Cancer Cell
Article

# Malignant Astrocytomas Originate from Neural Stem/Progenitor Cells in a Somatic Tumor Suppressor Mouse Model

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DOI 10.1016/j.ccr.2008.12.006

## SUMMARY

**Malignant astrocytomas are infiltrative and incurable brain tumors. Despite profound therapeutic implications, the identity of the cell (or cells) of origin has not been rigorously determined. We previously reported mouse models based on conditional inactivation of the human astrocytoma-relevant tumor suppressors *p53*, *Nf1*, and *Pten*, wherein through somatic loss of heterozygosity, mutant mice develop tumors with 100% penetrance. In the present study, we show that tumor suppressor inactivation in neural stem/progenitor cells is both necessary and sufficient to induce astrocytoma formation. We demonstrate in vivo that transformed cells and their progeny undergo infiltration and multilineage differentiation during tumorigenesis. Tumor suppressor heterozygous neural stem/progenitor cultures from presymptomatic mice show aberrant growth advantage and altered differentiation, thus identifying a pretumorigenic cell population.**

## INTRODUCTION

Gliomas are the most common primary malignancies of the central nervous system (CNS). Astrocytomas, which account for the majority of these tumors, exhibit histologic resemblance to astroglial cells. The most malignant form, glioblastoma multiforme (GBM), is one of the most lethal forms of cancer, with a median survival of about one year (Maher et al., 2001; Zhu and Parada, 2002). These highly infiltrative tumors are resistant to conventional radiation and chemotherapy, resulting in dismal survival outcomes that, in contrast to some forms of cancer, have improved only marginally in the past several decades (Stupp et al., 2005).

A variety of mutations have been described in human astrocytoma, and these frequently disrupt cell-cycle and apoptosis regulation (*INK4A*, *CDK4*, *RB*, *TP53*) and growth factor receptor signaling (*EGFR*, *PDGFR*, *PTEN*) (Furnari et al., 2007). These genetic lesions have been exploited in mice to generate animal models that phenocopy the human malignancy and thus allow for in vivo investigation of tumor development and their use in translational studies. A number of these mouse models involve introduction of oncogenic mutations in the germline or specific cell subpopulations in the brain. These include overexpression of active forms of Ras, Akt, epidermal growth factor receptor (EGFR), and platelet-derived growth factor (PDGF) as well as transforming antigens such as v-src and polyoma middle T-antigen, often in combination with mutations in tumor suppressors such as *Ink4A* or *Arf* (Fomchenko and Holland, 2006). The first endogenous genetic tumor suppressor mouse model was

### SIGNIFICANCE

Identification of the original cell that gives rise to a tumor and whether it is a limited cell type has crucial implications for understanding cancer development. This knowledge is also requisite for rigorous investigation of tumor initiation mechanisms. Using fully penetrant mouse models, we identify neural stem/progenitor cells as cancer-initiating cells and derive insight into the behavior of these tumors. We also report malignant astrocytoma mouse models wherein tumor suppressor inactivation at embryonic, early postnatal, or adult ages induces tumor formation and demonstrates the capacity of tumor cells to differentiate within the tumor. Our studies on presymptomatic mutant progenitor cultures indicate that the disease could be disseminating and acquiring growth advantage long before the onset of clinical manifestations.

based on heterozygous mice carrying *cis*-germline mutations in *Nf1*, a Ras GTPase-activating protein (RasGAP) and effector of receptor tyrosine kinase signaling, and *Trp53* (*p53*). Depending on genetic background, these mice develop brain tumors with varying penetrance (Reilly et al., 2000).

Further refinements have been made through cre/lox technology, wherein mouse strains with germline or somatic heterozygous mutations of the *p53, Nf1*, and *Pten* tumor suppressors develop high-grade astrocytomas with 100% penetrance (Kwon et al., 2008; Zhu et al., 2005a). *TP53* and *PTEN* mutations are among the most frequent mutations reported in astrocytomas (Furnari et al., 2007; Maher et al., 2001). Patients with germline mutations in *NF1*, called neurofibromatosis type 1, have increased susceptibility to astrocytomas (Gutmann et al., 2002), and recent detailed investigations of this 350 kb gene by the Cancer Genome Atlas Research Network indicate that along with *PTEN* and *TP53*, somatic *NF1* mutations are also prevalent in sporadic GBMs. In fact, these three genes are among the top five most mutated genes in human GBMs (McLendon et al., 2008).

Mouse models harboring a heterozygous germline or conditional somatic *p53* mutation combined with conditional somatic *Nf1* heterozygosity develop low- to high-grade (secondary) astrocytomas (Zhu et al., 2005a). Tumor formation is further accelerated into high-grade astrocytomas similar to primary GBM by additional loss of *Pten* (Kwon et al., 2008). These fully penetrant endogenous tumor suppressor-based mouse models develop tumors that are indistinguishable from the human malignancy based on known histologic and molecular criteria that define human astrocytomas.

The subventricular zone (SVZ) is an extensive germinal layer that concentrates neural and glial progenitors on the walls of the lateral ventricles of adult mammals (Alvarez-Buylla and Lim, 2004). In rodents, SVZ neural stem cells correspond to type B cells. These primary progenitors give rise to transient amplifying type C cells that undergo limited mitoses before differentiating into neuroblasts that migrate through the rostral migratory stream (RMS) and into the olfactory bulb (OB) (Doetsch et al., 1999). Neurogenesis also occurs in the subgranular zone (SGZ) of the dentate gyrus, which produces local neurons that incorporate into the granular cell layer (GL) (Gage, 2000; Zhao et al., 2008). In humans, the SVZ and SGZ have both been shown to harbor neural stem cells (Eriksson et al., 1998; Sanai et al., 2004). Recent studies have suggested the existence of additional, though minor, stem/progenitor niches elsewhere in the brain (Gould, 2007).

Historically, astrocytomas have been thought to arise from differentiated glia that undergo a process of dedifferentiation (Sanai et al., 2005; Sauvageot et al., 2007). However, whether mature differentiated astrocytes in their normal parenchymal environment are capable of initiating tumor formation in vivo has not been rigorously tested. The recent identification of adult neural stem cells, immature cells that divide throughout the lifetime of the individual, presents attractive targets for acquisition of tumor-causing mutations.

In the clinical setting, it is impossible to determine either the location of tumor origin or the nature of the cell type (or types) capable of generating these tumors. Similarly, because of astrocytoma's infiltrative nature from the early onset, it is impossible to determine the in vivo fate of tumor cells. Many theories have been put forth regarding these issues, including an astrocytic origin for GBM. Additionally, GBMs may contain highly heterogeneous tumor-derived cells that can differentiate. Despite the fundamental implications for development of therapies, direct examination or proof of these ideas remains to be provided.

Physiologically relevant genetic mouse models of high-grade astrocytomas provide one approach to address these critical questions. Examination of events preceding unassailable tumor development in our mouse models suggested that the tumors arise in the SVZ and possibly within stem/progenitor cells (Zhu et al., 2005a; Kwon et al., 2008). To directly examine whether neural stem/progenitors are the source of astrocytomas, we undertook a two-pronged approach. Using both genetic and stereotactic injection methods, we demonstrate that in the context of prevalent mutations and aberrant signaling pathways found in idiopathic glioma—*TP53*, *NF1*, and *PTEN*—embryonic, early postnatal, and, importantly, adult neural stem/progenitor cells can give rise to malignant astrocytomas in vivo, whereas more mature cell types cannot.

## RESULTS

### Genetic Tumor Suppressor Targeting of Neural Stem/Progenitor Cells Induces Astrocytomas

Our previous astrocytoma mouse models relied on heterozygous deletion of the tumor suppressors *Nf1*, *p53*, and/or *Pten* using a human *GFAP* promoter-controlled cre transgenic line (*hGFAP-cre*) to drive recombination (Zhuo et al., 2001), followed by spontaneous loss of heterozygosity (LOH) at these loci (Kwon et al., 2008; Zhu et al., 2005a). The *GFAP* promoter used in these conditional knockout mice is expressed in both stem cells and white matter astrocytes. Thus, whether the tumors arose from the neural stem/progenitors cells could not be determined.

To directly examine whether tumor suppressor depletion in neural stem/progenitors was sufficient to induce astrocytoma formation, we targeted neural stem/progenitor cells in vivo using a transgene that expresses a cre recombinase-modified estrogen receptor ligand-binding domain fusion protein (*cre-ER*$^{T2}$) under the control of the *Nestin* promoter/enhancer (Chen et al., 2009). Nestin is an intermediate filament protein that is widely used to mark neural stem/progenitor cells but not differentiated astrocytes, and the second intron enhancer allows for neural precursor-specific expression in the CNS (Lendahl et al., 1990; Zimmerman et al., 1994). Tamoxifen administration induces nuclear transfer of the cre-ER$^{T2}$ protein in nestin-expressing cells, where it can mediate *loxP*-dependent recombination (Feil et al., 1996). In a Rosa26 β-galactosidase (*R26-lacZ*) reporter background (Soriano, 1999), neural stem/progenitor cells and all of their progeny are indelibly marked by lacZ, which can be identified by X-gal staining (Figures 1A and 1B). Induction at embryonic day 13.5 (E13.5) produced a broad pattern of expression similar to the *hGFAP-cre* transgene that was used for the *Nf1-p53-Pten* conditional astrocytoma mouse models (Kwon et al., 2008; Zhu et al., 2005a), reflecting expression in embryonic telencephalic progenitors (Figure 1A). Induction at early postnatal stages exhibited marked and progressively reduced recombination in parenchyma (Chen et al., 2009), and by adult ages, lacZ staining in the forebrain and midbrain almost exclusively labeled the neural stem cells in the SVZ and its progeny along the RMS and OB, as well as progenitors in the SGZ and their progeny in the dentate gyrus (Figure 1B).

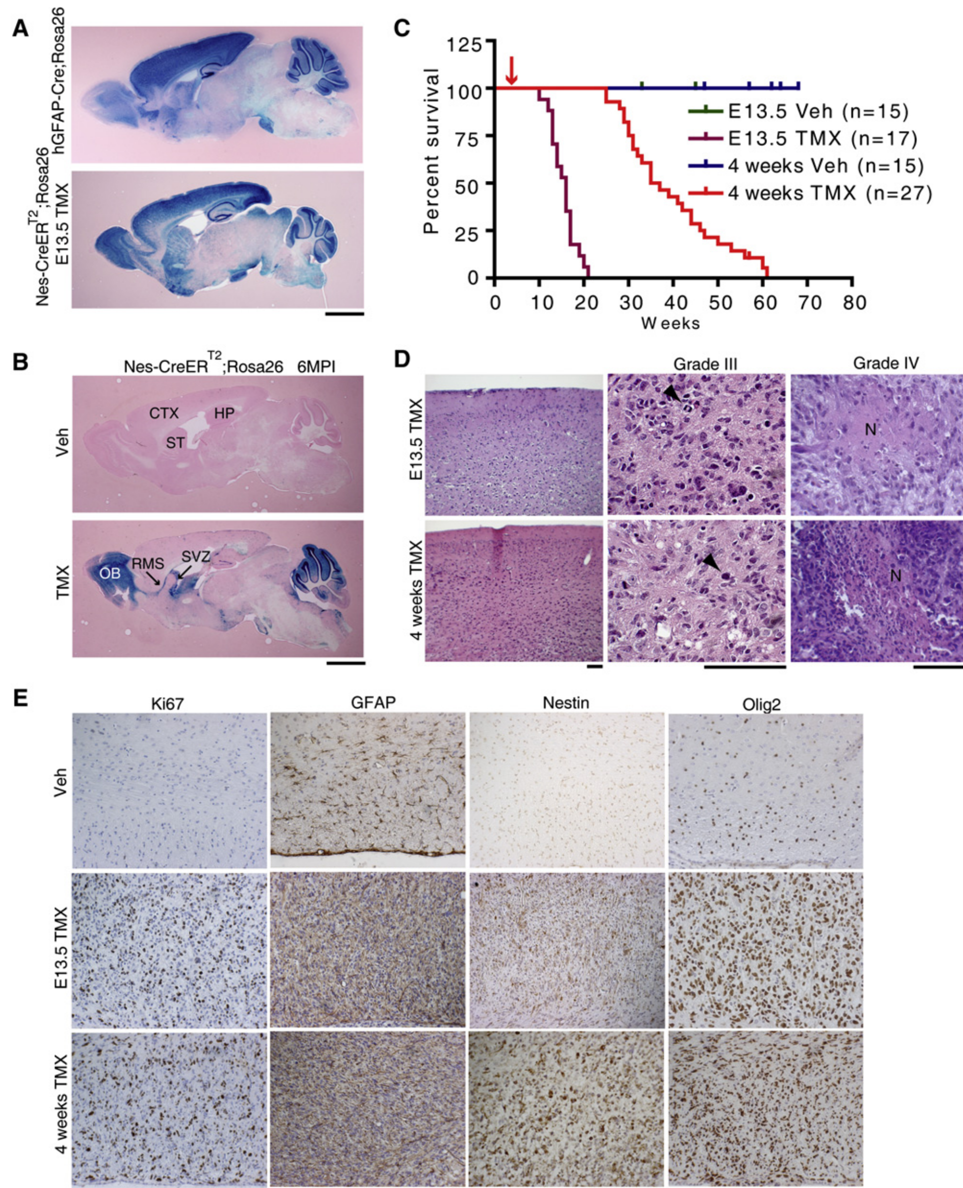


**Figure 1. Tumor Suppressor Inactivation in Nestin-Expressing Neural Stem/Progenitor Cells Induces High-Grade Astrocytoma Formation**

(A and B) *Nestin-cre-ER$^{T2}$* transgenic mice possess cre recombinase activity in the neural stem/progenitor niches.

(A) Representative images of X-gal-stained adult brain sections show that cre activity of *Nestin-cre-ER$^{T2}$;R26-lacZ* mice treated with tamoxifen at E13.5 recapitulates that of *hGFAP-cre* transgenic mice. Scale bar = 2 mm.

(B) Representative images of X-gal-stained brain sections at 6 months postinjection (MPI) reveal that tamoxifen induction at 4 weeks of age mainly restricts cre activity to neural stem cells and their progeny in the subventricular zone (SVZ), rostral migratory stream (RMS), and olfactory bulb (OB), as well as in the subgranular zone and granule cell layer of the dentate gyrus in the hippocampus (HP). Transgene expression is also found in the cerebellum. No staining is found in the adult cortex (CTX) or striatum (ST), or in vehicle-treated mouse brains. Scale bar = 2 mm.

(C) Mice with induced tumor suppressor inactivation (*Nestin-cre-ER$^{T2}$;Nf1$^{flox/+}$;p53$^{flox/flox}$;Pten$^{flox/+}$* or *Nestin-cre-ER$^{T2}$;Nf1$^{flox/flox}$;p53$^{flox/flox}$*) by tamoxifen treatment (TMX) have shortened survival compared to vehicle-treated (Veh) mice. Kaplan-Meier survival curves of *Nestin-cre-ER$^{T2}$* mice with tumor suppressor inactivation induced at either E13.5 or 4 weeks of age show median survival durations of 16 weeks and 46 weeks, respectively. Red arrow indicates timing of adult injection.

(D) Histologically identifiable high-grade astrocytomas develop in inducible mutant mice. Representative hematoxylin and eosin (H&E)-stained brain sections reveal formation of brain tumors in *Nestin-cre-ER$^{T2}$* mice with tumor suppressor inactivation induced at either E13.5 or 4 weeks of age. Grade III or IV astrocytomas with characteristic features of nuclear atypia, mitoses (arrowheads), and necrosis (N) are shown. Scale bars = 100 μm.

(E) Tumors express traditional markers of astrocytomas, including Ki67, GFAP, nestin, and Olig2. Scale bar = 100 μm.

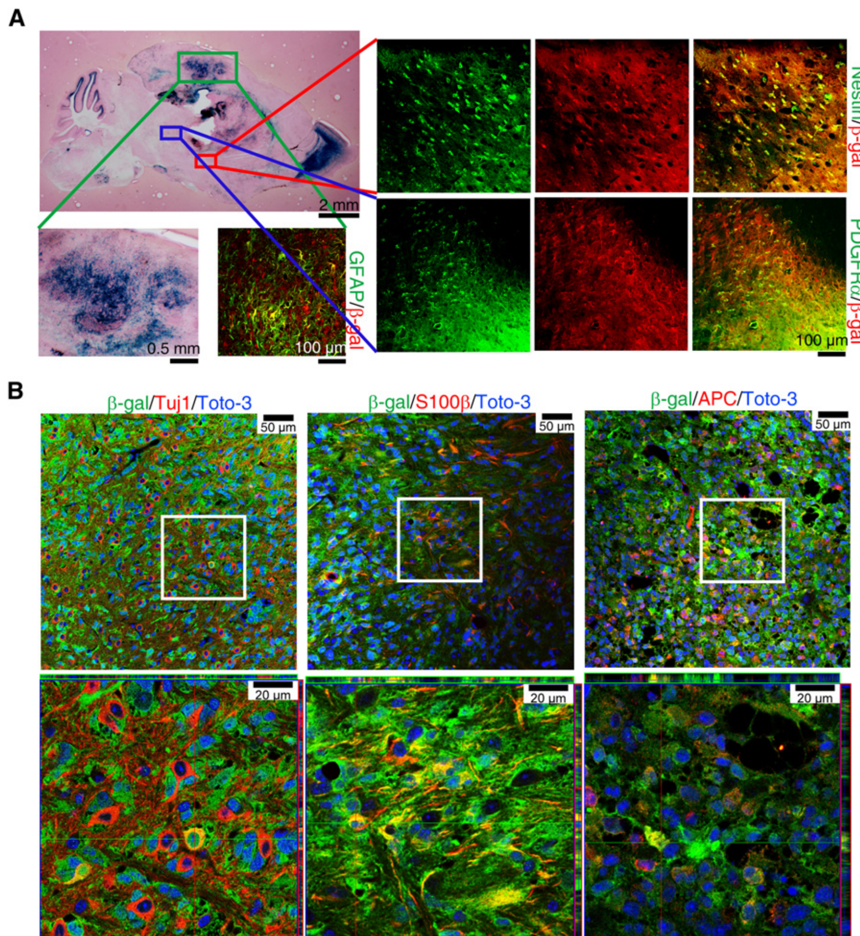


**Figure 2. Cancer-Initiating Cells Exhibit Infiltration and Differentiation During Tumor Development**

(A) β-galactosidase (β-gal)-positive mutant cells infiltrate into adjacent brain structures, away from their normal neural stem/progenitor niches. A representative X-gal-stained brain tumor section from a *Nestin-cre-ER$^{T2}$;Nf1$^{flox/+}$;p53$^{flox/flox}$;Pten$^{flox/+}$;R26-lacZ* mouse 8 months after tumor suppressor inactivation shows tumor cells found outside of their original locations in the SVZ/SGZ niches (compared to Figure 1B). β-gal-positive tumor cells, shown here expressing GFAP, nestin, and PDGFRα, are found elsewhere in the forebrain, such as the cortex and striatum.

(B) Marked astrocytoma cells expressing multilineage markers are found in the tumor bulk. β-gal-positive tumor cells in inducible mutant mice, shown here in the thalamus, express markers of neuronal (Tuj1) and glial (S100β and APC) differentiation. Toto-3 was used to mark cell nuclei.

We bred the inducible *Nestin-cre-ER$^{T2}$* mice to incorporate the tumor suppressor floxed alleles (either *Nf1$^{flox/+}$;p53$^{flox/flox}$;Pten$^{flox/+}$* or *Nf1$^{flox/flox}$;p53$^{flox/flox}$*) and injected pregnant females with tamoxifen at E13.5 or adult mice at 4 weeks of age. All tamoxifen-treated (TMX) mutant mice developed high-grade astrocytomas, while vehicle-treated (Veh) mice did not (Figure 1C). E13.5 TMX mutant mice developed tumors at a rate similar to the previously reported *hGFAP-cre;Nf1$^{flox/+}$;p53$^{-/flox}$;Pten$^{flox/+}$* mouse strain (Kwon et al., 2008), with a median survival of around 16 weeks. Mutant mice injected with tamoxifen at 4 weeks of age developed tumors with a median survival of around 46 weeks. Hematoxylin and eosin (H&E) staining of these tumors showed the classical features of diffusely infiltrating astrocytomas, including nuclear atypia and prominent mitoses as well as necrosis (Figure 1D). Both E13.5- and adult-treated mutant mice developed tumors diagnosed as grade III or IV (GBM) astrocytomas based on the World Health Organization classification system (see Figure S1A and Table S1 available online). These tumors had large numbers of Ki67+ cells, indicating robust proliferation, and were immunoreactive for GFAP, nestin, and Olig2 (Figure 1E), acknowledged markers of human astrocytic tumors (Furnari et al., 2007). Consistent with activation of the Ras and Akt signaling pathways by loss of *Nf1* and *Pten*, respectively, some tumor regions showed robust pERK and pAKT expression (Figure S1B). We further confirmed LOH of the tumor suppressor alleles in tumors by immunostaining and genotyping (Figure S2). Thus, cre-mediated somatic mutation of *Nf1*, *p53*, and *Pten* restricted to the neural/stem progenitor compartment is sufficient to replicate the high-grade astrocytoma phenotype previously observed using combinations of germline and somatic mutations and a less specific *hGFAP-cre* driver (Kwon et al., 2008; Zhu et al., 2005a). Furthermore, loss of *Nf1*, *p53*, and/or *Pten* was present in all tumors and is therefore apparently required for high-grade tumor induction (Kwon et al., 2008).

## Cancer-Initiating Cells Undergo Infiltration and Spontaneous Differentiation

The presence of the *R26-lacZ* reporter in the context of the floxed tumor suppressors allows for lineage tracing of cells as they undergo tumorigenic transformation. While normal neural stem cells and their progeny are principally restricted to the SVZ-RMS-OB and SGZ-GL (Figure 1B), tumors arising from inducible mutant mice were found in adjacent brain regions, including the cortex and striatum, as shown by X-gal staining (Figure 2A). The cre transgene is expressed in cerebellum, but only one tumor was found in cerebellum, and it resembled astrocytoma rather than medulloblastoma, which is the idiotypic tumor of this brain region. The β-galactosidase-positive tumor cells costained with the astrocytoma markers GFAP, nestin, and platelet-derived growth factor receptor α (PDGFRα; Figure 2A). Thus, mutant stem cells or their progeny migrate away from their normal niches and invade the parenchyma during tumor development.

Astrocytomas are heterogeneous tumors, with varying cellular morphologies and presence of immature and mature markers for all neural lineages. Upon examination of the tumor bulk, we found a variety of β-galactosidase-positive cells coexpressing

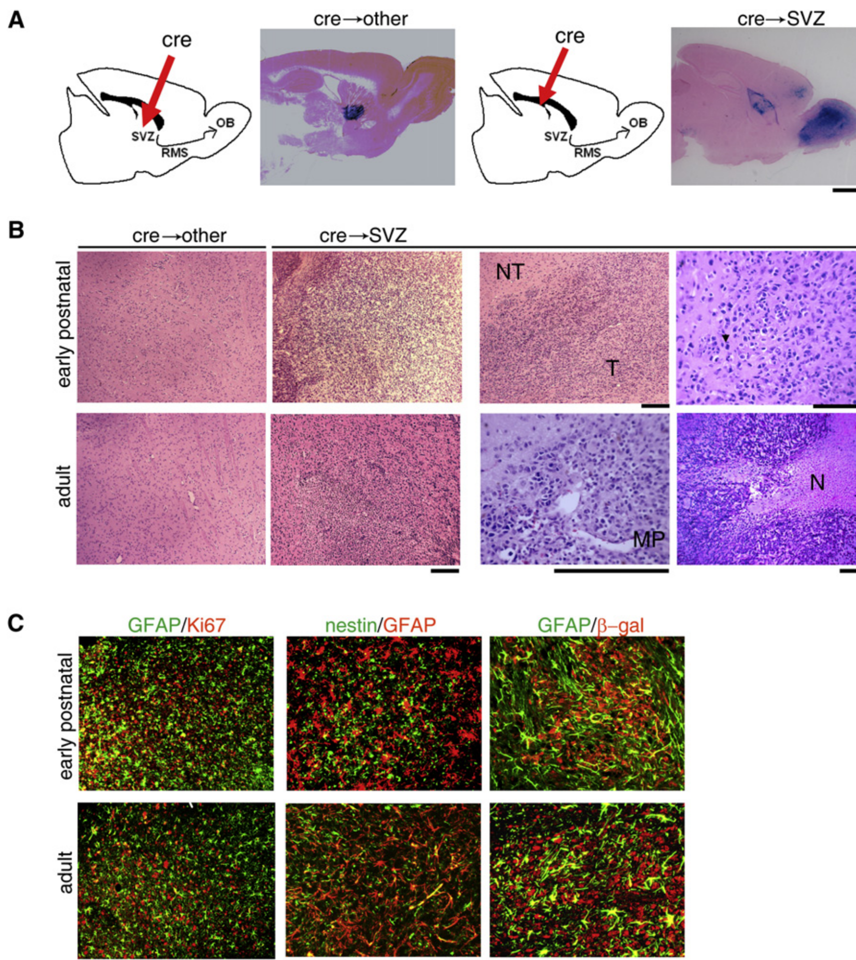


**Figure 3. Stereotactic Viral cre-Mediated Targeting of the SVZ Induces Astrocytoma Formation, Whereas Targeting of Non-neurogenic Regions Does Not**

(A) Schema of the viral cre injection experiments and representative X-gal staining images. Right: cre adenovirus targeting of the SVZ in *R26-lacZ* reporter mice marks neural stem/progenitors and their progeny in the RMS (arrowheads) and OB. Left: cre virus injection into nonneurogenic regions such as the striatum causes localized labeling at the site of injection, but no labeling at the RMS or OB. Scale bar = 2 mm.

(B) SVZ-targeted mutant mice develop histologically identifiable high-grade astrocytomas. Cre adenovirus was stereotactically injected into the SVZ of tumor suppressor floxed mice (*Nf1*$^{flox/flox}$;*p53*$^{flox/flox}$, *Nf1*$^{flox/flox}$;*p53*$^{flox/-}$, or *Nf1*$^{flox/+}$;*p53*$^{flox/flox}$;*Pten*$^{flox/+}$). Analysis of injected mouse brains by H&E staining shows infiltrative tumor cells in SVZ-injected mutant mice (cre→SVZ), whereas mice injected in other nonneurogenic brain regions (cre→other) do not develop tumors. Tumors from SVZ-targeted mutant mice show classic histopathologic characteristics of high-grade astrocytomas, including tumor (T) invasion into normal (nontumor, NT) regions, nuclear atypia and mitosis (arrowhead), microvascular proliferation (MP), and necrosis (N). Scale bars = 200 μm.

(C) Tumors from SVZ-targeted tumor suppressor floxed mice with *R26-lacZ* reporter show characteristic expression of the astrocytoma-associated markers Ki67, GFAP, and nestin. Scale bars = 200 μm.

markers of subsets of differentiated cells, such as the neuronal marker Tuj1 and the glial markers S100β and adenomatous polyposis coli (APC), as shown in a thalamic tumor in Figure 2B. These immunoreactive tumor cells morphologically resembled mature neurons, astrocytes, and myelin-ensheathing oligodendrocytes. In contrast to normal CNS cells, which show abundant PTEN expression (Kwon et al., 2008), these marker-positive cells were PTEN negative (Figure S2C), confirming that these "differentiated" cells were indeed cancer cells. These data provide formal evidence that tumor cells have the stem/progenitor capacity to exit the cell cycle and at least partially differentiate in situ. This may account for the heterogeneity of tumor cell types that is classically associated with high-grade astrocytomas.

## Stereotactic Viral cre-Mediated Targeting of the SVZ Induces Astrocytoma Formation

As an independent approach for targeting cre recombinase to the SVZ neurogenic niche, we adopted a stereotactic injection method. Numerous studies have used stereotactic targeting to study the function, lineage, and identity of neural stem cells by delivery of dyes, growth factors, or viral particles directly into the SVZ (Doetsch et al., 1999; Merkle et al., 2004, 2007; Yoon et al., 1996). Injection of cre recombinase-expressing adenovirus alone into the SVZ of *R26-lacZ* reporter mice results in labeled neural stem cells in the SVZ and their progeny as they travel through the RMS and into the OB (Figure 3A, right panels). In contrast, cre adenovirus injection into nonneurogenic regions such as the cortex or striatum causes only localized labeling in the area of the injection and no labeling in the RMS or OB (Figure 3A, left panels). In both cases, restricted staining can also be found along the needle tract.

We injected cre adenovirus into the SVZ of three strains of tumor suppressor floxed mice (*Nf1*$^{flox/flox}$;*p53*$^{flox/flox}$, *Nf1*$^{flox/flox}$;*p53*$^{flox/-}$, and *Nf1*$^{flox/+}$;*p53*$^{flox/flox}$;*Pten*$^{flox/+}$) either at postnatal day 1–2 or adult ages (4–8 weeks). We found that viral cre-mediated tumor suppressor inactivation in the SVZ at both early postnatal and adult ages induced astrocytoma formation (Figure 3; Table 1). Injected mutant mice developed brain tumors classified as grade III or IV astrocytomas starting at 6 months postinjection (Figure S3A; Table S2). These tumors exhibited the histopathologic hallmarks of high-grade astrocytomas, including diffuse infiltration, nuclear atypia, mitoses, microvascular proliferation, and necrosis (Figure 3B), and were indistinguishable from those of our previous studies (Kwon et al., 2008; Zhu et al., 2005a). The tumors were immunoreactive for Ki67, GFAP, and nestin (Figure 3C) as well as Olig2, pERK, and pAKT (Figure S3B). We further confirmed that the tumors underwent cre recombination by β-galactosidase immunostaining (Figure 3C). An internal

**Table 1. Astrocytoma Formation in Tumor Suppressor Floxed Mice by Stereotactic Viral cre Injection**

| Age at Injection | Experiment: Virus → Target Area | Tumor Incidence/Mice Targeted |
|---|---|---|
| Early postnatal | cre → SVZ | 10/10 |
| | cre → other regions | 1/12 |
| | controls[a] | 0/8 |
| Adult | cre → SVZ | 12/12 |
| | cre → other regions[b] | 0/20 |
| | controls[a] | 0/9 |

Early postnatal (postnatal day 1–2) or adult (4- to 8-week old) mutant mice were injected with cre adenovirus targeted to the subventricular zone (SVZ) or nonneurogenic brain regions such as the cortex and striatum. All adult SVZ-targeted mice developed tumors, whereas mice targeted in the nonneurogenic regions did not.

[a] Controls included Ad-GFP injected into the SVZ or cortex/striatum of tumor suppressor mutant mice and Ad-Cre injected into *R26-lacZ* mice alone.

[b] Nine out of 20 injections were intended SVZ injections that missed and did not result in LacZ+ SVZ-RMS-OB; 11 of 20 injections intentionally targeted cortex or striatum.

control and validation in these studies was a posteriori verification that intended injections into the SVZ resulted in *lacZ* lineage tracing of the SVZ-RMS-OB axis. We observed that only successful SVZ-RMS-OB targeted injections, as evidenced by X-gal staining in the tumor bulk, SVZ, and the olfactory bulb (Figure 4A) as well as β-galactosidase immunohistochemistry (data not shown), generated astrocytomas. We also confirmed loss of tumor suppressor alleles in tumors by PCR genotyping (Figure 4A).

Similar to the nestin-inducible mutant mice, tumors were found throughout most of the brain parenchyma (Figure 4A). Early postnatal-injected mutant mice seemed to develop more extensive tumors compared to adult-injected mutant mice, which developed more restricted, albeit still invasive, high-grade astrocytomas. Clear evidence of migration is shown by the presence of tumors away from the SVZ, such as in the cortex, hippocampus, and thalamus. In both cases, we found intense X-gal staining of the tumor regions, as well as in the SVZ-RMS-OB (Figure 4A).

As described in the tamoxifen-inducible tumors, β-galactosidase-positive tumor cells had morphological features of differentiated CNS cells and expressed mature markers, including the astrocytic marker GFAP, the oligodendrocytic marker myelin basic protein (MBP), and the neuronal marker NeuN (Figure 4C). As an independent method of confirming that these marker-positive cells were indeed tumor cells, we stained these tumors for PTEN, which is frequently suppressed in high-grade astrocytomas. We found that a number of these "differentiated" cells were PTEN negative (Figure 4C). We also found a rare population of β-galactosidase-positive tumor cells near the cortex that expressed calbindin (Figure 4C), which is normally expressed by a subset of OB neurons produced by the SVZ neural stem/progenitor cells (Merkle et al., 2007). These data demonstrate that cancer-initiating cells or their progeny have the capacity to migrate throughout the parenchyma, seed tumors, and give rise to differentiated cell types during tumor development.

### Viral cre-Mediated Targeting of Adult Nonneurogenic Brain Regions Does Not Induce Tumor Formation

The above results demonstrate that progenitor cells in the SVZ have the capacity to give rise to astrocytomas. However, these studies do not rule out the possibility that additional parenchymal cells might also harbor this capacity, or that tumors might arise from the few cells that are infected with the cre adenovirus along the injection track. Previous studies using other experimental systems, which involved in vitro manipulation or oncogenic transformation, have suggested that mature astrocytes can also give rise to gliomas (Bachoo et al., 2002; Dai et al., 2001; Uhrbom et al., 2002). In order to target tumor suppressor inactivation to cells outside the SVZ including astrocytes in vivo, we stereotactically delivered cre adenovirus into the cortex or striatum of 4- to 8-week-old tumor suppressor floxed adult mice injected in parallel with the previously described SVZ injections. In contrast to the successful SVZ injections, where 100% of the mice developed tumors, none of the animals injected in the cortex or striatum showed evidence of tumor formation (Figure 3B; Table 1), despite clear evidence of successful cre adenovirus infection as demonstrated by X-gal staining and PCR genotyping (Figure 4B) or β-galactosidase immunohistochemistry (Figure S4). H&E staining showed disorganization of the cortical or striatal architecture in the injection site (Figure 3B), while immunostaining showed the presence of GFAP-positive but Ki67-negative cells (Figure S4), which is indicative of fibrosis and astrogliosis (Zhu et al., 2005b). Cells near the injection site likewise stained positive for nestin and vimentin (Figure S4), consistent with reactive astrocytosis (Correa-Cerro and Mandell, 2007; Sofroniew, 2005), phenotypes that are similar to GFP adenovirus-injected control brains (data not shown).

Non-SVZ regions were also targeted at early postnatal ages, and the majority of these mice did not develop gliomas (Table 1). However, we did find 1 out of 12 cases where a non-SVZ-targeted neonatal floxed mouse developed tumors 7 months postinjection. This is consistent with prior reports of oncogenic transformation of early postnatal brain cells. The rarity of tumor induction in non-SVZ-injected mutant mice is consistent with the rare targeting of neural progenitor cells in the cortex or striatum that still exist at early postnatal ages (Seaberg et al., 2005) or, alternatively, the rare targeting of radial glia that project into the parenchyma and are the progenitors of SVZ adult stem cells (Merkle et al., 2007). Overall, these data indicate that while tumor suppressor targeting of SVZ neural stem/progenitor cells readily induces high-grade astrocytoma formation, more differentiated cell types are less susceptible to malignant transformation by the tumor suppressor mutations assessed in this study.

### Pretumorigenic Neural Stem/Progenitor Cells in the SVZ

The above studies demonstrate that neural stem/progenitor cells can spontaneously give rise to malignant astrocytomas in our tumor suppressor mouse models. This allows us to follow these cells as they evolve from normal to transformed cells, as well as to investigate the molecular events involved in tumor initiation. Hence, we went back to our original tumor mouse models, which we have previously shown to exhibit hyperplastic and migration defects in the SVZ at early stages in vivo (Kwon et al., 2008; Zhu et al., 2005a). We examined the properties of mutant SVZ cells as neurospheres, which is the classical assay for studying neural

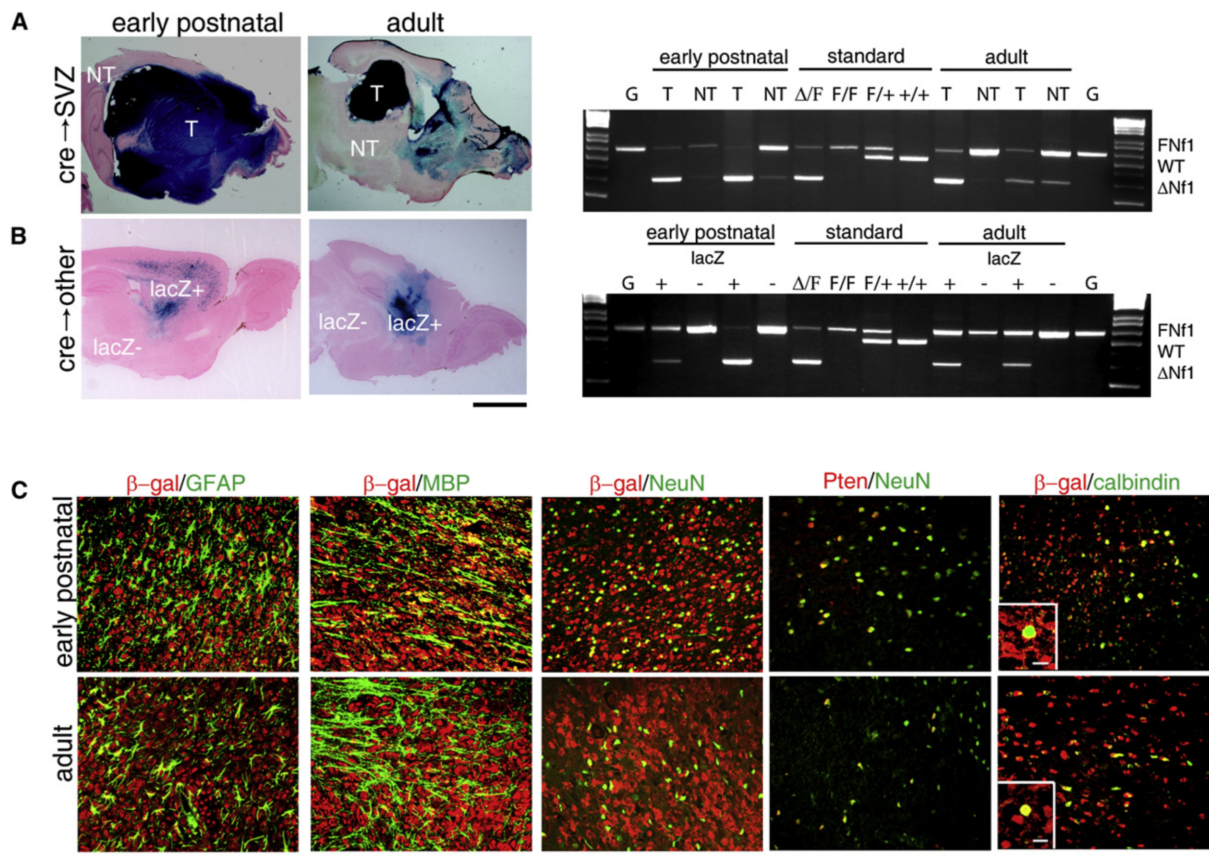


**Figure 4. Tumors Arising from SVZ-Targeted Mutant Mice Show Infiltration and Spontaneous Differentiation**

(A) Left: representative X-gal-stained brain sections from mutant mice targeted with cre adenovirus into the SVZ at early postnatal and adult ages show massive tumor formation, extensive infiltration and migration of tumor cells within the brain parenchyma, and labeling of the SVZ-RMS-OB axis. Right: LacZ-positive tumor (T) and lacZ-negative nontumor (NT) regions were dissected for PCR genotyping, which showed increased recombination of tumor suppressors—in this case, of floxed *Nf1* alleles in tumors. G, tail genomic DNA; F, floxed; Δ, recombined; +, wild-type (WT) allele.

(B) Left: X-gal-stained brain sections from mice targeted in nonneurogenic regions such as striatum show localized staining at the site of injection and along the needle tract, but no tumor formation. Right: PCR genotyping of lacZ-positive (lacZ+) and lacZ-negative (lacZ−) regions confirms successful recombination of floxed alleles in lacZ+ samples. Scale bar = 2 mm.

(C) Tumor cells from virus-injected mutant brains exhibit multilineage differentiation. Representative immunostaining images of high-grade astrocytomas from SVZ-targeted tumor suppressor floxed mice with *R26-lacZ* reporter injected at early postnatal and adult ages show expression of mature differentiation markers in a subset of β-gal-positive tumor cells, including GFAP for astrocytes, myelin basic protein (MBP) for oligodendrocytes, and NeuN for neurons. The majority of marker-positive cells within the tumor bulk (in this case, NeuN-positive cells) were also PTEN negative. A small number of β-gal-positive tumor cells near the cortex were positive for calbindin, a marker of a subset of differentiated OB neurons derived from the SVZ neural stem/progenitor cells. The insets show a β-gal/calbindin double-positive cell. Scale bar = 200 μm; inset scale bars = 10 μm.

stem/progenitor behavior in vitro (Reynolds and Weiss, 1992). Interestingly, we found that cultured SVZ neurospheres from tumor-prone mutant mice (Mut3: *hGFAP-cre;Nf1*$^{flox/+}$;*p53*$^{-/+}$ or Mut4: *hGFAP-cre;Nf1*$^{flox/+}$;*p53*$^{-/+}$;*Pten*$^{flox/+}$) at young ages prior to any histologic evidence of hyperplasia exhibited abnormal growth properties (Figure 5). Compared with wild-type controls, heterozygous mutant SVZ neurospheres displayed increased proliferation as shown by increased neurosphere diameter (Figure 5A) and bromodeoxyuridine (BrdU) incorporation (Figure 5B), as well as decreased apoptosis as shown by annexin V staining (Figure 5C). These cells likewise exhibited abnormal stem cell properties, including increased self-renewal capacity (Figure 5D), and altered differentiation potential with decreased astrocytic differentiation compared to normal cells (Figure 5E; Figure S5). To determine whether SVZ cells underwent LOH in the young mice months prior to tumor appearance, we performed PCR genotyping of the tumor suppressor alleles in the abnormal neurosphere cultures. We noted the presence of wild-type alleles for all tumor suppressors, indicating retention of heterozygosity (Figure 5F). These data indicate that in the absence of gross morphologic abnormalities, heterozygous tumor suppressor-deficient stem/progenitor cells already display abnormal growth and differentiation properties—processes that may serve as a prelude to tumor formation.

## DISCUSSION

### Neural Stem/Progenitor Cells as Astrocytoma-Initiating Cells

In this report, we describe the spatial and temporal restriction of in vivo gene targeting to the neurogenic niches of the brain through

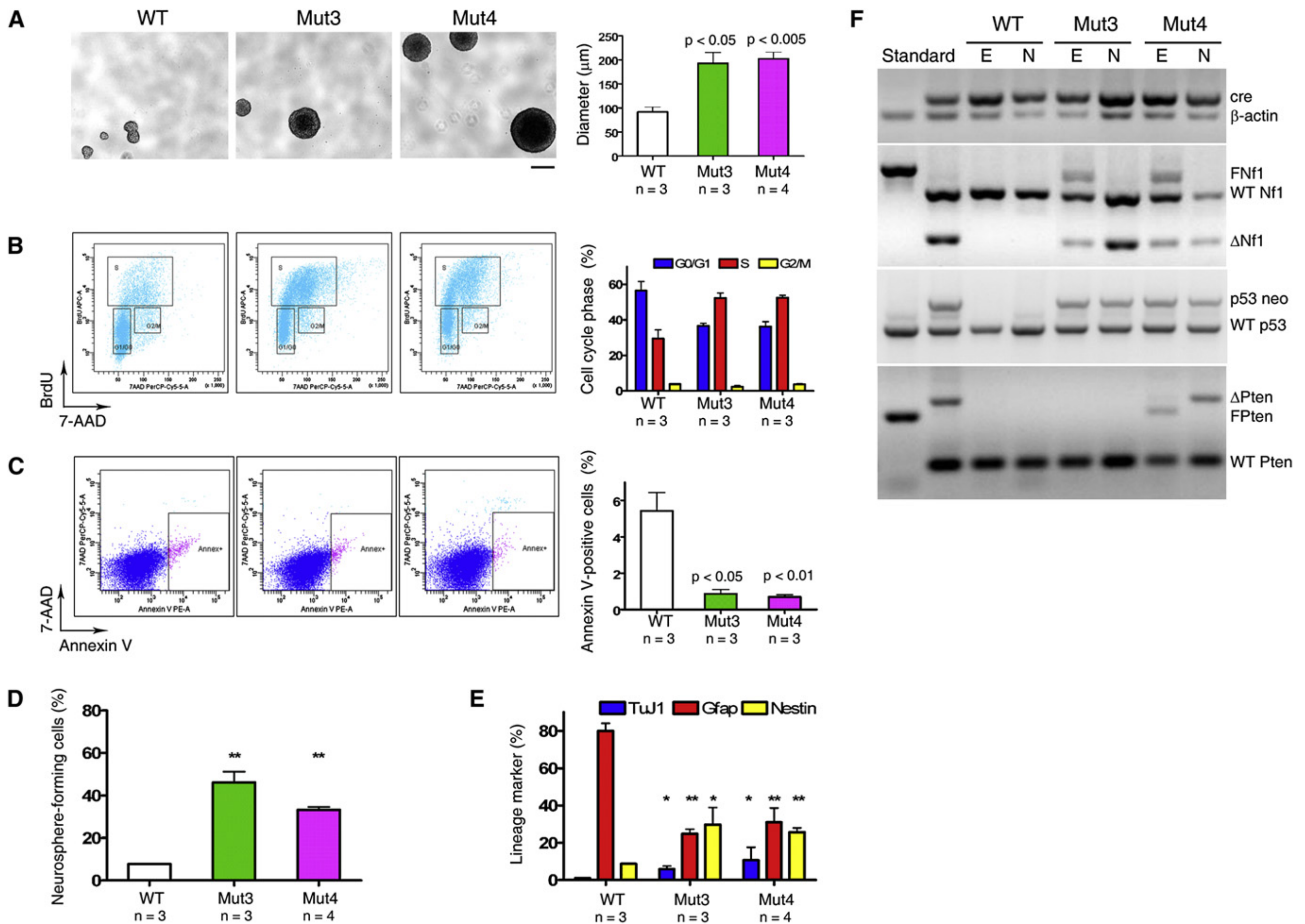


**Figure 5. Pretumorigenic SVZ Neural Stem/Progenitor Cells Exhibit Growth Advantage and Altered Differentiation**

(A) SVZ neurospheres from presymptomatic mutant mice exhibit hypertrophy. SVZ cells of 4-week-old mice were cultured as neurospheres. As shown in representative images, *Nf1/p53*-deficient (Mut3: *hGFAP-cre;Nf1*$^{flox/+}$;*p53*$^{-/+}$) or *Nf1/p53/Pten*-deficient (Mut4: *hGFAP-cre;Nf1*$^{flox/+}$;*p53*$^{-/+}$;*Pten*$^{flox/+}$) neurospheres were larger than those from WT neurospheres. Scale bar = 200 μm.

(B) Neurosphere proliferation was measured by 2 hr of bromodeoxyuridine (BrdU) pulsing in day 3 cultures and subsequent fluorescence-activated cell sorting (FACS) analysis. A significantly higher percentage of Mut3 and Mut4 cells were in S phase compared to WT cells ($p < 0.05$).

(C) Neurosphere cell death was measured by annexin V labeling and subsequent FACS analysis. Mut3 or Mut4 cells underwent significantly reduced cell death compared to WT cells. 7-aminoactinomycin (7-AAD) is a vital dye.

(D) SVZ neurospheres from presymptomatic mutant mice exhibit increased self-renewal potential compared to WT as shown by methylcellulose assay (**$p < 0.005$).

(E) Quantification of differentiated cells reveals significant increase in Tuj1- or nestin-positive cells and significant decrease in GFAP-positive cells in Mut3 and Mut4 cultures compared to those of WT control (*$p < 0.05$, **$p < 0.005$). All error bars in (A)–(E) are ± standard error of the mean (SEM).

(F) Neurosphere cultures from presymptomatic mutant mice exhibit retention of heterozygosity of tumor suppressor genes. Genomic DNA from the ear (E) or neurospheres (N) of 4-week-old Mut3 and Mut4 mice was subjected to semiquantitative PCR genotyping. Representative data for *cre*, *Nf1*, *p53*, and *Pten* status indicate retention of WT alleles in all Mut3 and Mut4 neurospheres.

the use of a tamoxifen-inducible *Nestin-cre-ER*$^{T2}$ transgene or by stereotactic viral-mediated cre recombinase delivery to the SVZ. The results were striking in that all adult mice subjected to SVZ targeting developed astrocytomas, thus establishing that mutation of these astrocytoma-relevant tumor suppressors in the neurogenic compartment in vivo is sufficient to induce tumor formation. Importantly, all viral injections into the SVZ were validated postmortem by lacZ staining to verify effective labeling of the RMS and OB, normal destination sites for progeny derived from the SVZ. In contrast, viral targeting of adult parenchyma where the vast majority of cells were differentiated did not yield tumors but instead local astrogliosis and localized lacZ staining, despite demonstration of recombination in glial cells. The inducible tumor model, on the other hand, showed that specifically targeting nestin-expressing neural stem and progenitor cells induces tumor formation. These data strongly support the idea that mutations in the stem/progenitor compartment account for the majority of these tumors and identify neural stem/progenitor cells as cancer-initiating cells in our fully penetrant astrocytoma mouse models. Furthermore, astrocytoma induction occurs efficiently in embryonic, early postnatal, and adult mice dependent on stem/progenitor cell targeting of the tumor suppressors.

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For all of the described tumor studies, the endpoint was selected to ensure adequate incubation of all manipulated cells, whether tumor suppressor-bearing or control. We ended the studies when specific cohorts exhibited morbidity that in all cases was verified to be the consequence of advanced astrocytoma. Therefore, we did not assess the natural history of tumor development or the genetic signature of the tumors since the present models are predicated on our previously extensively characterized tumor suppressor-based mouse models (Kwon et al., 2008; Zhu et al., 2005a). Moreover, we cannot rigorously distinguish between quiescent stem cells or actively dividing progenitor cells as the cells where LOH originates in our mouse models. Since the tumors arise months after targeting and, in the normal course of events, transient amplifying cells reach the OB and differentiate within two weeks (Petreanu and Alvarez-Buylla, 2002), we favor the hypothesis that the tumorigenic state preexists in the stem cell population but becomes phenotypically manifest once the cells enter the transient amplifying state. Experimental investigation of these distinctions will require more refined cell-type-specific promoters to drive cre-mediated recombination. It also remains to be determined whether the differential susceptibility of neural stem cells and astrocytes to transformation is dependent on local microenvironment.

### Cancer Stem Cells in Malignant Astrocytomas

The concept that some or many forms of cancer may be comprised of a subset of tumor-propagating cells and another subset of cells that cannot propagate the tumor has recently received increasing attention (Reya et al., 2001). Dirks and colleagues initially showed that human GBM xenografts into immunodeficient mice have such an identifiable subset of cancer-propagating cells or "cancer stem cells" (Singh et al., 2004). Cancer stem cells are thus technically defined in terms of their in vivo capacity for tumor initiation in serial transplantations and rely on retrospective isolation of these self-renewing cells (Dalerba et al., 2007). It is logical then to suggest that these cancer stem cells have characteristics in common with stem cells, but whether normal stem cells are the cells of origin of these tumors remained to be experimentally established. Our data indicate that normal neural stem/progenitor cells are cancer-initiating cells and can readily give rise to high-grade astrocytomas.

### Tumorigenic Stem/Progenitors Migrate and Differentiate Abnormally In Vivo

Astrocytomas are notorious for their infiltrative capacity, a property that clinically confounds complete surgical resection. We show here that in contrast to normal adult neural stem cells that are strictly confined to the SVZ or SGZ, tumors arising from tumor suppressor-deficient neural stem cells or their progeny are not restricted to these niches and actually migrate away from their normal locations, thus accounting for the presence of tumors elsewhere in the forebrain, including the cortex, striatum, hippocampus, and thalamus. This can also explain the presence of tumors in regions where the *hGFAP-cre* transgene is not expressed in conditional mutant mouse models (Kwon et al., 2008; Zhu et al., 2005a). Another distinct feature of human astrocytomas is the heterogeneity of cell types within these tumors. Because of their infiltrative nature, one interpretation is that "diverse" nontumor cells are present and surrounded by tumor cells. This may be the case to some degree, as genotyping of primary tumor tissue yields a faint wild-type or nonrecombined band (Figure 4A and data not shown), and the majority of cells expressing mature, differentiated markers are β-galactosidase negative (Figure 2B; Figure 4C), suggesting that normal cells were trapped within the tumor bulk. An additional alternative is that the tumor itself has a heterogeneous component of tumor-derived cells. This alternative has been indirectly supported by several lines of evidence, including in vitro differentiation and xenografting into immunodeficient mice. However, direct demonstration that the original tumor is heterogeneous in situ has been lacking. The power of mouse genetics permitted neural stem/progenitor compartment-specific tumor suppressor inactivation in the context of a cre-dependent *lacZ* reporter transgene. Thus, through morphologic assessment of lacZ-positive and PTEN-negative tumor cells in conjunction with labeling with lineage-specific markers, we found that a subset of tumor-derived cells have properties of astrocytes, oligodendrocytes, and neurons. We even found a rare subset of lacZ-positive tumors cells that express calbindin, which is normally expressed by a subset of OB neurons produced by the SVZ neural stem/progenitors, suggesting that the differentiation capacity of these cancer-initiating stem/progenitor cells is retained during tumor development.

The degree of differentiation is variable among individual tumor cells and between tumors from different individuals. However, since these "mature" cells may represent a less aggressive population of tumor cells, this observation suggests that differentiation therapy may provide a plausible approach to arresting tumor growth while avoiding killing "bystander" normal cells. In sum, the clinically relevant migration and differentiation capacity of astrocytomas fits well with a neural stem/progenitor cell origin uncovered here.

### Cell of Origin in Mouse and Human Gliomas

Other astrocytoma mouse models have used combinations of oncogenic overexpression and/or tumor suppressor inactivation to induce tumor formation. Several reports have shown that *Nestin* promoter-driven oncogenesis at early postnatal ages can give rise to astrocytomas, whereas *GFAP* promoter-driven oncogenesis has reduced penetrance depending on the initiating mutations (Holland et al., 2000; Uhrbom et al., 2002). The tumor cell of origin in these previous studies was inferred but not directly examined. Ex vivo expansion of cultured neural stem cells or neonatal astrocytes followed by transplantation into immunodeficient mice also gives rise to astrocytomas (Bachoo et al., 2002; Dai et al., 2001). Thus, to date, cells targeted for transformation have been derived from either embryonic or early postnatal brain cells. Moreover, in vitro manipulation to establish tumorigenicity is likewise problematic because it is well established that cell culture significantly alters the normal biological behavior of cells. Studies using oncogenic mutations may also provide supraphysiologic levels of activated oncogenes. In light of the present studies, we suggest that these mouse models may be targeting embryonic precursors present in neonatal brains, with the distinction that our models equally target adult stem/progenitor cells and induce high-grade astrocytomas with tumor suppressor inactivation. On the other hand, the Cancer Genome Atlas project has described *EGFR* receptor mutations as mutually exclusive

from *NF1* mutations in gliomas. Thus, it is possible that other mutations in neural stem/progenitors can likewise give rise to gliomas, or that gliomas with differing genetic signatures may originate from different cell subtypes. The finding that the SVZ contains a diverse set of neural stem cells that can give rise to specific progenitor subtypes (Merkle et al., 2007) provides some clues. Whether these heterogeneous stem cell populations are susceptible to the same mutations or give rise to different tumor subtypes also remains to be examined. The experimental approaches described herein will be useful in determining the cell of origin of other models using GBM signature mutations or pathways.

## Haploinsufficiency and Tumor Initiation

The mechanisms involved in tumor initiation remain poorly understood, and identification of the cell of origin allows us to follow normal SVZ cells as they undergo transformation and full-blown tumorigenesis. We were surprised by the finding that in culture, pretumorigenic neurosphere-forming cells from the SVZ already show growth advantage and apoptotic resistance while retaining heterozygosity. It is also surprising that at these early stages, the differentiation capacity of these mutant cells is altered, suggesting that the differentiation state of these immature cells is tightly coupled to self-renewal processes. This underscores the importance of the *Nf1*, *p53*, and *Pten* tumor suppressor pathways in regulating normal neural stem cell proliferation and self-renewal. It also has implications for therapeutic approaches, should the physiological relevance of our models continue to be validated. These data imply that there remains a persistent and primed source of pretumorigenic cells in the neurogenic niche that will require further investigation and therapeutic targeting.

## EXPERIMENTAL PROCEDURES

### Mice

All mouse experiments were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center at Dallas. Mice with conditional *Nf1*, *p53*, and/or *Pten* alleles with the *R26-lacZ* reporter were maintained on a mixed 129SvJ/C57BL/6/B6CBA background (Kwon et al., 2008). Mice harboring the floxed *Nf1* and *p53* alleles in *cis* were generated by crossing the $Nf1^{flox/+}$ strain to the $p53^{flox/+}$ strain to generate $Nf1^{flox/+}$;$p53^{flox/+}$ *trans* mice that were then crossed to wild-type mice. Generation and maintenance of Mut3 and Mut4 mice as well as genotyping for the flox, wild-type, and recombinant alleles were as described previously (Kwon et al., 2008; Zhu et al., 2005a).

### Tamoxifen Induction

Tumor suppressor floxed mice containing the *Nestin-cre-ER*$^{T2}$ transgene were injected intraperitoneally with vehicle (9:1 sunflower oil:ethanol mixture) (Sigma) or tamoxifen (Sigma) at a working concentration of 6.7 mg/ml. Pregnant mothers were injected with 1 mg tamoxifen at E13.5, whereas 4-week-old mice were injected with 83.8 mg/kg tamoxifen twice a day for 5 consecutive days (Chen et al., 2009).

### Adenovirus Injection

Tumor suppressor floxed mice at 4–8 weeks of age or postnatal day 1–2 were injected with cre- or GFP-expressing adenovirus as described previously (Doetsch et al., 1999; Merkle et al., 2004), with some modifications. Two hundred nanoliters of adenovirus (Ad-Cre, $2.0 \times 10^{12}$ plaque-forming units [pfu]/ml, University of Iowa Vector Core; Ad-GFP, $1.0 \times 10^{11}$ pfu/ml) was injected using a World Precision nanoinjector apparatus, according to the following coordinates (in mm anterior, lateral, and dorsal to the bregma): SVZ (0, 1.4, 1.6; 0.5, 1.1, 1.7; and 1, 1, 2.3); cortex (0, 3.5, 1.0); and striatum (0, 1.4, 2.6). For early postnatal injections, postnatal day 1 or 2 pups were injected with 40 nl of Ad-Cre or Ad-GFP as described previously (Merkle et al., 2004), with some modifications. The following coordinates (in mm anterior, lateral, and dorsal to the bregma) for a range of neonate weights were used: 1.4–1.5 g (1.5, 2.6, 1.4); 1.5–1.7 g (1.6, 2.7, 1.4); 1.7–1.9 g (1.7, 2.9, 1.5); and >1.9 g (1.7, 2.9, 1.7). All virus- and tamoxifen-injected mice were followed for development of neurologic abnormalities and harvested for histologic analysis.

### Histology and Tumor Analysis

Mice were perfused and fixed with 4% paraformaldehyde. Five micrometer sections were cut, and every fifth slide was stained with H&E. Brain sections were independently examined by S.A.L. and J.C. as well as D.K.B., a certified neuropathologist, and tumor diagnosis was determined based on World Health Organization criteria (Kleihues et al., 2002). Brains used for X-gal staining were postfixed in 2% PFA overnight. Half brains or 50 μm vibratome sections were stained in X-gal solution, and sections were counterstained with nuclear fast red as described previously (Luikart et al., 2005). In some cases, half brains stained with X-gal were subsequently processed and used for immunohistochemistry. For PCR genotyping, DNA extraction and PCR were performed using tumor and nontumor tissues as described previously (Kwon et al., 2008).

### Immunohistochemistry

Paraffin sections were deparaffinized, rehydrated, and subjected to citrate-based antigen retrieval. Primary antibodies were used against GFAP (DAKO, 1:1000; BD Biosciences, 1:200), Ki67 (Novocastra, 1:1000), nestin (BD Biosciences, 1:100), Olig2 (Chemicon, 1:1000), β-gal (ICN, 1:1000), NeuN (Chemicon, 1:500), MBP (Sternberger, 1:200), calbindin (Swant, 1:1000), vimentin (Zymed, 1:200), S100β (Sigma, 1:200), pERK (Cell Signaling, 1:400), pAKT (Cell Signaling, 1:100), PTEN (Cell Signaling, 1:100), and PDGFRα (Spring, 1:50). We used both immunofluorescence staining with Cy2, Cy3, or Cy5 (Jackson Labs, 1:400) and biotin-streptavidin Alexa Fluor-conjugated secondary antibodies (Molecular Probes, 1:1000), as well as horseradish peroxidase-based Vectastain ABC Kit (Vector Laboratories). Sections were examined using optical, fluorescence, and confocal microscopy (Olympus and Zeiss).

### Neurosphere Culture and Assays

Neurosphere cultures were established and maintained as described previously (Kwon et al., 2008). To measure neurosphere size, we seeded dissociated cells into 24-well plates and acquired all neurosphere images after 7 days of incubation. We measured neurosphere diameter using MetaMorph software (Universal Imaging Corporation). Proliferation and apoptosis in neurosphere cultures were quantified by flow cytometry using BrdU and annexin V analysis kits (BD Biosciences). Self-renewal assay was performed by plating dissociated cells with methylcellulose-containing media (final concentration 0.8%, Sigma) as described previously (Gritti et al., 1999). For differentiation, we seeded dissociated cells in eight-chamber slides coated with Matrigel (BD Biosciences, 1:20) and cultured in NeuroCult with differentiation medium (StemCell Technologies). Quantification was performed in at least n = 3 samples from each genotype, and Student's t test was used for statistical analysis.

## SUPPLEMENTAL DATA

The Supplemental Data include five figures and two tables and can be found with this article online at http://www.cancercell.org/supplemental/S1535-6108(08)00409-1.

## ACKNOWLEDGMENTS

The authors thank L. McClellan, S. McKinnon, A. Deshaw, S. Kennedy, J. Chandler, and P. Leake for technical assistance and Parada laboratory members, especially R. McKay, for helpful suggestions and discussions. Adenovirus was provided by the University of Iowa Vector Core. This work was supported in part by a Children's Tumor Foundation Young Investigator Award to S.A.L.; Basic Research Fellowships from the American Brain Tumor Association (in memory of Daniel J. Martinelli and Geoffrey J. Cunningham) to C.-H.K.; and NIH (5P50NS052606), US Department of Defense (W81XWH-05-1-0265), and

American Cancer Society (RP0408401) grants to L.F.P. L.F.P. is an American Cancer Society Research Professor.

Received: August 15, 2008
Revised: October 28, 2008
Accepted: December 10, 2008
Published: January 5, 2009

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