NEURAL STEM CELLS

IN BRAIN TUMOR DEVELOPMENT

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DEDICATION

To Marc

the one constant in my life during my graduate school years

who never doubted and always believed
who made my sorrows less sad and my joys fuller
whose presence made my existence complete

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To Marc

With whom, I am

And to Him, without Whom I am nothing…Whose mysteries defy wisdom, but nonetheless allows a peak into His genius.

NEURAL STEM CELLS

IN BRAIN TUMOR DEVELOPMENT

by

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NEURAL STEM CELLS IN BRAIN TUMOR DEVELOPMENT

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Malignant astrocytomas are highly invasive and incurable brain tumors. Mouse models that genetically resemble the human disease are valuable tools in understanding the pathogenesis of these malignancies. We previously reported mouse models based on conditional inactivation of the human astrocytoma-relevant tumor suppressors *Nf1, p53* and *Pten.* Through somatic loss of heterozygosity, these mice develop varying grades of astrocytic malignancy with 100% penetrance.

Studies on our tumor suppressor mouse models indicated a central role for neural stem cells and stem cell-like cancer cells in malignant astrocytoma formation. Using stereotactic viral cre-mediated approach, we demonstrate that targeting of tumor suppressor inactivating mutations in the subventricular zone (SVZ) where neural stem and progenitor cells reside is both necessary and sufficient to induce astrocytoma formation. We also show evidence of spontaneous differentiation and infiltration of these cancer-initiating cells *in situ* during tumor development.

These studies have so far shown that neural stem cells or its progeny can give rise to astrocytomas. Neural stem cells, which have unlimited self-renewal potential, produce transit amplifying cells, or progenitor cells, which undergo limited mitoses

before differentiating into more mature cell types. By genetically targeting transit amplifying cells using the *Ascl1-cre*^{ERT2} transgenic mouse, we show that tumor suppressor inactivation in the progenitor compartment alone induces malignant astrocytoma formation. Defects in proliferation, differentiation, and migration are likewise found several months prior to advanced disease. This establishes both neural stem *and* progenitor cells as cells of origin of malignant astrocytomas in our tumor suppressor mouse models.

In another study, we isolated and characterized a population of stem cell-like cancer cells from murine astrocytomas that are enriched for tumor cells compared to primary tumor tissue, exhibit aberrant stem cell properties, and are tumorigenic *in vivo*. We demonstrate resistance to a known chemotherapeutic agent and the migratory capacity of these cells. We also investigated the mechanisms involved in astrocytoma progression and maintenance by gene expression analysis. Genomic profiling of tumor-derived neurosphere-forming cells from conditional astrocytoma mouse models show prominent dysregulation of genes involved in neurodevelopmental processes and transcriptional regulation, particularly the hox transcription factors, in high-grade astrocytomas.

Taken together, we have demonstrated that neural stem and progenitor cells are the origins of malignant astrocytoma in tumor suppressor mouse models. We have established a system by which molecular mechanisms of tumor development can be further investigated and performed genomic profiling of tumor-derived neurosphere-forming cells, suggesting a possible role for homeobox transcription factors in malignant astrocytoma formation. These mouse models thus represent powerful tools

in understanding various aspects of cancer development that otherwise cannot be explored in humans. Further studies will provide a better understanding of the biology of these tumors and will hopefully pave the way for more effective therapeutic approaches for these devastating diseases.

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Alcantara S & Halos S (1998) Allele frequency distribution of the VNTR locus d1s80 and the genetic relationship of Filipinos to other populations *As. Pac. J. Mol. Biol. & Biotech.* 6(2): 121-130.

Lon CT, ***Alcantara S***, Luchavez J, Tsuyuoka R, Bell D. (2005) Positive control wells: a potential answer to remote-area quality assurance of malaria rapid diagnostic tests *Trans R Soc Trop Med Hyg*. 99 (7): 493-498.

Kwon CH, ***Alcantara S*,***Zhao D*, Chen J*, , Li Y, Burns D, Mason R, Lee E, Wu H and Parada LF (2008) Pten Haploinsufficiency accelerates formation of high grade astrocytomas *Cancer Res* 68 (9): 3286-3294. (*equal contribution)

Alcantara Llaguno S, Chen J, Kwon CH, & Parada LF (2008) Neural and cancer stem cells in tumor suppressor mouse models of malignant astrocytoma. *Cold Spring Harb Symp Quant Biol*. Vol 73.

Alcantara Llaguno S*, Chen J*, Kwon CH*, Jackson E, Li Y, Burns D, Alvarez-Buylla A, &. Parada LF (2009) Malignant astrocytomas originate from neural stem/progenitor cells in a somatic tumor suppressor mouse model. *Cancer Cell* 15 (1): 45-56. (*equal contribution)

Alcantara Llaguno S, Chen J, & Parada LF. Signaling in Malignant Astrocytomas: Role of Neural Stem Cells and its Therapeutic Implications, in preparation.

Alcantara Llaguno S, Chen J, & Parada LF. Stem Cell Origin of Malignant Astrocytomas, in preparation.

Alcantara Llaguno S, Li Y & Parada LF. Insulin-like growth factor receptor signaling is required for dentate gyrus development and adult neurogenesis, in preparation.

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BrdU	bromodeoxyuridine
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
DCX	doublecortin
DG	dentate gyrus
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
FDR	false discovery rate
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GEFs	guanosine exchange factors
GTP	guanosine triphosphate
GBM	glioblastoma multiforme
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
H&E	hematoxylin and eosin
MAPK	mitogen-activated protein kinase
NSC	neural stem cells
OB	olfactory bulb
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PDGF	platelet-derived growth factor
PI3K	phosphatidylinositide-3-kinase
PIP2	phosphatidylinositol-4,5-biphosphate
PIP3	phosphatidylinositol-3,4,5-triphosphate
PTEN	phosphatase and tensin homologue on chromosome 10
RMS	rostral migratory stream
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SGZ	subgranular zone
SVZ	subventricular zone
TCGA	The Cancer Genome Atlas
TD-NFC	tumor-derived neurosphere-forming cells
WT	wild type

Chapter One

Introduction

Malignant Astrocytomas and Tumors of the Central Nervous System

Gliomas are the most common primary malignancies in the central nervous system (CNS). 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 other forms of cancer, have improved only marginally in the past several decades (Stupp et al., 2005).

Gliomas exhibit histologic resemblance to glial cells, and are classified based on the predominant tumor cell type(s). Astrocytomas, which morphologically resemble astrocytes, the support cells of the central nervous system (CNS) that are involved in metabolism, ionic balance, maintenance of the blood brain barrier, and response to injury (Kandel et al, 2000), comprise the majority of these tumors. High-grade astrocytomas exhibit cellular heterogeneity, diffuse infiltration and widespread invasion throughout the brain, precluding complete surgical resection. Radiation and chemotherapy improve survival but are not curative (Maher et al., 2001; Zhu and Parada, 2002).

Astrocytic tumors are further classified based on histopathologic and clinical criteria into increasing degrees of malignancy---World Health Organization (WHO) grades I to IV (Kleihues et al., 2002). Grade I tumors are benign and are considered curable if surgically resectable whereas grade II tumors are low-grade malignancies that undergo early diffuse infiltration, and are associated with survival period of between 5 to 15 years. Both Grade III (anaplastic astrocytoma) and Grade IV (GBM)

tumors are highly malignant and invasive tumors that are lethal within years to months. Grade III lesions exhibit increased proliferation and anaplasia whereas Grade IV tumors exhibit more advanced features of malignancy, including necrosis and microvascular proliferation (Furnari et al., 2007; Louis, 2006).

GBMs can be further subtyped based on clinical presentation. Secondary GBMs, which usually occur in younger patients, present with a previous clinical history of a lower-grade lesion, whereas primary GBMs, predominantly seen in older patients, arise *de novo*. Secondary GBMs evolve from lower grade astrocytomas, with Grade II astrocytomas progressing to Grade III or IV within 5 to 10 years of diagnosis. Despite differing clinical histories, however, primary and secondary GBMs are phenotypically similar in terms of clinical and pathologic endpoint and carry equally poor prognosis. However, recent studies have shown differences in the genomic make-up of these GBM subtypes (Maher et al., 2006; Tso et al., 2006).

Unlike other malignancies, high-grade astrocytomas rarely metastasize outside the CNS, hence, tumor grade serves as the primary determinant of clinical outcome (Furnari et al., 2007; Maher et al., 2001).

Genetic Pathways in Malignant Astrocytomas

At the molecular level, a variety of mutations have been described in human astrocytomas. The classical genetic alterations generally target pathways involved in cell cycle and apoptosis regulation and growth factor receptor signaling, as illustrated in Figure 1.1. Frequent mutations in genes involved in these processes underscore the importance of mitogenic signaling through receptor tyrosine kinases coupled with

inactivation of critical negative regulators of cell proliferation and senescence in the acquisition of the malignant phenotype (Furnari et al., 2007; Louis, 2006; Zhu and Parada, 2002). Recent work by The Cancer Genome Atlas (TCGA) Research Network in characterizing the glioblastoma genome has provided further insight on the genetic changes and core pathways in high-grade astrocytomas (TCGA, 2008).

Cell cycle and apoptosis regulation. The retinoblastoma-mediated binding of the E2F family of transcription factors is an important block to unrestrained proliferation. RB-mediated cell cycle inhibition is overcome by genetic alterations in the *RB* gene itself, which is mutated in ~24% of high-grade astrocytomas. Functional inactivation of *RB* is also accomplished by amplification of the cyclin-dependent kinases *CDK4* and *CDK6*, and inactivation of its negative regulator $p16^{Ink4a}$ (Furnari et al., 2007). On the other hand, the p53 tumor suppressor, as a critical regulator of cell cycle progression and apoptosis, is frequently mutated in its DNA-binding region in both low- and high-grade astrocytomas (Zhu and Parada, 2002). Contrary to what was previously thought, *TP53 (P53)* mutations are common not only in secondary GBMs but also in primary GBMs (TCGA, 2008). *P53* loss of function also occurs by amplification of the p53 ubiquitin ligase *MDM2* and its related gene *MDM4*, or loss of function of $p14^{Arf}$, which antagonizes MDM2 (Furnari et al., 2007; TCGA, 2008).

Growth factor receptor signaling. Persistent activation of growth factor receptors, particularly receptor tyrosine kinases (RTKs), is a common feature of high-

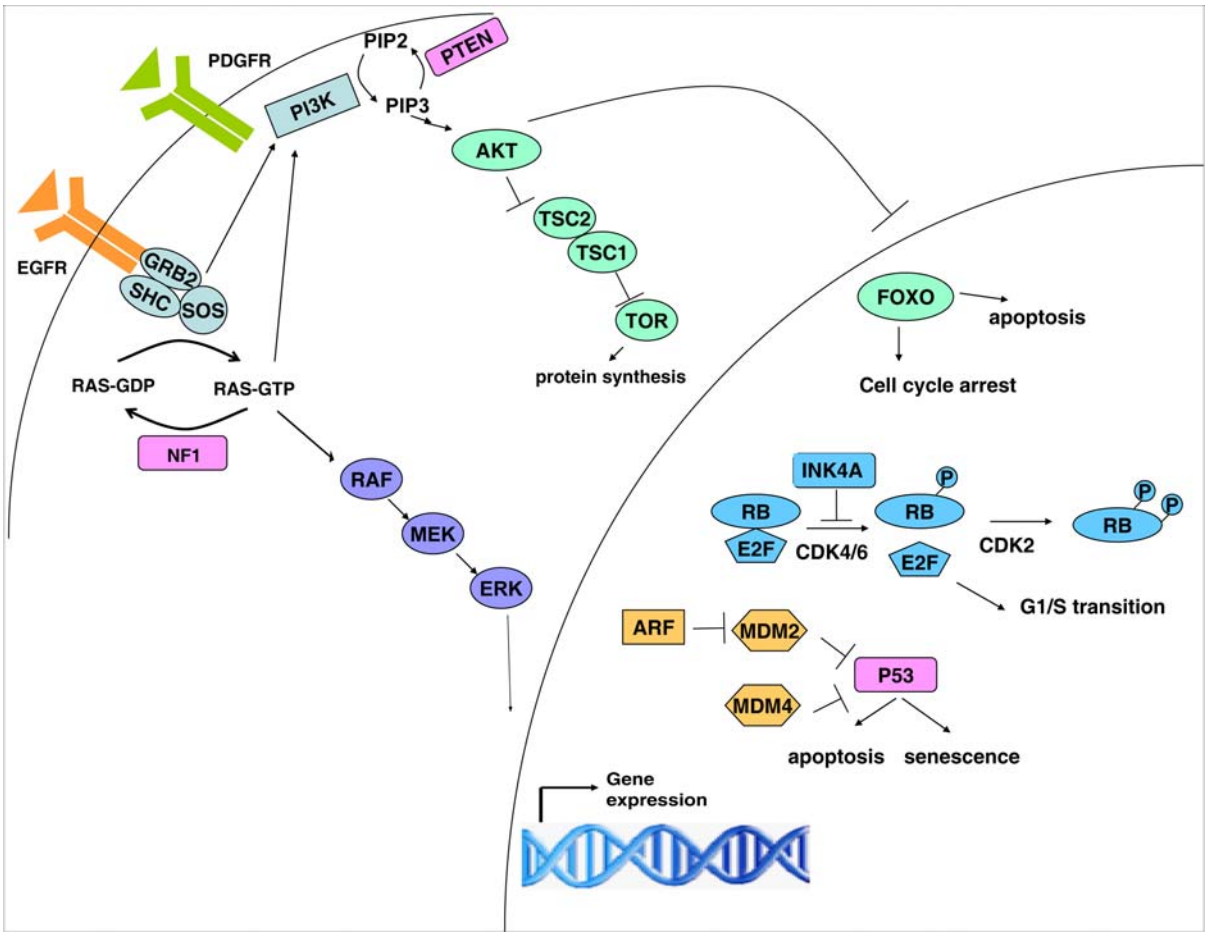

Figure 1.1. Genetic Pathways in Malignant Astrocytomas. Simplified schematic of genetic alterations frequently found in astrocytomas, including growth factor receptor signaling and cell cycle and apoptosis regulation. Overactivation of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) leads to stimulation of its downstream effectors, mainly Ras and phosphatidylinositide-3-kinase (PI3K) pathways, which are frequently activated in malignant astrocytomas. Ras signals through the Raf-Mek-Erk effector arm, among others, and can also directly activate the PI3K pathway. PI3K-mediated phosphorylation of PIP2 (phosphatidylinositol-4,5-biphosphate) to PIP3 phosphatidylinositol-3,4,5-triphosphate activates Akt, leading to activation of TOR (target of rapamycin) and inhibition of FOXO transcription factors, promoting cellular growth and survival. Nf1 and Pten are tumor suppressors that negatively regulate Ras and PI3K signaling, respectively. In the nucleus, G1/S progression is regulated by the retinoblastoma gene Rb, which binds the E2F family of transcription factors and is inhibited by cyclin-dependent kinases Cdk4/6 and Cdk2. On the other hand, the stability of the transcription factor p53, a master regulator of apoptosis and senescence pathways, is controlled by its ubiquitin ligase MDM2 and related gene MDM4. Ink4A and Arf positively regulate the activity of Rb and p53, which are commonly inactivated in astrocytomas.

grade astrocytomas. Epidermal growth factor receptor (*EGFR*) mutations comprise of amplifications, point mutations and deletions, including the most common, variant III deletion of the extracellular domain (EGFR-vIII mutant) (TCGA, 2008). Platelet-derived growth factor (PDGF) receptor and its ligands PDGF-A and PDGF-B are also overexpressed in low- and high-grade astrocytomas. These are also frequently co-expressed, suggesting a probable autocrine or paracrine loop of growth stimulation (Zhu and Parada, 2002). Other RTK genes, *ERBB2,* another member of the EGF receptor family, and *MET,* which encodes the hepatocyte growth factor receptor, have also been shown to be frequently mutated in GBMs (TCGA, 2008).

Receptor tyrosine kinases signal through adaptor proteins to its downsteam effectors Ras and phosphatidylinositide-3-kinase (PI3K). Active Ras signals through a variety of downstream effectors, chief amongst them the mitogen-activated protein kinase (MAPK) pathway regulating cell growth and proliferation, and the PI3K pathway. Ras cycles between the active guanosine triphosphate (GTP)-bound to the inactive guanosine diphosphate (GDP)-bound state. It is negatively regulated by GTPase-activating proteins (GAPs) such as p120GAP and positively by guanosine exchange factors (GEFs) such as SOS (Schubbert et al., 2007). The neurofibromatosis gene *NF1* encodes the protein neurofibromin, which contains a functional RasGAP domain, thereby inhibiting Ras activity (Le and Parada, 2007). Almost a quarter of all mutations screened by TCGA are *NF1* inactivating mutations and deletions, demonstrating *NF1* as a bona fide glioblastoma tumor suppressor (TCGA, 2008).

On the other hand, the PI3K pathway is an essential survival pathway for a variety of cancer cells. PI3K-mediated phosphorylation of phosphatidylinositol-4,5-

biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) sets up a cascade of events leading to activation of Akt and its downstream pro-growth and survival signals. This includes release of inhibition of TSC1/TSC2 (tuberous sclerosis 1 and 2) complex on TOR (target of rapamycin), and negative regulation of the forkhead (FOXO) transcription factors, which mediate apoptosis and cell cycle arrest. The tumor suppressor Pten (phosphatase and tensin homologue on chromosome 10) negatively regulates the PI3K pathway by dephosphorylating PIP3 back to PIP2 (Cully et al., 2006). *PTEN* mutations that target its phosphatase domain are frequent, as are mutations involving the catalytic (p110α) and regulatory (p85α) domains of PI3K (TCGA, 2008).

Tumor suppressor pathways in astrocytomas. Nf1, p53, and Pten represent tumor suppressor pathways that are frequently involved in human malignant astrocytomas. Patients with germline mutations in *NF1*, called neurofibromatosis type I, have increased susceptibility to astrocytomas, as much as five-fold higher compared to the general population (Gutmann et al., 2002). On the other hand, patients with astrocytomas have a 20-fold higher incidence of *NF1* mutations (Gutmann et al., 2002). Molecular analysis of *NF1*-associated astrocytomas show genetic changes such as *P53* mutations and *CDKN2A/P16* deletions, which are likewise found in sporadic malignant astrocytomas (Gutmann et al., 2003). Individuals with germline mutations in *p53* (Li Fraumeni) and *Pten* (Cowden disease) also have increased incidence of developing astrocytomas compared to the general population (Gutmann et al., 2002; Ichimura et al., 2004; Rasmussen et al., 2001).

These underscore a central role for these tumor suppressors in the development of malignant astrocytomas.

Mouse models of malignant astrocytoma

Signature genetic lesions found in human tumors have been exploited in the mouse to generate genetically engineered animal models that have greatly enhanced our understanding of astrocytoma development. Strategies have included gain-of-function approaches, such as overexpression of active forms of Ras, Akt, EGFR, PDGF, and transforming antigens v-src and polyoma middle T-antigen, often in combination with targeted deletions of Ink4A/Arf or Pten (Bachoo et al., 2002; Ding et al., 2001; Fomchenko and Holland, 2006; Furnari et al., 2007; Holland et al., 2000; Uhrbom et al., 2002; Weissenberger et al., 1997; Xiao et al., 2002). These mutations were induced in the germline or in specific cell populations and tumor development was observed with variable penetrance. The first endogenous genetic tumor suppressor mouse model was based on heterozygous mice carrying *cis* germline mutations in *Nf1* and *(Trp53) p53*. These mice developed high-grade astrocytomas with varying penetrance depending on genetic background (Reilly et al., 2000).

Table 1.1. Genetic configurations and phenotypes of *Nf1-p53-Pten* Tumor Suppressor Mouse Models*

Mutant	*cre*	*Nf1*	*p53*	*Pten*	Tumor grade[+]
Mut0	*hGfap-cre*	$Nf1^{f/+}$	$p53^{+/+}$	$Pten^{f/+}$	No tumors
Mut1	*hGfap-cre*	$Nf1^{f/f}$	$p53^{-/-}$	$Pten^{+/+}$	Low to high-grade astrocytomas
Mut2	*hGfap-cre*	$Nf1^{f/f}$	$p53^{-/+}$	$Pten^{+/+}$	Very rare high-grade astrocytomas
Mut3	*hGfap-cre*	$Nf1^{f/+}$	$p53^{-/+}$	$Pten^{+/+}$	Low to high-grade astrocytomas
Mut4	*hGfap-cre*	$Nf1^{f/+}$	$p53^{-/+}$	$Pten^{f/+}$	High-grade astrocytomas
Mut5	*hGfap-cre*	$Nf1^{f/+}$	$p53^{-/f}$	$Pten^{+/+}$	Low to high-grade astrocytomas
Mut6	*hGfap-cre*	$Nf1^{f/+}$	$p53^{-/f}$	$Pten^{f/+}$	High-grade astrocytomas

f=flox/loxP; +=wild type

[+]low grade=Grade II, high-grade=Grade III or Grade IV astrocytomas

*modified from Zhu et al., 2005 and Kwon et al., 2008

Further refinements were made through cre/lox technology, which permits for more selective spatial and temporal gene ablation (Feil, 2007). In order to better understand the biology of malignant astrocytomas, our laboratory previously developed mouse models based on tumor suppressor inactivation in specific cell types in the brain. We took advantage of a mouse transgenic expressing cre recombinase under the control of the *hGFAP* promoter (*hGFAP-cre)* (Zhuo et al., 2001) and combinations of tumor suppressor conditional alleles or germline mutations (Groszer et al., 2001; Jacks et al., 1994; Lin et al., 2004; Zhu et al., 2001). We first generated conditional mutant mice wherein *cis* heterozygous germline or somatic *p53* heterozygosity was combined with somatic *Nf1* heterozygosity driven by cre recombinase that is active in both neural stem cells and differentiated astrocytes (Zhu et al., 2005a). These mice developed astrocytomas with 100% penetrance, and were indistinguishable from the human malignancy based on known histologic and molecular criteria. This provided evidence that Nf1 and p53 loss-of-function is sufficient to initiate malignant astrocytoma formation. Variations in the genetic configurations of the tumor suppressor alleles also showed that p53 inactivation concomitant or prior to Nf1 inactivation is critical for tumor development. These are shown in Table 1.1 as Mut1 or Mut3 conditional mutant mice, which developed, with 100% penetrance, a spectrum of low to high-grade malignant astrocytomas, whereas Mut2 mutants very rarely developed tumors. Furthermore, when we added somatic *Pten* heterozygosity to the *Nf1-p53* mouse models, mutant mice (Mut 4 & Mut6) were found to develop high-grade astrocytomas with decreased latency of tumor formation (Kwon et al., 2008). We also found that *Nf1* and *Pten* somatic heterozygosity alone

was not sufficient for astrocytoma development (Mut0). These studies underscore the importance of the *Nf1*, *p53* and *Pten* tumor suppressors in malignant astrocytoma formation and progression.

Neural Stem Cells in the CNS

Neural stem cells are lifelong self-renewing cells in the CNS that exhibit multipotent differentiation into all neural cell types in the brain, including neurons, astrocytes and oligodendrocytes (Gage, 2000). In the adult mammalian brain, the two major neural stem cell niches are the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus (Zhao et al., 2008), as shown in Figure 1.2A. The SVZ is an extensive germinal layer adjacent to the ependyma that concentrates neural and glial progenitors on the walls of the lateral ventricles of adult mammals (Alvarez-Buylla and Lim, 2004; Doetsch et al., 1999). In rodents, SVZ neural stem cells correspond to type B cells that express the astrocytic marker glial fibriallary acidic protein, or GFAP, and are relatively quiescent (Doetsch et al., 1999). These primary precursors give rise to transient amplifying type C progenitor cells that undergo limited mitoses before differentiating into type A neuroblasts that migrate through the rostral migratory stream (RMS) and into the olfactory bulb (OB), where they integrate as granule neurons in the glomerular cell layer and periglomerular neurons in the glomerular layer (Alvarez-Buylla and Lim, 2004; Doetsch et al., 1999). Neuroblasts originating from the SVZ travel up to a distance of 5 mm in the rodent and within a span of about two weeks. Once it reaches its final destination, however, most newly born cells die within weeks, and the

survival of granule neurons becomes dependent on sensory input (Petreanu and Alvarez-Buylla, 2002).

Both Type B and C cells have been shown to express the intermediate filament marker nestin (Zimmerman et al., 1994), whereas transient amplifying cells have been identified by markers such as transcription factors Dlx2 (Doetsch et al., 2002) and Mash 1 or Ascl1 (Kim et al., 2007). Newly-born neurons are positive for doublecortin (DCX) and polysialylated neural adhesion molecule (PSA-NCAM) and differentiate into NeuN-expressing mature neurons (Zhao et al., 2008) (see Figure 1.2B).

Neurogenesis also occurs in the subgranular zone (SGZ) of the dentate gyrus, which produces local neurons that incorporate into the granular cell layer (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).

The self-renewal and differentiation of neural stem cells and its progeny are tightly regulated by a variety of intrinsic and extrinsic factors. Growth factors such as EGF and fibroblast growth factor (FGF) are well-known stimulators of neural stem cell proliferation *in vitro*. Classical developmental pathways such as Notch, bone morphogenic protein (BMP) and sonic hedgehog (Shh) signaling have been also shown to play important roles in maintaining the neurogenic niche (Alvarez-Buylla and Lim, 2004).

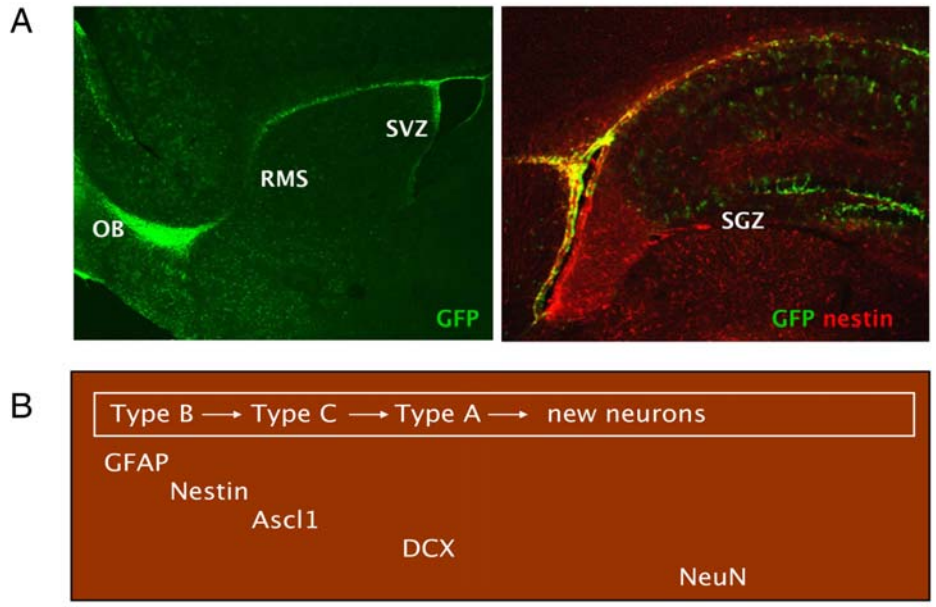

Figure 1.2. Neural Stem Cells and its Progeny in the Adult Rodent Brain. A. Left: A nestin-GFP reporter shows neural stem cells in the subventricular zone (SVZ) that give rise to transit amplifying cells that differentiate into newly born neurons that migrate through the rostral migratory stream (RMS) and olfactory bulb (OB). Right: The SVZ is shown with subgranular zone (SGZ) of the dentate gyrus. B. Simplified schematic of the lineage of SVZ neural stem cells. GFAP-positive neural stem cells or Type B cells undergo unlimited self-renewal and asymmetric cell division to give rise to rapidly-dividing transit amplifying (Type C) cells, which express Ascl1. The intermediate filament nestin is expressed in both Type B and C cells. Neural lineage-restricted type C cells differentiate into doublecortin (DCX)-positive immature neuroblasts which eventually mature into NeuN-positive neurons.

Nf1, p53, and Pten, like other tumor suppressors, have been shown to function as negative regulators of neural stem cell function. *Nf1* deficiency was shown to promote neural stem cell proliferation and survival (Dasgupta and Gutmann, 2005; Hegedus et al., 2007). Loss of *p53* increases proliferation in the SVZ neural stem/progenitors and provides a growth advantage compared to wild type cells (Gil-Perotin et al., 2006; Meletis et al., 2006), and *Pten* loss increases neural stem cell proliferation and self-renewal (Groszer et al., 2006; Groszer et al., 2001).

Neural Stem Cells and Cancer

Stem cells have been widely implicated in different types of cancers. These cells possess key characteristics which are somehow phenocopied by cancer cells. Cancer cells share important characteristics exhibited by stem cells, including unlimited replicative potential, diversity of progeny, telomere maintenance, and migratory properties (Sanai et al., 2005). In contrast to cancer cells, however, the function of stem cells at different stages of development is tightly regulated by diverse signaling pathways that impinge on various processes, including self-renewal, differentiation, and survival (Dalerba et al., 2007; Reya et al., 2001; Vescovi et al., 2006). Hence, it has been hypothesized that this population of undifferentiated cells that persist throughout the lifetime of an individual may play important roles in the natural progression of cancer.

Historically, the differentiated astrocyte has been thought to be the cell of origin of astrocytomas (Sanai et al., 2005; Sanson et al., 2004). On the other hand,

numerous studies have suggested that these tumors may arise from the transformation of neural precursor cells (Holland et al., 2000) or dedifferentiation of mature astrocytes (Bachoo et al., 2002; Uhrbom et al., 2002). None of these studies have been conclusive so far. Given the recent *in vivo* identification of precursor populations in the adult brain, a previously thought-of post-mitotic organ, the proposal that neural stem cells can give rise to these tumors is an attractive hypothesis, but one that has yet to be experimentally verified.

On the other hand, cancer cells with stem cell properties have been isolated from human cancers. These "cancer stem cells" have been operationally defined as a subpopulation of cells within tumors that maintain the self-renewing or propagating properties that confer the ability to initiate tumor formation in immunodeficient mice (Dalerba et al., 2007). These cells are thought to be responsible for the aggressive behavior, invasiveness, metastatic potential, and even resistance to conventional chemo- and radio-therapy of many tumors (Dalerba et al., 2007; Reya et al., 2001; Wang and Dick, 2005). In human astrocytomas, the presence of stem-like cancer cells has been reported and it has been suggested that the CD133+ fraction of GBMs comprise the population of self-renewing stem–like cancer cells with enriched tumorigenic capacity (Singh et al., 2004). These cancer stem cells have also been shown to be sensitive to bone morphogenetic protein signaling inhibition while being resistant to radiation therapy (Bao et al., 2006; Lee et al., 2008; Piccirillo et al., 2006). Many questions remain, however, as the molecular mechanisms that regulate cancer and normal neural stem cell behavior are still being unraveled, and efforts are underway to exploit these cells as possible therapeutic targets.

Chapter Two

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

Abstract*

Malignant astrocytomas are brain tumors that are locally infiltrative and incurable. However, despite profound therapeutic implications, the identity of the cell(s) of origin has not been rigorously determined. We previously reported mouse models based on conditional inactivation of human astrocytoma-relevant tumor suppressors *p53*, *Nf1*, and *Pten*, wherein through somatic loss of heterozygosity, mutant mice develop tumors at 100% penetrance. In the present study, we provide evidence that tumor suppressor inactivation in neural stem/progenitor cells leads to astrocytoma development in our mouse models. Stereotactic viral cre-mediated targeting of the subventricular zone (SVZ) at both early postnatal and adult ages induces astrocytoma formation, whereas targeting of non-neurogenic regions such as cortex and striatum does not. We also demonstrate that transformed cells and their progeny undergo infiltration and multi-lineage differentiation during tumorigenesis, recapitulating the invasive and heterogeneous nature of these tumors. This study thus identifies neural stem/progenitor cells as cancer-initiating cells, and suggests that more immature cells, in contrast to more differentiated cell types, are more susceptible to malignant transformation *in vivo*.

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Background

Astrocytomas have been traditionally 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, represents 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(s) capable of generating these tumors. Similarly, because of its infiltrative nature from the early onset, it is impossible to determine the *in vivo* fate of tumor cells. Many theories have been put forth, including an astrocytic origin, and further, that GBM contains highly heterogeneous tumor-derived cells. However, despite fundamental implications for development of therapies, direct examination or proof of these ideas remains to be established.

Physiologically relevant genetic mouse models of high-grade astrocytomas provide one approach to address these critical questions. Examination of events prior to 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 astrocytoma, we undertook a stereotactic injection approach and demonstrate that in the context of prevalent mutations found in idiopathic glioma---*TP53, NF1,* and *PTEN*---early

postnatal and, importantly, adult SVZ neural stem/progenitor cells can give rise to malignant astrocytomas *in vivo* whereas more mature cell types cannot.

Results

Stereotactic Viral cre-mediated Targeting of the SVZ Induces Astrocytoma Formation

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 deletion in neural stem/progenitor cells can induce astrocytoma formation, we targeted neural stem cells *in vivo* using a stereotactic injection approach. 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. Adenoviruses, in particular, have been successfully used to label neural stem cells in the SVZ (Doetsch et al., 1999; Merkle et al., 2007; Merkle et al., 2004; Yoon et al., 1996). Stereotactic targeting of neural stem cells is ideal because of its anatomically distinct location in the brain. Equally importantly, this method allows us to target differentiated cells in the brain parenchyma where neural stem cells do not reside.

In a Rosa26 β-galactosidase reporter background (Soriano, 1999), neural stem cells and all their progeny are indelibly marked by *lacZ*, which can be identified by X-gal staining. Injection of cre recombinase-expressing adenovirus into the SVZ of *R26R-stop-lacZ* reporter mice alone results in labeled neural stem cells in the SVZ and their progeny as they travel through the RMS and into the OB (Figure 2.1A, right panels). Double-labeling of GFAP-positive neural stem cells with β-galactosidase, as shown by immunostaining (data not shown) and the continuous labeling of the SVZ-RMS-OB axis several months post-injection, as opposed to transient labeling of progeny, suggest that the stem cells were definitively targeted. In contrast, cre adenovirus injection into non-neurogenic 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 2A, left panels). In both cases, restricted staining can also be found along the needle tract.

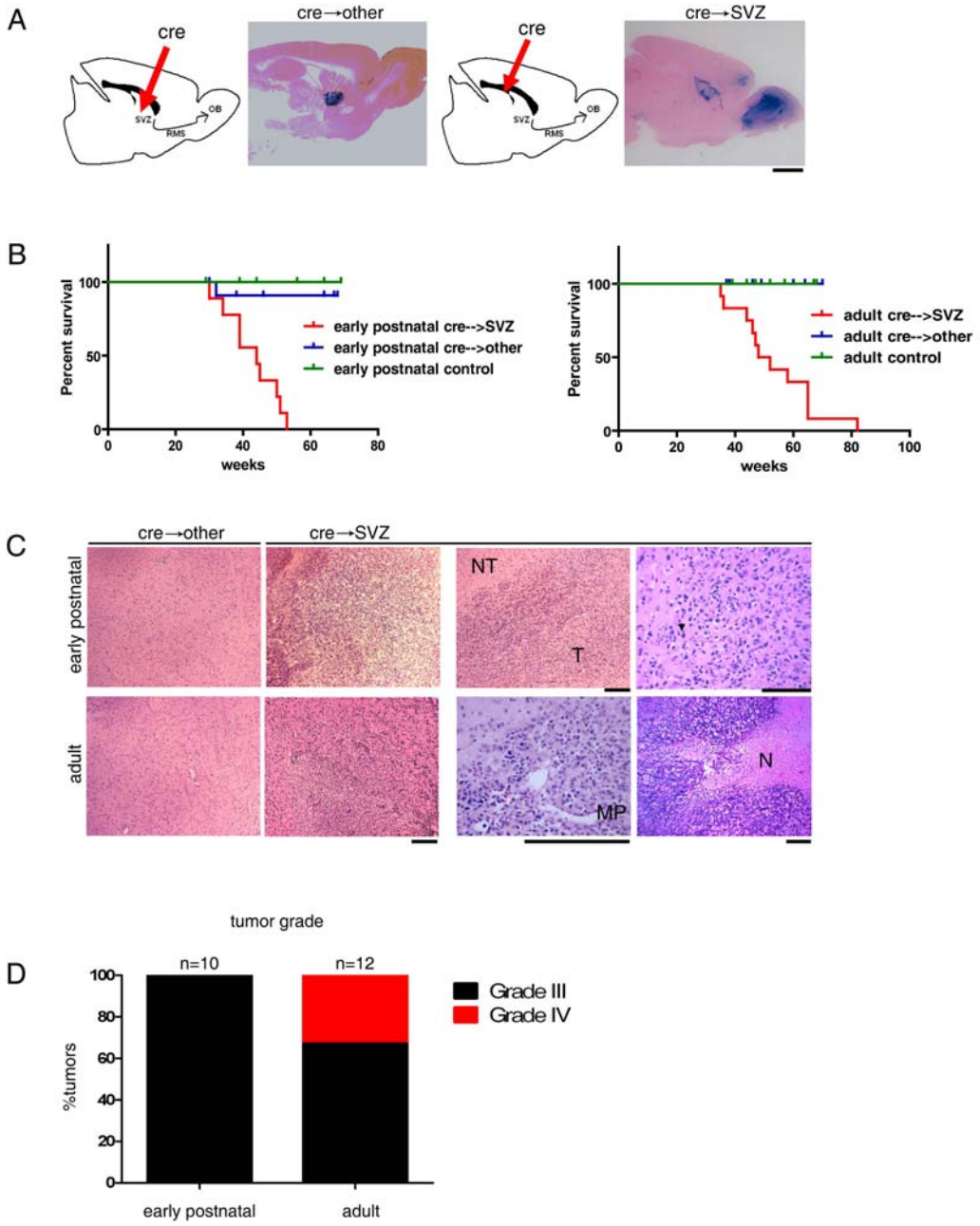

Figure 2.1. Stereotactic Viral cre-mediated Targeting of the SVZ, but not Non-neurogenic Regions, Induces Astrocytoma Formation. A. Schema of the viral cre injection experiments and representative X-gal staining images. Right, cre adenovirus targeting of the SVZ of *R26-lacZ* reporter mice marks neural stem/progenitors and its progeny in the RMS (arrowheads) and OB. Left, cre virus injection into non-neurogenic 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. Kaplan-Meier curves of experimental and control animals injected at early postnatal (left panel) and adult (right panel) ages. 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*$^{flox/+}$). All SVZ-targeted mutant mice have shortened survival compared to controls. C. SVZ-targeted mutant mice develop histologically identifiable high-grade astrocytomas. 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 non-neurogenic brain regions do not develop tumors (cre→other). Tumors from SVZ-targeted mutant mice show classic histopathologic characteristics of high-grade astrocytomas, including tumor (T) invasion into normal (non-tumor, NT) regions, nuclear atypia and mitosis (arrowhead), microvascular proliferation (MP), and necrosis (N). Scale bars, 200 μm. D. Mutant mice with SVZ-targeted injections of cre adenovirus at early postnatal ages develop Grade III astrocytomas, whereas mice injected at adult ages develop both Grade III and Grade IV astrocytomas.

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*$^{flox/-}$, or *Nf1*$^{flox/+}$;*p53*$^{flox/flox}$;*Pten*$^{flox/+}$) either at postnatal day 1-2 or adult ages (4-8 weeks). To target differentiated cell types, we also directly injected cre adenovirus into the cortex or striatum of tumor suppressor floxed mice. Mice bearing the conditional tumor suppressor alleles injected with GFP-expressing adenovirus were used as controls.

SVZ-targeted mutant mice had shortened survival compared to those targeted in the non-neurogenic regions, which lived as long as controls, except for one mouse that was injected at early postnatal age (Figure 2.1B). Injected mice were aged until the appearance of general (weight loss, anorexia) or neurologic (ataxia, seizures, paralysis) symptoms. Histologic analysis showed that viral cre-mediated tumor suppressor inactivation in the SVZ at both early postnatal and adult ages induced astrocytoma formation (Figure 2.1C-D, Table 1.1). One hundred percent of SVZ-injected mutant mice developed brain tumors classified as Grade III or IV astrocytomas starting six months post-injection. These tumors exhibited the classic histopathologic hallmarks of high-grade astrocytomas, including diffuse infiltration, nuclear atypia, mitoses, microvascular proliferation, and necrosis, and were indistinguishable from those of our previous studies (Kwon et al., 2008; Zhu et al., 2005a). These tumors had large numbers of Ki67+ cells, indicating robust proliferation, and were immunoreactive for GFAP, nestin and Olig2 (Figure 2.2), acknowledged markers in 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

2.2B). We further confirmed deletion of the tumor suppressor alleles in tumors by immunostaining and genotyping (Figure 2.3A and data not shown). 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 (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).

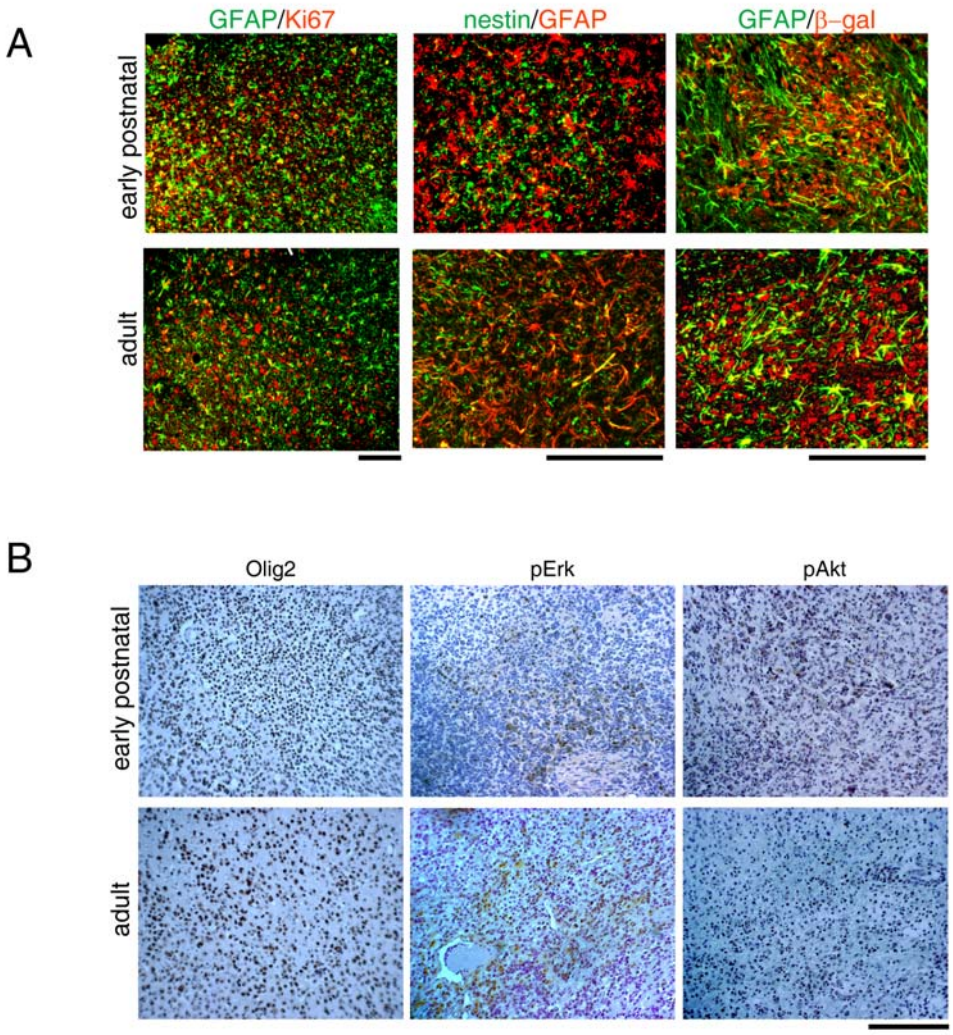

Figure 2.2. Tumors from SVZ-injected Mutant Mice express Traditional Markers of Astrocytomas.

A. Tumors from SVZ-targeted tumor suppressor floxed mice with *R26-lacZ* reporter show characteristic expression of astrocytoma-associated markers Ki67, GFAP, and nestin.

B. Some tumor regions show expression of Olig2, pErk, and pAkt. Scale bar, 200 μm.

Table 2.1. 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[1]	Cre→ SVZ	10/10
	Cre→ other regions[3]	1/12
	Controls[5]	0/8
Adult[2]	Cre→ SVZ	12/12
	Cre→ other regions[3,4]	0/20
	Controls[5]	0/9

[1]Postnatal day 1-2;
[2]4-8 weeks of age;
[3]Other non-neurogenic regions targeted include the cortex and striatum;
[4] 9 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;
[5]Controls include Ad-GFP injected into the SVZ or cortex/striatum of tumor suppressor mutant mice, and Ad-Cre injected into R26-lacZ mice alone

An internal 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 2.3A), 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 2.3A).

SVZ-derived tumors exhibit infiltration and heterogeneity

The presence of the *R26R-stop-lacZ* reporter in the context of the floxed tumor suppressors allows for lineage tracing of cells as they undergo tumorigenic transformation. While targeted SVZ neural stem cells and their progeny are initially restricted to the SVZ-RMS-OB axis (Figure 2.1A), tumors arising from SVZ-targeted mutant mice were found in adjacent brain regions, as shown by X-gal staining (Figure 2.3A). Evidence of migration is shown by the presence of tumors away from the SVZ in the adult-injected mice, such as in the cortex, hippocampus and thalamus. Tumors were found throughout most of the brain parenchyma in mutant mice injected at early postnatal ages, whereas adult-injected mutant mice develop more restricted, albeit still invasive, high-grade astrocytomas. In both cases, we found intense X-gal staining of the tumor regions, as well as in the SVZ-RMS-OB (Figure 2.3A).

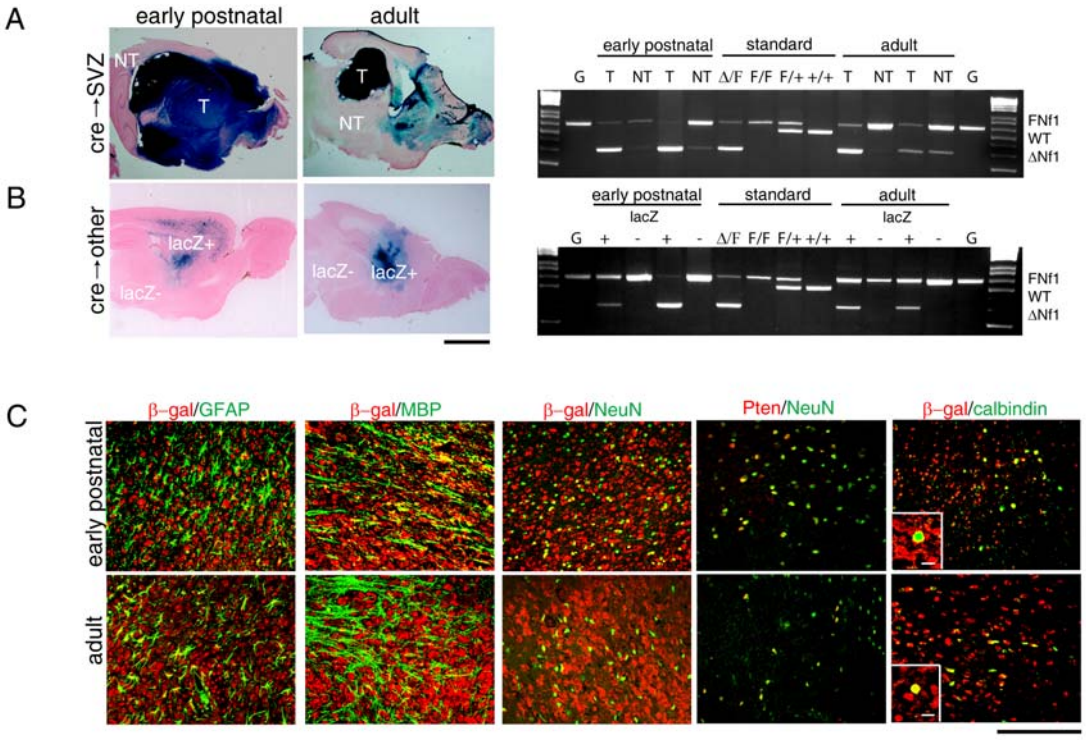

Figure 2.3. Tumors arising from SVZ-targeted mutant mice show infiltration and spontaneous differentiation.

A. 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, as well as labeling of the SVZ-RMS-OB axis. LacZ-positive tumor (T) and lacZ-negative non-tumor (NT) regions were dissected out 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, and +=WT=wild type alleles). B. X-gal stained brain sections from mice targeted in non-neurogenic regions such as striatum show localized staining at the site of injection and along the needle tract but no tumor formation. 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 multi-lineage 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-galactosidase-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-galactosidase-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. Scale bar, 200 μm. Insets show a β-galactosidase/calbindin double-positive cell. Scale bar, 10 μm.

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 co-expressing markers of subsets of differentiated cells, such the neuronal marker NeuN, astrocytic marker GFAP and oligodendrocytic marker myelin basic protein (MBP), as shown in a cortical tumor in Figure 2.3C. These immunoreactive tumor cells morphologically resembled mature neurons, astrocytes, and myelin-ensheathing oligodendrocytes. As an independent method of confirming that these marker-positive cells are indeed tumor cells, we stained these tumors for Pten, which is frequently suppressed in high-grade astrocytomas. In contrast to normal CNS cells which show abundant Pten expression (Kwon et al., 2008), these marker-positive cells were Pten-negative, confirming that these "differentiated" cells are indeed cancer cells (Figure 2.3C). We also found a rare population of β-galactosidase-positive tumor cells near the cortex that express calbindin, which is normally expressed by a subset of OB neurons produced by the SVZ neural stem/progenitor cells (Merkle et al., 2007). 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.

Viral cre-mediated Targeting of Adult Non-neurogenic Brain Regions Does Not Induce Tumor Formation

The above results demonstrate that precursor 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, suggest 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 20 four to eight 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 develop tumors, none of the animals injected in the cortex or striatum had evidence of tumor formation (Figure 2.1C, Table 2.1) despite clear evidence of successful cre adenovirus infection as demonstrated by X-gal staining and PCR genotyping (Figure 2.3B) or β-galactosidase immunohistochemistry (Figure 2.4). H&E staining showed disorganization of the cortical or striatal architecture in the injection site (Figure 2.1C,2.4), while immunostaining showed the presence of GFAP-positive but Ki67-negative cells (Figure 2.4), which is indicative of fibrosis and astrogliosis (Zhu et al., 2005b). Cells near the injection site likewise stained positive for nestin and vimentin (Figure 2.4), consistent with reactive astrocytosis (Correa-Cerro and Mandell, 2007; Sofroniew,

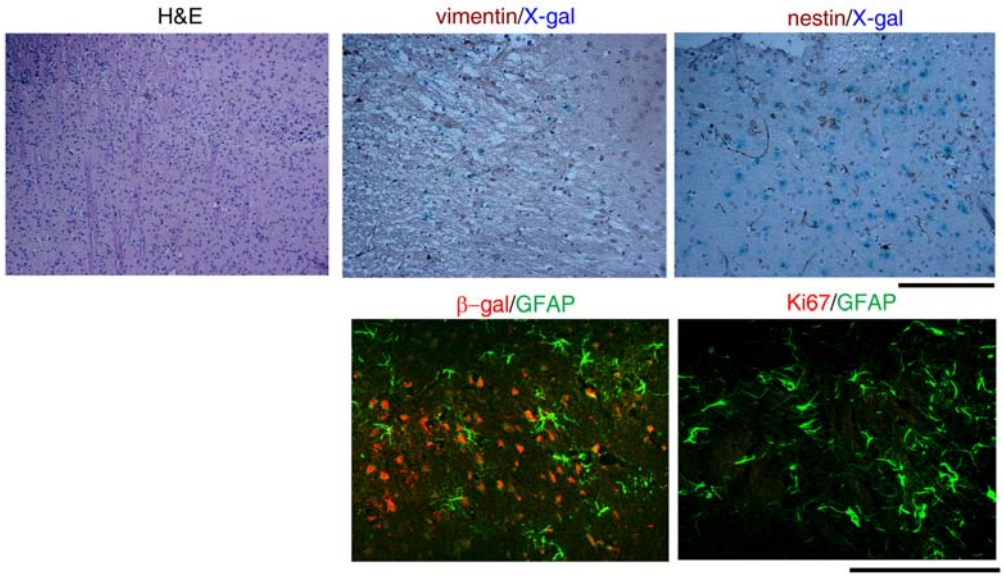

Figure 2.4 Targeting of Non-neurogenic Brain Regions in Mutant Mice Does Not Lead to Tumor Formation

Mutant mice injected with cre virus targeted to the striatum or cortex do not develop tumors, as shown by H&E staining. β-galactosidase-positive cells in the site of injection show localized expansion of Gfap-positive but Ki67-negative glial cells. Injected regions also show vimentin and nestin staining in the site of injection (shown here with X-gal-positive cells) consistent with astrogliosis. Scale bars, 200 μm.

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 do not develop gliomas (Table 2.1). However, we did find one out of twelve cases where non-SVZ-targeted neonatal floxed mice developed tumors 7 months post-injection. 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.

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 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. These results were confirmed by genetic studies by another graduate student in the laboratory, Jian Chen, who used *nestin-cre* inducible mice to drive tumor suppressor inactivation in neural stem and progenitor cells, resulting in astrocytoma formation. These experiments thus establish mutations of these astrocytoma-relevant tumor suppressors in the neurogenic compartment *in vivo* as 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 are differentiated did not yield tumors but, instead, local astrogliosis and localized lacZ staining, despite demonstration of recombination in glial cells. 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 early postnatal and adult mice dependent on stem/progenitor cell targeting of the tumor suppressors.

For all of the described tumor studies, the endpoint was selected to ensure adequate incubation of all manipulated cells, whether tumor suppressor-bearing or

controls. 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 extensively previously 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 pre-exists 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 shows 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 the 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 the 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” non-tumor 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 non-recombined band (Figure 2.3A and data not shown), and the majority of cells

expressing mature, differentiated markers are β-galactosidase-negative (Figure 2.3C), 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 *in situ*, the original tumor is heterogeneous 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 find 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. Hence, the clinically relevant migration and differentiation capacity of astrocytomas is consistent with the neural stem/progenitor cell origin uncovered here.

Cell of Origin in Mouse and Human Gliomas

Additional 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 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 gave rise to astrocytomas (Bachoo et al., 2002; Dai et al., 2001). Thus, to date, cells that were targeted for transformation were 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 (TCGA, 2008). 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.

Methods

Mice

All mouse experiments were approved 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/C57Bl6/B6CBA background (Kwon et al., 2008). Mice that harbor the *Nf1* and *p53* floxed 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. Genotyping for the flox, wild type and recombinant alleles of *Nf1* and *p53* have been previously described (Kwon et al., 2008; Zhu et al., 2005a).

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 in the literature (Doetsch et al., 1999; Merkle et al., 2004) with some modifications. Two hundred nL of adenovirus (Ad-Cre, 2.0×10^{12} pfu/mL, University of Iowa Vector Core; Ad-GFP, 1.0×10^{11} pfu/mL) was injected using a World Precision nanoinjector apparatus, according to the following coordinates - SVZ (0, 1.4, 1.6; 0.5, 1.1, 1.7; and 1, 1, 2.3), cortex (0, 3.5, 1.5), and striatum (0, 1.4, 2.6) mm anterior, lateral, and dorsal to the bregma. For early postnatal injections, postnatal day 1 or 2 pups were injected with 40 nL of Ad-Cre or Ad-GFP, as previously described (Merkle et al., 2004) with some modifications. The following coordinates for a range of neonate

weights were used: 1.4-1.5g (1.5, 2.6, 1.4); 1.5-1.7g (1.6, 2.7, 1.4); 1.7-1.9g (1.7, 2.9, 1.5); >1.9g (1.7, 2.9, 1.7) mm anterior, lateral and dorsal to the bregma. 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-μm sections were cut and every fifth slide was stained with H&E. Brain sections were independently examined by S.A.L. as well as D.K.B., a certified neuropathologist, and tumor diagnosis was determined based on the 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 by nuclear fast red, as previously described (Luikart et al., 2005). In some cases, half brains that were stained with X-gal were subsequently processed and used for immunohistochemistry. For PCR genotyping, DNA extraction and PCR was performed using tumor and non-tumor tissues, as previously described (Kwon et al., 2008).

Immunohistochemistry

Paraffin sections were deparaffinized, rehydrated, and subjected to citrate-based antigen retrieval. Primary antibodies against the following were used as follows: 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 using 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 Lab). Sections were examined using optical, fluorescence and confocal microscopy (Olympus and Zeiss).

Chapter Three

Tumor Suppressor Inactivation in Transit Amplifying Cells induces Malignant Astrocytoma Formation

Abstract

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. Using fully penetrant mouse models, we have previously reported subventricular zone (SVZ) neural stem/progenitor cells as cancer-initiating cells in malignant astrocytomas. Neural stem cells in the adult SVZ give rise to rapidly-dividing transit amplifying cells, or progenitor cells, which then differentiate into more mature cell types. Whether short-lived progenitor cells alone, but not lifelong self-renewing stem cells, can give rise to astrocytomas is not known.

By genetically targeting transit amplifying cells using the *Ascl1-cre*^{ERTM} transgenic mouse, we provide evidence that induction of tumor suppressor inactivating mutations in the progenitor compartment induces malignant astrocytoma formation. Analysis of mutant mice several months prior to advanced disease shows defects in proliferation, differentiation, and migration, suggesting that these are requisite processes prior to full blown tumor development. This establishes progenitor cells as cells of origin of malignant astrocytomas.

Background

Tumorigenesis is a multi-step process that involves accumulation of sufficient mutations within cells. Cancer cells progressively acquire capabilities that allow it to breach the anti-cancer defense mechanisms that are hardwired in cells and tissues (Hanahan and Weinberg, 2000). The unlimited self-renewal potential of stem cells allows these cells to acquire mutations during the lifetime of an individual, making these cells ideal targets for malignant transformation.

Neural stem cells play essential roles in the development and maturation of the CNS during embryogenesis and early postnatal stages. However, precursors for the different CNS cell types continue to persist in the adult brain. Astrocytes and oligodendrocytes are generated in response to injury and pathologic conditions, whereas new neurons are continually produced in the subgranular layer of the dentate gyrus and the subventricular zone of the lateral ventricles (Alvarez-Buylla and Lim, 2004; Menn et al., 2006; Zhao et al., 2008). In the SVZ, neural stem cells are GFAP-positive cells of the astroglial lineage that give rise to progenitor cells, or transit amplifying cells. These lineage-restricted progenitor cells are highly mitotic and responsive to EGF stimulation, and were previously shown to express the homeobox transcription factor Dlx2 (Doetsch et al., 2002). However, not much was known regarding its identity and lineage.

The basic helix-loop-helix transcription factor Ascl1 (previously Mash1) is known as a neuronal differentiation factor during early development. It is also involved in neuronal subtype specification in the developing central and peripheral nervous systems (Bertrand et al., 2002). Besides early neurogenesis, Ascl1 also plays

an important role in oligodendrocyte development (Parras et al., 2007). Ectopic expression of Ascl1 in neural stem cell cultures leads to preferential differentiation into neurons and oligodendrocytes but not astrocytes (Sugimori et al., 2007), suggesting that it may also play important roles in neural stem cell development in the adult. Recently, it was demonstrated that Ascl1-positive cells represent the lineage-restricted progenitor population in the adult mouse brain. Fate labeling experiments using the inducible *Ascl1-cre*^{ERTM} trangenic and *Ascl1*^{GFP} knock-in mice identified Ascl1 as a marker for transit amplifying cells in the SVZ and RMS, type 2a progenitor cells in the SGZ, as well as oligodendrocyte progenitor cells in the subcortical gray matter and near the corpus callosum (Kim et al., 2007).

The role of lineage-restricted progenitor cells or transit-amplifying cells in malignant astrocytoma formation is not known. In this study, we used the genetic approach to induce tumor suppressor inactivating mutations in these cells and demonstrate that targeting progenitor cells alone is sufficient to induce tumorigenesis in our mouse models.

Results

Ascl1-cre^{ERTM} transgenic mouse targets progenitor cells but not stem cells in the CNS

Our previous studies using stereotactic and genetic approaches identified the SVZ neural stem/progenitor cells as the cells of origin of astrocytoma in our tumor suppressor mouse models. These involved targeting tumor suppressor inactivating mutations in the SVZ neural stem/progenitor cells, which resulted in malignant astrocytoma formation (Alcantara Llaguno et al., 2009). Because inactivating mutations in stem cells are passed on to its progeny, both neural stem and progenitor cells are equally targeted in these experiments. Hence, it does not address the role of these rapidly-dividing cells in astrocytoma formation.

In order to specifically target the transit amplifying cells, we used a transgenic mouse line that expresses a cre recombinase-modified estrogen receptor ligand binding domain fusion protein (*cre-*^{ERTM}) under the control of the *Ascl1* regulatory elements (Kim et al., 2007). Ascl1 is a pro-neural transcription factor during early CNS development that has been recently shown to be expressed in adult lineage-restricted progenitor cells (Kim et al., 2007). Tamoxifen administration induces nuclear transfer of the cre-^{ERTM} protein in *Ascl1*-expressing cells where it can mediate *loxP*-dependent recombination (Feil et al., 1996). Cre recombinase expression in Ascl1-expressing cells and its progeny is evaluated by breeding the transgenic into a *R26-stop-lacZ* reporter strain. X-gal staining of tamoxifen-treated *Ascl1-cre^{ERTM}*; *R26-*

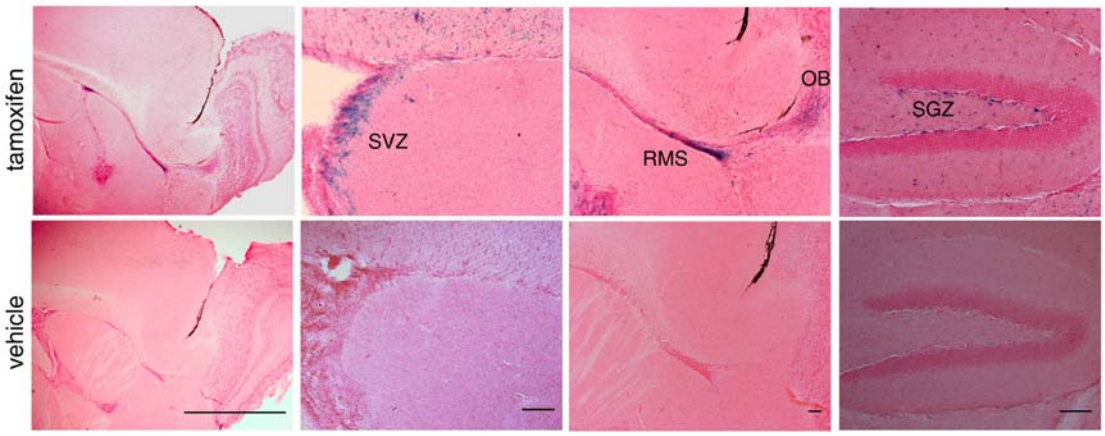

Figure 3.1. *Ascl1-cre*^{ERTM} transgenic mouse targets cre-mediated recombination in transit amplifying cells. X-gal staining of *Ascl1-cre*^{ERTM}*; R26-stop-lacZ* mouse brains treated with tamoxifen or vehicle two weeks post-induction. Blue lacZ+ cells are found in the SVZ, RMS and OB, as well as in the SGZ of the dentate gyrus. Scale bars, 200 μm except leftmost panels, 2mm.

stop-lacZ mouse brain sections shows labeled cells in the SVZ-RMS-OB as well as in the SGZ at two weeks post-induction**,** whereas vehicle-treated controls did not (Figure 3.1). There were also lacZ-positive cells near the corpus callosum, representing oligodendrocyte progenitor cells (data not shown). LacZ-positive cells also expressed markers of immature neuronal differentiation such as doublecortin and NeuN (data not shown).

At 3 months post-induction, we did not find any more lacZ+ cells in the SVZ, whereas most of the recombined cells were found in the RMS, OB and near the corpus callosum (control in Figure 3.2A, bottom panel). This is consistent with the fate of neural and oligodendrocyte progenitor cells in the SVZ (Doetsch et al., 1999; Menn et al., 2006). LacZ+ cells were also found in the granular layer of the dentate gyrus, while there were also some scattered staining in the cortex. These correspond to the various locations of transit amplifying cells in the SVZ and DG and its mature progeny, and are consistent with what was reported in the literature (Kim et al., 2007). The paucity of lacZ-positive cells is likewise consistent with targeting of the transit amplifying population as compared with the stem cell compartment, which produce new progeny continuously and hence produce more blue cells.

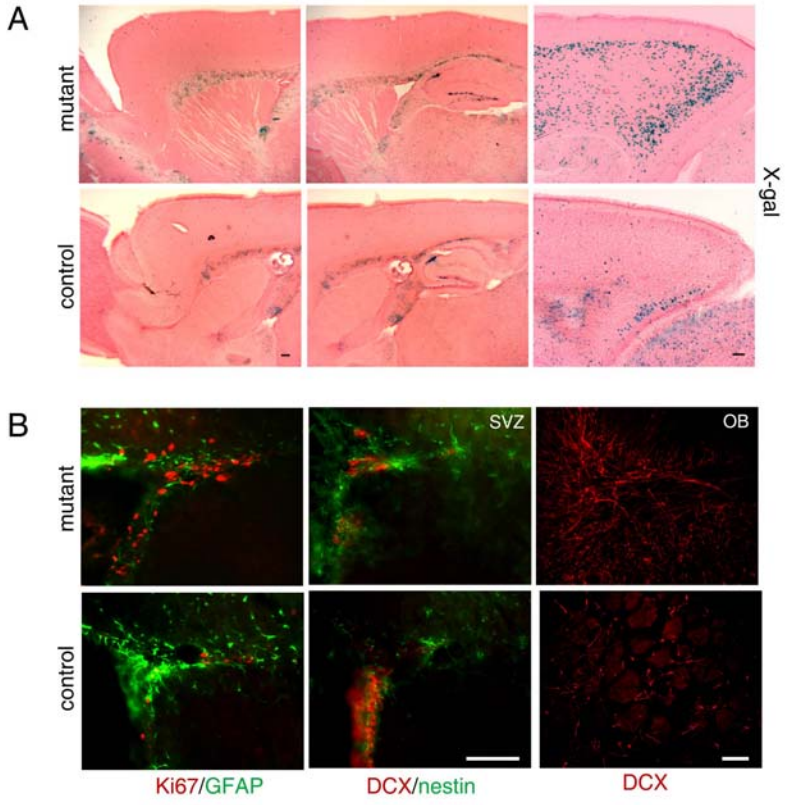

Figure 3.2. Defects in migration, proliferation and differentiation in *Ascl1* tumor suppressor floxed mice. *Ascl1-cre^{ERTM}; Nf1$^{flox/flox}$; p53$^{flox/flox}$* mice were administered with tamoxifen at 4 weeks of age and analyzed at 3 months post-induction. A. X-gal stained brain sections of mutants show more lacZ+ cells in the RMS-OB and along the corpus callosum in mutants as compared to a *Ascl1-cre^{ERTM}; R26-stop-lacZ* reporter control at the same age. There are also more lacZ-positive cells in the cortex. Scale bar, 100 μm. B. Mutants show increased proliferation as shown by increased Ki67+ cells in the SVZ as compared to controls. There are also more nestin-expressing cells in the SVZ. Doublecortin (DCX)-positive cells in the SVZ are fewer but are increased in the olfactory bulb (OB). Scale bar, 100 μm.

Defects in migration, proliferation and neurogenesis prior to full blown tumor development

To determine whether tumor suppressor deletion in transit amplifying cells leads to astrocytoma formation, we bred *Ascl1-cre^{ERTM}* transgenic mice to incorporate the tumor suppressor floxed alleles (*Ascl1-cre^{ERTM}; Nf1$^{flox/flox}$;p53$^{flox/flox}$; Ascl1-cre^{ERTM}; Nf1$^{flox/flox}$;p53$^{flox/-}$)* and treated adult mice with tamoxifen by oral gavage at 4 weeks of age. *Ascl1* tumor suppressor floxed mice treated with vehicle or tumor suppressor floxed mice treated with tamoxifen were used as controls.

At 3 months post-induction, *Ascl1* tumor suppressor floxed mouse brains exhibited defects in migration, proliferation and differentiation (Figure 3.2). X-gal staining of *Ascl1-cre^{ERTM}; Nf1*$^{flox/flox}$; *p53*$^{flox/flox}$*;R26R-stop-lacZ* brain sections show more lacZ+ cells in the RMS, olfactory bulb and corpus callosum compared to a similarly-aged *Ascl1-cre^{ERTM}; R26-stop-lacZ* control brain (Figure 3.2A). There were also more abundant lacZ-positive cells in regions such as the cortex and striatum, which were not found in controls. These recombined cells were negative for Ki67 (data not shown), suggesting that these cells may have migrated from its original location. In the SVZ, there were more cells that were positive for Ki67, as well as nestin, a stem and progenitor marker. On the other hand, immunostaining with doublecortin (DCX), an immature neuronal marker, showed that there were less newly born neurons in the SVZ but increased numbers in the olfactory bulb (Figure 3.2B). This shows that tumor suppressor deletion leads to enhanced differentiation of progenitor cells. The increased proliferation in the SVZ may be a secondary effect due to the increased turnover of progenitor cells. Aberrant migration of *Ascl1*-positive

tumor suppressor-deficient progeny also occurs prior to full blown tumor formation, similar to what was observed with the *nestin* tumor suppressor mutant mice (Alcantara Llaguno et al., 2009)

Genetic targeting of tumor suppressor mutations in progenitor cells leads to astrocytoma development

Ascl1 tumor suppressor floxed mutant mice were aged until they exhibited neurologic symptoms. As shown by the Kaplan-Meier curve (Figure 3.3A), mutant mice have decreased survival compared to their control littermates, with a median survival of 36 weeks. Analysis of symptomatic mutant mice reveal that *Ascl1* tumor suppressor floxed mice developed high-grade astrocytomas while control mice did not (Figure 3.3B). 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. Adult-treated mutant mice developed tumors diagnosed as Grade III or Grade IV (glioblastoma multiforme) astrocytomas based on the World Health Organization classification system. These tumors had large numbers of Ki67+ cells, indicating robust proliferation, and were immunoreactive for astrocytoma markers Gfap, nestin and Olig2 (Figure 3.3C). We also confirmed cre-mediated recombination in these tumors by β–galactosidase immunohistochemistry (data not shown). These data demonstrate that tumor suppressor inactivation in self-renewal-limited progenitor cells induces astrocytoma formation.

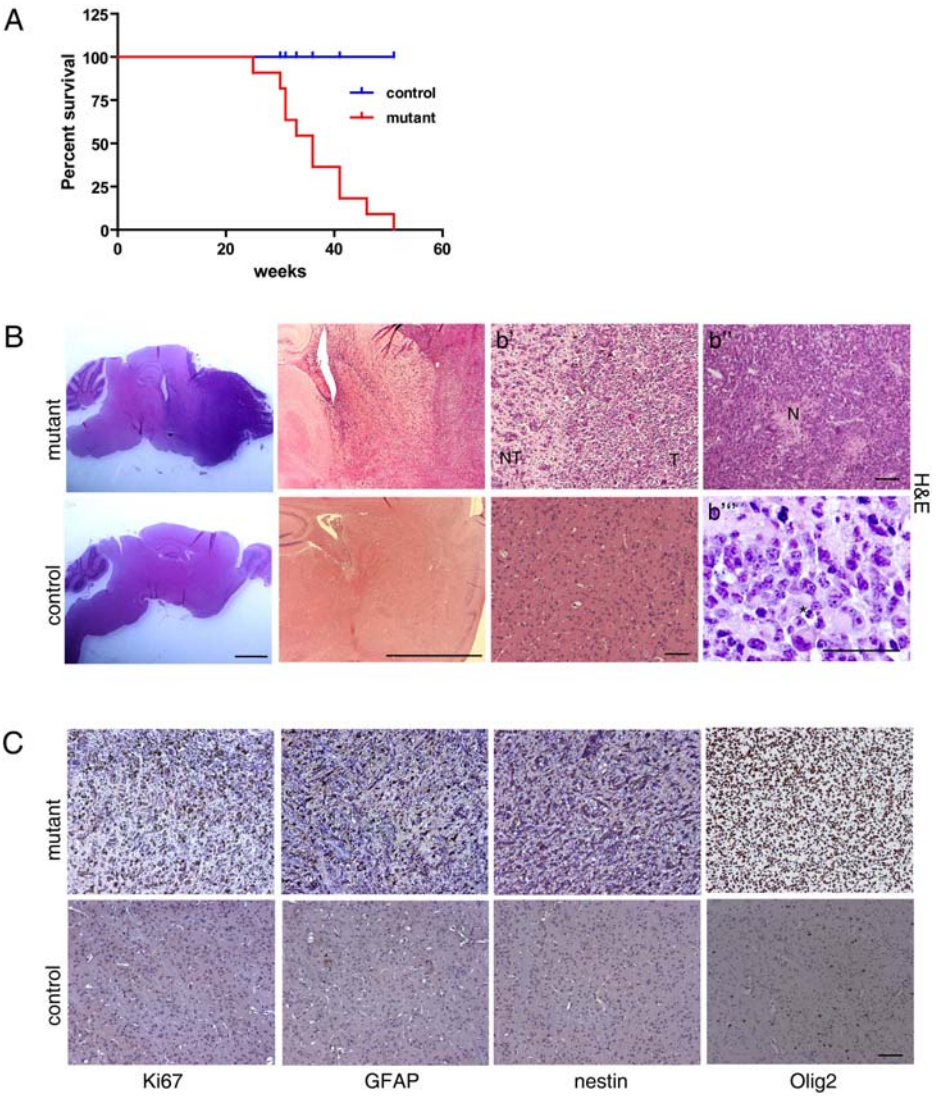

Figure 3.3. Tumor suppressor inactivation in transit amplifying cells leads to astrocytoma formation. A. Mice with induced tumor suppressor inactivation (*Ascl1-cre*^{ERTM};*Nf1*$^{flox/flox}$;*p53*$^{flox/flox}$) have shortened survival compared to control mice. Kaplan-Meier survival curves of *Ascl1-cre*^{ERTM} mice with tumor suppressor inactivation induced at 4 weeks of age show median survival of 36 weeks of age. B. Histologically identifiable high-grade astrocytomas develop in inducible mutant mice. Representative H&E-stained brain sections reveal formation of brain tumors in *Ascl1-cre*^{ERTM} mice with tumor suppressor inactivation induced at 4 weeks of age. High-grade astrocytomas with characteristic features such as infiltration of tumor cells (T) into adjacent normal (NT) regions (b'), necrosis (b'') and presence of mitotic figures (asterisk) are shown. Scale bars, 100 μm, except leftmost panels, 2 mm. C. Tumors express traditional markers of astrocytomas, including Ki67, Gfap, nestin, and Olig2. Scale bar, 100 μm.

Discussion

Lineage-restricted Progenitor Cells as Cancer-initiating Cells in Tumor Suppressor Mouse Models

In this study, we report the first specific targeting of lineage-restricted progenitor cells to determine their role in brain tumor formation. The use of the *Ascl1-cre^{ERTM}* transgenic line allows specific ablation of tumor suppressors in progenitor cells but not in stem cells. These transient amplifying cells undergo limited mitoses before differentiating into newly born cells. In the SVZ, it takes about two weeks for these cells to migrate through the RMS and into the OB, where they integrate into the existing circuitry and later on undergo activity-dependent apoptosis (Petreanu and Alvarez-Buylla, 2002). Consistent with the cre expression pattern of Ascl1, transit amplifying cells and its progeny are far less abundant as compared to stem cells, which have unlimited potential for self-renewal and generation of new CNS cells. In fact, the *Ascl1-cre^{ERTM}; R26R-stop-lacZ* reporter line shows very limited expression at 2 weeks and even more so at 3 months post-induction. Hence, it was a somewhat surprising result that tumor suppressor ablation in the progenitor compartment alone leads to malignant astrocytoma development. The tumors are phenotypically similar to high-grade astrocytomas based on histologic and molecular features. Cre-mediated recombination is very robust, such that the mice develop tumors as early as 7 months post-induction. It should be noted that these mice already have homozygous floxed *Nf1* alleles and homozygous floxed or floxed/null *p53* alleles. Hence, cre-mediated recombination leads to complete deletion of the *Nf1* and *p53* tumor suppressors. This singular event may be sufficient to drive the

tumorigenic cascade in progenitor cells. On the other hand, *Ascl1* tumor suppressor floxed mice seem to develop tumors faster than the equivalent *Nestin* tumor suppressor floxed mice (Alcantara Llaguno et al., 2009 and data not shown). Since both stem and progenitor cells are equally targeted in *Nestin* mutant mice, faster tumor induction in *Ascl1* mutants may indicate greater selective pressure for tumor suppressor-deficient progenitor cells to block the normal course of differentiation, leading to a more accelerated tumorigenic phenotype. This may suggest that if the initiating mutations are sufficient for transformation, manifestation of the malignant state is more direct if originated in progenitor cells.

Whether it is the neural or glial progenitors that are giving rise to the tumors cannot be definitively determined, however, since Ascl1 is expressed in both populations of cells. It will require even more restricted cell type-specific promoters to distinguish between these cells.

Progenitor Cell Origin of Cancer

The role of committed progenitors or other cell lineages as cancer-initiating cells in different forms of cancer is not well understood, partly because of the absence of cell type-specific markers that identify these cells. The lineage hierarchy of the hematopoietic system is the most well-characterized. Hence, most studies have been done by isolating different cell populations by sorting using different markers and subsequent introduction of oncogenic mutations. Committed progenitors induced by an activating fusion protein MLL-AF9 leads to development of acute myelogenous leukemia (Krivtsov et al., 2006). On the other hand, expression of the *BCR/ABL*

fusion gene and *BCL-2* in myeloid progenitors but not hematopoietic stem cells induces acute myelogenous leuekemia (Jaiswal et al., 2003). Introduction of *BCR-ABL* alone, however, only results in myeloproliferative disease, suggesting that additional mutations may be required to induce full blown disease in progenitor cells. In a *Kras* model of juvenile myelomonocytic leukemia and T-cell lymphoblastic leukemia/lymphoma, hematopoietic stem cells were suggested to be the initial target, whereas lineage-specific progenitors the final potential targets for final leukemic transformation (Zhang et al., 2009). These studies suggest that when initiating mutations are sufficient to cause tumorigenesis, i.e. combination of mutations, then transformation of progenitor cells is a probable event. However, unless a single mutation is powerful enough to cause tumorigenesis, ie. *Kras* model just described, a combination of mutations may be needed for full blown tumorigenesis to occur.

The mouse models used in this study are already homozygous floxed for the *Nf1* and *p53* tumor suppressors. Hence, cre-mediated recombination ensures complete deletion of both tumor suppressors. This may be sufficient to induce transformation in the progenitor cells in a shorter period of time. As shown in the 3 months post-induction data, there are less doublecortin-positive newly born neurons in the SVZ but more at the olfactory bulb. This indicates that progenitor cells undergo increased turnover (increased mitosis and subsequent differentiation) leading to increased rate of neurogenesis. This is also accompanied by increased proliferation in the stem and progenitor regions. Since recombined cells have already exited the SVZ, increased proliferation in the SVZ may be a compensatory mechanism to replenish differentiating progenitor cells. On the other hand, increased proliferation in the

progenitor cells may allow the cells to acquire additional mutations that aid in transformation during a short period of time. This demonstrates a link between proliferation and differentiation states of initiating cells during early stages of tumor development.

Stem Cells vs. Progenitor Cells as Cells of Origin of Astrocytoma

Our studies demonstrate that dividing cells such as stem and progenitor cells are the most likely targets of tumorigenesis. This was accomplished using combinations of mutations that are known to be present in human cancers. However, it may be reasonable to assume that normal individuals do not acquire combinations of critical tumor-causing mutations with one hit. It may take time to accumulate mutations required for normal cells to undergo transformation, as supported by the increased incidence of brain tumors in the elderly population. This may suggest that the initial event may more likely occur in the stem cell than in the progenitor compartment.

On the other hand, it will be interesting to determine the impact of tumor suppressor-deficient progenitor cells on wild type stem cells. Since the fate of the quiescent stem cells is linked to the progenitor cells, the hypothesis would be that these cells may decrease production of lineage-committed progenitors if these cells are proliferating more rapidly. Another issue is how transformed progenitor cells acquire unlimited self-renewal in order to block its normal course of differentiation. The acquisition of this stem cell trait has been suggested in a mouse model of chronic myelogenous leukemia (CML), whereby leukemic stem cells isolated from CML

derived from committed progenitors displayed reactivation of a subset of genes that are highly expressed in hematopoietic stem cells (Krivtsov et al., 2006). Whether this reactivation of stem cell genes in transformed progenitor cells occurs in our mouse models will provide valuable insight into mechanisms of disease progression.

Model for Neural Stem and Progenitor Origin of Malignant Astrocytomas

Our stereotactic and genetic experiments have demonstrated that neural stem *and* progenitor cells are cancer-initiating cells in our tumor suppressor mouse models. This model of neural stem and progenitor origin of malignant astrocytomas is depicted in Figure 3.4. Neural stem and progenitor cells are the cell types that are most susceptible to malignant transformation whereas post-mitotic differentiated cells are less likely to give rise to these tumors. Whether progenitor cells require stem cell signature traits for tumorigenesis to occur or transformed stem cells are manifest phenotypically as progenitor-like cells are also depicted.

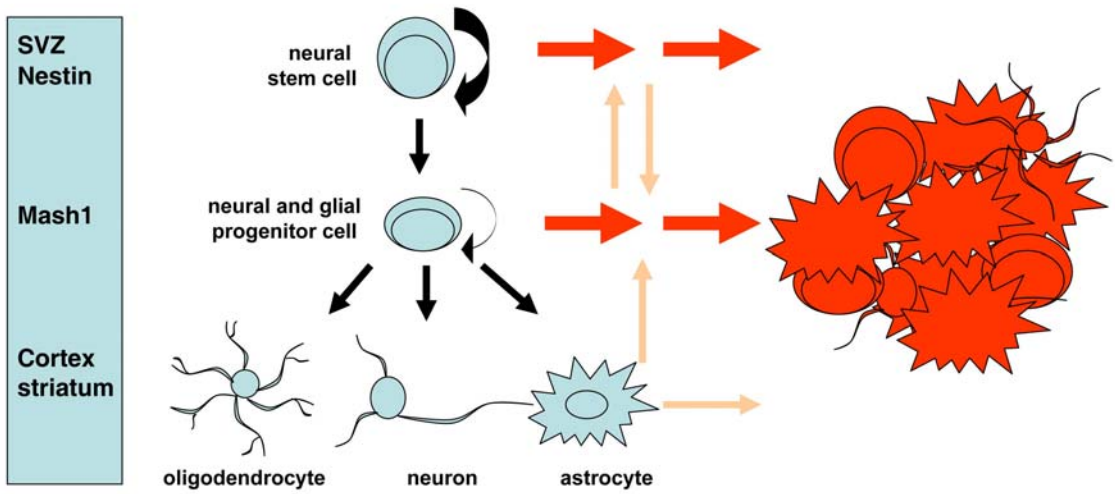

Figure 3.4. Neural Stem and Progenitor Cells as Cells of Origin of Malignant Astroctyomas. Working model proposes that the lifelong self-renewing stem cells and rapidly-dividing transit amplifying cells or progenitor cells are astrocytoma-initiating cells in tumor suppressor mouse models. These are supported by both stereotactic injection (SVZ, cortex, striatum) and genetic (*Mash1 or Ascl1, Nestin)* experiments that target these immature cell types, resulting in tumor formation. Whether neural stem cells transition to a progenitor-like state or vice versa prior to full blown formation is also posited.

Methods

Mice

All mouse experiments were approved 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/C57Bl6/B6CBA background (Kwon et al., 2008).

Tamoxifen Induction

Tumor suppressor floxed mice containing the *Ascl1-cre*^{ERTM} transgene was administered with tamoxifen (Sigma) or vehicle (9:1, sunflower oil: ethanol mixture) by oral gavage at a working concentration of 50 mg/mL. Four-week-old mutant mice (*Ascl1-cre*^{ERTM}*;Nf1*$^{flox/flox}$*;p53*$^{flox/flox}$ or *Ascl1-cre*^{ERTM}*;Nf1*$^{flox/flox}$*;p53*$^{flox/-}$) were administered with 500 mg/kg tamoxifen once a day as a single dose or for 2 consecutive days. Conditional (*Nf1*$^{flox/flox}$*;p53*$^{flox/flox}$ or *Nf1*$^{flox/flox}$*;p53*$^{flox/-}$) or mutant mice given tamoxifen or vehicle, respectively, were used as controls.

Histology and Tumor Analysis

Mice were perfused and fixed with 4% paraformaldehyde. Five-μm sections were cut and every fifth slide was stained with H&E. Brain sections were independently examined by S.A.L. and D.K.B., a certified neuropathologist, and tumor diagnosis was determined based on the World Health Organization criteria (Kleihues et al., 2002). Brains used for X-gal staining were postfixed in 2% PFA

overnight. Fifty-μm vibratome sections were stained in X-gal solution, and sections were counterstained by nuclear fast red, as previously described (Luikart et al., 2005). For PCR genotyping, DNA extraction and PCR was performed using tumor and non-tumor tissues, as previously described (Kwon et al., 2008).

Immunohistochemistry

Paraffin sections were deparaffinized, rehydrated, and subjected to citrate-based antigen retrieval. In other cases, 50-μm vibratome sections were used for staining. Primary antibodies against the following were used as follows: 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), Doublecortin (Santa Cruz, 1:200), NeuN (Chemicon, 1:500) and MBP (Sternberger, 1:200). We used both immunofluorescence staining using 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 Lab). Sections were examined using optical, fluorescence and confocal microscopy (Olympus and Zeiss).

Chapter Four

Characterization and Genomic Profiling of Tumor-derived Neurosphere-Forming Cells from Astrocytoma Mouse Models

Abstract

Malignant astrocytomas are frequently incurable due to its infiltrative nature at the early onset and resistance to conventional therapies. The mechanisms involved in its malignant behavior are not well understood. Our laboratory has previously developed mouse models based on conditional inactivation of human astrocytoma-relevant genes *Nf1*, *p53*, and *Pten*, wherein mutant mice develop tumors with 100% penetrance. Using our tumor suppressor mouse models, we isolated tumor-derived neurosphere-forming cells (TD-NFC) and show that this enriched population of stem cell-like cancer cells exhibit enhanced self-renewal, aberrant differentiation and tumorigenicity in an orthotopic transplantation model. We also demonstrate its resistance to a known chemotherapeutic drug and its migratory capacity in the brain. We also performed gene expression analysis of TD-NFCs which revealed the predominance of neurodevelopmental signaling genes and transcriptional regulators. The prevalence of the hox family of transcription factors in two strains of tumor suppressor mouse models suggests a significant role for these genes in high-grade astrocytomas.

Background

Malignant astrocytomas are highly invasive and lethal brain tumors. Understanding the processes involved in the progression and maintenance of the malignant phenotype is essential for designing novel therapeutic approaches for this incurable disease.

These tumors are also highly heterogeneous. While predominantly astrocytic in character, there are a variety of cells types within the tumor mass, including cells that are immunoreactive for immature, neuronal and oligodendrocytic markers. The variety of tumor cell types within astrocytomas suggest the presence of cancer cells with an immature phenotype that is capable of giving rise to different neural lineages. This suggests that a subpopulation of tumor cells may exhibit more stem cell-like properties compared to the rest of the tumor bulk.

In humans, several reports have indicated the presence of stem cell-like cancer cells in GBMs and their possible roles in invasion, metastasis and resistance to existing therapies (Bao et al., 2006; Dalerba et al., 2007; Singh et al., 2004). These studies on human astrocytomas and analysis of our own tumor suppressor mouse models suggest a role for stem-like cancer cells in malignant astrocytoma formation.

In order to investigate the signaling pathways involved in malignant astrocytoma initiation, progression and maintenance, we performed gene expression profiling on tumor-derived neural stem/progenitor cells using our tumor suppressor mouse models, and uncovered novel developmentally regulated genes and transcription factors that may potentially be important for malignant astrocytoma formation.

Results

Tumor-derived neurosphere-forming cells from mouse astrocytomas exhibit growth and survival advantage and are tumorigenic in vivo

In order to enrich for cancer cells with more immature phenotypes in our mouse tumors, we used the neurosphere formation assay as a tool for enriching stem-like cancer cells. This ability to form three-dimensional spherical bodies when grown in serum-free media supplemented with growth factors has been the classical method for studying neural precursor function *in vitro* (Reynolds and Weiss, 1992), and has since been widely used in the field of neurogenesis as well as in other organ systems to characterize stem cell-like properties (Singec et al., 2006). We believe that this represents a more homogenous population of precursor-like cancer cells compared with primary tumor tissue, which not only contain differentiated cell types but also non-tumor cells as well. This system allows us to directly compare tumor-derived cells with normal neural stem cells and provides us with sufficient material to manipulate these cells in future studies.

Dissociated primary tumor cells from symptomatic Mut3 mice (*hGFAP-cre;Nf1*$^{flox/+}$*; p53*$^{-/+}$) grown in serum-free conditions supplemented with growth factors such as epidermal growth factor and basic fibroblast growth factor produced more neurospheres than normal neural stem cells from age-matched controls (Figure 4.1A). These tumor-derived neurosphere-forming cells (TD-NFCs) showed increased self-renewal potential when dissociated to form secondary and tertiary neurospheres (Figure 4.1B). They were also able to differentiate into the astrocytic, neuronal and

oligodendrocytic lineages when placed in serum-containing media (Figure 4.1C). Genotyping for the tumor suppressors also revealed that these cells represent a more homogeneous population of tumor cells, as shown by the presence of only p53-null alleles in these cultures, in contrast to primary tumor tissue, which still contain the wild type p53 allele (Figure 4.1D). When treated with the alkylating agent temozolomide, which is currently used as an adjuvant chemotherapeutic drug in patients with GBM (Stupp et al., 2005), these cells also showed increased survival as compared to normal neural stem cells or astrocytes (Figure 4.1F). Moreover, these tumor-derived neurosphere-forming cells gave rise to malignant astrocytomas when orthotopically transplanted into immunodeficient mice (Figure 4.1E-e'). Transplanted tumors were phenotypically similar to primary tumors (Figure 4.1E-e'') whereas lineage tracing of transplanted tumor cells bearing the *R26-stop-lacZ* reporter allele demonstrated the capacity of these cells to migrate, especially in stem cells niches such as the SVZ-RMS-OB axis (Figure 4.1E-e''', left and middle panels) and the SGZ of the dentate gyrus (Figure 4.1E-e''', right panel).

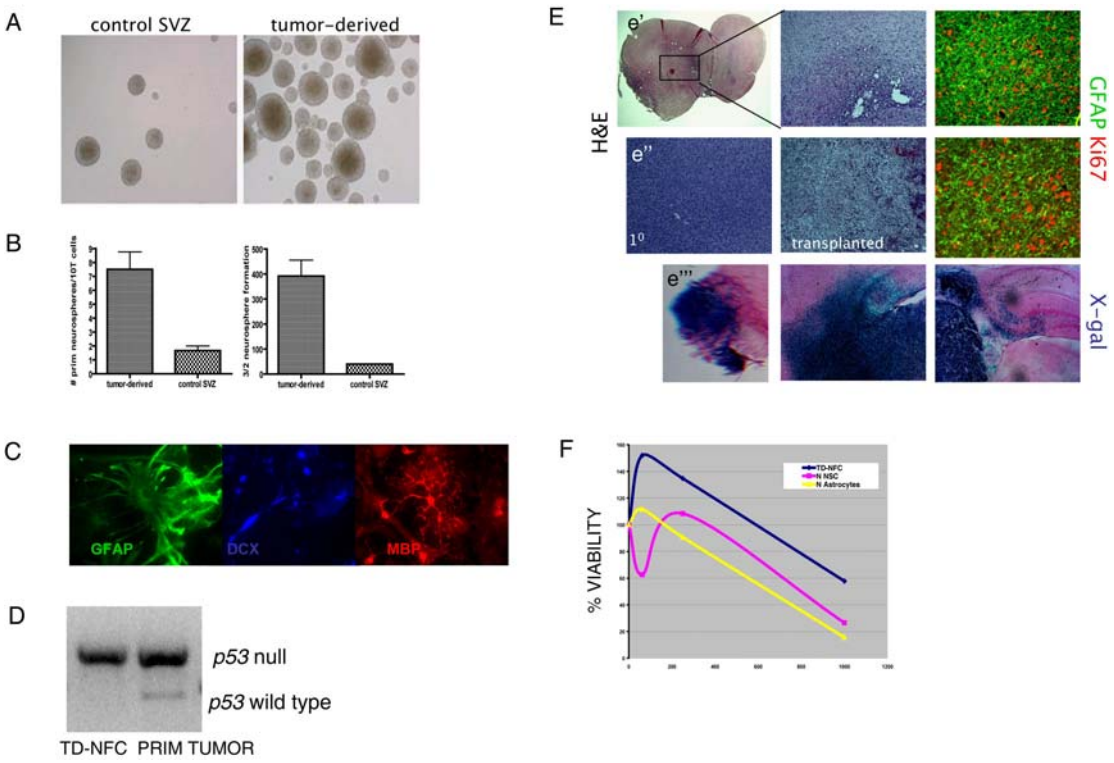

Figure 4.1. Tumor-derived neurosphere-forming cells exhibit growth and survival advantage and are tumorigenic *in vivo*. A. Primary tumor tissue from symptomatic mutant mice were dissected and cultured in serum-free conditions. SVZ from normal, age-matched, littermate mice were used as controls. Murine gliomas readily give rise to neurospheres, as do control SVZ cells. B. Tumor-derived neurosphere-forming cells (TD-NFC) exhibit greater self-renewal capacity than control SVZ. C. TD-NFCs have multipotent differentiation potential. Under serum-containing media, they differentiate into GFAP+ astrocytes, doublecortin+ neurons and MBP+ oligodendrocytes. The majority of differentiated cells are GFAP+. D. TD-NFCs are a more homogenous population of cancer cells. Polymerase chain reaction (PCR) genotyping shows TD-NFCs have undergone loss of heterozygosity of tumor suppressor alleles, in this case, of p53, as compared to primary tumor tissue (Prim Tumor), which contains both knockout and wild type alleles. E. TD-NFCs were transplanted into immunodeficient nu/nu mice. Transplanted nude mice developed infiltrating high-grade astrocytomas, as shown by H&E staining, and are immunoreactive for Gfap and Ki67, known glioma markers (e'). Transplanted tumors phenocopy parental tumors. H&E & immuno-staining shows the same histologic tumor type and grade in both transplanted and original tumors (e''). Transplanted TD-NFCs migrate to known neurogenic niches (e'''). X-gal staining of transplanted cells bearing the *R26-lacZ* reporter shows that transplanted TD-NFCs migrate into neural stem/progenitor regions such as the subventricular zone-rostral migratory stream-olfactory bulb (SVZ-RMS-OB) and the subgranular zone (SGZ) in the dentate gyrus. No tumors were found in normal neural stem/progenitor-injected mice. F. TD-NFCs exhibit greater resistance to temozolomide treatment compared to

normal neural stem/progenitor cells or astrocytes. Cells were subjected to increasing concentrations of temozolomide (TMZ) and cell viability was measured after 48-72 hours. Normal neural stem/progenitors are also more resistant compared to differentiated astrocytes.

Transcriptional profiling of stem-like cancer cells in astrocytoma mouse models reveal a possible role for homeobox transcription factors in malignant astrocytomas

In order to determine the signal transduction pathways that are important in advanced astrocytomas, we performed genomic profiling using tumor derived-neurosphere-forming cells derived from symptomatic mutant mice. These were compared with SVZ neurosphere cultures from age-matched control littermate mice. Cells were harvested at passage 0 and total RNA was used for analysis using Illumina Mouse Whole Genome expression beadchips, which contain more than 40,000 probes and interrogate almost 20,000 unique genes.

We first performed gene expression profiling of Mut3 tumor-derived neurosphere-forming cells vs. SVZ cells from control littermates. Using 2-fold change and false discovery rate (FDR) of less than 5% as cut-off, we were able to identify more than 1000 up-regulated and 1200 downregulated genes. As shown by the cluster analysis and heat map representation of differentially expressed genes between mutants and controls, the genomic profile of tumor-derived neurosphere-forming cells is vastly different as compared to control neural stem/progenitor cells (Figure 4.2A). Interestingly, we found that 14% of these genes are involved in developmental processes, particularly ectoderm development (Figure 4.3A, Table 4.1). Genes involved in embryogenesis and mesoderm development were likewise represented. Twenty percent were involved in signal transduction, while genes involved in nucleic acid and protein metabolism accounted for almost a third. Based on molecular

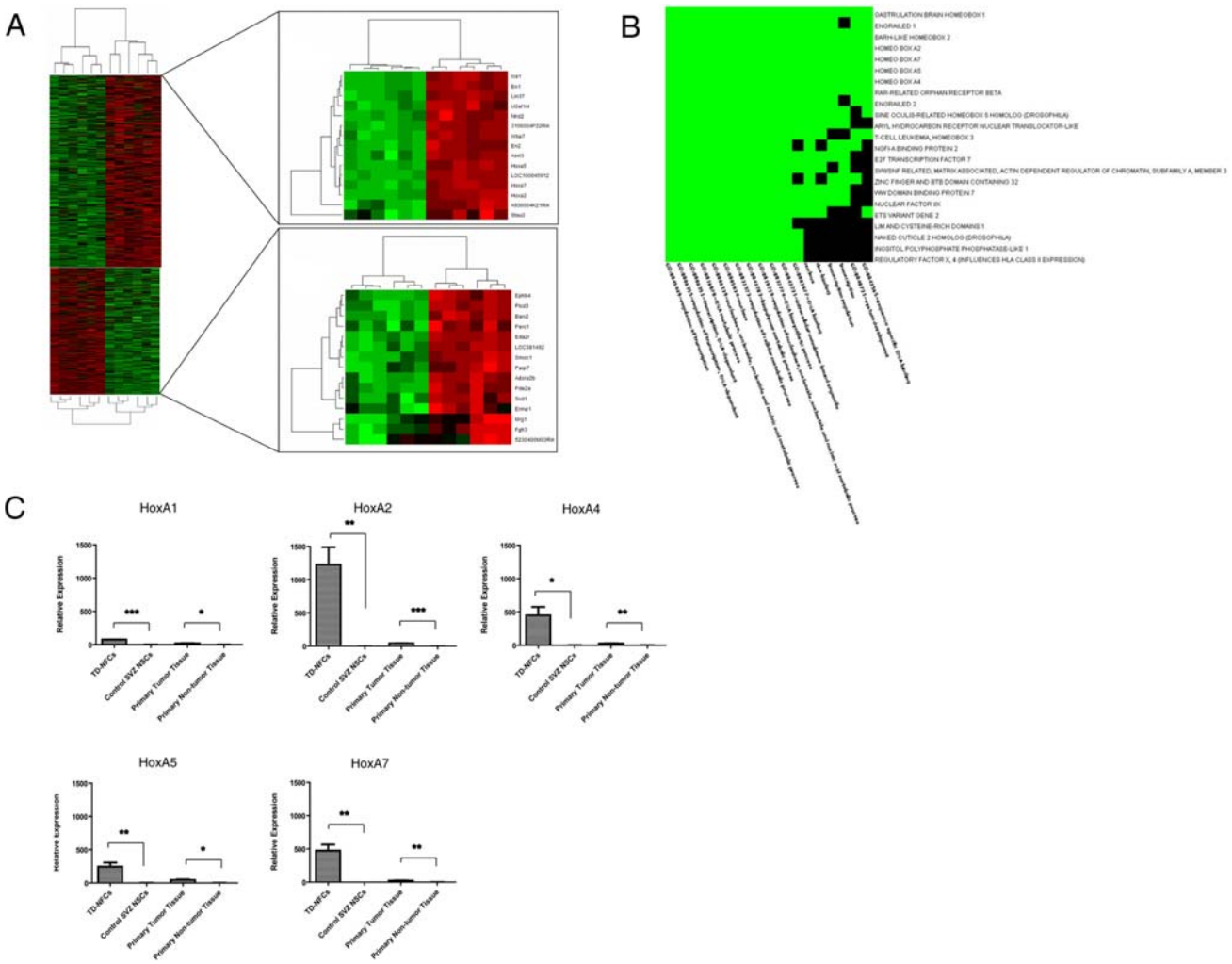

Figure 4.2. Genomic profiling of Mut3 tumor-derived neurosphere-forming cells demonstrate upregulation of transcriptional regulators such as the Hox transcription factors. A. Heat map showing differentially expressed genes in Mut3 TD-NFCs. Right panels are blown-up heat maps of the top 15 upregulated (in red) and downregulated (in green) genes. B. Heat map showing the top clustered genes in Mut3 TD-NFCs, mostly transcriptional regulators, and their respective molecular functions (green-reported, black-not reported). C. Real time PCR analysis confirmed the significant upregulation of Hoxa1, Hoxa2, Hoxa4, Hoxa5, and Hoxa7 in Mut3 TD-NFCs and primary tumor tissue as compared to controls.

function, about 9% are transcription factors, 7% are regulatory molecules while 8% are receptors. Genes involved in Wnt, angiogenesis, PDGF, integrin, and cytokine signaling were the most represented pathways.

Functional classification of the top most differentially expressed genes produced 5 gene clusters (Table 4.2), representing genes involved in transcriptional regulation, represented in a heat map in Figure 4.2B, as well as genes with transmembrane domains and receptors.

A closer inspection of the differentially expressed genes in Mut3 TD-NFCs revealed the predominance of the homeobox transcription factors, particularly hox transcription factors. As shown in Figure 4.2A, 3 of the top 15 most upregulated genes are Hox genes. Table 4.3, on the other hand, shows the different hox genes that are differentially expressed with their corresponding FDR-specific p values (Q value). There are a total of 8 Hox genes that are upregulated (5 Hoxa genes, 3 Hoxd genes) in Mut3 TD-NFCs, suggesting a significant role for this class of genes in advanced astrocytomas. We validated the relative expression of differentially expressed genes in tumor-derived neurosphere-forming cells as compared to controls using real time PCR, a representative of which is shown in Figure 4.2C, showing the fold change in Hoxa1, Hoxa2, Hoxa4, Hoxa5, and Hoxa7 transcript levels.

Transcriptional profile of stem-like cancer cells in astrocytoma mouse models show functional similarities

Enriching for cancer cells that possess stem cell-like properties by culturing cells in serum-free medium with growth factors may result in tissue culture artifacts

Table 4.1. Developmentally-regulated genes in Mut3 TD-NFCs

Biological process	*No. of genes*	*Genes*
Anterior/posterior patterning	7	Hhip1, Foxg1, Foxn2, Odz, Nanos3, Prickle1, Odz2
Determination of dorsal/ventral axis	3	Chl1, Prickle1, Sdccag331
Ectoderm Development	88	Tnc, Elavl3, Chl1, Hhip, Ntn2l, Ednra, Mical2,9530064J02, Negr1, BC060632, Smpdl3b, Efna5, Slit2, Mag, , Irx1, Ntrk2,C030027L06Rik, Ifrd1, Fgfr3, Irx3, Nrp,Nrp1, Ncam2, Egr2, Rtn3, Prrx1,Ephb6, Epha2, Lhx2, Ngfr, Cntn1, Cdh6, Unc5b, Celsr3, Nedl2, Olfm2, Pcdhb12, Fgfr1, Mtss1, Plxna2, Notch4, Pcdhb3, Pcdh1, 2610040L17Rik,Odz2, Notch3, Asah3l, Sema4a, D930029E11Rik, 9330161A03Rik,Stmn3, Ptprd, 2810402K13Rik,Klhl25, Fat1, Pcdhb10, Pcdhb6, Pcdhb2, Nell2, Irx5, Smurf1, D130058E05Rik,Gbx2, Odz2, Nradd, Ephb4, Smpdl3a, Otx1, Epha5, Rps6ka1, Ncam1, Dcc, Adora2b, En1, Hoxd10, Hoxd8, Vax2, Tlx3, Hoxa1, Foxg1, En2, Hoxa2, Hoxa4, Hoxa7, Hoxd9, Hoxa5, Hes5, Fabp3, Barx2, Crabp2, Rbp1, Dcx, Fabp5, Hlx, Foxn2
Embryogenesis	11	Hhip, Celsr3, Plk4, Rorb, Fat1, Kit, Dhh, Gsc, Foxg1, Foxn2, Nos3
Endoderm Development	2	Hhip, BC042423
Fertilization	2	Tyro3, Mertk
Gametogenesis	12	Lrp5, Adcyap1r1, Lrp4,6430526J12Rik, Tes, Mtap7, Vldlr, Stard5, Khdrbs3, Dnahc7, Kit, Bax, Nos3
Meiosis	2	Nos3, Hfm1
Mesoderm Development	69	Pcsk6, Hhip, Angpt2, Scube2, Ltbp4, Melk, Col5a1, Drp2, Csrp2, Klf4, Nrp,Nrp1, Farp2, Ltbp3, Vegfa, Ephb6, Traf4, Epha2, Dmd, Vegfc, 2610524G07Rik, Amotl2, Pax1, Flt1, Tbx2, Myh9, Sufu, Iap, Nfatc4, 2610040L17Rik,Odz2, Ebi3, Fbln2, Cdon, Jak2, Anxa2, Egr3, Col18a1, Dtnbp1, Smurf1, Angptl6, Amotl1, Bves, Evc2, Fbln1,Odz2, Efemp2, Ephb4, Nfe2l2 , Phex, Epha5, Rps6ka1, Tuft1, Clec11a, Hhex, Bax, Sdc4, Crip2, Tlx3, Pdlim7, Foxg1, Etv2, Dlx2, Socs4, Nkx2-2, Nkx6-2, Barx2, Mgp, Bax, Hlx, Foxn2
Segment Specification	11	Lfng, Porcn, Sdccag33l, Foxg1, Dlx2, Hoxa2, Hoxa4, Hoxa7, Hoxa5, Foxn2, Nos3
Other developmental process	11	Timp1, Hhip, Pard6a, Mrg1, 4930507C10Rik, Fbxw4, Ptprd, Six5, Gdap1, Gdap1l1, Tspan2

Table 4.2. Gene clusters in Top Differentially Expressed Genes in Mut3 TD-NFCs

Gene cluster	Functional Gene Annotation	Genes
G1	Transcriptional regulation	Arntl, Hoxa2, En1, Gbx2, Hltf, Nfix, Rorb, Zbtb32, Nab2, Hoxa7, Etv2, Hoxa4, En2, Lmcd1, Inppl1, Hoxa5, E2f7, Wbp7, Barx2, Six5, Tlx3, Rfx4
G2	Transmembrane	Slc6a15, Emb, Sdc4, Sema4b, Jam2, Paqr7, Gpr85
G3	Kinase activity	Egfr, Araf, Mylk, Rps6kl, Cdk10, Ephb4, Fgfr3
G4	Transmembrane	Paqr7, Marveld1, Pcdhb3, Pcdha7, Gdpd5, Evc2, Mfsd3
G5	Receptor activity	Adora2b, Paqr7, Gpr85, Gper, Lgr5,Slc19a2

Table 4.3. Hox genes are differentially expressed in murine malignant astrocytomas

Gene Name	Mut3 TD-NFCs		Mut3 Primary Tumor Tissue		Mut6 TD-NFCs	
	Fold change	Q value	Fold change	Q value	Fold change	Q value
Hoxa1	2.82	0.59	7.63	0	6.42	0
Hoxa2	33.82	0	35.72	0	13.45	3.67
Hoxa4	19.45	0	7.18	0	7.28	0.34
Hoxa5	105.66	0	120.12	0	130.69	0
Hoxa7	52.35	0	156.68	0	23.53	3.67
Hoxd4	-	-	16.56	0	-	-
Hoxd8	11.81	0.59	69.87	0	-	-
Hoxd9	12.42	0.59	13.62	0	-	-
Hoxd10	2.12	1.54	-	-	-	-

that may adversely affect the gene expression profile of tumor-derived neurosphere-forming cells as compared to what actually occurs *in vivo*. Genomic studies of human astrocytomas likewise use patient tumor samples compared with normal brain samples. To determine whether the neurosphere system mimics what occurs *in vivo*, we also performed gene expression analysis of Mut3 primary tumor tissue vs. non tumor tissue from control mice. As expected, there were more upregulated (4744 in Mut3 primary tumor tissue vs. 1046 in Mut3 TD-NFCs) and downregulated (4392 vs. 1232) genes when comparing primary tumor with non-tumor tissue. However, we also found a lot of overlap in terms of the differentially expressed genes in cultured and primary tumor tissue. Analysis of differentially expressed genes involved in core pathways and biologic and disease processes between Mut3 TD-NFCs and primary tumor tissue revealed 219 common genes, which is about 40% of Mut3 TD-NFC genes that are also differentially expressed in primary tumor tissue, as shown in Figure 4.3B (top panel). Moreover, similar to the TD-NFC gene expression data, the top clustered differentially expressed genes in primary tumor tissue were involved in signal transduction, nucleic acid metabolism, protein metabolism and developmental processes (Figure 4.3A). In terms of molecular function, we also see the same pattern of nucleic acid binding factors, transcription factors, receptors, and regulatory molecules as the most predominant genes, while pathway analysis of these genes reveal the importance of the Wnt, PDGF, integrin, inflammation and angiogenesis signaling in Mut 3 primary tumor tissue, similar to Mut3 cultured cells. We also confirmed up-regulation of the same Hoxa and Hoxd genes, except Hoxd4 and Hoxd10 (Table 4.3).

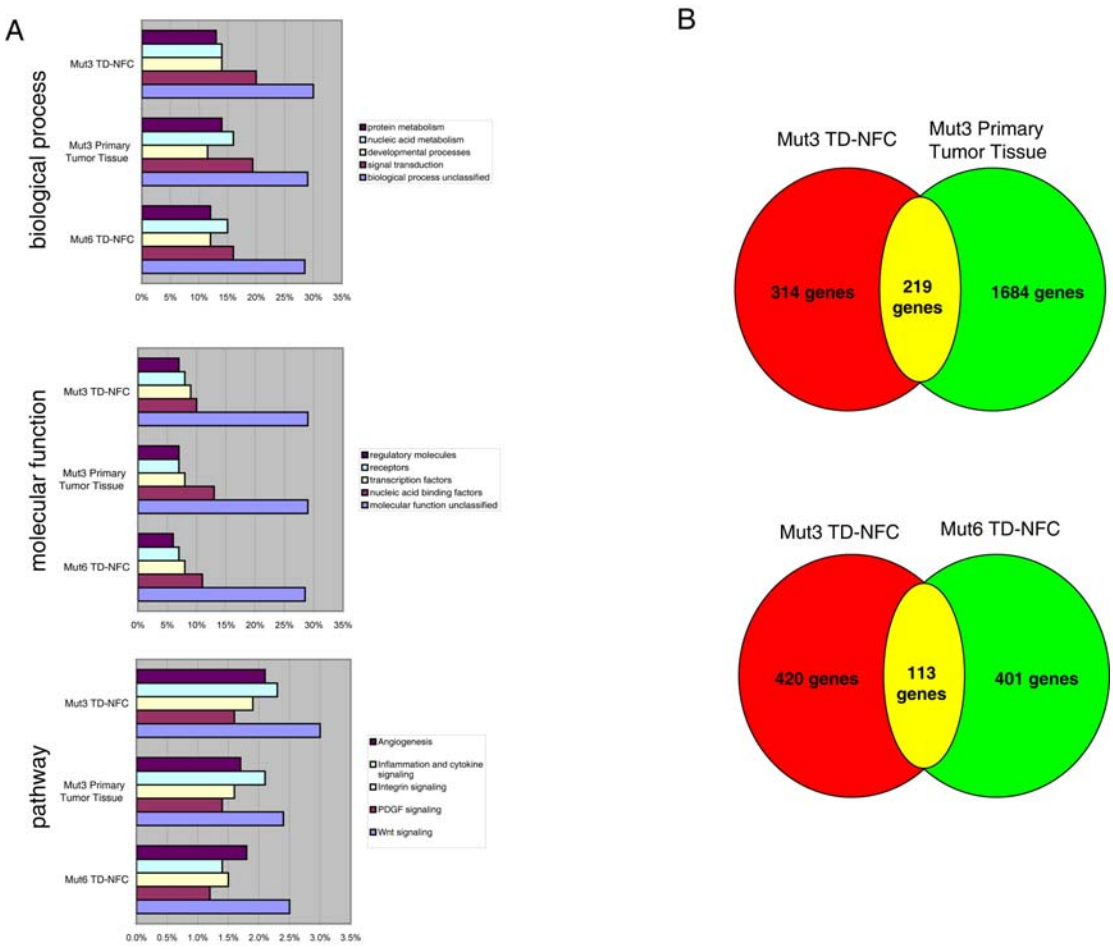

Figure 4.3. Comparison of gene expression profiles of Mut3 tumor-derived neurosphere-forming cells (TD-NFCs) with primary tumor tissue and Mut6 TD-NFCs. Up- and down-regulated genes from Mut3 TD-NFCs were compared with those from Mut3 primary tumor tissue and Mut6 TD-NFCs. A. Differentially expressed genes from these three data sets have similar profiles in terms of biological process, molecular function, and pathways involved. B. Analysis of differentially expressed genes involved in biological and disease processes as well as core pathways revealed that Mut3 TD-NFCs have 40% (219/533) common genes with Mut3 primary tumor tissue, whereas 20% (113/533) of its genes are in common with Mut6.

On the other hand, the Mut6 tumor suppressor mouse model (*hGFAP-cre;Nf1*$^{flox/+}$*; p5*$^{flox/-}$*;Pten*$^{flox/+}$) adds a heterozygous *Pten* allele with the existing *Nf1* and *p53* mutations. This results in accelerated formation of high-grade astrocytomas (Kwon et al., 2008). Whereas Mut3 mice develop tumors after 4-6 months of age up to more than a year of age, the majority of Mut6 mice die before 4 months of age. Tumors from these mice, however, are usually smaller in size and less necrotic and hemorrhagic as compared to Mut3 tumors. Similar to the Mut3 TD-NFC microarray experiments, we also performed genomic profiling of Mut6 TD-NFCs. As shown in Figure 4.3B, almost a quarter of Mut3 genes were shared with Mut6 TD-NFCs. Interestingly, there are also functional similarities in the differentially expressed genes in both models (Figure 4.3A). Common genes between Mut3 and Mut TD-NFCs clustered around transcriptional regulators, kinases, and transmembrane-encoding genes (data not shown), similar to the top clustered genes in Mut3 TD-NFCs (Figure 4.3B, Table 4.2) .In both Mut3 and Mut6 tumor-derived neurosphere-forming cells, there is a preponderance of signal transduction and developmental signaling pathways, as well as nucleic acid binding factors. We also see the same pattern of genes involved in terms of molecular function and signaling pathways. The number of up-regulated (1006 in Mut3 vs. 1046 in Mut6) and down-regulated (1276 vs. 1232) genes is also at par with each other.

On the other hand, we found upregulation of the same Hoxa genes (*Hoxa1, Hoxa2, Hoxa4, Hoxa5, Hoxa7)* in Mut6 TD-NDCs (Table 4.3). In contrast with Mut3 TD-NFCs, however, Mut6 TD-NFCs showed no upregulation of the Hoxd genes, which we also validated by real time PCR (data not shown). Along with other unique

genes in Mut6 TD-NFCs that are not found in Mut3 TD-NFCs (data not shown), this indicates that differences in initiating mutations lead to different paths to malignancy.

Discussion

Stem cell-like cancer cells in tumor suppressor mouse models

In this study, we isolated and characterized tumor-derived neurosphere-forming cells from our tumor suppressor mouse models. We showed that these cells are enriched for tumor cells, exhibit stem cell properties, and are tumorigenic *in vivo*. We also demonstrated two clinically relevant properties of these tumors. These cells showed better survival when exposed to temozolomide, a known GBM adjuvant chemotherapeutic drug, as compared to normal neural stem cells and even more so against normal astrocytes, which is reflective of the therapeutic resistance exhibited by these cancer cells. We also demonstrated its migratory capacity, especially in the neural stem cells niches of the SVZ-RMS-OB and SGZ, where we found labeled tumor cells months after transplantation, consistent with the infiltrative nature of these tumors.

While these represent a population of cells that are enriched for stem/progenitor-like cancer cells compared to primary tumor tissue, there are also immature differentiated cell types, similar to normal neural stem/progenitor neurosphere cultures. This is not a pure population of so-called “cancer stem cells” nor is it ascribed as such. It is, however, a reproducible system by which we can characterize and analyze the more immature astrocytoma cells, compare them with normal neural stem cells---the cells of origin of these tumors in our mouse models, and manipulate these cells for both *in vitro* and *in vivo* assays to evaluate the functional importance of candidate genes.

Transcriptional profiling in murine astrocytomas

Gene expression analysis revealed thousands of up- and down-regulated genes in tumor-derived neurosphere-forming cells. Similar to gene expression studies in human glioblastomas, analysis of primary tumor tissue, which may also be contaminated with non-tumor brain cells, vs. normal brain tissue yields four-fold more genes, around 8000+ genes that are differentially expressed. Our analysis on TD-NFCs compares enriched stem/progenitor-like cancer cells with normal neural stem cells, and hence, may have produced a more focused set of genes that are relatively over- or under-expressed in the stem cell compartment. On the other hand, this may exclude a large number of genes that are inherently different between normal differentiated cells (e.g. neurotransmitters, synaptic proteins, myelin-associated proteins), and proliferating tumor cells, as these very mature cell types do not thrive in neurosphere-forming conditions.

It is interesting that we found a similar pattern of functionally classified genes in Mut3 TD-NFCs, Mut3 primary tumor tissue and Mut6 TD-NFCs in terms of biological processes, molecular function and pathways involved. We also found common and unique genes between these different groups. The considerable similarities between Mut3 cultured vs. primary tumor tissue gene expression profiles indicate that cancer signaling mechanisms are retained to a certain degree *in vitro*. On the other hand, Mut3 TD-NFCs also showed considerable similarities but also significant differences with Mut6 TD-NFC gene expression. The addition of a *Pten* mutation thus leads to both convergent and divergent pathways to malignant astrocytoma formation. Overall, these *in vitro* and *in vivo* data will allow us to cross

check genes that are physiologically relevant and the most important for brain tumor development. Ultimately, these murine expression data should be directly compared and analyzed with human microarray data to determine evolutionarily conserved genes and the most significant signaling pathways to tumor development. It will also be interesting to compare Mut3 and Mut6 gene expression profiles with those of primary and secondary GBMs.

Developmentally regulated and stem-cell-related genes in malignant astrocytomas

Genomic profiling revealed clusters of developmentally regulated genes, particularly those involved in ectodermal development and neurogenesis. There were also genes involved in embryogenesis and mesoderm development. This is similar to the pattern of genes found in murine hematopoietic stem cells, which shared a portion of their genetic program with embryonic and neural stem cells (Ivanova et al., 2002). An embryonic stem cell-like signature was also reported in poorly differentiated human tumors including glioblastoma (Ben-Porath et al., 2008). These include genes expressed in embryonic stem cells, targets of Nanog, Oct4, Sox2 and associated transcription factors, Polycomb and Myc targets. We found that a subset of genes that are in this ES signature are expressed in our murine astrocytomas, including *Hhip1*, *Rorb, Dhh, Gsc, Foxg1* and all the *Hox* genes. These suggest that some form of reactivation of developmental signaling networks occurs in high-grade astrocytomas.

Role of Hox transcription factors in astrocytoma development

Hox genes are homeobox transcription factors that are widely conserved throughout evolution. These genes have been shown to be important in cell fate determination processes, as well as pathologic conditions such as cancer. Its role in malignant brain tumors, however, is not known (Moens and Selleri, 2006). It has only been recently shown that several Hox genes are highly expressed in human glioblastoma (Abdel-Fattah et al., 2006). More importantly, it has been reported that gene expression profiling of human GBMs revealed a distinct "stem cell signature" that is dominated by Hox genes (Murat et al., 2008). In the same paper, it was reported that the level of Hox gene expression was predictive of survival in patients with GBM. This is consistent with survival analysis of a subset of Hox genes in the Cancer Genome Atlas database (https://cma.nci.nih.gov/cma-rembrandt/), which show shortened survival in brain tumor patients with higher expression of hox genes *HOXA1*, *HOXA4*, *HOXA5*, *HOXA7*, and *HOXD10* (data not shown). Higher enrichment for these embryonic stem cell-like gene clusters was also detected in poorly undifferentiated GBMs but not in lower-grade astrocytomas, which also correlated with poor survival (Ben-Porath et al., 2008), suggesting that expression of these genes is correlated with a more undifferentiated tumor phenotype. This strongly suggests an important role for these genes in high-grade astrocytoma formation.

While Hox genes are usually known for their roles in embryonic patterning and morphogenesis, stem cells, especially hematopoietic stem cells, have also been shown to be regulated by these genes (Iacovino et al., 2008). Whether it plays a role in normal neural stem cell development, whether at embryonic, early postnatal or

adult ages, is not known and will provide clues to its function in tumorigenesis. These genes are also widely expressed in a wide variety of tumor cell types, including head and neck, breast and lung carcinomas (Argiropoulos and Humphries, 2007). It has been shown to be overexpressed in leukemic stem cells (Krivtsov et al., 2006), and was recently shown to be required for its survival (Faber et al., 2009). It is an intriguing prospect what its possible roles are not only in contributing to the malignant phenotype in astrocytomas, but also whether this same gene signature can be commonly turned on in different organ cancers.

Materials and Methods

Neurosphere cultures

Neurosphere cultures from the SVZ of non-symptomatic Mut3 (*hGFAP-cre;Nf1*$^{flox/+}$*; p53*$^{-/+}$) and control (wild type, *Nf1*$^{flox/+}$*; p53*$^{-/+}$*; hGFAP-cre)* mice were established, as previously described (Alcantara Llaguno et al., 2009). Neurospheres cultures were also established from symptomatic mutant (Mut3: *hGFAP-cre;Nf1*$^{flox/+}$*; p53*$^{-/+}$ and Mut6: *hGFAP-cre;Nf1*$^{flox/+}$*; p5*$^{flox/-}$*;Pten*$^{flox/+}$) and littermate control animals (wild type, *Nf1*$^{flox/+}$*; p53*$^{-/+}$*; hGFAP-cre)*, as previously described, with some modifications. Primary tumor tissue that is distinct from the SVZ were dissected from symptomatic mutant mice, while the lateral walls of the lateral ventricles were dissected from control mice. Dissected tissue was digested by mechanical trituration and enzymatic digestion with DNase I (250 u/mL), papain (2.5 u/mL), and neutral protease (1u/mL). Cells were washed and plated at a density of 20 cells/μL on ultra-low attachment plates (Corning) in media containing EGF (20 ng/mL), bFGF (20 ng/mL), and B27 without Vitamin A (Gibco) and cultured under low (5%) O2 conditions.

Neural stem cell assays

To assess for self-renewal potential, primary neurospheres were dissociated and plated at a density of 5 cells/uL, and the number of neurospheres generated after 7 days were counted, as previously described. The number of secondary, tertiary and quaternary neurospheres were likewise counted following serial passaging. To assess for multipotentiality, dissociated neurospheres were plated in matrigel-coated 4-well

chamber slides in medium containing 10% fetal bovine serum. Cells were immunostained after 7-14 days of differentiation, as described (Kwon et al., 2008). To test drug resistance, dissociated tumor-derived neurosphere-forming cells, along with normal neural stem cells and astrocytes, were plated in 96-well plates and treated with increasing doses of temozolomide (Sigma) and cell viability was measured after 48-72 hours using the Adenosine-5'-triphosphate (ATP) bioluminescence assay (Roche), as per manufacturer's instruction.

Intracranial transplantation

Cells were harvested and resuspended in L15 medium (Gibco). For intracranial transplantations, we injected 1-2x10^5 cells into the striatum (0 AP, +1.4mm ML, -2.6mm DV) of 4 to 8 week-old immunodeficient *nu/nu* mice (Charles River) using a Hamilton syringe. Mice were examined for development of tumors using standard histological analysis.

RNA extraction and genomic profiling

Neurospheres were harvested at passage 0, 7 to 10 days post-plating, and immediately flash frozen in liquid nitrogen. RNA was extracted and concentrated using the RNeasy Mini and Minelute CleanUp Kits (Qiagen) respectively, as per manuacturer's instructions. Samples were submitted to the UT Southwestern Microarray Core for cDNA synthesis and purification, aRNA synthesis and purification, hybridization and scanning of chips. We used Illumina Sentrix Mouse WG6-V1 and -V2 expression beadchip arrays, which uses over 47,000 probes

covering the entire mouse genome. This platform was chosen primarily because of lower starting RNA requirements and cost, while providing outstanding data quality with an average 30-fold redundancy.

Statistical Analysis of Microarray Data

Statistical analysis of the microarray data was done in collaboration with Yang Xie and Jingsheng Yan of the UT Southwestern Cancer Center Biostatistics and Bioinformatics Core. The Illumina BeadArray microarray data was summarized using the Illumina Beadstudio software and then processed using the Model-based Background Correction for Beadarray (MBCB) method (Ding et al., 2008). Quantile-quantile normalization (Bolstad et al., 2003) was performed on the log transformed gene expression data. Hierarchical clustering and histogram were used for quality control. Differentially expressed genes were identified using the Samr package (Significance Analysis of Microarray) (Tusher et al., 2001) in R statistical software. The multiple testing issues were addressed by implementing q-values to estimate the gene-specific false discovery rate (FDR). In this study, genes with FDR less than 0.05 were considered as differentially expressed. Pathway analysis was done using Ingenuity Pathway Analysis (www.ingenuity.org), Panther (www.pantherdb.org), Database for Annotation, Visualization and Integrated Discovery or DAVID (david.abcc.ncifcrf.gov) and Gene Set Enrichment Analysis or GSEA (www.broad.mit.edu/gsea).

Quantitative Polymerase Chain Reaction

Validation of microarray results was done by real time PCR. We will use the Applied Biosystems 7500 system using both Sybr Green- and Taqman-based probes for RNA message relative quantification, according to the manufacturer's instructions.

Chapter Five

Perspective and Future Directions

The use of genetic mouse models in studying human disease has greatly facilitated our understanding of cancer. Using tumor suppressor mouse models, mutations in human malignant astrocytomas were validated as *bona fide* tumor-initiating mutations that lead to full-blown tumor development. Cooperativity between these tumor-initiating genes was also uncovered. The work described here detailed studies on the astrocytoma cell of origin in these tumor suppressor mouse models as well as the gene expression profile of these tumors, providing insight into the role of neural and stem-like cancer cells in tumor initiation and progression.

The mechanisms involved in early astrocytoma development are not well understood. This has been previously hampered by the absence of experimental systems by which to follow tumor development faithfully from the early onset until progression into advanced disease. It also requires that animal models have a fairly high degree of penetrance of tumor formation. Requisite knowledge of the original cell that undergoes the initial transforming event as well as the initiating mutations required for the tumorigenesis cascade to be set in motion are crucial for understanding mechanisms of cancer development.

Our tumor suppressor mouse models are ideal tools for studying the development of cancer from its inception. These mutant strains are indistinguishable from their wild type littermates at birth and progressively develop tumors during mid to late adulthood with 100% penetrance. *Nf1*, *p53*, and *Pten* mutations are amongst the five most mutated genes in human glioblastoma (TCGA, 2008), and we have shown that deletion of the *Nf1, p53* and/or *Pten* tumor suppressors is sufficient in driving malignant astrocytoma formation in brain cells (Kwon et al., 2008; Zhu et al.,

2005a). We have also already established that the SVZ neural stem and progenitor cells as the origin of these tumors (Alcantara Llaguno et al., 2009).

Hence, future studies will involve gene expression analysis of mutant neural stem/progenitor cells at progressive stages of tumor development. It will also be interesting to determine the contribution of genes involved in regulating cell cycle progression, apoptosis and senescence as well as DNA damage and repair during transformation.

For the genomic studies on full blown astrocytomas, comparison of our mouse tumor microarray data with the human data will identify evolutionarily conserved pathways which may be the most important in carcinogenesis. *In vitro* and *in vivo* loss- and gain-of-function studies can also be done to demonstrate the functional role of candidate genes, particularly the hox genes in astrocytoma self-renewal, differentiation, survival, and tumorigenicity. This can be accomplished by using second generation microRNA-adapted short hairpin RNA (shRNAmir) cloned into lentiviral vectors for both short- and long-term knockdown. Vector-based shRNAmirs are more versatile compared to the traditional short interfering RNAs (siRNA), as it allows for both transient transfection and stable integration, and its infection-based delivery is suitable for hard-to-transfect primary cell lines and *in vivo* testing. It also produces more efficient knockdowns even compared to the first generation short hairpin RNAs (shRNAs) (Chang et al., 2006; Fewell and Schmitt, 2006; Silva et al., 2005). Short-term knockdowns will be done by transient transfection of lentiviral vector-inserted shRNAmir constructs into primary neural stem cell cultures, whereas longer-term knockdowns will be accomplished by infection with shRNAmir-

expressing lentivirus. The same strategy can be used for gain-of-function experiments: transfect cDNA clones directly into primary neural stem cell cultures for transient overexpression, whereas stable expression will be done by infecting cells with lentivirus expressing the gene of interest. These functional studies on the signaling pathways that are important in advanced tumorigenesis will be crucial in finding pharmacologic inhibitors of brain tumor cells.

Further advances in the fields of neurodevelopment and cancer biology will provide more sophisticated tools in studying these malignancies. The interface between human and mouse cancers will need to be more fully exploited in order to rapidly translate basic science knowledge into practical clinical applications. The hope is that a more integrated understanding of the molecular and physiological processes underlying this complex disease will provide us with the means of finally cracking open some of the many secrets of brain tumor formation, ultimately resulting in more targeted and effective therapies.

Bibliography

Abdel-Fattah, R., Xiao, A., Bomgardner, D., Pease, C.S., Lopes, M.B., and Hussaini, I.M. (2006). Differential expression of HOX genes in neoplastic and non-neoplastic human astrocytes. J Pathol *209*, 15-24.

Alcantara Llaguno, S., Chen, J., Kwon, C.H., Jackson, E.L., Li, Y., Burns, D.K., Alvarez-Buylla, A., and Parada, L.F. (2009). Malignant astrocytomas originate from neural stem/progenitor cells in a somatic tumor suppressor mouse model. Cancer Cell *15*, 45-56.

Alvarez-Buylla, A., and Lim, D.A. (2004). For the long run: maintaining germinal niches in the adult brain. Neuron *41*, 683-686.

Argiropoulos, B., and Humphries, R.K. (2007). Hox genes in hematopoiesis and leukemogenesis. Oncogene *26*, 6766-6776.

Bachoo, R.M., Maher, E.A., Ligon, K.L., Sharpless, N.E., Chan, S.S., You, M.J., Tang, Y., DeFrances, J., Stover, E., Weissleder, R., *et al.* (2002). Epidermal growth factor receptor and Ink4a/Arf: convergent mechanisms governing terminal differentiation and transformation along the neural stem cell to astrocyte axis. Cancer Cell *1*, 269-277.

Bao, S., Wu, Q., McLendon, R.E., Hao, Y., Shi, Q., Hjelmeland, A.B., Dewhirst, M.W., Bigner, D.D., and Rich, J.N. (2006). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature *444*, 756-760.

Ben-Porath, I., Thomson, M.W., Carey, V.J., Ge, R., Bell, G.W., Regev, A., and Weinberg, R.A. (2008). An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet *40*, 499-507.

Bertrand, N., Castro, D.S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. Nat Rev Neurosci *3*, 517-530.

Bolstad, B.M., Irizarry, R.A., Astrand, M., and Speed, T.P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics *19*, 185-193.

Chang, K., Elledge, S.J., and Hannon, G.J. (2006). Lessons from Nature: microRNA-based shRNA libraries. Nat Methods *3*, 707-714.

Correa-Cerro, L.S., and Mandell, J.W. (2007). Molecular mechanisms of astrogliosis: new approaches with mouse genetics. J Neuropathol Exp Neurol *66*, 169-176.

Cully, M., You, H., Levine, A.J., and Mak, T.W. (2006). Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. Nat Rev Cancer *6*, 184-192.

Dai, C., Celestino, J.C., Okada, Y., Louis, D.N., Fuller, G.N., and Holland, E.C. (2001). PDGF autocrine stimulation dedifferentiates cultured astrocytes and induces oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes *in vivo*. Genes Dev *15*, 1913-1925.

Dalerba, P., Cho, R.W., and Clarke, M.F. (2007). Cancer stem cells: models and concepts. Annu Rev Med *58*, 267-284.

Dasgupta, B., and Gutmann, D.H. (2005). Neurofibromin regulates neural stem cell proliferation, survival, and astroglial differentiation *in vitro* and *in vivo*. J Neurosci *25*, 5584-5594.

Ding, H., Roncari, L., Shannon, P., Wu, X., Lau, N., Karaskova, J., Gutmann, D.H., Squire, J.A., Nagy, A., and Guha, A. (2001). Astrocyte-specific expression of activated p21-ras results in malignant astrocytoma formation in a transgenic mouse model of human gliomas. Cancer Res *61*, 3826-3836.

Ding, L.H., Xie, Y., Park, S., Xiao, G., and Story, M.D. (2008). Enhanced identification and biological validation of differential gene expression via Illumina whole-genome expression arrays through the use of the model-based background correction methodology. Nucleic Acids Res *36*, e58.

Doetsch, F., Caille, I., Lim, D.A., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell *97*, 703-716.

Doetsch, F., Petreanu, L., Caille, I., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (2002). EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. Neuron *36*, 1021-1034.

Eriksson, P.S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A.M., Nordborg, C., Peterson, D.A., and Gage, F.H. (1998). Neurogenesis in the adult human hippocampus. Nat Med *4*, 1313-1317.

Faber, J., Krivtsov, A.V., Stubbs, M.C., Wright, R., Davis, T.N., van den Heuvel-Eibrink, M., Zwaan, C.M., Kung, A.L., and Armstrong, S.A. (2009). HOXA9 is required for survival in human MLL-rearranged acute leukemias. Blood *113*, 2375-2385.

Feil, R. (2007). Conditional somatic mutagenesis in the mouse using site-specific recombinases. Handb Exp Pharmacol, 3-28.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., and Chambon, P. (1996). Ligand-activated site-specific recombination in mice. Proc Natl Acad Sci U S A *93*, 10887-10890.

Fewell, G.D., and Schmitt, K. (2006). Vector-based RNAi approaches for stable, inducible and genome-wide screens. Drug Discov Today *11*, 975-982.

Fomchenko, E.I., and Holland, E.C. (2006). Mouse models of brain tumors and their applications in preclinical trials. Clin Cancer Res *12*, 5288-5297.

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., *et al.* (2007). Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev *21*, 2683-2710.

Gage, F.H. (2000). Mammalian neural stem cells. Science *287*, 1433-1438.

Gil-Perotin, S., Marin-Husstege, M., Li, J., Soriano-Navarro, M., Zindy, F., Roussel, M.F., Garcia-Verdugo, J.M., and Casaccia-Bonnefil, P. (2006). Loss of p53 induces changes in the behavior of subventricular zone cells: implication for the genesis of glial tumors. J Neurosci *26*, 1107-1116.

Gould, E. (2007). How widespread is adult neurogenesis in mammals? Nat Rev Neurosci *8*, 481-488.

Groszer, M., Erickson, R., Scripture-Adams, D.D., Dougherty, J.D., Le Belle, J., Zack, J.A., Geschwind, D.H., Liu, X., Kornblum, H.I., and Wu, H. (2006). PTEN

negatively regulates neural stem cell self-renewal by modulating G0-G1 cell cycle entry. Proc Natl Acad Sci U S A *103*, 111-116.

Groszer, M., Erickson, R., Scripture-Adams, D.D., Lesche, R., Trumpp, A., Zack, J.A., Kornblum, H.I., Liu, X., and Wu, H. (2001). Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene *in vivo*. Science *294*, 2186-2189.

Gutmann, D.H., James, C.D., Poyhonen, M., Louis, D.N., Ferner, R., Guha, A., Hariharan, S., Viskochil, D., and Perry, A. (2003). Molecular analysis of astrocytomas presenting after age 10 in individuals with NF1. Neurology *61*, 1397-1400.

Gutmann, D.H., Rasmussen, S.A., Wolkenstein, P., MacCollin, M.M., Guha, A., Inskip, P.D., North, K.N., Poyhonen, M., Birch, P.H., and Friedman, J.M. (2002). Gliomas presenting after age 10 in individuals with neurofibromatosis type 1 (NF1). Neurology *59*, 759-761.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hegedus, B., Dasgupta, B., Shin, J.E., Emnett, R.J., Hart-Mahon, E.K., Elghazi, L., Bernal-Mizrachi, E., and Gutmann, D.H. (2007). Neurofibromatosis-1 regulates neuronal and glial cell differentiation from neuroglial progenitors *in vivo* by both cAMP- and Ras-dependent mechanisms. Cell Stem Cell *1*, 443-457.

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.

Iacovino, M., Hernandez, C., Xu, Z., Bajwa, G., Prather, M., and Kyba, M. (2008). A Conserved Role for Hox Paralog Group 4 in Regulation of Hematopoietic Progenitors. Stem Cells Dev.

Ichimura, K., Ohgaki, H., Kleihues, P., and Collins, V.P. (2004). Molecular pathogenesis of astrocytic tumours. J Neurooncol *70*, 137-160.

Ivanova, N.B., Dimos, J.T., Schaniel, C., Hackney, J.A., Moore, K.A., and Lemischka, I.R. (2002). A stem cell molecular signature. Science *298*, 601-604.

Jacks, T., Remington, L., Williams, B.O., Schmitt, E.M., Halachmi, S., Bronson, R.T., and Weinberg, R.A. (1994). Tumor spectrum analysis in p53-mutant mice. Curr Biol *4*, 1-7.

Jaiswal, S., Traver, D., Miyamoto, T., Akashi, K., Lagasse, E., and Weissman, I.L. (2003). Expression of BCR/ABL and BCL-2 in myeloid progenitors leads to myeloid leukemias. Proc Natl Acad Sci U S A *100*, 10002-10007.

Kim, E.J., Leung, C.T., Reed, R.R., and Johnson, J.E. (2007). *In vivo* analysis of Ascl1 defined progenitors reveals distinct developmental dynamics during adult neurogenesis and gliogenesis. J Neurosci *27*, 12764-12774.

Kleihues, P., Louis, D.N., Scheithauer, B.W., Rorke, L.B., Reifenberger, G., Burger, P.C., and Cavenee, W.K. (2002). The WHO classification of tumors of the nervous system. J Neuropathol Exp Neurol *61*, 215-225; discussion 226-219.

Krivtsov, A.V., Twomey, D., Feng, Z., Stubbs, M.C., Wang, Y., Faber, J., Levine, J.E., Wang, J., Hahn, W.C., Gilliland, D.G., *et al.* (2006). Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature *442*, 818-822.

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.

Le, L.Q., and Parada, L.F. (2007). Tumor microenvironment and neurofibromatosis type I: connecting the GAPs. Oncogene *26*, 4609-4616.

Lee, J., Son, M.J., Woolard, K., Donin, N.M., Li, A., Cheng, C.H., Kotliarova, S., Kotliarov, Y., Walling, J., Ahn, S., *et al.* (2008). Epigenetic-mediated dysfunction of the bone morphogenetic protein pathway inhibits differentiation of glioblastoma-initiating cells. Cancer Cell *13*, 69-80.

Lin, S.C., Lee, K.F., Nikitin, A.Y., Hilsenbeck, S.G., Cardiff, R.D., Li, A., Kang, K.W., Frank, S.A., Lee, W.H., and Lee, E.Y. (2004). Somatic mutation of p53 leads to estrogen receptor alpha-positive and -negative mouse mammary tumors with high frequency of metastasis. Cancer Res *64*, 3525-3532.

Louis, D.N. (2006). Molecular pathology of malignant gliomas. Annu Rev Pathol *1*, 97-117.

Luikart, B.W., Nef, S., Virmani, T., Lush, M.E., Liu, Y., Kavalali, E.T., and Parada, L.F. (2005). TrkB has a cell-autonomous role in the establishment of hippocampal Schaffer collateral synapses. J Neurosci *25*, 3774-3786.

Maher, E.A., Brennan, C., Wen, P.Y., Durso, L., Ligon, K.L., Richardson, A., Khatry, D., Feng, B., Sinha, R., Louis, D.N., *et al.* (2006). Marked genomic differences characterize primary and secondary glioblastoma subtypes and identify two distinct molecular and clinical secondary glioblastoma entities. Cancer Res *66*, 11502-11513.

Maher, E.A., Furnari, F.B., Bachoo, R.M., Rowitch, D.H., Louis, D.N., Cavenee, W.K., and DePinho, R.A. (2001). Malignant glioma: genetics and biology of a grave matter. Genes Dev *15*, 1311-1333.

Meletis, K., Wirta, V., Hede, S.M., Nister, M., Lundeberg, J., and Frisen, J. (2006). p53 suppresses the self-renewal of adult neural stem cells. Development *133*, 363-369.

Menn, B., Garcia-Verdugo, J.M., Yaschine, C., Gonzalez-Perez, O., Rowitch, D., and Alvarez-Buylla, A. (2006). Origin of oligodendrocytes in the subventricular zone of the adult brain. J Neurosci *26*, 7907-7918.

Merkle, F.T., Mirzadeh, Z., and Alvarez-Buylla, A. (2007). Mosaic organization of neural stem cells in the adult brain. Science *317*, 381-384.

Merkle, F.T., Tramontin, A.D., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (2004). Radial glia give rise to adult neural stem cells in the subventricular zone. Proc Natl Acad Sci U S A *101*, 17528-17532.

Moens, C.B., and Selleri, L. (2006). Hox cofactors in vertebrate development. Dev Biol *291*, 193-206.

Murat, A., Migliavacca, E., Gorlia, T., Lambiv, W.L., Shay, T., Hamou, M.F., de Tribolet, N., Regli, L., Wick, W., Kouwenhoven, M.C., *et al.* (2008). Stem cell-related "self-renewal" signature and high epidermal growth factor receptor expression associated with resistance to concomitant chemoradiotherapy in glioblastoma. J Clin Oncol *26*, 3015-3024.

Parras, C.M., Hunt, C., Sugimori, M., Nakafuku, M., Rowitch, D., and Guillemot, F. (2007). The proneural gene Mash1 specifies an early population of telencephalic oligodendrocytes. J Neurosci *27*, 4233-4242.

Petreanu, L., and Alvarez-Buylla, A. (2002). Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. J Neurosci *22*, 6106-6113.

Piccirillo, S.G., Reynolds, B.A., Zanetti, N., Lamorte, G., Binda, E., Broggi, G., Brem, H., Olivi, A., Dimeco, F., and Vescovi, A.L. (2006). Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. Nature *444*, 761-765.

Rasmussen, S.A., Yang, Q., and Friedman, J.M. (2001). Mortality in neurofibromatosis 1: an analysis using U.S. death certificates. Am J Hum Genet *68*, 1110-1118.

Reilly, K.M., Loisel, D.A., Bronson, R.T., McLaughlin, M.E., and Jacks, T. (2000). Nf1;Trp53 mutant mice develop glioblastoma with evidence of strain-specific effects. Nat Genet *26*, 109-113.

Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001). Stem cells, cancer, and cancer stem cells. Nature *414*, 105-111.

Reynolds, B.A., and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science *255*, 1707-1710.

Sanai, N., Alvarez-Buylla, A., and Berger, M.S. (2005). Neural stem cells and the origin of gliomas. N Engl J Med *353*, 811-822.

Sanai, N., Tramontin, A.D., Quinones-Hinojosa, A., Barbaro, N.M., Gupta, N., Kunwar, S., Lawton, M.T., McDermott, M.W., Parsa, A.T., Manuel-Garcia Verdugo, J., *et al.* (2004). Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature *427*, 740-744.

Sanson, M., Thillet, J., and Hoang-Xuan, K. (2004). Molecular changes in gliomas. Curr Opin Oncol *16*, 607-613.

Sauvageot, C.M., Kesari, S., and Stiles, C.D. (2007). Molecular pathogenesis of adult brain tumors and the role of stem cells. Neurol Clin *25*, 891-924, vii.

Schubbert, S., Shannon, K., and Bollag, G. (2007). Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer *7*, 295-308.

Seaberg, R.M., Smukler, S.R., and van der Kooy, D. (2005). Intrinsic differences distinguish transiently neurogenic progenitors from neural stem cells in the early postnatal brain. Dev Biol *278*, 71-85.

Silva, J.M., Li, M.Z., Chang, K., Ge, W., Golding, M.C., Rickles, R.J., Siolas, D., Hu, G., Paddison, P.J., Schlabach, M.R., *et al.* (2005). Second-generation shRNA libraries covering the mouse and human genomes. Nat Genet *37*, 1281-1288.

Singec, I., Knoth, R., Meyer, R.P., Maciaczyk, J., Volk, B., Nikkhah, G., Frotscher, M., and Snyder, E.Y. (2006). Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. Nat Methods *3*, 801-806.

Singh, S.K., Hawkins, C., Clarke, I.D., Squire, J.A., Bayani, J., Hide, T., Henkelman, R.M., Cusimano, M.D., and Dirks, P.B. (2004). Identification of human brain tumour initiating cells. Nature *432*, 396-401.

Sofroniew, M.V. (2005). Reactive astrocytes in neural repair and protection. Neuroscientist *11*, 400-407.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet *21*, 70-71.

Stupp, R., Mason, W.P., van den Bent, M.J., Weller, M., Fisher, B., Taphoorn, M.J., Belanger, K., Brandes, A.A., Marosi, C., Bogdahn, U., *et al.* (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med *352*, 987-996.

Sugimori, M., Nagao, M., Bertrand, N., Parras, C.M., Guillemot, F., and Nakafuku, M. (2007). Combinatorial actions of patterning and HLH transcription factors in the spatiotemporal control of neurogenesis and gliogenesis in the developing spinal cord. Development *134*, 1617-1629.

TCGA (2008). Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature *455*, 1061-1068.

Tso, C.L., Freije, W.A., Day, A., Chen, Z., Merriman, B., Perlina, A., Lee, Y., Dia, E.Q., Yoshimoto, K., Mischel, P.S., *et al.* (2006). Distinct transcription profiles of primary and secondary glioblastoma subgroups. Cancer Res *66*, 159-167.

Tusher, V.G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A *98*, 5116-5121.

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.

Vescovi, A.L., Galli, R., and Reynolds, B.A. (2006). Brain tumour stem cells. Nat Rev Cancer *6*, 425-436.

Wang, J.C., and Dick, J.E. (2005). Cancer stem cells: lessons from leukemia. Trends Cell Biol *15*, 494-501.

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.

Xiao, A., Wu, H., Pandolfi, P.P., Louis, D.N., and Van Dyke, T. (2002). Astrocyte inactivation of the pRb pathway predisposes mice to malignant astrocytoma development that is accelerated by PTEN mutation. Cancer Cell *1*, 157-168.

Yoon, S.O., Lois, C., Alvirez, M., Alvarez-Buylla, A., Falck-Pedersen, E., and Chao, M.V. (1996). Adenovirus-mediated gene delivery into neuronal precursors of the adult mouse brain. Proc Natl Acad Sci U S A *93*, 11974-11979.

Zhang, J., Wang, J., Liu, Y., Sidik, H., Young, K.H., Lodish, H.F., and Fleming, M.D. (2009). Oncogenic Kras-induced leukemogeneis: hematopoietic stem cells as the initial target and lineage-specific progenitors as the potential targets for final leukemic transformation. Blood *113*, 1304-1314.

Zhao, C., Deng, W., and Gage, F.H. (2008). Mechanisms and functional implications of adult neurogenesis. Cell *132*, 645-660.

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

Zhu, Y., Harada, T., Liu, L., Lush, M.E., Guignard, F., Harada, C., Burns, D.K., Bajenaru, M.L., Gutmann, D.H., and Parada, L.F. (2005b). Inactivation of NF1 in CNS causes increased glial progenitor proliferation and optic glioma formation. Development *132*, 5577-5588.

Zhu, Y., and Parada, L.F. (2002). The molecular and genetic basis of neurological tumours. Nat Rev Cancer *2*, 616-626.

Zhu, Y., Romero, M.I., Ghosh, P., Ye, Z., Charnay, P., Rushing, E.J., Marth, J.D., and Parada, L.F. (2001). Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain. Genes Dev *15*, 859-876.

Zhuo, L., Theis, M., Alvarez-Maya, I., Brenner, M., Willecke, K., and Messing, A. (2001). hGFAP-cre transgenic mice for manipulation of glial and neuronal function *in vivo*. Genesis *31*, 85-94.

Zimmerman, L., Parr, B., Lendahl, U., Cunningham, M., McKay, R., Gavin, B., Mann, J., Vassileva, G., and McMahon, A. (1994). Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. Neuron *12*, 11-24.