise in the lateral ventricle and invade the parenchyma (Supplementary Fig. S5). In one animal, it seemed that independent tumors were present in the lateral ventricles, and migratory tumor cells were seen in the olfactory bulb (Supplementary Fig. S6). We speculate that a subset of tumors in this model may be oligoclonal, arising simultaneously and sometimes migrating from the subventricular zone to the olfactory bulb similarly to normal neural stem cells (40). IHC data indicated that ependymal cells, subependymal cells, and cells >1 mm from the ventricle were transfected (Fig. 1). Although we determined that the tumor cell lines derived from the SB model expressed nestin, CD133, and CD15 (data not shown), it is not clear whether expression of these stem cell markers is a consequence of dedifferentiation or transformation of putative neural progenitor cells. This is an active area of investigation in our laboratory.

![Figure 5](http://www.aacrjournals.org/)

**Figure 5.** Characterization of the NRAS/shP53/EGFRvIII model. **A,** large tumor in the right hemisphere infiltrating the left hemisphere (left). Middle, ×20 magnification of region marked by the black box in A showing single cell infiltrates invading normal brain tissue. Right, representative mitotic figures (arrows). **B,** representative images of pseudopalisading necrosis, nestin, and EGFRvIII IHC are shown (black arrows, few EGFRvIII-positive cells). **C,** representative images of GFAP, S100, and NeuN staining are shown where T marks the bulk tumor mass and NB marks normal brain.
Human MG evolves with the host immune system, a different circumstance than that modeled by injecting glioma cells into the brain of adult mice. MG patients often develop tolerance to tumor antigens facilitated by an elevated fraction of regulatory T cells (41). Nonetheless, spontaneous antigenic immune responses have been documented after successful treatment with radiation and chemotherapy (42, 43). In the current study, tumors developed in immunocompetent mice presumably tolerized to foreign antigens used to drive MG formation and Treg frequency was not assessed. Future studies will address these features. We have previously shown that neonatal mice are tolerized to challenge with human neoantigens we delivered using SB. The penetrance of tumor development dropped in mice older than 3 days despite equivalent transfection efficiency and we could not induce tumors with SV-40-Lg1/INRAS in adult mice (experiments 18 and 19; Table 1). Additionally, M7 cell transplants were rejected in 2/5 immunocompetent C57BL/6 mice (data not shown) but not in SCID mice (Supplementary Fig. S3). The SB-induced somatic tumors could be initiated in GEM for immunology studies, creating “humanized” models that express human tumor antigens and human MHC class I (45). Likewise, human RTK genes could drive tumor growth so that small molecule drugs or humanized antibodies could be tested on the human RTK to diminish artifacts with species specificity. This SB-induced somatic model offers a new tool to understand the biology of MG and realize effective targeted therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 5/15/2008; revised 11/9/2008; accepted 11/10/2008.

Grant support: NIH/NINDS 1R21-NX055738-01A2 (J.R. Ohlfest), Randy Shaver Foundation (J.R. Ohlfest), Minnesota Medical Foundation (J.R. Ohlfest), NIH/NIDA T32 DA02616 (S. Decker and W.C. Low), NIH/National Cancer Institute R01CA113636-01A (D.A. Largaespada), Minnesota Department of Employment and Economic Development SPAF-05-0013-P-FY06 (D.A. Largaespada).

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We thank Aaron Sarver for bioinformatics support in analyzing pyrosequencing data.

References

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