Overexpression Models: Lentiviral Modeling of Brain Cancer

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ABSTRACT

Glioblastoma multiforme (GBM) is one of the most common and most malignant of the brain tumors. Gliomas can be classified into four different grades according to their histologic characteristics; the most aggressive of the gliomas is glioblastoma multiforme (grade IV). Despite optimal treatment, the median survival is only 12 to 15 months. In the past few years, important advances were made in understanding the biology and pathology of malignant gliomas. A mouse model of brain tumors using inducible lentiviral vectors is described here. In this approach, a lenti-vector with *lox*P sites flanking the gene of interest (oncogene) is injected into mice expressing Cre recombinase under the control of a brain-specific promoter. The steps to perform cell-type/region-specific injection of Cre-*lox*P-controlled lentiviral vectors in the brain of adult mice are described here in detail. *Curr. Protoc. Mouse Biol.* 3:121-139 © 2013 by John Wiley & Sons, Inc.

Keywords: gliomas • lentivirus gene transfer • genetic alterations • Cre-*lox*P system • gene delivery

INTRODUCTION

Glioblastomas are the most common and lethal form of intracranial tumors. They account for ∼70% of the 22,500 new cases of malignant primary brain tumors that are diagnosed in adults in the United States each year (Furnari et al., 2007; CBTRUS, 2008; Wen and Kesari, 2008). Although relatively uncommon, malignant gliomas are associated with disproportionately high morbidity and mortality. These cancers exhibit a relentless malignant progression characterized by widespread invasion throughout the brain, resistance to traditional and newer targeted therapeutic approaches, destruction of normal brain tissue, and certain death. The median age of patients at the time of diagnosis is 64 years. Despite optimal treatment and improving standard of care, the median survival is only 12 to 15 months for patients with glioblastomas.

The laboratory mouse has been used extensively in cancer research, and gliomas are no exception. For many years, people have been modeling gliomas using different technologies and animal species. The major strategies that have been used thus far successfully are chemical mutagen–induced models, xeno- or allograft transplantation–induced models, germline genetic modification–induced models, and somatic genetic modification–induced models. All four strategies share the same goal—to develop animal models that accurately recapitulate human gliomas. These animal models are powerful tools that will hopefully provide excellent opportunities for identifying novel therapeutic targets for this disease and for testing potential new drugs and therapeutic strategies in vivo.

Malignant transformation in gliomas results from the sequential accumulation of genetic aberrations leading to disregulation of the cell cycle or mitogenic signaling pathways. The RB and p53 pathways, which regulate the cell cycle primarily by governing the G1-S phase transition, are major targets of inactivating mutations in glioblastomas (Furnari et al., 2007). The receptor tyrosine kinase, Ras, AKT, and PI3 kinase pathways

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involved in cell proliferation have also undergone changes ranging from point mutations to deletions leading to rampant and unregulated growth in glioblastomas. The complete genomic sequencing (through The Cancer Genome Anatomy project) of >206 human glioblastomas (McLendon et al., 2008; Parsons et al., 2008) revealed that 66%, 70%, and 59% harbored somatic mutations in the core components of the RB, p53, and RTK/RAS PI3 signaling pathways. In fact, 74% of glioblastomas sequenced harbored aberrations in all three pathways, thus confirming that alterations in these three pathways are a core requirement for glioblastoma formation. To understand the role of some or all of the altered genes found in the human gliomas, in recent years important advances have been made in the construction of transgenic mouse models harboring glioma-relevant mutations or combinations of mutations. In several cases, transgenic mice develop gliomas with many of the features of the human disease (Weissenberger et al., 1997; Uhrbom et al., 1998; Holland et al., 2000; Uhrbom et al., 2002; Holmen and Williams, 2005; Zhu et al., 2005; Charest et al., 2006).

To address the function of certain genetic loci in glioma formation, gain-of-function or loss-of-function germline modifications can be achieved by transgenic techniques (see reviews in Aguzzi et al., 1995 and Gao et al., 1999). Gain-of-function modifications can be expressed in specific cell populations using tissue-specific promoters to drive the expression of the oncogenes. For loss-of-function modifications, gene targeting techniques can cause deletion of specific genes in certain cell populations. Combining gain-of-function and loss-of-function by breeding of mouse lines allows determination of cooperative effects between oncogenes and tumor suppressor genes. Many concurrent genetic alterations are possible, but extensive breeding will be required to generate all the combinations. When tissue-specific promoters are driving expression of the oncogene, all of the cells in the organ will turn on the expression of the gene of interest at the same time. This is not so in human cancers, where tumors are probably initiated by genetic alterations acquired by one cell or a few cells in the organ.

In contrast to early murine glioma models (e.g., oncogene-bearing transgenic mice or tumor suppressor gene knockouts), newer models utilize the conditional regulation of gene expression. This last strategy for modeling human gliomas is somatic cell genetic modification using retroviral vectors. One system, the avian leukosis virus (ALV)–based system, is composed of two parts, the ALV-based replication-competent avian retrovirus (RCAS) vectors and transgenic mice that express the RCAS receptor tv-a from a tissue-specific promoter. The RCAS vectors are a family of retroviral vectors derived from the SR-A strain of Rous sarcoma virus (RSV), a member of the avian sarcoma-leukosis virus (ASLV) family. These vectors will only infect mammalian cells if they express tv-a, and so the gene transfer in this system is limited to cells that express the transgene promoter (Holland and Varmus, 1998). Another system, which will be the focus here, is based on inducible-lentiviral vectors (LV) using the Cre-*lox*P recombinase system. This approach uses the injection of LVs that are able to transduce both dividing (such as neural-progenitor cells) and non-dividing (such as terminally differentiated astrocytes) post-mitotic cells into adult brain cells. The LVs carry *lox*P sites that flank a cassette carrying multiple polyadenylation signals (to Stop transcription) preceding an oncogene, so that Cre recombinase can delete the Stop cassette and express the gene in a cell-type and region-specific manner (Marumoto et al., 2009). To achieve deletion in only a few cells, the LV-containing Stop sequences flanked by *lox*P sites, which will prohibit expression of the oncogene is injected to defined areas of the brain of Cre transgenic mice, so that only those cells infected with the LV delete the Stop cassette and express the oncogene. The versatility of this system may be supplemented by combining with a loss-of-function phenotype generated by adding shRNAs targeting a variety of tumor suppressor genes into the same vector. A flow chart summarizing the steps to follow and the procedures described in this article is shown in Figure 1.

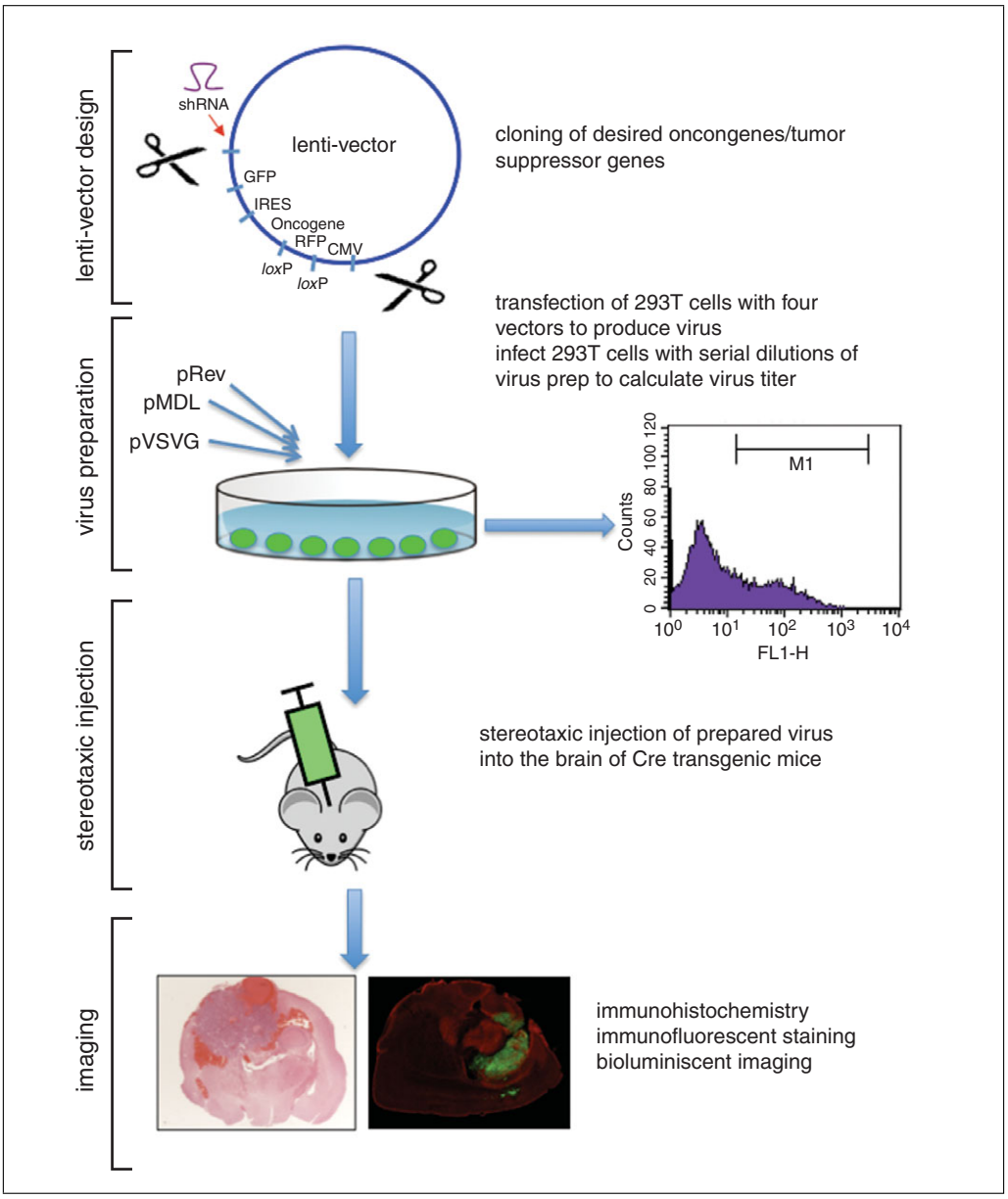

Figure 1 Flow chart summarizing the steps to induce glioma tumors by lentivirus injection into adult mouse brain. The first step consists of designing and cloning the desired oncogene/tumor suppressor genes in the lentiviral vector backbone. Once the lentivector has been validated in vitro, the next step will be to transfect 293T cells to prepare high-titer lentivirus (biological titer is confirmed by flow cytometry analysis). The concentrated lentivirus is stereotaxically injected into the brain of CRE-transgenic mice to induce the glioma tumors. Finally, the obtained tumors can be analyzed by different imaging techniques such as immunohistochemistry, immunofluorescence, or biolumminiscence imaging in vivo. GFP, green fluorescent protein; IRES, internal ribosome entry site; RFP, red fluorescent protein; CMV, cytomegalovirus.

Overview of Lentiviral Vectors

Lentiviral vectors derived from HIV-1 are capable of transducing a wide variety of dividing and non-dividing cells, integrate stably into the host genome, and result in long-term expression of the transgene (Trono, 2002). The third generation lentiviral vector system consists of four plasmids. The transfer vector contains the transgene to be delivered in a lentiviral backbone containing all the cis-acting sequences required for genomic RNA production and packaging. The packaging system involves three additional plasmids (pMDL, pRev, and pVSVG), which provide the required trans-acting

factors, namely Gag-Pol, Rev, and the envelope protein VSV-G (Vesicular Stomatitis Virus glycoprotein), respectively. Plasmid pMDL encodes a Gag-Pol precursor protein that is eventually processed into an integrase, reverse transcriptase, and structural proteins.

While the structural proteins are an absolute requirement of particle production, integrase and reverse transcriptase (packaged into the viral particle) are involved in events subsequent to infection. The presence of VSV-G in the viral envelope membrane confers upon the viral particle the ability to transduce a broad range of cell types, including primary cells, stem cells, and early embryos (Naldini et al., 1996; Lois et al., 2002; Pfeifer et al., 2002). While VSVG is usually the default choice, use of other envelope proteins results in targeting of viral particles to particular cell types (Verhoeyen and Cosset, 2004). In addition, an important safety feature is provided by a deletion of the promoter-enhancer region in the 3′LTR (SIN vectors). During reverse transcription, the proviral 5′LTR is copied from the 3′LTR, thus transferring the deletion to the 5′LTR; the deleted 5′LTR of the integrated provirus is therefore transcriptionally inactive, preventing subsequent viral replication or mobilization in the transduced cell (Miyoshi et al., 1998; Zufferey et al., 1998). When these four plasmids are transfected into 293T HEK cells, viral particles accumulate in the supernatant and high-titer viral preparations can be obtained by ultracentrifugation.

Overview of RNAi

RNAi has forcefully emerged as a pathway constituting a whole new level of control of gene expression. The pathway has been under intense study and details of its basic biological mechanism are described elsewhere (Denli and Hannon, 2003). Briefly, short, 21- to 23-nucleotide double-stranded small interfering RNAs (siRNAs) are incorporated into a multi-component nuclease complex, RNA-induced silencing complex (RISC), which selects and degrades mRNAs that contain a target complementary to the antisense strand of the siRNA (Fjose et al., 2001; Hannon, 2002). In mammalian systems, siRNAs can be delivered by (1) transient transfection of synthetic siRNAs (Elbashir et al., 2001) or (2) transient or stable transfection (if viral vectors are used, transduction) of constructs expressing small hairpin RNAs (shRNAs) expressed from pol III promoters (Brummelkamp et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2002; Rubinson et al., 2003; Tiscornia et al., 2003). More recently, methods have been developed for using pol II promoters for expression of shRNAs in the context of microRNA precursors (McManus et al., 2002; Stegmeier et al., 2005). The shRNA is processed by the endogenous microRNA pathway, resulting in the production of a mature siRNA and subsequent degradation of the target mRNAs.

BASIC PROTOCOL 1 DESIGN AND CLONING OF LENTIVECTORS EXPRESSING ONCOGENES AND shRNAs

Description of the pTomo LV and cloning strategy of the oncogenes (e.g., H-RasV12) has been described previously (Marumoto et al., 2009). In this protocol, a description on how to clone shRNAs in LVs and how these two cloning strategies can be combined is provided. Figure 2A shows a schematic diagram of the LV used in this particular mouse model. Additionally, guidance for validation of lentiviral constructs, which dovetails with transfection procedures covered in Basic Protocol 2, is provided.

For additional discussion on silencing construct design, cloning, and validation, see Critical Parameters and Troubleshooting.

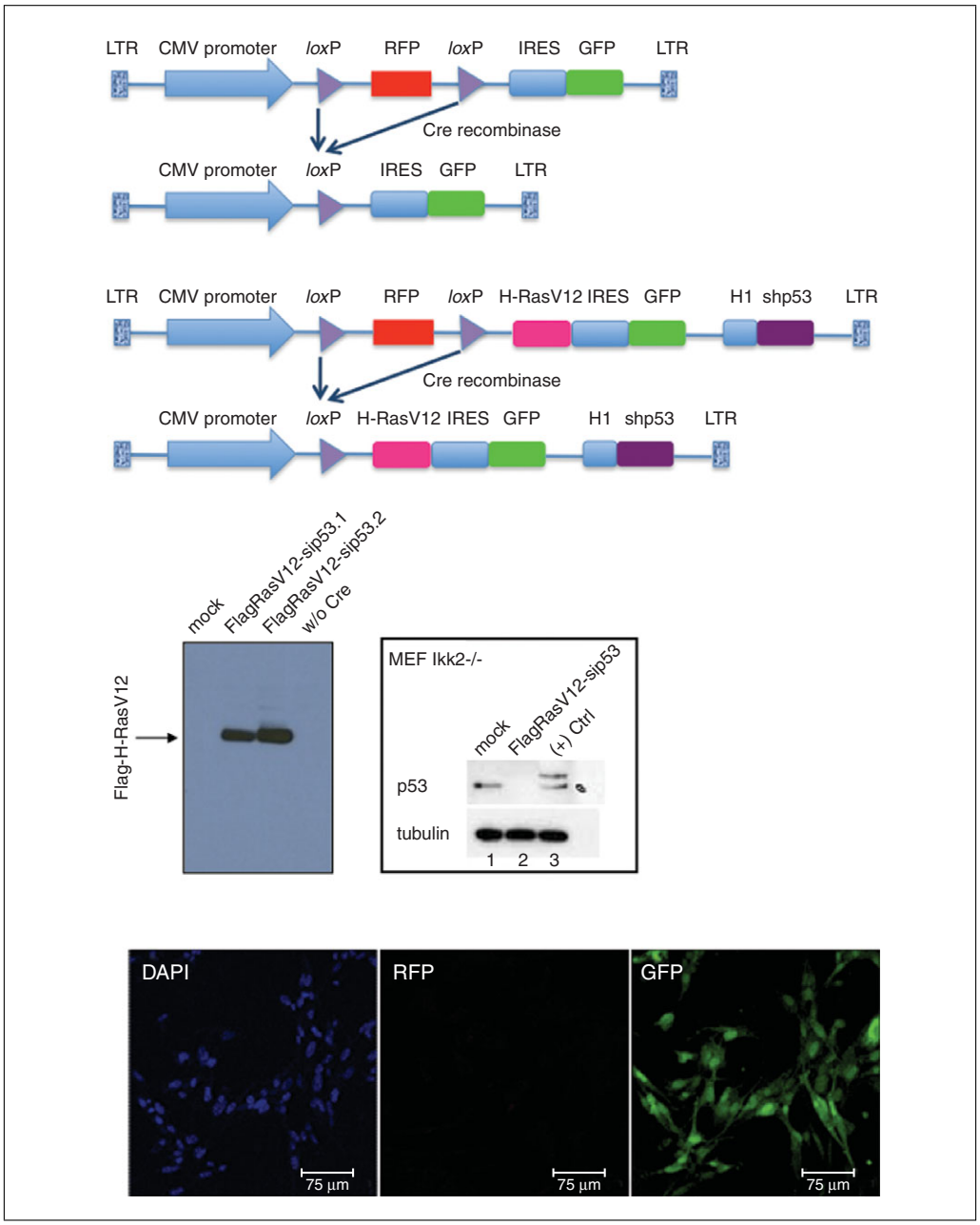

Figure 2 (**A**) Schematic representation of the lentiviral vector. The upper two schema show the basic pTomo mock construct (Marumoto et al., 2009) and the lower two schema show the H-RasV12-shp53 lentivector described. The floxed RFP fragment keeps the translation of the H-RasV12 in an "off" state. Only in the presence of Cre recombinase, the floxed RFP cassette is cut out resulting in the expression of the H-RasV12 gene. The shRNA under the H1 promoter targets the p53 protein. (**B**) A western blot showing expression of Flag H-RasV12 expression induced by Cre recombinase and silencing of p53. In the left-side blot, MEF (mouse embryonic fibroblasts) Ikk2−/− were infected with mock virus (lane 1), H-RasV12-shp53 (1 and 2 indicate different clones) plus Cre-expressing lentivirus (LVs) (lanes 2 and 3), and without (w/o) Cre LV (lane 4). Cell lysates were processed for western blot using anti-Flag antibody. For the right-side blot, MEF Ikk2−/− cells were infected with mock virus (lane 1), H-RasV12-shp53 plus Cre (lane 2), and in lane 3 a positive control for p53 was run. Tubulin detection was used as loading control. (**C**) Immunofluorescent microscopy showing H-RasV12 expression (GFP) induced by Cre recombinase. Primary astrocytes isolated from GFAP-Cre mice (which express Cre) were infected with H-RasV12-shp53. The infected cells express GFP but lost expression of RFP. Blue = Dapi (nuclear staining); green = GFP.

Materials

Primers
Pol III promoters plasmids (pH1, phU6 or pmU6) and appropriate lentiviral vectors (Verma Laboratory, Salk Institute for Biological Studies, *verma@salk.edu*)
Advantage GC-2 polymerase mix (BD Biosciences) with GC melt or *Taq* polymerase with 7% DMSO
Appropriate enzymes
Thermal cycler

Additional reagents and equipment for PCR amplification (Kuslich et al., 2008), cloning (Finney et al., 2001), and sequencing (Shendure et al., 2011)

1. Select a target within the gene to be silenced. For example, for mouse p53: 5′- GGAGCTATTACACATGTAC-3′, the following Web site is used to predict shRNA candidates (*http://sfold.wadsworth.org/cgi-bin/sirna.pl*), but many others exist.

2. Design primers to amplify the silencing cassette; use pH1 (or phU6, pmU6) as template. The forward primer must contain an *Nhe*I or a compatible site such as *Xba*I, *Spe*I, or *Avr*II flanking the 5′ of the pol III promoter. The reverse primer must have a 22-nucleotide stretch at its 3′ end capable of annealing to the 3′ end of the sense strand of the pol III promoter and a 5′ tail including the entire shRNA, transcriptional stop signal (T5), and *Xba*I site sequences as follows: *Xba*I site – T5 – sense – loop – antisense – H1 complimentary sequence. The same design will work with the hU6 and mU6 promoters with appropriate promoter-specific complementary sequences; although, U6 promoters require that position number one in the target sequence is a G.

3. For the target suggested above, design the following 3′ reverse primer: 5′-CTGTCTAGACAAAAAGGAGCTATTACACATGTAC**TCTCTTGAA**GTACAT GTGTAATAGCTCc***GGGGATCTGTGGTCTCATACA-3′***, where the sequence in italics is complementary to the H1 promoter, the *Xba*I site is underlined, the loop is bold and underlined and nucleotide +1 is in small caps. Replace the sense and antisense parts with equivalents from the new shRNA candidates.

4. Amplify the silencing cassette using the 5′ forward primer and the 3′ reverse primer (final primer concentration is 10 μM) described above, use 10 ng template pH1 (pmU6 or phU6 with appropriate forward primer) to PCR amplify the silencing cassette. It is essential to add DMSO or other similar agents to weaken hydrogen bonding and prevent formation of hairpin structure. Amplify the product on a thermal cycler using the following PCR parameters:

1 cycle:	3 min	94°C
30 cycles:	30 sec	94°C
	30 sec	55°C
	40 sec	72°C
1 cycle:	10 min	72°C

The authors use the Advantage GC-2 polymerase mix (BD Biosciences) with GC melt additive or Taq polymerase with 7% DMSO to prevent hairpin formation.

5. Clone and sequence-verify the silencing cassette.

PCR amplification results in a ∼300-bp fragment containing the entire pol III promoter plus shRNA cassette. The complete nucleotide sequence of the insert should be determined, as mutations in the hairpin, potentially introduced by PCR, can have considerable impact on silencing efficiency.

The insert can be cloned into a PCR cloning vector (e.g., pGEM-T, Promega) and, following XbaI digest, the insert can be subsequently transferred into the lentivector (or, alternatively, the XbaI-digested PCR product can be cloned directly into the lentivector followed by sequencing of the hairpin).

The insert must be digested with XbaI, gel purified, ligated into the lentivector (previously linearized with NheI, dephosphorylated, and gel purified), and transformed.

6. Validate the lentivectors by sequencing the oncogene or cloning shRNA into the vector, as well as validating the insertion by appropriate restriction enzymes.

   Simple transfection of the lentiviral vector and Cre-LV (lentivector expressing Cre) will help to verify the switch from an RFP-plus GFP-expressing cell (without Cre) to a cell expressing only GFP when Cre recombinase is co-transfected. Protein lysate can also be prepared from these transfected cells and used to check expression of the inserted oncogene (similar to checking expression of Flag-H-RasV12 using anti-Flag antibody, see Fig. 2B). The same line of experiments can be done to validate the shRNA in the final construct using a cell line that is known to express the target mRNA to be silenced.

PRODUCTION OF LENTIVIRAL VECTORS

BASIC PROTOCOL 2

This protocol describes step-by-step instructions on how to produce high-titer infectious lentiviral vector particles. This protocol assumes that lentivectors (silencing and controls) have been made and validated according to Basic Protocol 1. The lentiviral vectors with the desired targeting cassettes are mixed with three packaging vectors (pMDL, pRev, and pVSVG), transfected into packaging cells, and the resulting viruses are harvested and titered. Successful execution of these procedures will produce viruses suitable for in vitro and in vivo use (see Basic Protocol 3 for application in vivo).

Materials

293T HEK mammalian cells (ATCC #CRL-11268)
DMEM plus 10% FBS
10% poly-L-lysine (SIGMA, cat. no. P-4832) in PBS, filter sterilize and store indefinitely at 4°C
Plasmids:
  Lentiviral transfer vector (Verma Laboraory, Salk Institute for Biological Studies, *verma@salk.edu*)
  Lentiviral packaging vectors: pMDL, pRev, and pVSVG (Addgene)
  Endotoxin-free maxipreps (Endo-free Maxiprep Kit, Qiagen, cat. no. 12632, or equivalent)
  1 μg/μl resuspended DNA
2.5 M $CaCl_2$ stock solution: 36.75 g $CaCl_2$ in 70 ml ddH_2O, bring to final volume of 100 ml, dispense into 1.5-ml microcentrifuge tubes, and store indefinitely at −20°C
2× BBS solution (50 mM BES/280 mM NaCl/1.5 mM Na_2HPO_4): 16.36 g NaCl, 10.65 g BES (Calbiochem, cat. no. 391334), 0.21 g Na_2HPO_4, add ddH_2O up to 900 ml; dissolve, titrate to pH 6.95 with 1 M NaOH, bring volume up to 1 liter, filter to sterilize, and store 14-ml aliquots indefinitely at 4°C
Hanks' balanced salt solution (HBSS, GIBCO, cat. no. 14175)
20% sucrose in 1× HBSS (filter sterilize and store indefinitely at 4°C)

12 × 15–cm tissue culture dishes
50-ml tubes
37°C, 3% to 5% CO_2 tissue culture incubator
Fluorescent microscope
37°C, 10% CO_2 tissue culture incubator
0.45-μm filter units, 500-ml capacity (Corning, cat. no. 430773)
Conical bottom ultracentrifugation tubes (Beckman, cat. no. 358126)

Beckman ultracentrifuge and Beckman SW28 rotor (or equivalent)
Aspirator
Round-bottom ultracentrifugation tubes (Beckman, cat. no. 326819)
Beckman SW55 swinging bucket rotor (or equivalent)
1.5-ml screw-cap microcentrifuge tubes
12-well plates

CAUTION: Working with lentiviral vectors requires Biosafety level II containment. For safety procedures regarding handling of lentiviral vector preparations, see Biosafety in Microbiological and Biomedical Laboratories, 4th edition, published by the Centers for Disease Control (CDC), which can be found at *http://www.cdc.gov/od/ohs/biosfty/bmb14/bmb14toc.htm*.

Day 1: Seed 293T HEK cells

1. For a 12 × 15–cm dish lentiviral preparation (yields 200-µl suspension of ~10^6 viral particles/µl), start the seeding procedure from two 12 × 15–cm dishes of confluent 293T HEK cells grown in DMEM plus 10% FBS.

   These dishes should not have been previously coated with poly-L-lysine, as this makes trypsinization difficult.

2. Pre-coat 12 × 15–cm dishes with poly-L-lysine to enhance cell adherence to the substrate. Add 10 ml of poly-L-lysine per dish, incubate 15 min at room temperature, siphon off the liquid, and proceed immediately.

3. Seed 293T HEK cells by splitting 293T cells from two confluent 15-cm dishes to twelve 15-cm dishes using DMEM plus 10% FBS. Swirl cells thoroughly to obtain even distribution across the surface of the dish.

   Cells should preferably be of low passage number; do not use cells after passage 20 or if growth is slow.

Day 2: Transfect 293T cells with plasmid mix

4. Observe the dishes seeded on day 1 (cells should be 30% to 40% confluent at time of transfection, but should still have room to undergo one to two cell divisions).

5. Prepare the plasmid mix for the $CaPO_4$ precipitation method. Dispense the following four plasmids into a 50-ml tube; for a 12 × 15–cm dish preparation, use 270 µg of transfer vector, 176 µg of pMDL (Gag/Pol), 95 µg of pVSV-G, and 68 µg of pREV.

6. Prepare 13.5 ml of 0.25 to 0.3 M $CaCl_2$ (dilute with filtered ddH_2O) and add to the plasmid mix.

7. Add 13.5 ml of 2× BBS solution to $CaCl_2$ plus plasmids mix.

   Accurate pH of the BBS solution is critical for the efficiency of transfection.

8. Mix gently by inverting several times and incubate 15 min at room temperature.

9. Add the transfection mixture (spreading in drops) to each plate (2.25 ml per plate). Swirl the plates gently and incubate overnight (16 to 20 hr) in a 37°C, 3% to 5% CO_2 tissue culture incubator.

   IMPORTANT NOTE: *Incubation at 3% to 5% CO_2 is absolutely required, as $CaPO_4$ precipitation (and therefore transfection efficiency) is highly dependent on pH of growth medium.*

Day 3: Change medium

10. Observe the cells (cells should be reaching confluency and should show >90% transfection efficiency as judged by GFP+ cells). Discard plates that are showing <70% transfection efficiency as virus titer is significantly reduced with lower efficiencies.

11. Remove medium. Add 15 ml of fresh DMEM plus 2% FBS to each plate and incubate overnight in a 37°C, 10% CO_2 tissue culture incubator.

Day 4: Collect first harvest of supernatant

CAUTION: From this point on, supernatants and suspensions contain infectious lentiviral particles.

12. On the next morning, collect and pool supernatant from dishes. Add 15 ml of fresh DMEM plus 2% FBS to each dish. Incubate dishes overnight in a 37°C, 10% CO_2 tissue culture incubator. Store supernatant at 4°C.

Day 5: Collect second harvest of supernatant

13. On the next morning, collect supernatant from dishes. Pool supernatant from the first and second harvests. Clear the supernatant of cell debris by filtering through a 0.45-μm filter.

    Filtered supernatants can be stored for up to 3 days at 4°C before proceeding to further concentration. At this point the plates should be discarded.

Day 6: Concentrate viral preparation

14. Concentrate viral particles by ultracentrifuging the supernatant 2 hr at 70,000 × *g*, 20°C, using conical tubes and a swinging bucket rotor.

    The authors use conical tubes and centrifuge using a Beckman SW28 rotor (capacity for six tubes) 2 hr at 19,400 rpm, 20°C. Fill each tube with 30 ml of supernatant—the total volume of supernatant from a 12 × 15–cm dish preparation is 360 ml; therefore, processing the full volume will require two consecutive spins.

15. Pour off the supernatant and allow the remaining liquid to drain by resting the inverted tubes on paper towels. Siphon off remaining droplets using an aspirator to remove all liquid from the pellet.

    The pellet should be barely visible as a small translucent spot.

16. Resuspend all pellets in a total of 1 ml of 1× HBSS. Rinse the tubes with a second 1 ml volume of 1× HBSS and pool both volumes. Store solution for 2 to 3 days at 4°C until final centrifugation or store longer at −20°C.

    The crude virus prep can be used as is for tissue culture experiments but for brain injection, an additional purification is required.

Day 7: Purify virus through a sucrose cushion

17. Centrifuge the preparation through a sucrose cushion. Pipet 1.5 ml of 20% sucrose (in HBSS) into tube and then carefully overlay combined volume of 2 ml (from step 16) without mixing the two phases.

18. Add 1× HBSS to the tubes as needed to fill and balance tubes before centrifugation (the combined volume should be 4.5 ml).

19. Centrifuge tubes 2 hr at 50,000 × *g*, 20°C.

    The authors use Beckman round-bottom tubes and a Beckman SW55 swinging bucket rotor (2 hr at 21,000 rpm, 20°C).

20. Pour off the supernatant, allow liquid to drain by resting the tube inverted on a paper towel and siphon off all remaining droplets with an aspirator.

21. Resuspend the clear, translucent pellet in 50 to 100 μl of 1× HBSS.

22. Rinse the tube with an additional 50 to 100 μl of 1× HBSS. Transfer to a 1.5-ml screw-cap microcentrifuge tube, and shake on a low-speed vortex for 15 to 30 min.

    The suspension will range from clear to slightly milky.

23. Clear insoluble debris by spinning for 10 sec on a tabletop microcentrifuge. Allow microcentrifuge to reach maximum speed (typically 13,000 rpm or 20,000 rcf) and then power down.

24. Prepare 20-μl aliquots and store 2 months at −20°C (for short term) or >2 months at −80°C (for long term). Avoid repeated freeze-thaw cycles.

Determine titer

At this point it is important to determine the titer of the viruses to make sure that the same amount of infectious particles per virus is used in a given experiment. The titer is defined as number of infectious particles per milliliter or microliter. For consistency, each individual prep should be titered using the same method.

25. Plate 293T cells in a 12-well plate at 20,000 cells per well.

26. On the next day, transduce each well with serial five-fold dilutions of each virus starting with 1 μl per well (six wells per virus). Count the number of cells per well at the time of transduction.

27. Two days later, harvest each well for FACS analysis of percentage of GFP-positive cells per well. Calculate titer by multiplying the %GFP-positive, at well showing between 10% and 30% positive, with number of cells per well at the time of transduction. Multiply this number with the dilution factor and the result is the number of infectious particles per microliter (IU/μl).

BASIC PROTOCOL 3

STEREOTAXIC INJECTION OF LENTIVIRUS INTO THE BRAIN

In this protocol, a step-by-step description on how to inject the lentivirus into the brain of an adult mouse is provided. The instructions in this particular protocol have broad applications, but it is assumed that the procedures in Basic Protocols 1 and 2 have been successfully carried out, and thus that titered, working lentivirus stocks have been prepared and are ready to use.

Materials

Anesthetics: 10 mg/ml ketamine and 1 mg/ml xylazine in 0.9% saline
8- to 16-week-old GFAP-Cre mice
Artificial tears (eye lubricant ointment, Butler, cat. no. AHS NDC 11695-1418-6)
Betadine surgical scrub
70% ethanol
Sterile PBS
Hydrogen peroxide (Sigma, cat. no. H-1009)
Virus solution (see Basic Protocol 2)
Tissue adhesive (3M Vetbond, cat. no. 1469SB)

Biosafety level-2 facility
Warming pad
Electric hair trimmer or shaver
Stereotaxic instrument (KOPF model 900)
Cotton swabs
Surgical tools
Microsyringes (Hamilton, cat. no. 87925)
33-G needles for microsyringes (Hamilton, cat. no. 7762-06)
Marker pen
Electric drill and drill burr, size no. 1 (Henry Shein, cat. no. 100-7176)
4-0 nylon thread

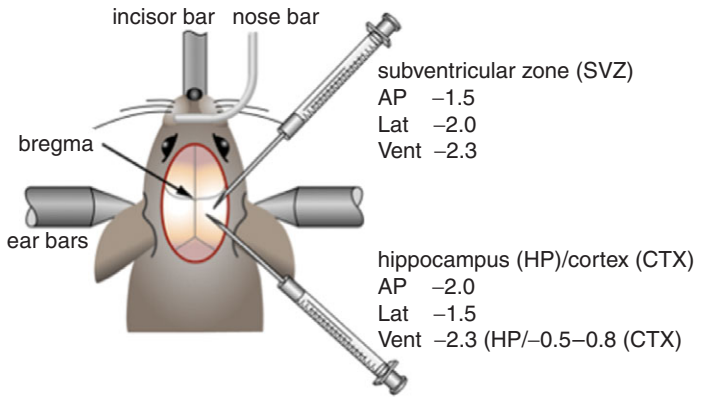

Figure 3 Diagram showing the position of the mouse head on the stereotaxic instrument and the specific coordinates for injection of the virus in different locations of the brain. AP = anterior/posterior, lat = lateral, vent = ventral, HP = hippocampus, CTX = cortex, SVZ = subventricular zone.

1. In a Biosafety level 2 facility, inject anesthetics (100 mg ketamine/10 mg xylazine per 1 kg body weight) into 8- to 16-week-old GFAP-Cre mice i.p. Place mice on the warming pad and allow 5 to 10 min for the anesthetics to take effect (until mice do not respond to toe pinches).
2. Shave a small area on the tip of the head of the mouse using a trimmer (see Fig. 3).
3. Place the mouse correctly onto the stereotaxic instrument and apply eye lubricant ointment to prevent eyes from over-drying. Sterilize the scalp using a cotton swab, first with betadine/iodine and then with 70% ethanol.
4. Cut a small incision (1 cm) into the scalp and wipe the area with clean cotton swabs and PBS. Clean tissue, periostium (shiny part), from skull using 1:1 hydrogen peroxide/PBS solution; clean the area well with cotton swabs and PBS.
5. Attach the microsyringe to the stereotaxic frame and position the tip of the needle over the Bregma.
6. Move the needle tip to −1.5 mm lateral and −2.0 mm posterior for the injection into the hippocampus (dentate gyrus), −2.0 mm lateral and −1.5 mm posterior for the injection into the subventricular zone, and −1.5 lateral and −2.0 mm anterior for the injection into the cortex. Mark the positions with a marker pen. See Figure 3 for a schematic representation of the brain coordinates for the injections in the aforementioned locations.
7. Lift the needle tip and make a small hole on the skull using an electric drill. If needed, clean the area with PBS.
8. Proceed to load the virus solution (1 µl of 1×10^9 IU/ml virus preparation). Move the tip down (ventral) by 2.3 mm for the hippocampus and subventricular zone injections and 0.5 to 0.8 mm for the cortex injection.
9. Start to inject the virus very slowly (0.1 µl/30 sec) to maintain the site of the injection localized and to avoid damaging the normal brain tissue.
10. When the virus injection ends, leave the needle for 5 min to prevent the injected solution from flowing back through the needle track.

11. Finally, close the skin by sewing with 4-0 nylon thread and by applying a thin layer of glue (VetBond). Place the mouse back into the cage on the warming pad until it is fully alert.

12. Observe the mice injected with the virus until they start to show symptoms of tumor, e.g., walking disturbance, loss of weight, and enlarged head. Refer to Anticipated Results for a detailed description of symptoms and timeline of tumor development.

BASIC PROTOCOL 4

TISSUE PROCESSING AND IMAGING

The latency of GFAP-Cre mice injected in the hippocampus with H-RasV12-shp53 virus is ~8 to 10 weeks. Mice that succumb to the disease show enlarged heads and kyphosis (hunched back; see Fig. 4A). At this point, mice should be euthanized and

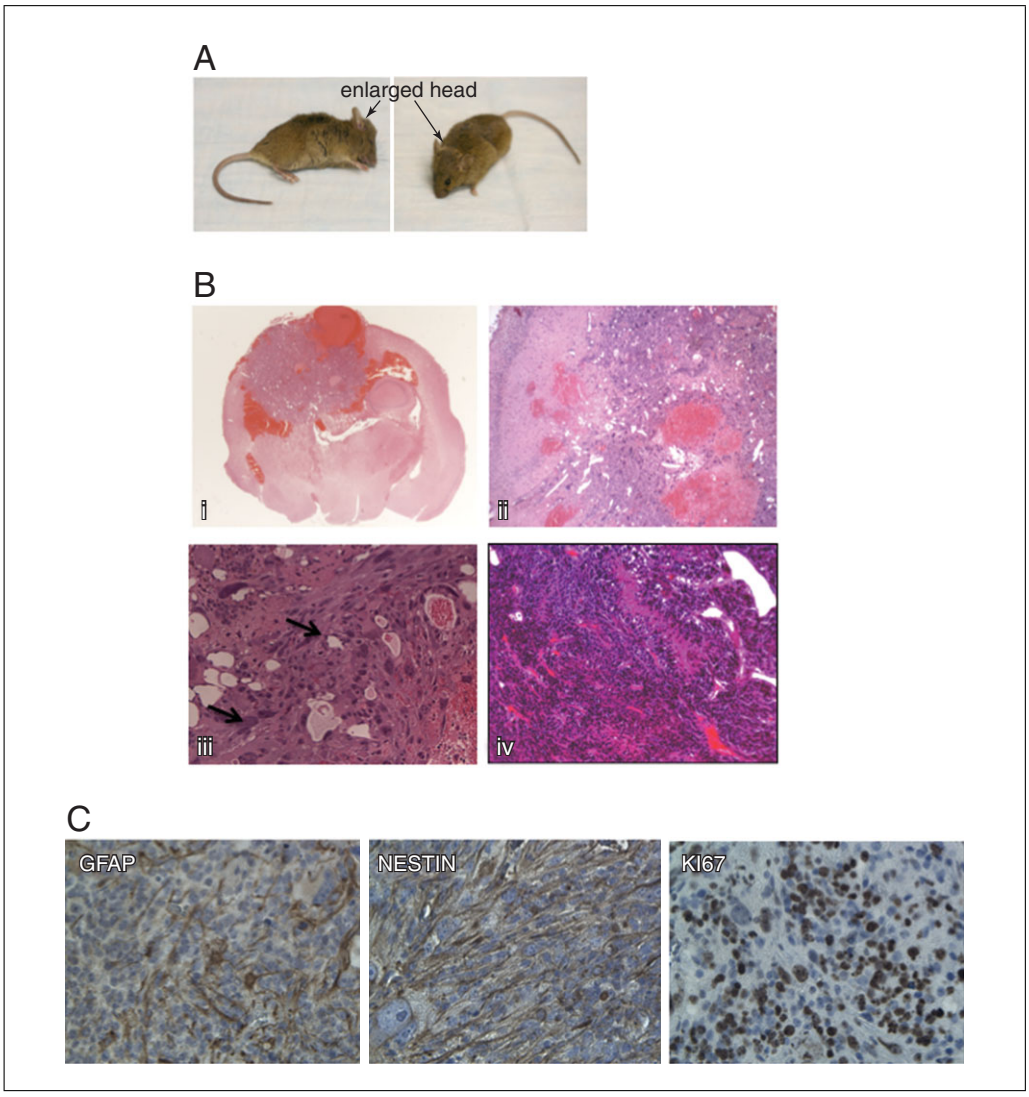

Figure 4 Brain tumors induced by H-RasV12-shp53 lentivirus injected in GFAP-Cre mice. (**A**) Images of GFAP-Cre mice showing tumor formation (enlarged head). (**B**) Hematoxilin and eosin (H&E) staining showing the common features of glioblastomas: (1) hemorrhage (magnification 4×), (2) necrotic areas surrounded by high density cellular regions (magnification 10×), (3) perivascular infiltration and multinucleated giant cells (arrows; magnification 40×), and (4) pseudopalisading (magnification 10×). (**C**) Representative images of immunohistochemical analysis using the indicated antibodies. Glial fibrilary acidic protein (GFAP). Magnification 40×.

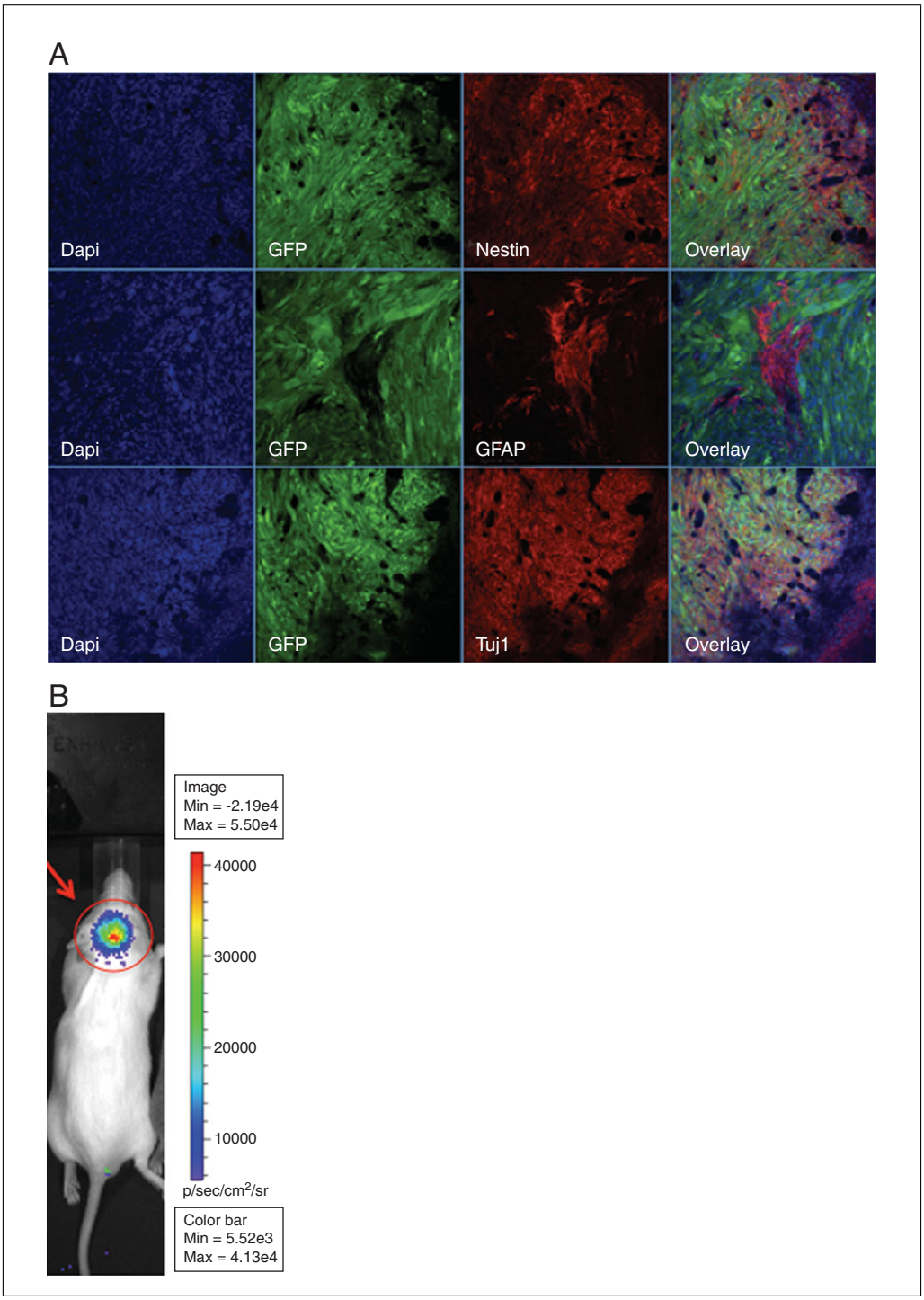

Figure 5 (**A**) Representative confocal microscopy images of 40-μm tumor sections labeled with the indicated antibodies. Magnification 20×. (**B**) Bioluminiscent image (BLI) of luciferase-label tumor bearing GFAP-Cre mouse (red arrow and circle points to the tumor). The GFAP-Cre mouse was injected with H-RasV12-shp53 (luciferase) into the hippocampus and 8 weeks later the tumor was visualized by BLI.

perfusion/fixation with 0.9% NaCl followed by 4% paraformaldehyde in PBS should be performed to collect the brain.

Brain samples can be embedded, sectioned, and stained with hematoxylin and eosin (H&E) or stained for different CNS markers (immunohistochemistry, as previously described in Gage et al., 1995). The samples can also be cryopreserved and cut into

sections with a cryostat or microtome and sections stained with immunofluorescent antibodies (as previously described in Gagneux et al., 2003) and analyzed by confocal microscopy.

The H&E staining will help classify the brain tumors obtained by the lenti-injections. Figure 4B shows an example of a brain tumor showing all the common features of a glioblastoma multiforme (GBM; Grade IV): multiform or variable appearance with evidence of old and recent hemorrhage; necrosis with palisading around necrotic foci; endothelial proliferation, perivascular infiltration; multinucleated giant cells; and highly vascular tumor.

Immunohistochemistry will help to determine the most common cell types usually found in glioma tumors: astrocytes (glial fibrillary acidic protein, GFAP–positive, +ve), neurons (β-tubulin III/Tuj1 +ve), and oligodendrocytes (O4 or myelin basic protein, MBP, +ve). Glial-derived neoplasms also express Nestin, the expression level of which is higher in high-grade than in low-grade gliomas (Dahlstrand et al., 1992). The proliferation index can be quantified by immunohistochemical analysis of Ki67 staining (Fig. 4C). Taking advantage of the GFP reporter in this vector, the same antibodies used for immunohistochemistry can be used in immunofluorescent staining and analyzed by confocal microscopy (Fig. 5A). This way it is possible to identify which markers are mostly expressed by the GFP+ tumor cells. As an example and using this technique, Dr. Yasushi Soda from the authors' laboratory found that GFP+ tumor cells can transdifferentiate into endothelial cells and form new blood vessels (Soda et al., 2011).

Finally, the tumors can be monitored by bioluminescent imaging if the fluorescent reporter, GFP, is replaced with the luciferase gene (Fig. 5B). In brief, mice bearing luciferase-labeled GBM tumors are injected with 3 mg per mouse of freshly prepared luciferin substrate. The mice are then anesthetized with isofluorane and imaged using the Xenogen IVIS 100 imaging system, 10-min post-intraperitoneal injection of luciferin at a 1-min acquisition time in a small binning mode (Fig. 5B). Biophotonic tumor imaging is a very convenient method when the effect of a new drug or therapeutic approach is tested on these tumors (Agemy et al., 2011).

COMMENTARY

Background Information

Different strategies have been developed to achieve conditional expression or deletion of a specific gene in a tissue and site-specific manner. One of the strategies uses the Cre-*lox*P system. Mouse models of GBM have been generated using the Cre-*lox*P conditional knockout system. One group used the combined deletion of *Nf1* and *Trp53* tumor suppressors lost by Cre-mediated recombination and germ-line mutation, respectively (GFAP-Cre; $Nf1^{f/+}$; $Trp53^{\pm}$; Zhu et al., 2005). In this system, the addition of a third mutation in Pten (GFAP-Cre; $Nf1^{f/+}$; $Trp53^{\pm}$; $Pten^{f/-}$) led to more malignant tumors (Kwon et al., 2008). Another group used the combined deletion of *Trp53* and *Pten* using GFAP-Cre mice (Zheng et al., 2008). In a subsequent generation of glioma mouse models, targeting of neural stem/progenitor cells was achieved using a Nestin-CreER driver. CreER is a fusion protein consisting of Cre recombinase linked to a modified estrogen receptor ligand-binding domain and can mediate *lox*P recombination when CreER is translocated into the nucleus upon treatment of the mice with tamoxifen (Chen et al., 2009).

Using the Cre-*lox*P strategy, numerous concurrent genetic alterations are achievable, these genetic alterations are known, and the targeted cells are also defined by the promoter driving Cre, but the requirement for extensive breeding to generate the desired combination makes the strategy time consuming and costly. In addition, all the cells expressing Cre will be genetically modified at the same time, which is not the case in human cancers where one or a few cells acquire the genetic alterations that lead to transformation and consequent development of tumors.

The last strategy, somatic cell gene transfer, differs from the germ-line gene modification approach in that the viral vector used to generate the tumors can transfer the gene of interest in a small number of cells and, most importantly, after birth (adult mice). These models can use different oncogenes and tumor suppressor genes to initiate the tumors, as well as different systems to generate these mutations, including viral-mediated technology and Cre recombinase transgenics. Holland and colleagues used the RCAS-TVA system to activate oncogenes in the mouse brain; the TVA expression was under the control of either the Nestin promoter or the GFAP promoter (Holland et al., 2000).

Here, the generation of brain tumors by Cre-inducible lentiviral vectors is described. It has been previously shown that injection of lentivirus in the hippocampus of GFAP-Cre mice generated 60 infected cells, and was sufficient to cause GBM development, showing advantages over the germ-line genetic modification techniques. Additionally, the vectors described allow the transfer of gene combinations using a single virus injection, giving the possibility to investigate the cooperative effect of overexpressing or knocking down various genes in the same cells simultaneously.

Critical Parameters and Troubleshooting

Basic Protocol 1

Selection of siRNA target sequences and design of shRNAs

The effectiveness of a particular siRNA is largely unpredictable and presumably reflects both mechanistic constraints of the RNAi pathway and accessibility of the target sequence within the tertiary structure of the target mRNA. A number of algorithms predict effective siRNA sequences (Reynolds et al., 2004) and many are available online at no cost (e.g., see *http://www.ambion.com* or *http://sfold.wadsworth.org*). In general, the target sequence should be 19 to 21 bases long, but lengths of up to 28 bases have been reported (Paddison et al., 2002). Longer targets should be avoided, as longer dsRNA molecules can trigger a protein kinase R (PKR) response (Clemens and Elia, 1997). A database search is recommended to filter out candidate targets that are present in other genes to avoid off-target silencing. GC content should be between 40% and 55%. shRNAs to be driven by the H1 promoter can begin with any base, but the U6 promoter requires a G as its first base. shRNAs can be directed to 5′UTR, ORF, or 3′UTR of the target mRNA as desired. Strings of identical bases should be avoided to circumvent stalling by the RNA polymerase III (in particular, 5 T's constitute a pol III transcriptional termination signal). As a loop, the 9-bp sequence (TTC AAG AGA, Brummelkamp et al., 2002) is generally used, but a variety of loops have been reported.

Cloning lentiviral silencing vectors

When an efficient shRNA candidate has been identified, it is cloned into the final lentiviral vector. High titer viral preparations should be tested by transduction of a cell line expressing the target protein followed by measuring target expression. The final validation of the lentiviral silencing vector against the endogenous target is crucial. It is important to realize that over-expression of any siRNA can cause some non-specific downregulation of gene expression; therefore, inclusion of adequate controls is critical. The best control is a lentiviral vector carrying a silencing cassette expressing a scrambled shRNA against no known target (alternatively, a lentiviral vector carrying a silencing cassette directed against a different, non-related target is acceptable). Several multiplicities of infection (MOI) should be tested in the transduction protocol (MOI is defined as the number of infectious particles per cell). Precise determination of target downregulation efficiency will require testing homogeneously transduced cell populations (Fig. 2C shows an example of a good transduction), which can be obtained by FACS sorting for GFP-positive cells.

For cloning hairpins into a lentiviral vector (Singer et al., 2005), the most convenient procedure is to PCR amplify the silencing cassette from a template pol III promoter by using a 5′ forward primer upstream of the pol III promoter and a 3′ reverse primer that includes the entire shRNA sequences (in 5′ to 3′ orientation of the primer: 5T's, antisense sequence, loop, sense sequence) followed by 22 bases complementary to the last 22 bp upstream of the +1 transcriptional start site of the pol III promoter. An *Nhe*I-compatible restriction site is included at the 5′ end of both forward and reverse primers. PCR amplification will result in a DNA fragment containing an shRNA expression cassette that can be cloned into a basic cloning vector (e.g., PGEM-T, Promega, or equivalent), tested, and then transferred to the lentiviral vector, or cloned into the lentiviral

vector directly at a unique *Nhe*I site positioned at the 3′ LTR (Fig. 2A).

Simultaneous silencing of two different genes

It is possible to silence two genes at the same time by cloning of two independent shRNA cassettes in the same vector. Efficacy of each cassette should be determined separately and then again in the context of the final vector. It is recommended to express each hairpin using a different pol III promoter (e.g., hH1 and hU6 or mU6) to avoid competition between identical promoters. In the authors' hands, positioning the stronger U6 promoter at the center of the viral vector and H1 promoter at the 3′ LTR site is preferred.

Validation of shRNAs

Typically, several shRNAs need to be generated and tested for every target gene. It is recommended that candidate shRNAs be cloned directly into lentiviral vector plasmid before validation. Initial screening is best achieved by co-transfection of an shRNA expressing plasmid and a vector expressing tagged (myc, FLAG, etc.) cDNA of the target into 293T HEK cells followed by western blotting against the tag. Alternatively, viral vector particles are used to transduce a cell type of interest and the effectiveness of target downregulation can be followed by analysis of target mRNA by quantitative RT-PCR, northern blot, or analysis of target protein levels by western blot against the endogenous target gene (Fig. 2A and B).

Basic Protocol 2

The most critical parameter in this protocol is to obtain an appropriate titer of the virus to be able to transduce oncogenes/tumor suppressor genes in a small number of cells in the brain. In the authors' hands, a good titer for injections is 1×10^9 IU/ml, considering that only 1 μl of this virus solution will be injected into the mouse brain.

Basic Protocol 3

If the mice do not show any symptoms of bearing brain tumors after the indicated time period (for H-RasV12-shp53 the latency is 8 to 10 weeks), one possible explanation is that a very low number of cells were infected or there were no cells infected at all. To confirm the approximate number of cells successfully infected with the lenti-vector, mice can be euthanized 7 days after the injection of the virus and the brain sections can be analyzed for the presence of GFP-positive cells. If no GFP-positive cells are present in the area injected (e.g., hippocampus), one possibility is that the needle was clogged during the injection procedure and no virus was injected. Another possibility is that a low titer virus was used for the injections and a very low number of cells were infected; a new batch of higher-titer virus should be tested.

If GFP-positive cells are only found outside of the target area, the mice were probably incorrectly mounted onto the stereotaxic frame. To find good stereotaxic coordinates, inject a dye (e.g., gel-loading dye for electrophoresis) into the mice at the same age as the ones used before. The size of the brain is different between transgenic lines and also at different ages.

Anticipated Results

GFAP-Cre transgenic mice injected with H-RasV12-shp53 in the hippocampus will start to show symptoms of disease in ∼8 to 10 weeks post-injection. These symptoms were described before but include mainly enlarged heads, hutched backs, and walking disturbances. At this point, the mice should be euthanized and the brain tissue collected as described before. The percent of success rate using this combination of oncogene/tumor suppressor gene is 100% and highly reproducible. If other oncogene/tumor suppressor genes are being used, the latency of tumor formation may vary. When testing new combinations of genes, usually one representative mouse from the 5 injected mice is euthanized at 8 weeks after the injection of the virus. Even if the latency is longer, a small lesion should be visible at this time point. When no evidence of tumor formation or any symptoms appear after 6 months of the injection day, leave the mice for another 6 to 8 months and only then collect the brain tissue for analysis (H&E staining). The same considerations are taken when different locations in the brain are being tested or new transgenic Cre lines are injected with the virus.

Time Considerations

The lenti-vector design (Basic Protocol 1) usually will require 1 week of work and will depend on the molecular biology skills of the investigator. Cloning takes 1 day, then bacteria are transfected and colonies are picked the next day and grown overnight to be able to proceed with mini-preps on the third day. If positive colonies are found (correct insert into the vector and sequencing results are positive), then the plasmid should be expanded

(maxi-prep), which usually takes 1 additional day. The required time for the validation of the obtained plasmid/lentivector will depend on the technique chosen (e.g., qPCR, western blot) but usually takes between 2 and 3 days.

The second step is virus preparation (Basic Protocol 2), which takes 7 days. It is possible to store the harvested viral supernatant on day 5 over the weekend (e.g., if the starting day was on a Monday) and proceed with centrifugations (day 6) on the following Monday. Once the virus is ready, the next step is stereotaxic injections into the mouse brain (Basic Protocol 3). The surgery procedure itself can take >30 min per mouse and this time also will vary with the skills of the investigator. It is possible to gain some time by placing the mice to sleep first and while the anesthesia takes effect, organizing the equipment and surgical tools required for the procedure. Virus injection for a total of five mice can take ~5 hr of work, from the beginning of the surgery until all the mice recover. With experience, this time can be shortened, and having two stereotaxic instruments can also help. By the time the first mouse is injected in the brain and the needle is still in place for another 5 min, the next mouse can be placed and prepared for the surgery in the second instrument. Finally, once the virus is injected, all that remains is to wait for symptoms of tumor development. This time will vary depending on the combination of oncongenes and tumor suppressor genes tested, but for the H-RasV12-shp53 virus described here, signs of tumor development start between week 8 and 9 after the injection. If luciferase was incorporated into the original vector, then bioluminescent imaging is an option and mice can be monitored for tumor development earlier.

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Literature Cited

Agemy, L., Friedmann-Morvinski, D., Kotamraju, V.R., Roth, L., Sugahara, K.N., Girard, O.M., Mattrey, R.F., Verma, I.M., and Ruoslahti, E. 2011. Targeted nanoparticle enhanced proapoptotic peptide as potential therapy for glioblastoma. *Proc. Natl. Acad. Sci. U.S.A.* 108:17450-17455.

Aguzzi, A., Brandner, S., Isenmann, S., Steinbach, J.P., and Sure, U. 1995. Transgenic and gene disruption techniques in the study of neurocarcinogenesis. *Glia* 15:348-364.

Brummelkamp, T.R., Bernards, R., and Agami, R. 2002. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296:550-553.

CBTRUS. 2008. Stastical report: Primary brain tumors in the United States, 199-2002. Central Brain Tumor Registry of the United States, 2000-2004 (Accessed July 7, 2008, at *http://www.cbtrus.org/reports/2007-2008/2007report.pdf*).

Charest, A., Wilker, E.W., McLaughlin, M.E., Lane, K., Gowda, R., Coven, S., McMahon, K., Kovach, S., Feng, Y., Yaffe, M.B., Jacks, T., and Housman, D. 2006. ROS fusion tyrosine kinase activates a SH2 domain-containing phosphatase-2/phosphatidylinositol 3-kinase/mammalian target of rapamycin signaling axis to form glioblastoma in mice. *Cancer Res.* 66:7473-7481.

Chen, J., Kwon, C.H., Lin, L., Li, Y., and Parada, L.F. 2009. Inducible site-specific recombination in neural stem/progenitor cells. *Genesis* 47:122-131.

Clemens, M.J. and Elia, A. 1997. The double-stranded RNA-dependent protein kinase PKR: Structure and function. *J. Interferon. Cytokine Res.* 17:503-524.

Dahlstrand, J., Collins, V.P., and Lendahl, U. 1992. Expression of the class VI intermediate filament nestin in human central nervous system tumors. *Cancer Res.* 52:5334-5341.

Denli, A.M. and Hannon, G.J. 2003. RNAi: An ever-growing puzzle. *Trends Biochem. Sci.* 28:196-201.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411:494-498.

Finney, M., Nisson, P.E., and Rashtchian, A. 2001. Molecular cloning of PCR products. *Curr. Protoc. Mol. Biol.* 56:15.4.1-15.4.11.

Fjose, A., Ellingsen, S., Wargelius, A., and Seo, H.C. 2001. RNA interference: Mechanisms and applications. *Biotechnol. Annu. Rev.* 7:31-57.

Furnari, F.B., Fenton, T., Bachoo, R.M., Mukasa, A., Stommel, J.M., Stegh, A., Hahn, W.C., Ligon, K.L., Louis, D.N., Brennan, C., Chin, L., DePinho, R.A., and Cavenee, W.K. 2007. Malignant astrocytic glioma: Genetics, biology, and paths to treatment. *Genes Dev.* 21:2683-2710.

Gage, F.H., Coates, P.W., Palmer, T.D., Kuhn, H.G., Fisher, L.J., Suhonen, J.O., Peterson, D.A., Suhr, S.T., and Ray, J. 1995. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc. Natl. Acad. Sci. U.S.A.* 92:11879-11883.

Gagneux, P., Cheriyan, M., Hurtado-Ziola, N., van der Linden, E.C., Anderson, D., McClure, H., Varki, A., and Varki, N.M. 2003. Human-specific regulation of alpha 2-6-linked sialic acids. *J. Biol. Chem.* 278:48245-48250.

Gao, X., Kemper, A., and Popko, B. 1999. Advanced transgenic and gene-targeting approaches. *Neurochem. Res.* 24:1181-1188.

Hannon, G.J. 2002. RNA interference. *Nature* 418:244-251.

Holland, E.C. and Varmus, H.E. 1998. Basic fibroblast growth factor induces cell migration and proliferation after glia-specific gene transfer in mice. *Proc. Natl. Acad. Sci. U.S.A.* 95:1218-1223.

Holland, E.C., Celestino, J., Dai, C., Schaefer, L., Sawaya, R.E., and Fuller, G.N. 2000. Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice. *Nat. Genet.* 25:55-57.

Holmen, S.L. and Williams, B.O. 2005. Essential role for Ras signaling in glioblastoma maintenance. *Cancer Res.* 65:8250-8255.

Kuslich, C.D., Chui, B., and Yamashiro, C.T. 2008. Overview of PCR. *Curr. Protoc. Essential Lab. Tech.* 00:10.2.1-10.2.31.

Kwon, C.H., Zhao, D., Chen, J., Alcantara, S., Li, Y., Burns, D.K., Mason, R.P., Lee, E.Y., Wu, H., and Parada, L.F. 2008. Pten haploinsufficiency accelerates formation of high-grade astrocytomas. *Cancer Res.* 68:3286-3294.

Lois, C., Hong, E.J., Pease, S., Brown, E.J., and Baltimore, D. 2002. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 295:868-872.

Marumoto, T., Tashiro, A., Friedmann-Morvinski, D., Scadeng, M., Soda, Y., Gage, F.H., and Verma, I.M. 2009. Development of a novel mouse glioma model using lentiviral vectors. *Nat. Med.* 15:110-116.

McLendon, R., Friedman, A., Bigner, D., Van Meir, E.G., Brat, D.J., Mastrogianakis, M., Olson, J.J., Mikkelsen, T., Lehman, N., Aldape, K., Alfred Yung, W.K., Bogler, O., Vandenberg, S., Berger, M., Prados, M., Muzny, D., Morgan, M., Scherer, S., Sabo, A., Nazareth, L., Lewis, L., Hall, O., Zhu, Y., Ren, Y., Alvi, O., Yao, J., Hawes, A., Jhangiani, S., Fowler, G., San Lucas, A., Kovar, C., Cree, A., Dinh, H., Santibanez, J., Joshi, V., Gonzalez-Garay, M.L., Miller, C.A., Milosavljevic, A., Donehower, L., Wheeler, D.A., Gibbs, R.A., Cibulskis, K., Sougnez, C., Fennell, T., Mahan, S., Wilkinson, J., Ziaugra, L., Onofrio, R., Bloom, T., Nicol, R., Ardlie, K., Baldwin, J., Gabriel, S., Lander, E.S., Ding, L., Fulton, R.S., McLellan, M.D., Wallis, J., Larson, D.E., Shi, X., Abbott, R., Fulton, L., Chen, K., Koboldt, D.C., Wendl, M.C., Meyer, R., Tang, Y., Lin, L., Osborne, J.R., Dunford-Shore, B.H., Miner, T.L., Delehaunty, K., Markovic, C., Swift, G., Courtney, W., Pohl, C., Abbott, S., Hawkins, A., Leong, S., Haipek, C., Schmidt, H., Wiechert, M., Vickery, T., Scott, S., Dooling, D.J., Chinwalla, A., Weinstock, G.M., Mardis, E.R., Wilson, R.K., Getz, G., Winckler, W., Verhaak, R.G., Lawrence, M.S., O'Kelly, M., Robinson, J., Alexe, G., Beroukhim, R., Carter, S., Chiang, D., Gould, J., Gupta, S., Korn, J., Mermel, C., Mesirov, J., Monti, S., Nguyen, H., Parkin, M., Reich, M., Stransky, N., Weir, B.A., Garraway, L., Golub, T., Meyerson, M., Chin, L., Protopopov, A., Zhang, J., Perna, I., Aronson, S., Sathiamoorthy, N., Ren, G., Wiedemeyer, W.R., Kim, H., Won Kong, S., Xiao, Y., Kohane, I.S., Seidman, J., Park, P.J., Kucherlapati, R., Laird, P.W., Cope, L., Herman, J.G., Weisenberger, D.J., Pan, F., Van Den Berg, D., Van Neste, L., Mi Yi, J., Schuebel, K.E., Baylin, S.B., Absher, D.M., Li, J.Z., Southwick, A., Brady, S., Aggarwal, A., Chung, T., Sherlock, G., Brooks, J.D., Myers, R.M., Spellman, P.T., Purdom, E., Jakkula, L.R., Lapuk, A.V., Marr, H., Dorton, S., Gi Choi, Y., Han, J., Ray, A., Wang, V., Durinck, S., Robinson, M., Wang, N.J., Vranizan, K., Peng, V., Van Name, E., Fontenay, G.V., Ngai, J., Conboy, J.G., Parvin, B., Feiler, H.S., Speed, T.P., Gray, J.W., Brennan, C., Socci, N.D., Olshen, A., Taylor, B.S., Lash, A., Schultz, N., Reva, B., Antipin, Y., Stukalov, A., Gross, B., Cerami, E., Qing Wang, W., Qin, L.X., Seshan, V.E., Villafania, L., Cavatore, M., Borsu, L., Viale, A., Gerald, W., Sander, C., Ladanyi, M., Perou, C.M., Neil Hayes, D., Topal, M.D., Hoadley, K.A., Qi, Y., Balu, S., Shi, Y., Wu, J., Penny, R., Bittner, M., Shelton, T., Lenkiewicz, E., Morris, S., Beasley, D., Sanders, S., Kahn, A., Sfeir, R., Chen, J., Nassau, D., Feng, L., Hickey, E., Weinstein, J.N., Barker, A., Gerhard, D.S., Vockley, J., Compton, C., Vaught, J., Fielding, P., Ferguson, M.L., Schaefer, C., Madhavan, S., Buetow, K.H., Collins, F., Good, P., Guyer, M., Ozenberger, B., Peterson, J., and Thomson, E. 2008. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 455:1061-1068.

McManus, M.T., Petersen, C.P., Haines, B.B., Chen, J., and Sharp, P.A. 2002. Gene silencing using micro-RNA designed hairpins. *RNA* 8:842-850.

Miyagishi, M. and Taira, K. 2002. U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat. Biotechnol.* 20:497-500.

Miyoshi, H., Blomer, U., Takahashi, M., Gage, F.H., and Verma, I.M. 1998. Development of a self-inactivating lentivirus vector. *J. Virol.* 72:8150-8157.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., and Trono, D. 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 272:263-267.

Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J., and Conklin, D.S. 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* 16:948-958.

Parsons, D.W., Jones, S., Zhang, X., Lin, J.C., Leary, R.J., Angenendt, P., Mankoo, P., Carter, H., Siu, I.M., Gallia, G.L., Olivi, A., McLendon, R., Rasheed, B.A., Keir, S., Nikolskaya, T.,

Nikolsky, Y., Busam, D.A., Tekleab, H., Diaz, L.A., Jr., Hartigan, J., Smith, D.R., Strausberg, R.L., Marie, S.K., Shinjo, S.M., Yan, H., Riggins, G.J., Bigner, D.D., Karchin, R., Papadopoulos, N., Parmigiani, G., Vogelstein, B., Velculescu, V.E., and Kinzler, K.W. 2008. An integrated genomic analysis of human glioblastoma multiforme. *Science* 321:1807-1812.

Pfeifer, A., Ikawa, M., Dayn, Y., and Verma, I.M. 2002. Transgenesis by lentiviral vectors: Lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. *Proc. Natl. Acad. Sci. U.S.A.* 99:2140-2145.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W.S., and Khvorova, A. 2004. Rational siRNA design for RNA interference. *Nat. Biotechnol.* 22:326-330.

Rubinson, D.A., Dillon, C.P., Kwiatkowski, A.V., Sievers, C., Yang, L., Kopinja, J., Rooney, D.L., Zhang, M., Ihrig, M.M., McManus, M.T., Gertler, F.B., Scott, M.L., and Van Parijs, L. 2003. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Genet.* 33:401-406.

Shendure, J.A., Porreca, G.J., Church, G.M., Gardner, A.F., Hendrickson, C.L., Kieleczawa, J., and Slatko, B.E. 2011. Overview of DNA sequencing strategies. *Curr. Protoc. Mol. Biol.* 7.1.1-7.1.23.

Singer, O., Marr, R.A., Rockenstein, E., Crews, L., Coufal, N.G., Gage, F.H., Verma, I.M., and Masliah, E. 2005. Targeting BACE1 with siRNAs ameliorates Alzheimer disease neuropathology in a transgenic model. *Nat. Neurosci.* 8:1343-1349.

Soda, Y., Marumoto, T., Friedmann-Morvinski, D., Soda, M., Liu, F., Michiue, H., Pastorino, S., Yang, M., Hoffman, R.M., Kesari, S., and Verma, I.M. 2011. Transdifferentiation of glioblastoma cells into vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 108:4274-4280.

Stegmeier, F., Hu, G., Rickles, R.J., Hannon, G.J., and Elledge, S.J. 2005. A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 102:13212-13217.

Tiscornia, G., Singer, O., Ikawa, M., and Verma, I.M. 2003. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc. Natl. Acad. Sci. U.S.A.* 100:1844-1848.

Trono, D. 2002. Lentiviral Vectors, vol. 261. Springer-Verlag, Berlin-Heidelberg.

Uhrbom, L., Hesselager, G., Nister, M., and Westermark, B. 1998. Induction of brain tumors in mice using a recombinant platelet-derived growth factor B-chain retrovirus. *Cancer Res.* 58:5275-5279.

Uhrbom, L., Dai, C., Celestino, J.C., Rosenblum, M.K., Fuller, G.N., and Holland, E.C. 2002. Ink4a-Arf loss cooperates with KRas activation in astrocytes and neural progenitors to generate glioblastomas of various morphologies depending on activated Akt. *Cancer Res.* 62:5551-5558.

Verhoeyen, E. and Cosset, F.L. 2004. Surface-engineering of lentiviral vectors. *J. Gene Med.* 6:S83-S94.

Weissenberger, J., Steinbach, J.P., Malin, G., Spada, S., Rulicke, T., and Aguzzi, A. 1997. Development and malignant progression of astrocytomas in GFAP-v-src transgenic mice. *Oncogene* 14:2005-2013.

Wen, P.Y. and Kesari, S. 2008. Malignant gliomas in adults. *N. Engl. J. Med.* 359:492-507.

Zheng, H., Ying, H., Yan, H., Kimmelman, A.C., Hiller, D.J., Chen, A.J., Perry, S.R., Tonon, G., Chu, G.C., Ding, Z., Stommel, J.M., Dunn, K.L., Wiedemeyer, R., You, M.J., Brennan, C., Wang, Y.A., Ligon, K.L., Wong, W.H., Chin, L., and DePinho, R.A. 2008. p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation. *Nature* 455:1129-1133.

Zhu, Y., Guignard, F., Zhao, D., Liu, L., Burns, D.K., Mason, R.P., Messing, A., and Parada, L.F. 2005. Early inactivation of p53 tumor suppressor gene cooperating with NF1 loss induces malignant astrocytoma. *Cancer Cell* 8:119-130.

Zufferey, R., Dull, T., Mandel, R.J., Bukovsky, A., Quiroz, D., Naldini, L., and Trono, D. 1998. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J. Virol.* 72:9873-9880.