Development and Characterization of a Focally Induced Mouse Model of Glioblastoma Multiforme Originating From Terminally Differentiated Astrocytes

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ABSTRACT

Ryan Bash: Development and Characterization of a Focally Induced Mouse Model of Glioblastoma Multiforme

(Under the Direction of Terry A. Van Dyke)

Glioblastoma multiforme (GBM) is a devastating tumor of the central nervous system and is the most frequently occurring primary brain tumor in adults. While much progress has been made in reducing the mortality associated with other forms of cancer, morbidity from GBM remains unchanged. The lack of progress in treatment of GBM highlights the continuing need for accurate preclinical animal models as a means of understanding pathogenesis and providing a resource for testing of potential therapies.

To model the development of GBM, we have developed a system using genetically engineered mouse (GEM) models in which silent mutant alleles can be converted to active oncogenes or inactive tumor supressors using the cre-lox recombination system. Through stereotactic injection of replication incompetent lentivirus expressing Cre-recombinase into the cortex of these animals, we have assessed the impact of known effectors of astrocytoma to induce and advance tumors originating from terminally differentiated astrocytes in the context of surrounding normal brain tissue.

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List of Abbreviations

4-OHT	4-hydroxy-tamoxifen
AA	anaplastic astrocytoma
CC	corpus callosum
CDK4	cyclin dependent kinase 4
CNS	central nervous system
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
GAP	RasGTPase activating protein
GBM	glioblastoma, glioblastoma multiforme
GEM	genetically engineered mouse
GFAP	glial fibrillary acidic protein
H&E	hematoxylin and eosin
HGA	high grade astrocytoma
IF	immunofluorescence
IHC	immunohistochemistry
IP	intraperitioneally
LOH	loss of heterozygosity
LSL	loxP-stop-LoxP cassette
LV	lateral ventricle
mTOR	mammalian target of rapamycin
NSCLC	Non-Small Cell Lung Cancer

OB	olfactory bulb
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PNET	primitive neuroectodermal tumors
PI3K	phosphatidylinositol 3’-kinase
Pten	phosphatase and tensin homology on chromosome ten
Rb	retinoblastoma
Rb_f	retinoblastoma family members
RMS	rostral migratory stream
RT-PCR	reverse transcription- polymerase chain reaction
RTKs	Receptor tyrosine kinases
SIN	self-inactivating
Stat	signal transducer and activator of transcription
SV40	simian virus 40
SVZ	sub ventricular zone
SVZa	anterior sub ventricular zone
T121	first 121 amino acids of SV40 large T antigen
TGFα	transforming growth factor-α
TUNEL	terminal deoxynucleotidyltransferase mediated dUTP-end labeling
VEGF	vascular endothelial growth factor
WHO	world Health Organization
Wt	wild type

um	-	micro meter

LIST OF SYMBOLS

α	alpha
β	beta
Δ	delta
μ	micro
ζ	zeta

Chapter I

Introduction

FEATURES AND GRADING OF GLIOMA

Gliomas are the most commonly occurring tumor arising from the central nervous system. In adult mammals, glial cells retain the ability for limited proliferation after development is completed, although neurons do not outside of specific regions (1-4). Gliomas are classified according to the cell type comprising the majority of the tumor and include astrocytomas, oligodendrogliomas and oligo-astrocytomas. Astrocytomas are diffuse gliomas that maintain the morphological and molecular characteristics of astrocytes and account for >60% of all primary brain tumors(5). Because gliomas rarely metastasize outside the CNS, histological classification of the tumors within the brain is the primary determinant of malignancy.

Pilocytic astrocytoma, or grade I astrocytoma, is the most common brain tumor in children and young adults. It accounts for about 5.7% of all gliomas(5). This tumor is found in the cerebellum, optic nerve, chiasm and hypothalamus. Typical histological features of pilocytic astrocytoma are bipolar cells (having two extended processes on opposing poles) with Rosenthal fibers (elongated eosinophilic bundles) that are immuno-reactive to GFAP (glial fibrillary acidic protein) and loose multi-polar cells with microcysts and granular bodies (rounded eosinophilic structures within a cell) that stain poorly with GFAP. GFAP is a component of the astrocytic intermediate filament that is used as a marker for mature astrocytes. In the normal

brain, astrocyte proliferation is minimal. Pilocytic astrocytoma is a slow-growing tumor with a low rate (<1%) of mitosis, and no necrosis. This type of tumor is well circumscribed, is usually not infiltrative and has no tendency to be malignant(6). Genetically, pilocytic astrocytoma is associated with neurofibromatosis type 1(NF1), especially optic pathway gliomas. It is curable using surgery with or without radiation therapy, and 80% of patients survive more than 20 years after diagnosis.

Low-grade astrocytomas, World Health Organization (WHO) grade II, are slow-growing tumors, accounting for ~40% of gliomas(5). They are characterized by a high degree of cellular differentiation and diffuse infiltration of the surrounding tissue. The mean survival can range from 6-8 years, with a high rate of curative treatment, depending on the extent of surgery, location, proliferative potential of the neoplasm, and the accumulation of multiple genetic alterations.

Anaplastic astrocytoma, WHO grade III, is an intermediate grade tumor that appears to develop from low-grade astrocytomas and accounts for 7.5% of gliomas(5). Localized hypercellularity produces a discernible mass within the surrounding brain tissue. This focal hypercellularity exists in combination with atypical nuclei and less differentiated cells that are frequently not morphologically identifiable as astrocytes(6). Typical treatment includes surgical resection when practical, followed by radiation therapy. The mean survival from these tumors is 2-3 years, with key prognostic factors being progression to GBM (decrease in survival) and the presence of an oligodendroglial component (increase in survival)(6;7).

Glioblastoma multiforme (GBM), WHO Grade IV, is the most malignant and common manifestation of astrocytic tumors accounting for more than 50% of

cases(5). Approximately 90% of GBM's arise without evidence of a less severe precursor lesion, and typically affect older patients(8;9). The other 10% develop from pre-existing diffuse or anaplastic astrocytomas, and typically arise in younger patients(8;9). Despite having similar histological presentation and prognosis, primary and secondary GBM typically harbor differing genetic alterations. Primary GBMs are characterized by a loss of heterozygosity 10q in 70% of cases, *PTEN* (phosphatase and tensin homologue) mutations (25%), *EGFR* (epithelial growth factor receptor) amplification (36%),and $p16^{INK4a}$ deletion (31%)(8-11). In secondary GBM, *TP53* mutations are the most frequently occurring alterations and are present in 60% of precursor lesions, followed by loss of heterozygosity 10q (70%) upon progression to GBM(8-11). Progression to GBM from anaplastic astrocytoma is characterized histologically by regional necrosis, and/or microvascular proliferation in addition to an increased proliferative index(12). Despite the presence of a central focus, infiltrative growth usually precludes complete surgical resection and makes eradicating tumor cells with chemotherapy or radiation therapy nearly impossible without extensive damage to surrounding brain parenchyma. GBM is associated with an exceptionally poor prognosis and a mean survival time of 9-12 months(6;12;13).

Recent large scale efforts have been made to accurately and systematically identify molecular aberrations present in human GBM(14;15). In particular, The Cancer Genome Atlas (TCGA) Research Network has reported an interim analysis of 206 GBM's isolated from human patients identifying changes in DNA copy number, gene expression and methylation(15). The most frequent alterations were

in RTK/Ras/PI(3)K signaling (88%), p53 signaling (87%) and RB signaling (78%). A similar study was perfomed independently for 22 human GBMs and obtained similar results(14). Both studies provide comprehensive analyses of molecular biological data leading to an improved understanding of the molecular causes of GBM.

Despite years of active research including clinical trials for pathway-targeted therapies, effective management of astrocytomas and mortality rates from them have not changed significantly over since the advent of radiation therapy. However, our understanding of the molecular and genetic alterations likely underlying these devastating tumors has greatly increased due to the analysis of human tumors and derivative cell lines and to the development of mouse models. These models are vital tools that will be used to explore the development and progression of these diseases, as well as aid in testing potential targets for therapies in the clinic.

GENETICS OF GLIOMA

pRb

pRb and the paralogous proteins, p107 and p130 (collectively referred to as pRb_f) play a critical role in regulating the E2F family of transcription factors. During cellular quiescence and the G1 phase, hypophosphorylated Rb associates with specific E2F proteins (E2F1-3)(16). Together these complexes actively repress E2F-dependent S phase gene transcription(17;18) through recruitment of histone deacetylases and other chromatin remodeling factors(19-21). Cell cycle progression is initiated by mitogenic signals that stimulate cyclin D/CDK4, 6 and cyclin E/CDK2

complexes to phosphorylate Rb. Hyperphosphorylation of Rb releases it from the E2F transcriptional complex that then directs transcriptional DNA-replication machinery and nucleotide biosynthesis, thereby facilitating cell cycle progression (Fig. 1.1). Cyclin-dependent kinase inhibitors, including the INK4 and p21 proteins, provide an additional layer of regulation by negatively regulating CDK function(22). While inherited Rb mutation in humans predisposes children to retinoblastoma and osteosarcoma(23), somatic loss of Rb function, or aberrations in other functions along the Rb pathway (Fig. 1.2), such as loss of p16INK4a or activation of CDK4/cyclinD, have been detected in the majority of human sporadic cancers(24-27). The observation that these mutations are mutually exclusive in a single tumor is genetic evidence for a linear tumor suppression pathway. pRb pathway mutations have been detected in 68-78%(14;15) of human GBM's.

While genetic engineering strategies in the mouse provide tools to investigate the genetic requirements of tumor onset and progression(28), the analysis of cell-specific somatic pRb inactivation is challenging. Rb deficiency causes embryonic lethality in mice, while heterozygous and chimeric mice develop only a small subset of tumors including pituitary and thyroid tumors(29;30). The limited tumor spectrum is believed to be the result of compensation by p107 and p130 in most cell types(30). Therefore, to analyze pRb pathway inactivation at the most downstream step by allele disruption would require 4-6 mutant alleles, one of which would be conditional, making further modeling of additional events prohibitive. Thus, a strategy was developed to dominantly interfere with pRb, p107 and p130 by cell specific expression of a truncated SV40 T-antigen, containing only the amino-terminal 121

amino acids and referred to as T_{121}. T_{121} binds to pRb, p107 and p130 and inactivates their ability to negatively regulate E2F's(31-35). Using a single transgenic allele expressing this dominant interfering protein the Van Dyke lab has thus far inactivated pRb function in several cell types in the mouse, predisposing them to tumorigenesis(36-39). In each case, aberrant proliferation is induced and is accompanied by apoptosis, initiating the process of tumorigenesis.

PTEN/PI3K

The lipid Phosphatase and Tensin Homolog (PTEN) functions as a tumor suppressor and is inactivated in a wide variety of human malignant neoplasms including glioblastoma, breast, and prostate(40;41). Loss of the chromosome region 10q23.3 containing PTEN is known to occur frequently in GBM(15). PTEN directly antagonizes the activity of the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR pathway(40;42;43). This is an evolutionarily conserved signaling cascade influencing multiple cellular functions including proliferation, growth, survival and migration(44). Activation of PI3-kinase by growth factor or extracellular matrix (ECM)-dependent signaling leads to synthesis of phosphatidylinositol 3,4,5-phosphate (PIP-3) that is dephosphorylated by PTEN to phosphatidylinositol (4,5)-diphosphate (PIP-2)(45). PIP-3 is responsible for recruiting AKT and phosphoinositide dependent kinase-1 (PDK1) to the plasma membrane by binding to their pleckstrin homology (PH) domains(44). At the plasma membrane AKT is activated by phosphorylation of its activation loop by PDK1(46) and phosphorylation

of its hydrophobic motif by Mammalian Target of Rapamycin Complex 2 (mTORC2), which is rapamycin-insensitve(47). Activated AKT is then capable of phosphorylating multiple proteins responsible for a variety of cellular processes. AKT phosphorylation of the forkhead (FOXO) family of transcription factors negatively regulates their activity by inducing their release from DNA, promoting cell survival and increasing proliferation by preventing activation of the proapoptotic genes Fas ligand (Fas-L), tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and Bcl-2 interacting mediator of cell death (Bim)(48-50). Activated Akt also downregulates p27 levels and inactivates Gsk-3, resulting in stabilization of cyclin D1, thus leading to increased cellular proliferation(51). Phosphorylation of the tuberosclerosis complex TSC1/TSC2 by AKT inhibits its ability to negatively regulate Ras homolog enriched in brain (RHEB), activating the rapamycin-sensitive mTOR complex (mTORC1)(52). The kinase activity of mTOR in the mTORC1 complex regulates protein synthesis and ribosome biogenesis via eukaryotic translation initiation factor 4E (4EBP1) and ribosomal S6 kinase(53).

PTEN has also been shown to have a role in cell mobility *in vivo* and *in vitro*. It downregulates cell spreading and focal adhesion in fibroblast and glioma cell lines(54). Human glioma cell lines lacking PTEN have been found to be highly invasive. Restoration of PTEN in these lines greatly reduces their capacity for invasion(55;56). It has also been shown that a CNS-specific knockout of PTEN disrupts the organized structure of cerebellum presumably as a result of aberrant migration(57). While loss of PTEN is clearly plays an important part in glioma progression, its multiple roles remain to be completely explored.

Ras

Although K-Ras mutation is rare in human brain tumors, it does appear to be frequently activated by RTKs(15;58). Many labs have demonstrated a role for K-Ras or H-ras in high-grade astrocytomas. Transgenic mice harboring a GFAP-regulated Ha-Ras^{V12} allele developed astrocytoma at severities dependent upon transgene expression level(59). However in another study(60) K-ras^{G12D} (a constitutively active point mutant) was not sufficient to induce glioma. These conflicting results may be attributable to functional and regional differences in K-ras and H-ras expression, or a difference in potency of the 2 Rases. It is also potentially attributable to methodological differences between the two studies, H-ras being expressed broadly throughout the brain and K-ras being delivered strictly to a small portion of the cortex.

The NF1 tumor suppressor gene is a negative regulator of Ras activity, converting it from a GTP-bound to a GDP-bound state. Astrocyte-specific inactivation of *Nf1* using crosses to specific Cre strains is not sufficient to predispose mice to astrocytoma(61). However it has recently been found that NF1 is deleted or harbors inactivating mutations in 23% of GBM samples(15), emphasizing the importance of the negative Ras regulation.

K-ras is a downstream effector of several receptor tyrosine kinases, including EGFR and PDGFRα which are frequently activated by mutation and/or amplification in human high grade astrocytomas. Although gain-of-function mutations in EGFR occur frequently in high-grade gliomas (~40% of GBM cases)(62), transgenic and

somatic delivery approaches to overexpress wildtype and mutant forms of EGFR in astrocytes have not generated astrocytoma, but do increase the number of glial cells(59;63). Because EGFR mutations are observed mainly in high grade gliomas, being mutated or amplified in 45% of GBM's(15) and largely absent in low-grade disease, it is thought that it is important for disease progression rather than initiation. The relevant downstream effector pathways in disease etiology are largely unknown. However KRas can regulate several pathways including MAPK, PKC, JNK and PI3K.

MOUSE MODELS OF GLIOMA

Retroviral RCAS/tv-a Models

Delivery of retrovirus carrying oncogenes has become a useful technique in altering the somatic genetics of postnatal mice(64;65). The use of recombinant retrovirus is effective and often more convenient than generating transgenic mice. The avian retrovirus (RCAS) cannot infect normal murine cells because these cells do not express the cognate receptors for the virus. Holland, Varmus and colleagues established transgenic mouse lines that express avian retroviral receptors under the control of a GFAP (Gtv-a) or nestin promoter (Ntv-a)(65). Nestin is an intermediate filament protein expressed broadly in the developing CNS, and restricted in the adult to a limited neural precursor population. Neonatal transgenic brains were then infected with recombinant avian retrovirus carrying the gene of interest. Using neonates is important as the CNS cells are still proliferating and are capable of being

infected. Akt and constitutively active K-Ras^{G12D} together, but not either alone, were capable of inducing glioma with high frequency(66). PDGFβ itself was sufficient to induce GBM formation, which was accelerated in an INK4a-Arf deficient background(64). On the other hand, several common genetic alterations failed to induce glioma. For example, even on INK4a-Arf or p53 deficient backgrounds, overexpressing EGFR induced less severe glioma-like lesions than overexpressing PDGF, Akt and Ras(63;64;66) . Interestingly, p53 deficiency did not cooperate with PDGFβ to accelerate glioma formation(64). This approach also shed light on the possible origin of the tumor cells. Gene transfer into mice that express the receptor targeted to neural progenitor cells (Ntv-a) caused more aggressive gliomas and with a higher frequency than mice expressing the receptor targeted to differentiated glial cells (Gtv-a)(64). For example, the combination of Akt and activated K-Ras allele only induced GBM in Ntv-a, but not Gtv-a mice(66). These results indicate that neural progenitor cells may be more predisposed than differentiated astrocytes to tumorigenesis upon oncogenic stimuli. However, because recombinant RCAS virus can only integrate into proliferating cells, the high proliferation rate of the progenitors (as in Ntv-a mice) could also be influencing the tumor phenotype. Another potential use of this approach is to evaluate the differentiation status of tumor cells during tumor progression. When PDGF or polyoma virus middle T antigen was delivered, mice developed mixed oligodendrocytoma and astrocytic glioma at varying frequencies(64;66). These results indicated that certain genetic alterations either have distinct roles in dedifferentiation of tumor cells or have a more profound impact on distinct glial lineages in this case.

Transgenic Ras-driven Models

Using a mouse ES cell transgenic approach, mice harboring Ha-*ras*^{V12} under the control of the GFAP promoter developed glioma(59). The phenotype is dependent on the transgene expression level. A high expressing line of mice develops GBM at two weeks, and is therefore not germline transmittable, while the low expression line develops low to high grade glioma (Grade II at 80%, Grade III at 20%). A variety of genetic mutations that frequently occur in human gliomas, such as Rb pathway and PTEN mutations, have also been found in tumors developing in this model. However, whether these mutations correlate with tumor progression and at what frequency was not reported(59).

The Ha-*ras* ^{V12} study provided an interesting contrast to the previous study, in which somatic delivery of *K-Ras*^{G12D} itself was not sufficient to induce glioma(63). The contrasting results may be due to functional and regional differences of H-Ras and K-Ras expression. *K-Ras*^{G12D} was delivered to the cortex of neonatal mice while Ha-*ras*^{V12} was expressed in much broader regions. Alternatively, this discrepancy may also reflect differences in the methodology. Since somatic delivery of *K-Ras*^{G12D} only infected a small number of cells, Dai and Holland proposed that the likelihood of developing secondary events that accelerate tumorigenesis is low(64). In contrast, numerous genetic alterations were found in the Ha-*ras*^{V12} transgenic mice(59). Thus, the RCAS delivery system may not identify the genetic alterations occurring during selective evolution subsequent to initiating events(64).

Further improvement in the somatic delivery approach will allow us to investigate the number of tumor cells necessary to develop secondary mutations vital for tumor progression and to observe the competition between tumor cells that develop different secondary mutations.

The *NF1* tumor suppressor gene is a negative regulator of Ras activity (GAP), converting it from a GTP-bound to a GDP-bound protein(67). It is located on the same chromosome as the *p53* gene (human:17, Mouse:11) separated by 7 cM. This configuration makes it difficult to combine the nullizygosities of the two by conventional genetic crosses in mouse. Hence, Jacks, Parada and colleagues selected the rare "*cis*" heterozygous null offspring from a cross between *p53* and *Nf1* null mice(68). The "*cis*" mice develop low to intermediate grades of glioma with varying frequency depending on the background strains. In a separate study, Parada and colleagues demonstrated that inactivating both alleles of NF1 (p53 intact) in astrocytes is not sufficient to induce glioma(61). Since p53 null mice do not develop glioma, these studies clearly demonstrate that collaboration between the two is critical for glioma initiation. The background dependence of the phenotype indicates the existence of modifier loci, which should also be of great interest.

Considering the high incidence of Ras gain-of-function mutations in other types of solid human tumors(69), it is surprising that Ras mutations have rarely been found in human gliomas. Yet many transgenic mouse models overexpressing Ras develop glioma. These discrepancies between human gliomas and mouse studies are not well understood.

RTK Signaling Aberrations

Although gain-of-function mutations of EGFR occur frequently in high-grade human gliomas (~40% in GBM)(62;70;71), transgenic and somatic delivery approaches to overexpress WT and mutant forms of EGFR in the mouse have not generated severe tumor development as expected, but only increased the number of glial cells (gliosis)(63;72) . EGFR knockout mice have a reduced number of GFAP expressing astrocytes and exhibit neuron degeneration(73-75). Still, these knockout results need to be interpreted carefully since trophic support from other cell types during CNS development is well known. Given the success in generating gliomas with other RTK/Ras perturbations (PDGFR, H-Ras and K-Ras) these data are intriguing. One explanation for the lack of tumorigenesis in this model is that EGFR mutations may only collaborate with other genetic mutations to accelerate tumorigenesis. Consistent with this hypothesis, somatic delivery of EGFR to the *INK4a-ARF* deficient mice background increased the frequency of glioma-like legions compared to the WT or p53 deficient mice(63). However, other mutations may be necessary to generate more malignant gliomas together with EGFR mutations. Another possibility is that the timing of EGFR mutations may be critical. Since EGFR mutations usually occur in high-grade human gliomas, preceding mutations may be needed to “prime” the tumor cells to accommodate upregulation of EGFR functions. Finally, EGFR upregulation may require a different targeted population from the ones used in the above studies to exert its effects(76;77). The EGF/EGFR signaling pathway has been proven vital for neural stem cell survival

(NSCs) in the CNS(76;77). Since EGFR signaling is important for CNS neural progenitor cell proliferation, these cells may be affected more by EGFR mutation than differentiated cells. Supporting this hypothesis, TGF-α (EGFR related ligand) mutant mice, both hypomorphic and KO, have a reduced proliferation rate in the adult SVZ(78).

To examine the role of EGFR in the pathogenesis of oligodendroglioma, transgenic mice were generated expressing *v-erbB,* a transforming homologue of *EGFR*, under control of the S100β promoter(79). S100β is expressed in oligodendroglia and astrocytes and is present in a wide variety of glial tumors. The S100β-v-erbB mice developed low-grade oligodendroglioma but showed no signs of astrocytoma. When crossed onto a heterozygous *ink4a/arf* or *p53* background, the mice developed high-grade tumors, which maintained the characteristics of oligodendroglioma. Comparative genomic hybridization in primary tumor samples as well as tumor cell lines revealed loss of distal portions of mouse chromosome 4, a region orthologous to human chromosome 1p, a region, which is commonly lost in human oligodendroglioma.

Rb Inactivation

The Rb pathway is the most frequently mutated pathway in human gliomas including GBM. Transgenic models have been generated to inactivate pRb family members (pRb, p107, p130)(80) using cell specific promoters. In the mouse, Rb family proteins have overlapping functions in suppressing tumorigenesis(81-83). To

overcome this problem and eliminate the need for the generation of multiple knockout animals, a truncated SV40 T antigen, named $T_{121,}$ was used(84). Encoding the N-terminal portion of the large T antigen, $T_{121,}$ contains the sequence motif that binds to and inactivates all three Rb family members and triggers the J domain to disrupt the protein complex formed by Rb_f and their associated proteins (Fig. 1.2)(85-87).

Prior work in the Van Dyke lab targeted T_{121} to astrocytes via the human glial fibrillary acidic protein (*GFAP*) regulatory region to test the hypothesis that astrocytes are the cell of origin for gliomas in human tumors. GFAP is a marker for astrocytes in the adult brain. A 2.2 kilobase 5'- flanking sequence derived from the human GFAP (hGFAP) gene was shown to direct astrocyte-specific transcription(88). Most mice generated with the GFAP promoter driving expression of T_{121} died perinatally due to severe glioma-like lesions of astrocytes and neural progenitor cells(36). Notably the cellular population of the subventricular zone was expanded, coupled with diffuse hypercellularity throughout the brain. Secondary structures including perivascular and perineuronal satellitosis were noted.

In an effort to bypass the perinatal lethality associated with the expression of the multi-copy T_{121} transgene under the control of the GFAP promoter, a conditional approach based on the Cre-LoxP system was used to reduce the transgene expression level(36). After a single cross of the conditional transgenic (GZT_{121}) to β-actin-Cre mice, mice of this lineage (*TgG(ΔZ)T*$_{121}$) (Fig. 1.4) developed malignant anaplastic astrocytoma as adults with 100% penetrance. However, high grade glioblastoma was not observed even after prolonged periods of time.

An effort was also made to determine the role of progenitor cells in astrocytoma formation using a similar approach. In this case T_{121} expression was driven by the nestin promoter. Nestin is an intermediate filament in the human central nervous system that has been characterized as a marker for neural progenitor cells that have the ability to give rise to multiple cell types(89;90). Expression of the intermediate filament is under strict temporal and spatial control in undifferentiated cells of the CNS. Nestin expression has been observed in mice as early as embryonic day 7.75 and is down regulated as cells differentiate and migrate throughout the brain(91). The subventricular zone (SVZ) of the brain is an area thought to be a source of neural stem cells (NSC) in adult brains. No founder animals were generated using the nestin promoter to drive T_{121} expression, suggesting that an embryonic lethal phenotype was induced (Xiao and Van Dyke, unpublished). It was hypothesized that the embryonic lethality was due to the high copy number of the transgene. Again, a Cre-loxP conditional system was used to bypass perinatal lethality and reduce the transgene to a single copy. T_{121} expression was restricted to neuroprogenitor cells beginning at embryonic day 11.5(92). Transgenic embryos showed aberrant central nervous system (CNS) cell proliferation and apoptosis by embryonic day 13.5. Defects in cortical development were evident at this time, including depletion of neural progenitors and aberrant cellular migration. Juvenile and adult brain morphologies were reproducibly abnormal. These aberrations resulted in highly penetrant behavioral phenotypes, including ataxia and seizures. The data indicated that inactivation of pRb_f in radial

glial cells, a population of neuroprogenitor cells, leads to specific disruptions in CNS cell patterning, but is not responsible for any type of CNS tumor.

In mice, it appears that pRb_f inactivation can only constitute an initiating event in the development of astrocytoma due to the long latency (>3 months) between pRb inactivation and the onset of life-threatening disease(36). It is well established in human tumors that additional genetic events contribute to disease progression. One such event that commonly occurs in the most malignant form of astrocytoma, GBM, is loss of PTEN. Studies performed in the Van Dyke lab showed that PTEN heterozygosity accelerated astrocytoma development(36;93). Because loss of PTEN is embryonic lethal(51), conditional alleles were generated in which loxP sites flank exons 4 and 5 (designated *Pten*$^{fl/fl}$), encoding a phosphatase domain critical for regulating the PI3K pathway. Cre-mediated excision of exon 4 and exon 5 results in a PTEN null allele, producing an unstable transcript. This system has been used to show that loss of PTEN results in increased cell survival and invasiveness (36;93). A summary of these models may be found in Table 1.1.

RATIONALE FOR ENGINEERING IMPROVED MOUSE MODELS OF GBM

While much progress has been made using the model systems described above, it is possible to improve on them by developing a model that features pertinent genetic alterations expressed at physiologically relevant levels in a small, localized population of mature astrocytes. One major improvement of these models may come from the refinement of somatic delivery strategies. Human tumors are

likely initiated from a single cell that acquires critical secondary alterations to gain a growth advantage. A somatic delivery system limits the number of cells with induced events and is a closer mimic of the single cell of origin scenario. Furthermore, this strategy is more convenient to study the impacts of multiple genetic alterations than the traditional approach, reducing the amount of breeding necessary in traditional GEM's. Finally, it can be used to study many important but unresolved issues in CNS tumor progression, especially in gliomagenesis, such as cell origins and regional predilection of tumor development, and tumor-microenviroment interaction. Recent improvements in viral vectors, particularly recombinant lentivirus, further enhance the efficiency of gene delivery. In the current project, a stereotaxic injection of self-inactivating lentiviral vector is used to deliver Cre-recombinase into the cerebral cortex of existing GEM models, inactivating pRb_f, activating Kras receptor tyrosine kinase, and inactivating the tumor suppressor PTEN, leading to GBM with short latency and high penetrance.

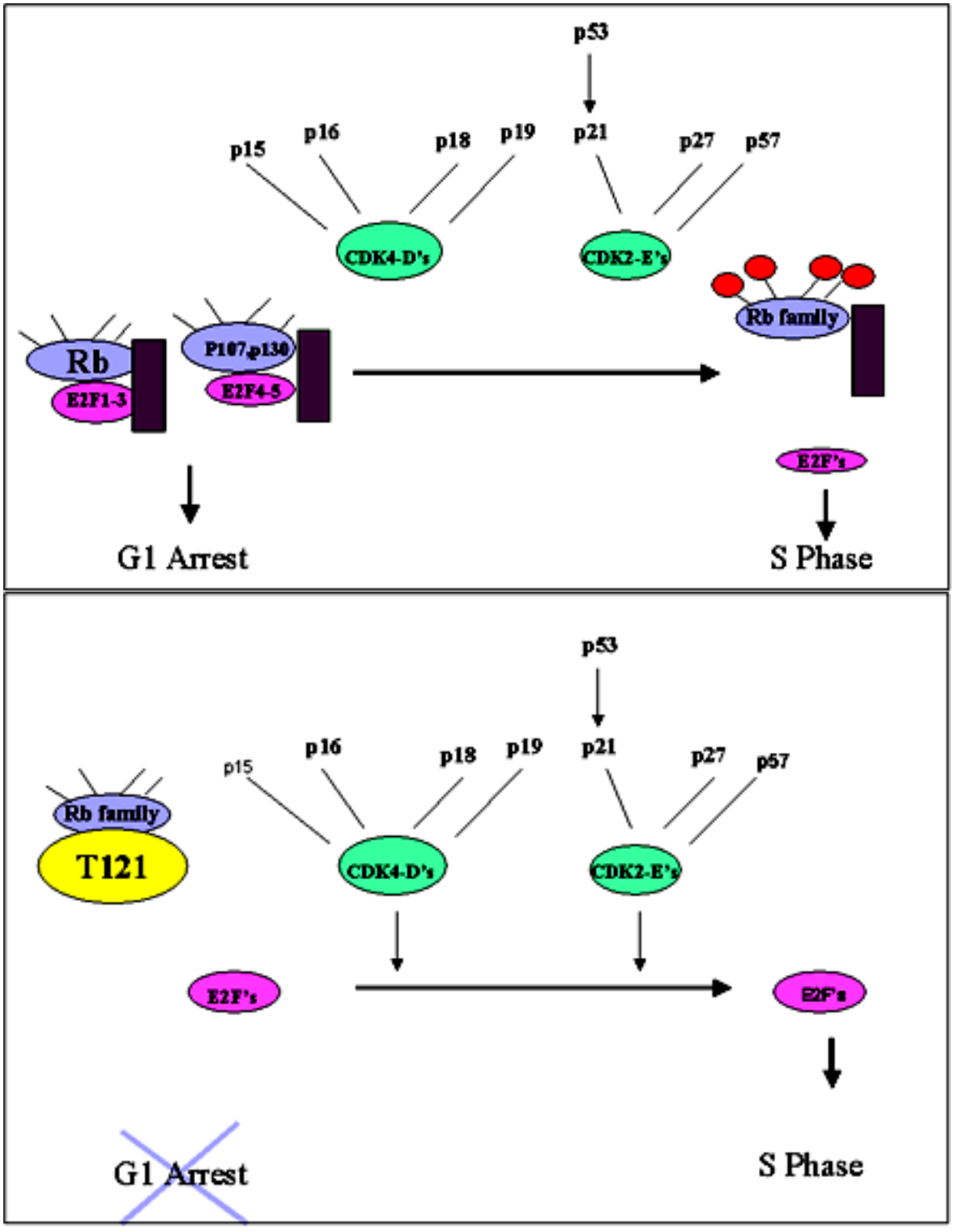


Figure1.1 RB regulation of the cell cycle. Upper Panel. Hyperphosphorylation of pRB family members by CDK's induces appropriate entry into the S phase of the cell cycle through E2F activation. The lower panel shows T_{121} binding to and inactivating pRb family

members allowing E2F transcription factors to drive S phase entry independently of normal signaling.

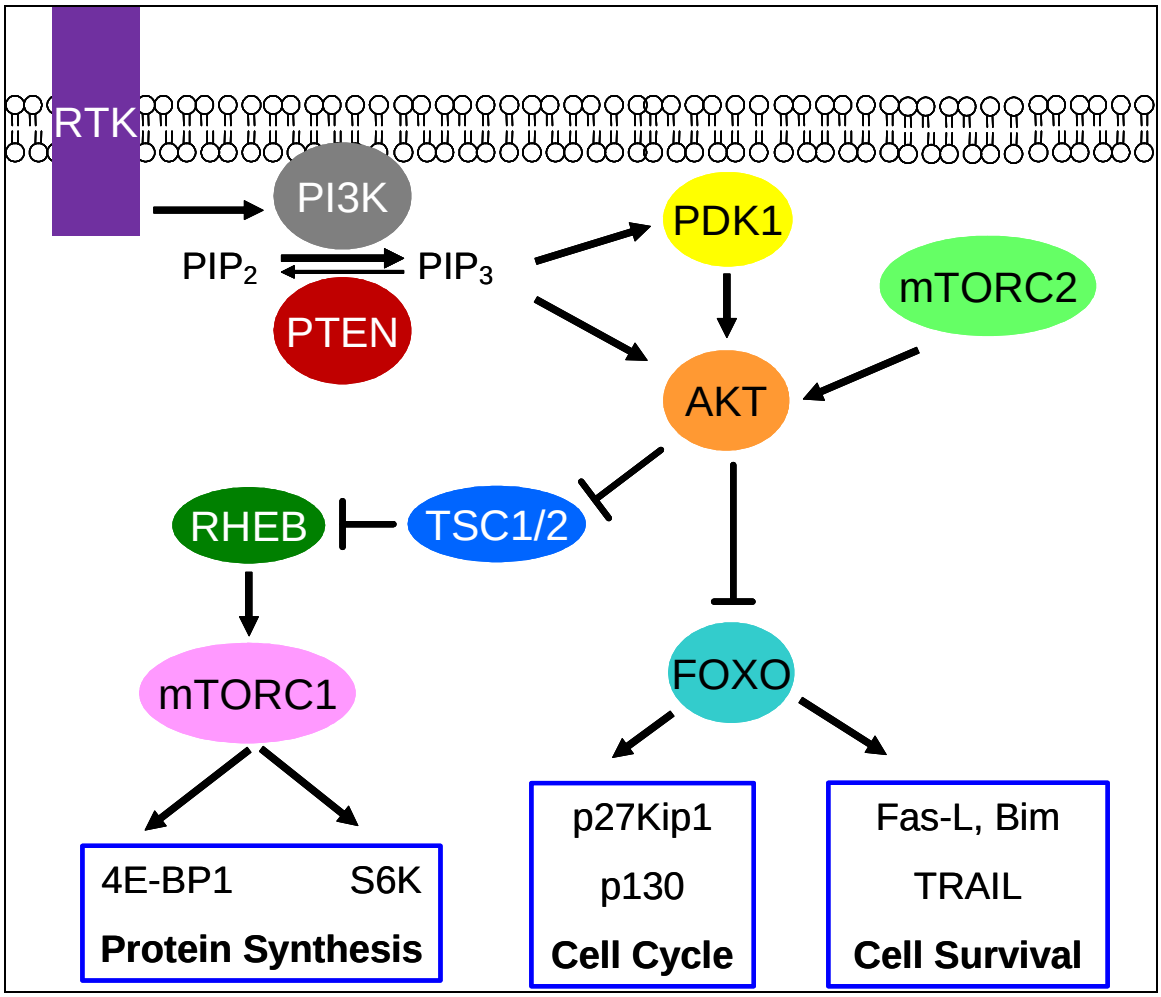


Figure 1.2. Relevant PTEN/PI3K signaling. Multiple downstream effectors of PI3K regulate diverse cell functions. Many of these are important in controlling cell migration and invasion, protein synthesis, cell cycle entry and apoptosis.

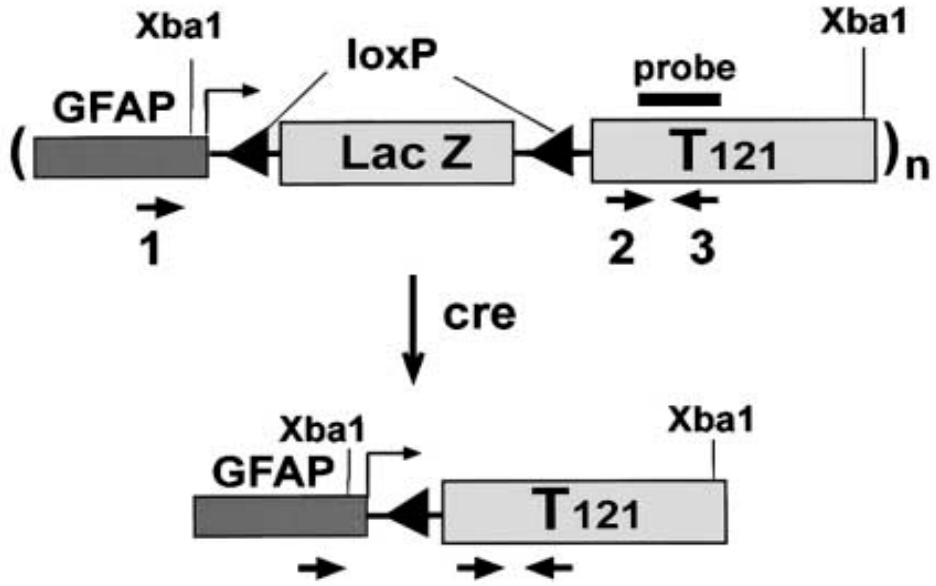


Figure 1.3. GZT_{121} Transgene Design. In this configuration, T_{121} expression is controlled by a fragment of the human GFAP promoter and is abrogated until the LacZ cassette is deleted by Cre-mediated recombination. Upon recombination in cells where the GFAP promoter is active, the T_{121} protein binds to and inactivates pRB family to promote inappropriate S phase entry.

Table 1.1 Summary of glioma mouse models

Glioma models	CNS Tumors	Phenotype
TgGFAP& Nestin–tva	Astrocytoma including GBM	1. Dependent on infection with RCAS containing mutant EGFR 2. GBM induced by overexpression of PDGF, Middle T-antigen, and Akt with constitutively active K-Ras^{G12D}, but not by b-FGF and EGFR overexpression (Holland et al. 1998)
TgGFAP-Ha-Ras	Astrocytoma including GBM	1. Low-grade astrocytoma to GBM, multi-focal and highly penetrant, severity depends on transgene expression level 2. Secondary mutations found in Rb, p53 and PTEN pathway (Ding et al. 2001)
Cis NF1;p53 null	Grade II and III gliomas	1.Grade II and III gliomas in cis heterozygotes. 2.Severity and penetrance dependent on background strain (Cichowski and Jacks 2001)
TgGFAP--(ΔZ)--T_{121}	Grade III gliomas	1. Grade III gliomas at 100% penetrance. 2. Tumor progression accelerated on PTEN but not p53 deficient backgrounds (*germline*) 3. PTEN deficiency (*somatic*) reduces apoptosis, enhances invasion and angiogenesis (Xiao et al. 2002)
TgGFAP-Src	Grade II and III gliomas	1. Low and high grade gliomas developed in ~20% of transgenic mice 2. Retarded growth, cerebellar ataxia and some hydrocephalus (Weissenberger et al. 1997)

Chapter II

Focal induction of GBM

ABSTRACT

Currently, there is no effective treatment for astrocytoma, including the most severe manifestation, glioblastoma multiforme (GBM). In order to develop therapies targeted specifically to this disease, it is important to develop animal models which will be used to determine the molecular pathogenesis of the disease. The Van Dyke Lab and others have developed genetically engineered mouse (GEM) models which target pathways frequently deregulated in human astrocytomas. Specifically, conditionally inactive/active alleles of *RB*, *KRAS* and *PTEN* were used. These genetic alterations were induced in the adult mice by Cre recombinase, which was delivered into mature cortical astrocytes via stereotactic injection of a self-inactivating (SIN) lentiviral vector. Animals with focal *RB* inactivation alone developed minimal focused hyperplasia. Mice with *RB* inactivation combined with constitutively active *KRAS* developed GBM (grade IV) with long latency. The additional loss of *PTEN* produced tumors with histopathological features of GBM with a shorter latency. These results support the role of *RB* in tumor initiation and the roles of *PTEN* and *KRAS* in tumor progression. This model also provides a

highly-penetrant, inducible system in which to study multiple aspects of disease progression from a single focus.

INTRODUCTION

Astrocytomas are the most commonly occurring malignant primary brain tumors in humans. At this time, there are no effective treatments available for these diseases(12) and therapeutic regimens are largely palliative in nature. Overall survival for all grades of astrocytoma have not changed significantly in the past fifty years(12;70). GBM is the most severe manifestation of astrocytoma, WHO Grade IV, and is characterized by the presence of increased mitotic activity, necrosis and vascular proliferation(12). GBM appears to arise via two distinct genetic pathways(12;94). The progression of grade II or III astrocytoma to GBM is referred to as secondary GBM and may occur over a period of months to decades. Secondary GBM often retains features of the lower grade precursor including *TP53* mutations, PDGFR-α overexpression and *RB* family alterations (pRB, p107 and p130) (12;94;95). GBM may also develop without any prior clinical history of brain tumor in the patient and is referred to as primary GBM. Clinical cases of primary GBM display aberrations in cell-cycle regulatory factors, including the *RB* family members (20-30%), *PTEN* deletion and high frequencies (30-70%) of *EGFR* overamplification. *KRAS* is not frequently mutated but is hyperactivated in many

cases of high grade astrocytoma(96;97), most probably as the result of the activation of upstream receptor tyrosine kinases (RTK) such as EGFR and/or PDGFR-α(70).

Unfortunately, the complexity of the brain and it's prominence in basic human function prevent the detailed dissection of the molecular pathogenesis, in particular the role of tumor microenvironment, of astrocytomas based on the analysis of human tumor samples alone. Relevant animal models have already played a key role in determining the factors involved in tumor initiation and progression(36;63;83;93;98) and are becoming increasingly important in the preclinical development of targeted therapies(99;100). While much has been learned from genetically engineered mouse models using germline and somatically-induced mutations, it is necessary to narrow the range of cells affected by the pertinent mutations. In so doing, the progression of events from initiation to terminal disease can be determined in detail. The tool we have chosen to narrow our population of affected cells is stereotaxic injection of a lentiviral vector expressing Cre-Recombinase into the cerebral cortex of the mouse. Lentiviral vectors are considered an advantageous vehicle for gene delivery because of features including a large packaging capacity, low immune response in the host target, the ability to enter nondividing cells and wide host cell tropism through pseudotyping of the envelope glycoprotein. They may also be engineered to be self-inactivating providing safety by eliminating spread from the initially infected population as well as limiting gene expression temporally and spatially(101;102). This feature is particularly important because sustained levels of high Cre-recombinase expression have been shown to be toxic in brain tissue(103). With the somatic induction of

genetic alterations in existing GEM models using lentiviral vectors to deliver Cre recombinase to the brain, we have developed a model which faithfully mimics disease initiation and progression as it is thought to occur in human GBM. We have induced clinically pertinent alterations (Rb inactivation, Pten loss and Kras activation at physiologically relevant levels) in a focused population of quiescent astrocytes in the adult animal, while leaving the surrounding tissue unaffected. We have found that mice treated in this manner develop GBM with high penetrance and a short latency providing an excellent model for the study of disease progression and molecular pathogenesis.

RESULTS

Development of a focal induction model for GBM

Because the *RB* family is frequently misregulated in human astrocytoma, the Van Dyke lab previously engineered *TgGZT*$_{121}$ mice in which the function of pRb family members can be conditionally inactivated in astrocytes(36). T_{121}, an N-terminal fragment of SV40 T-antigen, binds to and inactivates pRb and the related proteins, p107 and p130 without altering p53 function(104).

To achieve spatial and temporal control of T_{121} expression, we used a self-excising lentiviral vector, introduced via stereotactic injection, to deliver Cre-recombinase to mature astrocytes specifically in the cerebral cortex. A single loxP site in the 5' LTR of the single-stranded viral RNA is copied to the 3' LTR upon

reverse transcription and integration of the provirus into the host genome(101-103)(Fig. 2.2). The vector contains an internal promoter from cytomegalovirus (CMV), used to drive transcription of immediate-early genes and thought to be constitutively active in a wide range of host cells. The CMV promoter is used to drive expression of Cre-recombinase in infected cells. Upon infection, the Cre-recombinase remains active until it acts on the loxP sites in the integrated viral DNA, removing the genetic material necessary for its own production. This short pulse of Cre is sufficient to activate conditional genetic alterations in the $TgGZT_{121}$ mice without generating any long term cellular lethality within the sensitive neural tissue(103) (Fig. 2.3).

This same system was also used to induce other major events thought to occur during astrocytoma progression. It has been previously shown that astrocyte-specific *PTEN* inactivation accelerates pRb inactivation-induced astrocytoma development through the promotion of anti-apoptotic mechanisms, invasion and angiogenesis. However PTEN inactivation, through Cre mediated excision of exons 3 and 4, did not contribute to disease progression in these models(36;93). These data suggest that additional oncogenic events are required for the transition from high grade astrocytoma to GBM. It is possible that activation of receptor tyrosine kinase (RTK) pathways might function in this capacity (15). In order to test this, the effects of activated RTK were previously modeled using a constitutively activated KRAS allele (K-ras^{G12D}), which has been shown to induce astrocytoma formation upon somatic vector-mediated gene transfer to neural progenitors in combination with activated Akt(66). In contrast to this model, physiological levels of K-ras^{G12D}

were desired and obtained through use of a conditional knock-in strategy using $KRAS^{+/lsl\text{-}G12D}$ mice(83). Upon expression of Cre-recombinase, the excision of a loxP-stop-loxP (lsl) cassette occurs, achieving expression of the constitutively active $K\text{-}ras^{G12D}$.

Thus, in order to achieve pRb_f inactivation, constitutive $K\text{-}ras^{G12D}$ activation and/or *PTEN* deletion specifically in a localized population of adult astrocytes, 3 month old $TgGZT_{121}$, $KRAS^{+/lsl\text{-}G12D}$, $PTEN^{+/fl}$ and $PTEN^{fl/fl}$ mice were injected with 1μl of PTK-627 lentivirus (~$1x10^6$ particles) to induce the following genotypes in a small population of adult astrocytes: ($TgG(\Delta Z)T_{121}$) (T), ($TgG(\Delta Z)T_{121}$, $PTEN^{+/-}$) ($TP^{+/-}$) or ($K\text{-}ras^{G12D}$, $PTEN^{+/-}$) ($RP^{+/-}$) or ($TgG(\Delta Z)T_{121}$, $K\text{-}ras^{G12D}$) (TR) or ($TgG(\Delta Z)T_{121}$, $K\text{-}ras^{G12D}$ and $PTEN^{+/-}$ or $PTEN^{-/-}$) ($TRP^{+/-}$ and $TRP^{-/-}$) (Fig. 2.1 and Table 2.1).

Deletion of the floxed *lacZ*/stop cassette and the presence of viral DNA in treated GZT_{121} animals were confirmed by PCR (Fig. 2.3E). In the untreated hemisphere, recombination could not be detected and no viral DNA was found. Expression of T121 protein was readily detected by immunohistochemistry (IHC) as early as 5 days after viral injection and remained present through terminal stages (Fig. 2.4), but was undetectable in mock-treated littermates injected with a virus expressing GFP without Cre-recombinase.

Immunofluorescence (IF) staining was used to confirm astrocyte-specific T_{121} expression (Fig. 2.3). In GZT_{121} mice, T_{121} was observed near the injection site and was localized in both the nucleus and cytoplasm. T_{121} also showed overlapping expression with GFAP protein in the cytoplasm as expected.

Focal Rb inactivation induces limited pathogenesis

One cohort of Tg*GZT*$_{121}$ mice was stereotactically injected with the PTK-627 lentiviral vector and allowed to age for 3 weeks or 6 months to analyze both acute and long term effects of Rb inactivation. At 3 weeks T_{121} expression was observed by IHC along the injection track, in the surrounding cortex and the corpus callosum. These data indicate that the virus is capable of infecting GFAP positive cells and inducing recombination within the transgene (Fig. 2.3).

Two Tg*GZT*$_{121}$ animals were aged for a period of 6 months post-injection and were sacrificed without any outward signs of illness. Following gross dissection, there was no discernible mass present or visible scarring from needle insertion. Upon histological examination, small clusters of T_{121} positive cells were visible at the injection site as well as several hundred μm distant (Fig. 2.4). Colabeling experiments showed that T_{121} positive cells were GFAP positive as well, confirming that recombination in our target cells was achieved(Fig. 2.3). Secondary structures of Scherer (perineuronal satellitosis) were present, but no other features of high grade disease were observed in the older cohort (Fig. 2.4). While this finding differs from previous results demonstrating that pRB loss alone is sufficient for tumor initiation(36), it is not surprising considering the greatly reduced number of cells harboring mutations compared to previous experiments. Focal Ras activation and PTEN ablation were not tested on their own, given that these alterations induced throughout the brain do not generate notable disease (Zhang and Van Dyke, unpublished).

pRb loss and Ras activation are necessary for tumor progression

As in previous work, a combinatorial approach was taken to determine the roles of additional mutations in disease progression in the context of focal induction. Three out of six mice with combined pRb inactivation and PTEN heterozygosity developed low grade (grade II) tumors at the site of injection over a period of 4-12 months. These tumors showed minimal infiltration, were non-lethal and possessed secondary structures of Scherer, exhibiting perineuronal satellitosis and perivascular satellitosis. Interestingly, two out of three $TP^{-/-}$ animals developed similar features over a period of 10 weeks (Fig. 2.5). Longitudinal studies were not pursued with these animals due to the lack of progression to high grade disease. It was also found that no significant disease developed in animals with combined Kras activation and PTEN inactivation. These animals were aged for a period of up to 9 months with no outward or histological signs of disease.

The combination of pRb inactivation and K-ras upregulation dramatically increased the severity of tumors found in treated animals. Three out of five subjects developed lethal, high-grade astrocytomas over a period of 5 to 12 months. Two of these three were classified as GBM (astrocytoma grade IV) with a mean survival time of 322 days, greatly increased mitotic index and a significantly larger and more invasive tumor mass possessing multiple foci of necrosis (Fig. 2.6). These masses appear to have formed at the site of injection, as well as developing leptomeningeal spread, possibly resulting from a lack of contact inhibition on the surface of the brain. The GBM's which developed were bi-hemispheric, suggesting aggressive spread of

tumorigenic cells. Upon histological examination, the tumor boundary with surrounding parenchyma appeared to be largely discrete in H&E stained sections (Fig. 2.6C). However, immunohistochemical staining for T_{121} revealed a very infiltrative tumor margin (Fig. 2.6D). Both of the GBM's which appeared possessed dilated vasculature and necrotic regions. T_{121} expression was abundant within the tumor bulk, although it was not present within every cell (Fig 2.7), suggesting reversion to a more primitive phenotype which does not express GFAP or recruitment of surrounding cells. It is also possible that expression of the transgenic and native GFAP promoters has been uncoupled, possibly receiving differing epigenetic signaling. These tumors possessed immunohistochemical markers associated with human GBM's. They are largely GFAP positive, consistent with tumors arising from an astrocytic lineage. They are also partially Nestin positive, consistent with a sub-population of precursor cells. The tumors were largely NeuN negative, indicating that they lack a neural component, also consistent with human GBM's (Fig. 2.7).

Proliferation within these tumors was quantified at ~44% using Image J software to identify cells immunohistochemically positive for Ki-67 (a protein marker strictly associated with cell proliferation) in three randomly chosen fields (Fig. 2.8A). Virtually no proliferating cells could be located outside of the tumor region. Apoptosis within the tumor was also quantified at ~3%, by TUNEL assay (Terminal deoxyuridine triphosphate nick-end labeling) in 3 random fields (Fig. 2.8B). Again, virtually no apoptotic cells were present outside of the main tumor mass.

High-grade tumor formation is accelerated in triple mutant animals

The inclusion of PTEN reduction or ablation to pRB loss and K-ras activation greatly reduced the latency of lethal high-grade astrocytomas (HGA). $TRP^{+/-}$ animals survived an average of 201 days. They also developed HGA at a higher frequency (10/13 subjects) than their TR counterparts (Fig. 2.10). Five of these HGA's progressed to GBM. These animals developed tumors with a similar appearance to TR mice, but with much shorter latency. $TRP^{-/-}$ mice developed lethal GBM's even more quickly, with a mean survival time of 131 days (Table 2.2). 8/10 developed HGA. Six of these eight progressed to GBM (Fig. 2.10). These tumors contained areas of pseudopallisading necrosis, high mitotic index, vasodilation and secondary structures of Scherer (Fig. 2.9). The tumor margin is also much more diffuse than in TR brains, suggesting a higher capacity for invasion. There is no clear boundary, and T_{121} positive cells can be seen diffusely infiltrating the surrounding tissue (Fig. 2.9B). This result consistent with prior data demonstrating the role of PTEN loss in increasing migration and invasion in both in vitro and in vivo systems(36;93). This diffuse invasion is consistent with human tumors, and one of the most confounding complications in treatment. A steady increase in tumor severity is evident in the reduced survival of the mice as additional relevant oncogenic events are included (Fig. 2.11).

DISCUSSION

Somatic delivery is a useful tool to study clonal expansion in animal models

An important feature of human tumor progression is the clonal expansion of a subset of tumor cells, which gain a growth advantage by acquiring additional genetic/epigenetic mutations(105). In genetically engineered mouse models, combinations of genetic alterations are usually introduced by mating. The somatic delivery approach described has several advantages over the more commonly used traditional knockout and xenograft models. First, it introduces genetic mutations in adult mice, avoiding confounding developmental defects and circumventing possible lethality. Prior models using the *GFAP* promoter to drive Cre expression can generate brain defects unrelated to tumor formation due to GFAP's expression in a precursor cell type. In the current model, Cre is briefly expressed only in infected, mature and quiescent cells, also avoiding any deleterious effects associated with long-term expression. Secondly, it limits the area of the mutations to a carefully selected region, allowing the issue of tumor microenvironment to be addressed. The developing tumor can be studied in the context of intact brain tissue, granting more insight into how external factors effect progression. Finally, somatic induction through viral delivery allows multiple combinations of mutations to be induced in the same pool of cells. Because of the variations between transgenic lines, finding the same expression pattern of transgene in independent lines (even under the same promoter) can be challenging. This problem is bypassed with accurate stereotaxic injections which allow the same portion of the brain to be targeted with excellent reproducibility.

Multiple mutations are required for astrocytoma initiation and progression

Clinical analysis of human brain tumor samples has shown that multiple genetic defects are present in any given case of GBM. Recently described GEM's also appear to support the hypothesis that multiple mutations are required to generate advanced disease. The current model also supports this hypothesis, with no single mutation independently leading to astrocytoma. It was found that physiological levels of $Kras^{G12D}$ were capable of inducing advanced tumors in the context of pRb inactivation with significant latency, requiring nearly a full year to produce GBM. Notably, proliferation and apoptosis were elevated in these tumors. Ras pathway activation may also be necessary for solid tumor formation, as another downstream effector (VEGF) is responsible for the angiogenesis required to support a growing mass. PTEN loss and pRb inactivation resulted in low-grade (Grade II) disease and did not progress within the time course of this study. PTEN inactivation and $Kras^{G12D}$ produced no discernible disease after a period of 9 months. However, the combination of all three events produced a localized tumor which replicated many of features seen in human GBM's. This result suggests a synergistic relationship between Ras pathway activation and PTEN loss, as these high-grade astrocytomas form much more quickly and at an increased frequency. It remains to be determined if this is a direct result inhibition of apoptosis, due to deregulation of the PI3K signaling cascade. This pathway is also responsible for regulating invasion and migration through its downstream effectors MTOR and PKCζ. This particular effect of PTEN loss can clearly be seen in the diffuse tumor margin present in the triple mutant animals.

Future Directions

Several aspects of the effects on signaling remain unaddressed. Levels of p-Erk and p-Akt have not been determined within these tumor samples. It has been shown that $Kras^{G12D}$ can also activate p-Akt, which is additionally regulated by Pten. It would seem that p-Akt does not reach saturation until Pten is completely ablated. This could be quantified through direct immunohistochemical assay for p-Akt as well as apoptotic index in $TRP^{-/-}$ brains. A decrease in apoptosis would be expected.

Another interesting question that remains to be addressed is the propensity for other regions of the brain, aside from the cortex, to undergo tumorigenesis upon focal induction of oncogenic stimuli. Cortical injections were chosen because of high transgene expression levels, and the mouse cortex most closely resembles the human cerebral hemispheres, where the majority of gliomas occur. Future studies may use this focal induction model to assess other regions of the brain for susceptibility to tumorigenesis, as well as the testing of preclinical therapies upon tumor establishment.

MATERIALS AND METHODS

Generation of transgenic mice with conditional alleles

GZT_{121} mice were produced as described(36). Offspring were screened by PCR with primers: antisense GCA TCC CAG AAG CTC CAA AG, sense GAA TCT TTG CAG CTA ATG GAC C. The PCR product is bp in length. PCR conditions are 94^{o} C for 2 minutes followed by 35 cycles of 94^{o} C 20 seconds, 62^{o} C 45 seconds,

72^{o} C 2 45 seconds, and a final extension of 72^{o} C for 2 minutes. The recombined allele (ΔZ) was detected using primers: antisense TTC ATA AAG CCC TCG CAT CCC AGG, sense GAA TCT TTG CAG CTA ATG GAC C. The PCR product is bp in length. PCR conditions are 94^{o} C for 2 minutes followed by 35 cycles of 94^{o} C 30 seconds, 64^{o} C 30 seconds, 72^{o} C 2 30 seconds, and a final extension of 72^{o} C for 2 minutes.

Conditional *Pten* alleles were developed as described(36;93). Offspring were screened by PCR with primers: antisense AAA AGT TCC CCT GCT GAT GAT TTG T, and sense TGT TTT TGA CCA ATT AAA GTA GGC TGT G. The PCR products are 435 bp (wt) and 310 bp (floxed). PCR conditions are 94^{o} C for 2 minutes followed by 35 cycles of 94^{o} C 20 seconds, 62^{o} C 45 seconds, 72^{o} C 2 minutes, and a final extension of 72^{o} C for 2 minutes.

Conditional *KRAS*$^{+/lslG12D}$ mice were generated as described by the Tyler Jacks Lab(98). Detection of the mutant *Kras*^{G12D} allele was performed by PCR genotyping using primers: antisense AGC TAG CCA CCA TGG CTT GAG TAA GTC TGC A, sense CCT TTA CAA GCG CAC GCA GAC TGT AGA. The PCR product is ?bp in length. PCR conditions are 94^{o} C for 3 minutes followed by 35 cycles of 94^{o} C 30 seconds, 60^{o} C 30 seconds, 72^{o} C 2 minutes, and a final extension of 72^{o} C for 5 minutes.

Presence of the viral DNA at the injection site was also confirmed by PCR analysis. Detection was performed by PCR analysis using primers: antisense TCTACCACACACAAGGCTACTTCCC, sense ACGAAGTTATCCTAGCCTGCAGG. The PCR product is 102bp in length. PCR conditions are 94^{o} C for 3 minutes

followed by 35 cycles of 94^{o} C 30 seconds, 60^{o} C 30 seconds, 72^{o} C 2 minutes, and a final extension of 72^{o} C for 5 minutes.

Stereotactic injection

Stereotactic injections followed standard protocols as described(77). In brief, 2-3 month old mice were anesthetized with Avertin and placed into a stereotaxic frame (KOPF). One microliter of recombinant lentivirus PTK-627 (~10^{9} particles per ml) was delivered with an infusion pump (Sage Laboratory, Orion Research) at a rate of 0.1 μl/min. Coordinates used were: (A, L, D)=0,1, and 1.2 mm from the Bregma suture. These coordinates target the cerebral cortex, a known location for mature astrocytes where there is no evidence for neural precursors in adult animals.

Tissue processing and H&E staining

Mice were euthanized in a CO_2 chamber. Brain tissue was removed and fixed in 10% formalin for 20 to 24hr, then stored in 70% EtOH. Fixed tissues were paraffin embedded and sectioned at 4-5μM. Hematoxylin and eosin staining was performed as previously described (39).

IHC staining procedure

Paraffin sections were deparaffinized with xylenes and rehydrated with ethanol/H_2O, followed by a PBS wash. For antigen retrieval, sections were treated with citrate buffer by microwave boiling for 15 minutes, then were cooled to room temperature. Slides were washed twice with double-distilled water for 5 minutes

then quenched in 3% H_2O_2 with ddH_2O for 10 min. After washing, the slides were blocked with 5% goat serum/ PBS-T (0.1MPBS, 0.5% Tween 20) for 30min at room temperature. Primary antibody was diluted in 5% serum/ PBS-T and the slides were incubated at room temperature for 1hr (GFAP staining) or at 4°C overnight for all others. Slides were washed and incubated with a 1:333 dilution of biotinylated secondary antibody (Vector Laboratories) in 2% serum/ PBS-T for 30 min. After washing, the slides were incubated with Vector's Vectastain elite AB solution, and the color developed using Vector's NovaRED substrate kit. Slides were counter stained using hematoxylin for 2min, or methyl green for 10 minutes, rinsed briefly in double-distilled water, and then rinsed in Li_2CO_3 for 1-3 seconds. After washing, the slides were dehydrated and mounted.

Primary antibodies used were: Dako Rabbit anti-GFAP 1:1000, Chemicon Mouse anti-NeuN 1:500, BD Pharmingen Mouse anti-Nestin 1:500, BD Pharmingen Moues anti-Ki-67 1:500, Calbiochem Mouse anti-SV40 1:200.

Immunofluorescence

Mice were perfused with PBS at 4^{o}C followed with 4% paraformaldehyde (PFA) in PBS at 4^{o}C. Brains were removed and post-fixed for 24 hours in 4% PFA at 4^{o}C. 40 μm sections were prepared using a vibratome. Free floating sections were washed 3 X 10 minutes in 0.1M PBS, blocked in 5% normal goat serum (Vector Laboratories) in 0.1% Triton X 100, 0.1M PBS for 30 minutes and incubated with the both primary antibodies diluted in 5% goat serum/PBS overnight at 4˚ C with shaking. Sections were washed 3 X 10 minutes in PBS and then incubated in

secondary antibodies for 30 minutes with shaking at 4˚C. Samples were mounted, coverslipped using anti-fade mounting media (Vector Laboratories Inc.) and imaged. The primary antibodies used were the same as listed above.

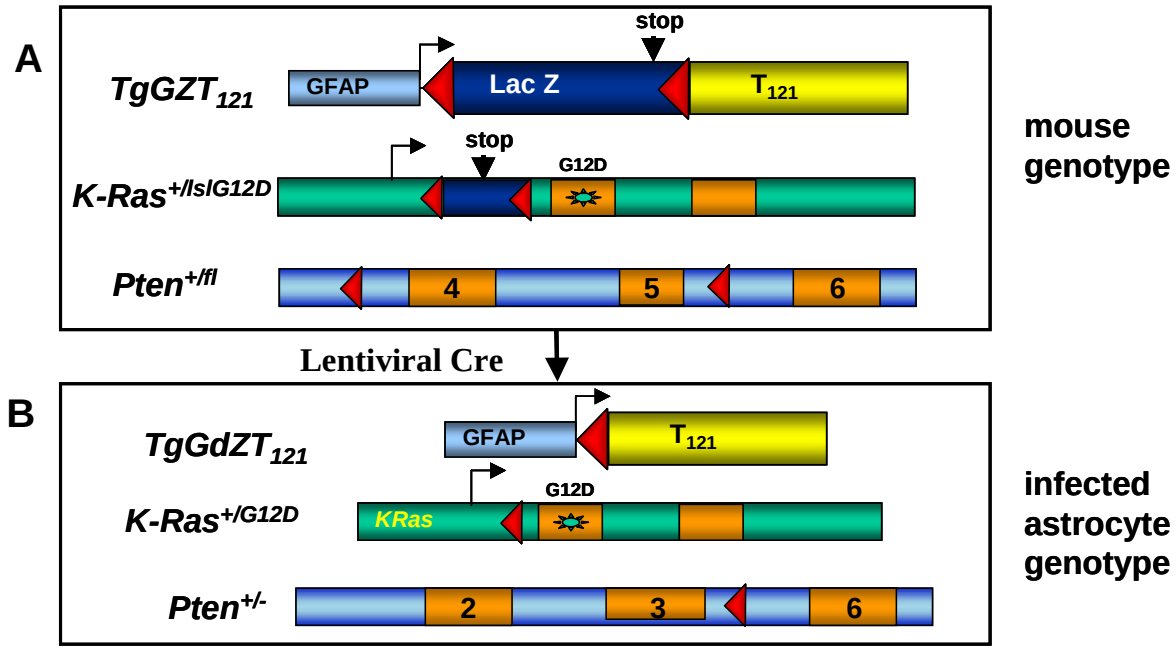


Figure 2.1 Relevant Genetic Constructs
Panel A illustrates the relevant mouse genetic constructs prior to Cre expression. In this configuration T_{121} and activated Kras are not expressed. Wildtype PTEN is produced. Panel B illustrates the genotypes after Cre-mediated recombination. T_{121} and activated Kras are expressed. The PTEN transcript is truncated and does not form a functional protein.

Table 2.1 Genotypic Abbreviations Used

Abbreviation	Genotype after Viral Recombination
T	$TgG(\Delta Z)T_{121}$
R	$Kras^{+/\Delta lsl\text{-}G12D}$
$P^{+/-}$	$PTEN^{+/-}$
$P^{+/-}$	$PTEN^{-/-}$
TR	$TgG(\Delta Z)T_{121}$:$Kras^{+/\Delta lsl\text{-}G12D}$
$TP^{+/-}$	$TgG(\Delta Z)T_{121}$:$PTEN^{+/-}$
$TP^{-/-}$	$TgG(\Delta Z)T_{121}$:$PTEN^{-/-}$
$TRP^{+/-}$	$TgG(\Delta Z)T_{121}$:$Kras^{+/\Delta lsl\text{-}G12D}$:$PTEN^{+/-}$
$TRP^{-/-}$	$TgG(\Delta Z)T_{121}$:$Kras^{+/\Delta lsl\text{-}G12D}$:$PTEN^{-/-}$

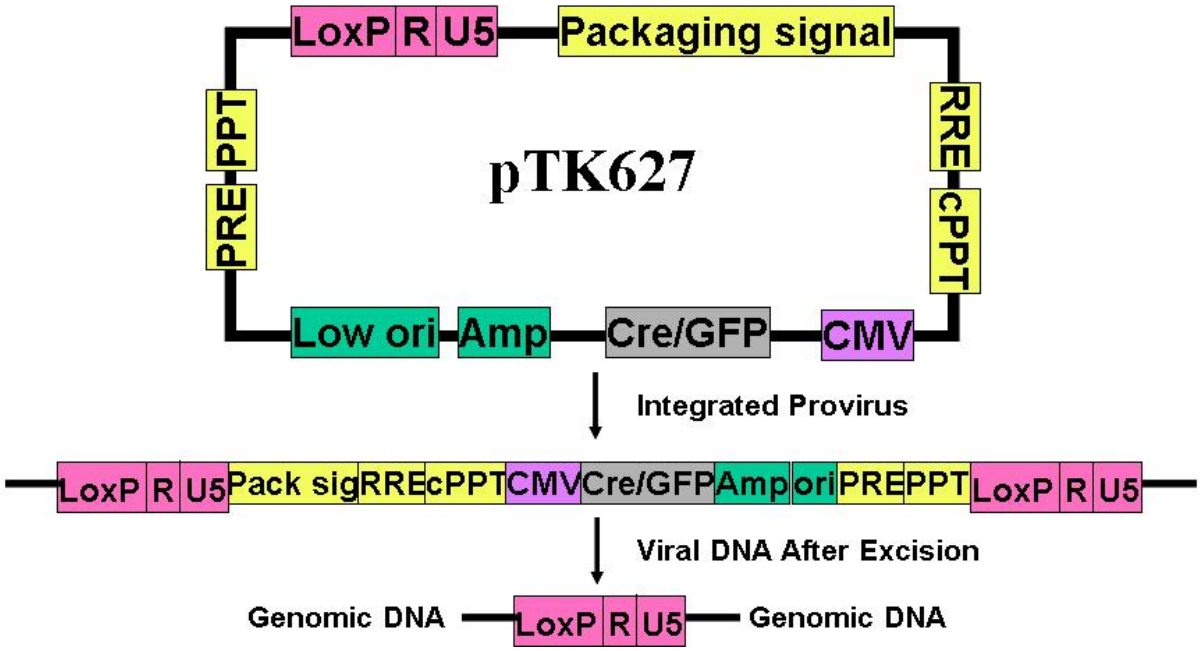


Figure 2.2 PTK-627 lentiviral structure
The top portion of the panel shows the plasmid used as part of the 3 plasmid transient transfection system in the host packaging cell line. The cell line produces replication incompetent VSVG pseudotyped virus that may be concentrated to very high titers. The middle portion illustrates the integrated provirus in the host genome following reverse transcription and duplication of the LTR. The bottom portion indicates the remaining exogenous sequences in infected cells following a brief period of Cre expression.

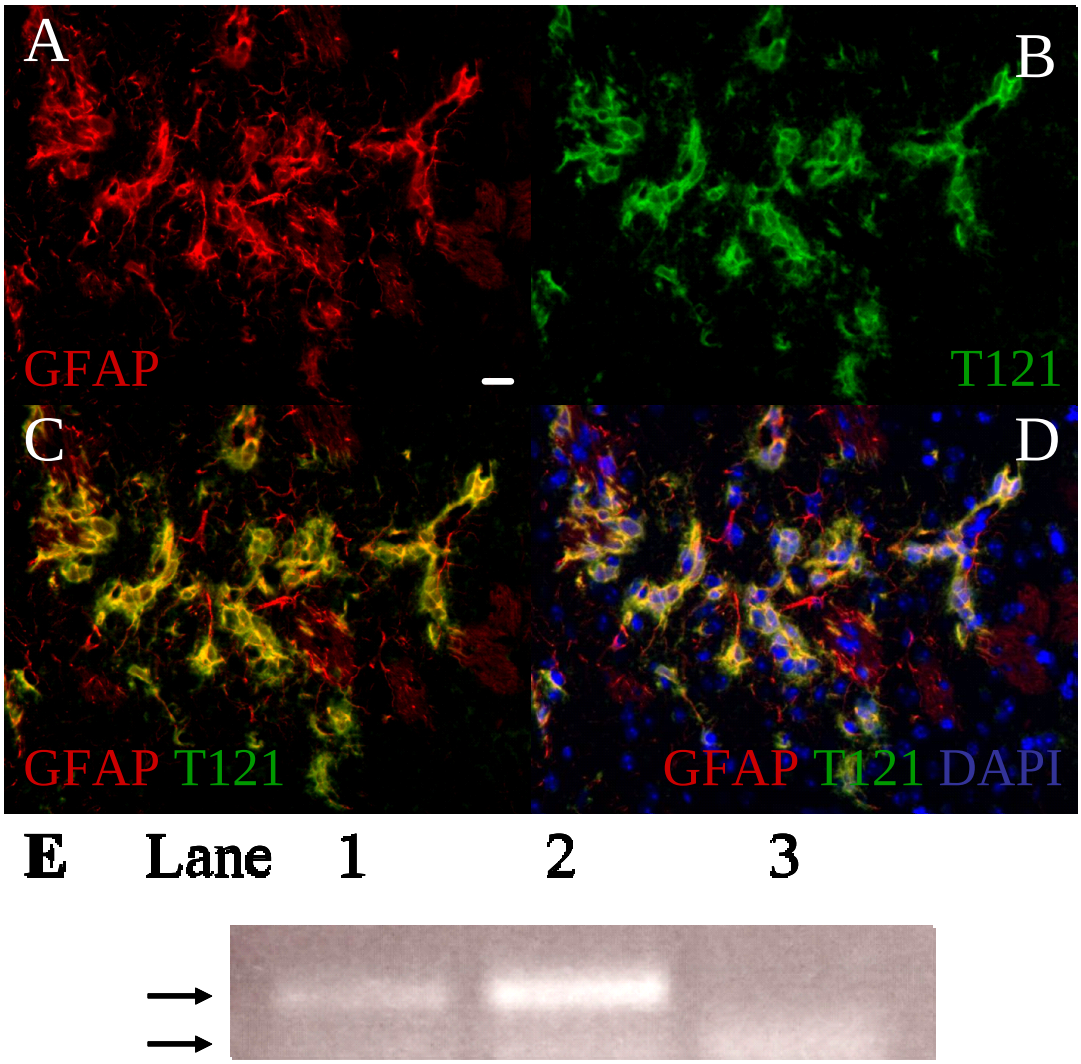
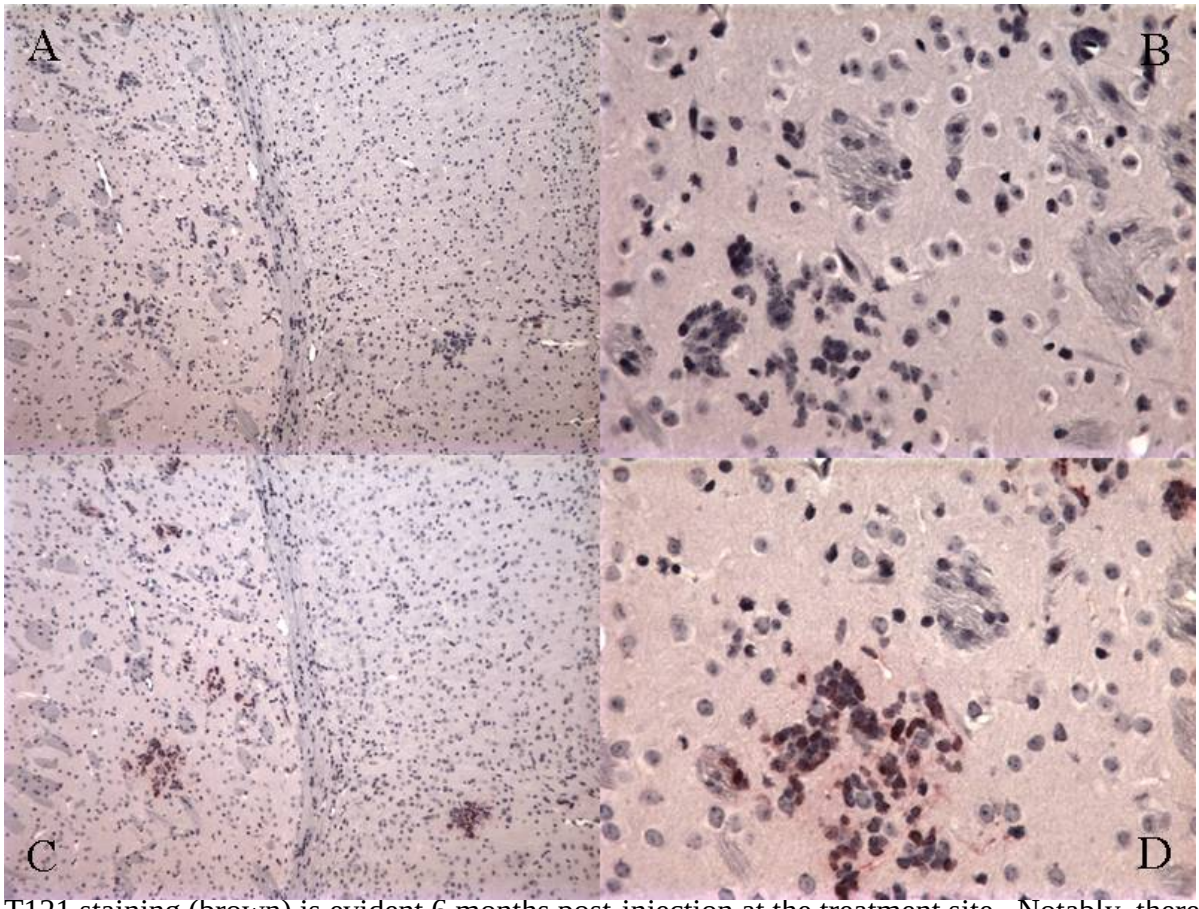


Figure 2.3 T_{121} Expression is Restricted to Astrocytes
GFAP intermediate filament expression in the adult cortex is a marker of adult astrocytic phenotype (A). T121 protein expression is specifically localized only in cells which also express GFAP (C), indicating appropriate regulation of the transgene in our target cells 19 days post-injection. T121 expression was not found in any location in the brain other than the injection site. Panel E demonstrates PCR evidence of viral DNA at the injection site. Upper arrow indicates 102 bp viral recombination product. Lower arrow indicates primer dimer band. Lane 1 is DNA from an induced GZT121 animal. Lane 2 is DNA isolated from a TRP tumor. Lane 3 is DNA isolated from the same animal, but derived from the untreated hemisphere. Scale bar = 20 μm.



T121 staining (brown) is evident 6 months post-injection at the treatment site. Notably, there are several clusters of positive cells up to 2mm away from the site of injection. Panel **A**: 100x magnification T_{121} negative control **B**: 400x magnification T_{121} Negative control **C**: 100x magnification T_{121} **D**: 400x magnification T_{121}.

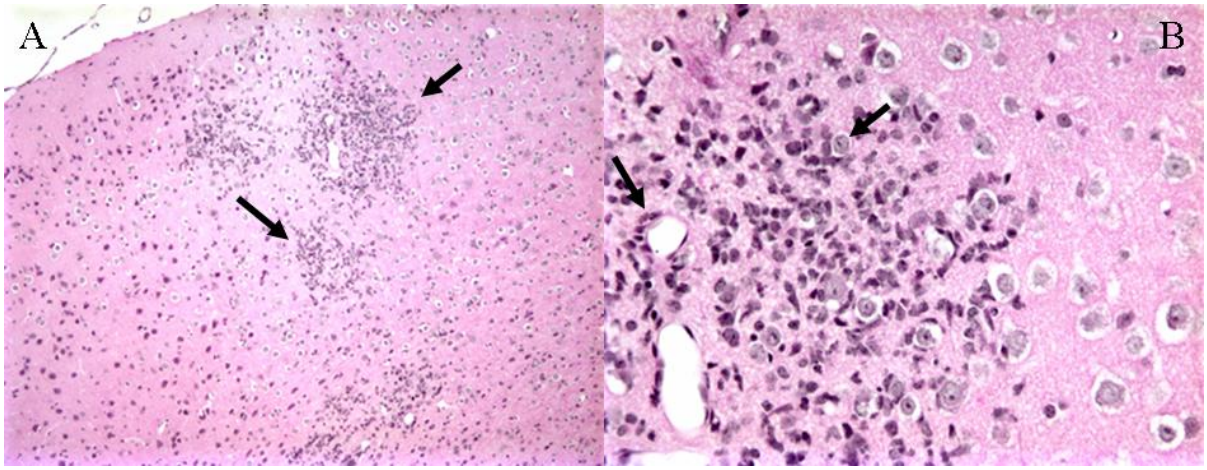


Figure 2.5 RB loss and PTEN Reduction Results in Focal Hyperplasia
Panel A is a low power image (100x magnification) demonstrating localized cell clusters, indicated by arrows, appearing 6 months post-injection in $TP^{+/-}$ animals. Panel B is a high power image (400x magnification) showing specific secondary structures of astrocytoma, perivascular and perineuronal satellitosis (arrows).

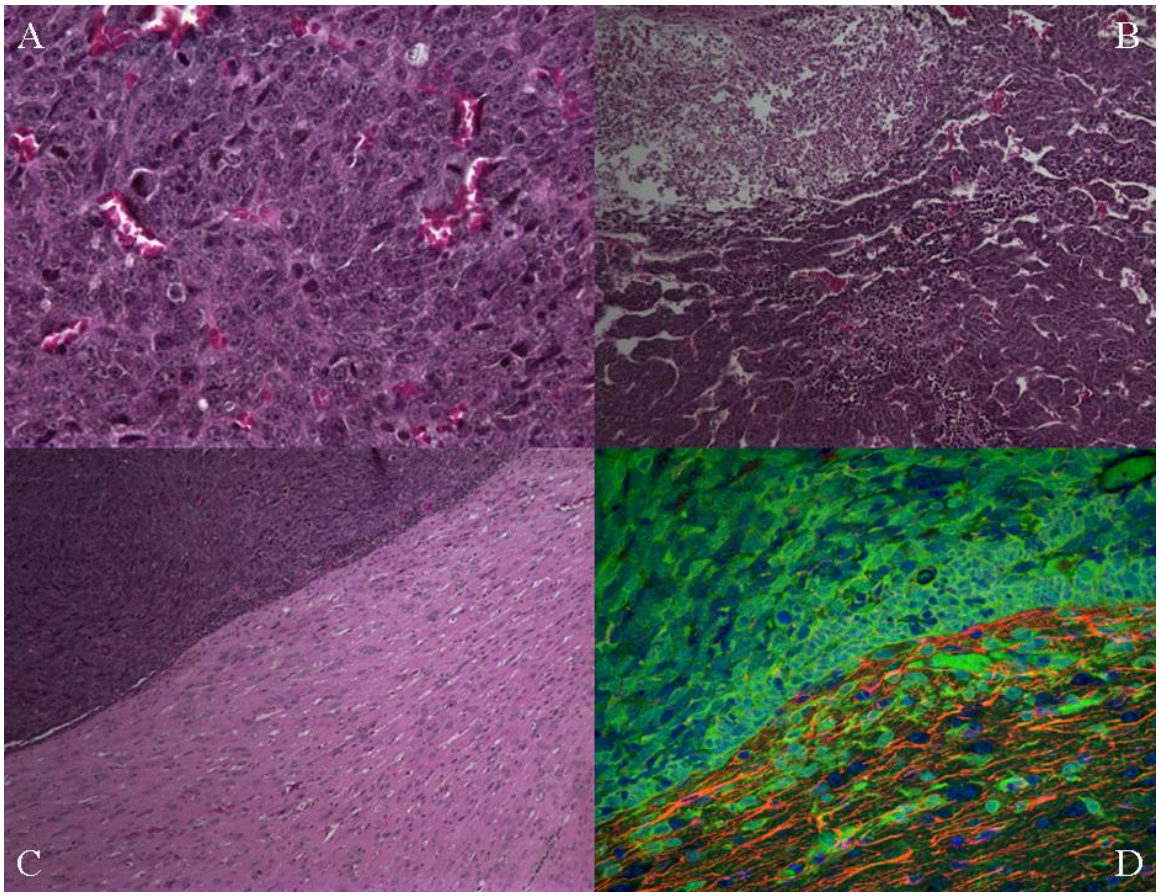
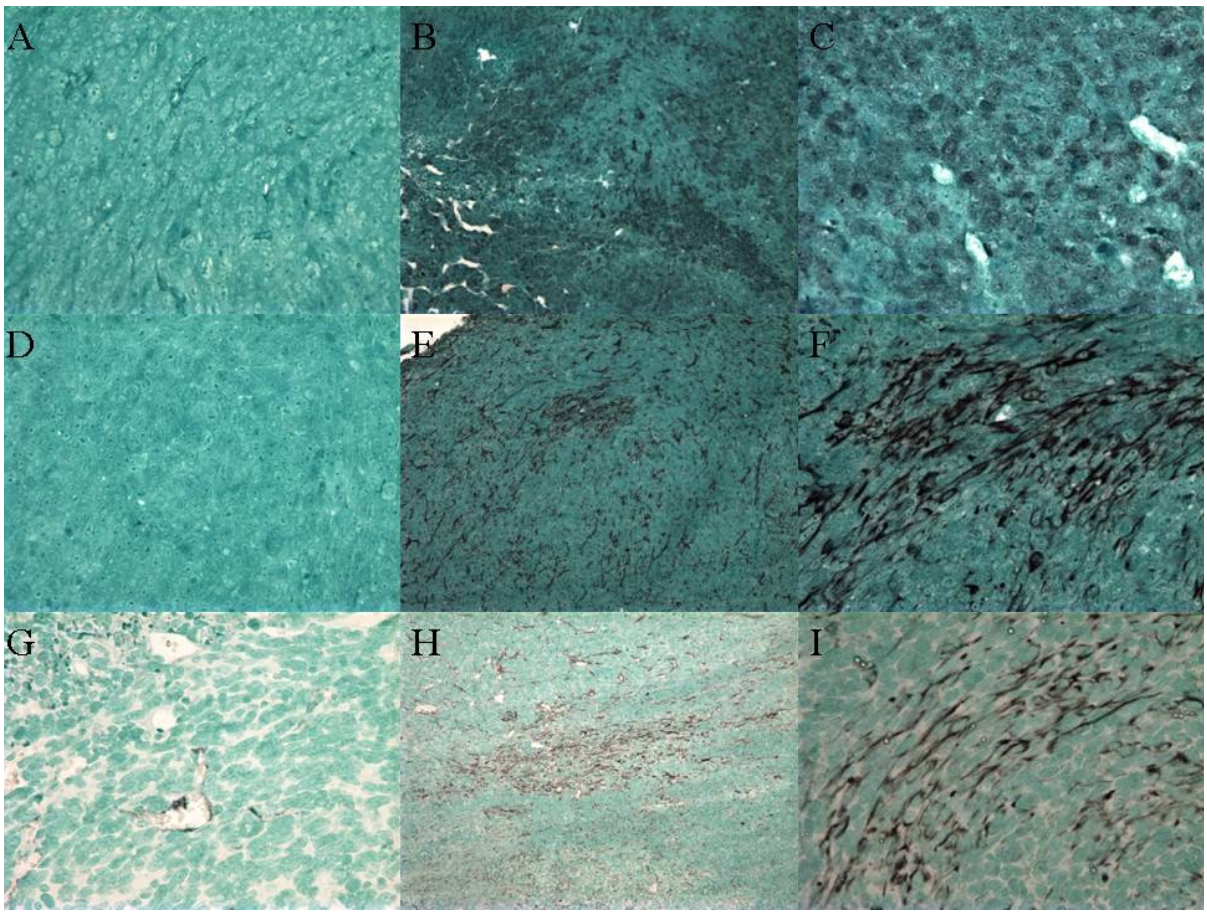


Figure 2.6 Features of TR GBM's
Panel A is a 400x magnification showing abnormal vasculature, including vasodilation. Several mitotic cells are visible. Panel B is a 100x magnification showing a large area of necrosis next to several foci of incipient necrosis. Panel C is a 100x magnification illustrating the apparently sharp tumor margin in contact with normal brain parenchyma. Panel D is a 400x magnification demonstrating invading tumor cells at the margin of the lesion. T_{121} is pictured in green, GFAP is pictured in red and DAPI is pictured in blue.



TR GBM's are immunohistochemically positive for T_{121} as well as other markers associated with human GBM, including Nestin and GFAP. Panel **A**: 100x T_{121} negative control **B**: 100x T_{121} **C**: 400x T_{121} **D**: 100x GFAP negative control **E**: 100x GFAP **F**: 400x GFAP **G**: 100x Nestin negative control **H**: 10x Nestin **I**: 400x Nestin.

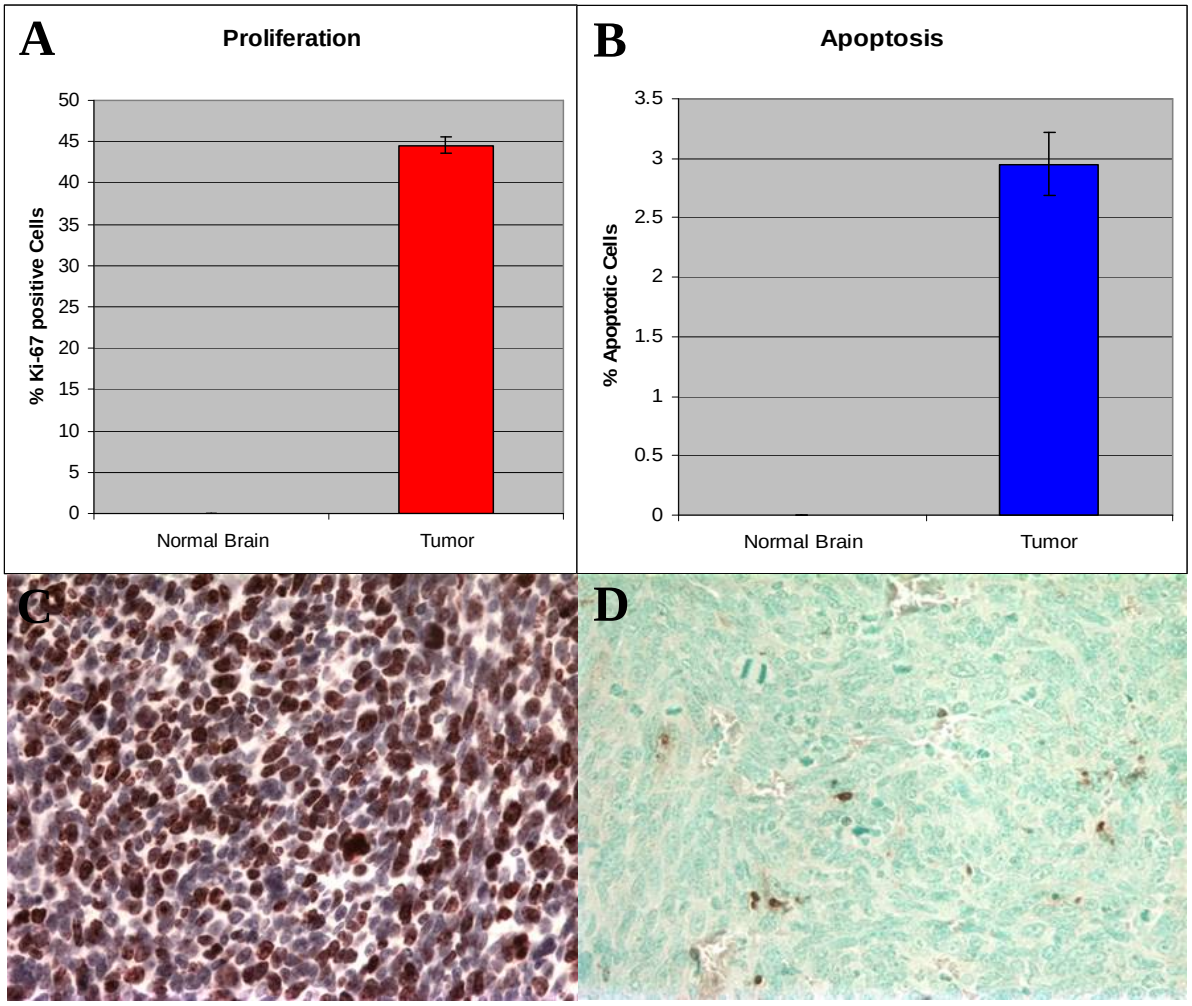


Figure 2.8 Proliferation and Apoptosis in TR animals
Proliferation was evaluated by Ki-67 immunoreactivity within the tumor. >44% of cells were positive for this marker (**A** and **C**). In contrast, normal adult mouse brain has virtually no proliferating cells outside of specific compartments. Apoptosis was also elevated within the tumors as evaluated by TUNEL assay (**B** and **D**).

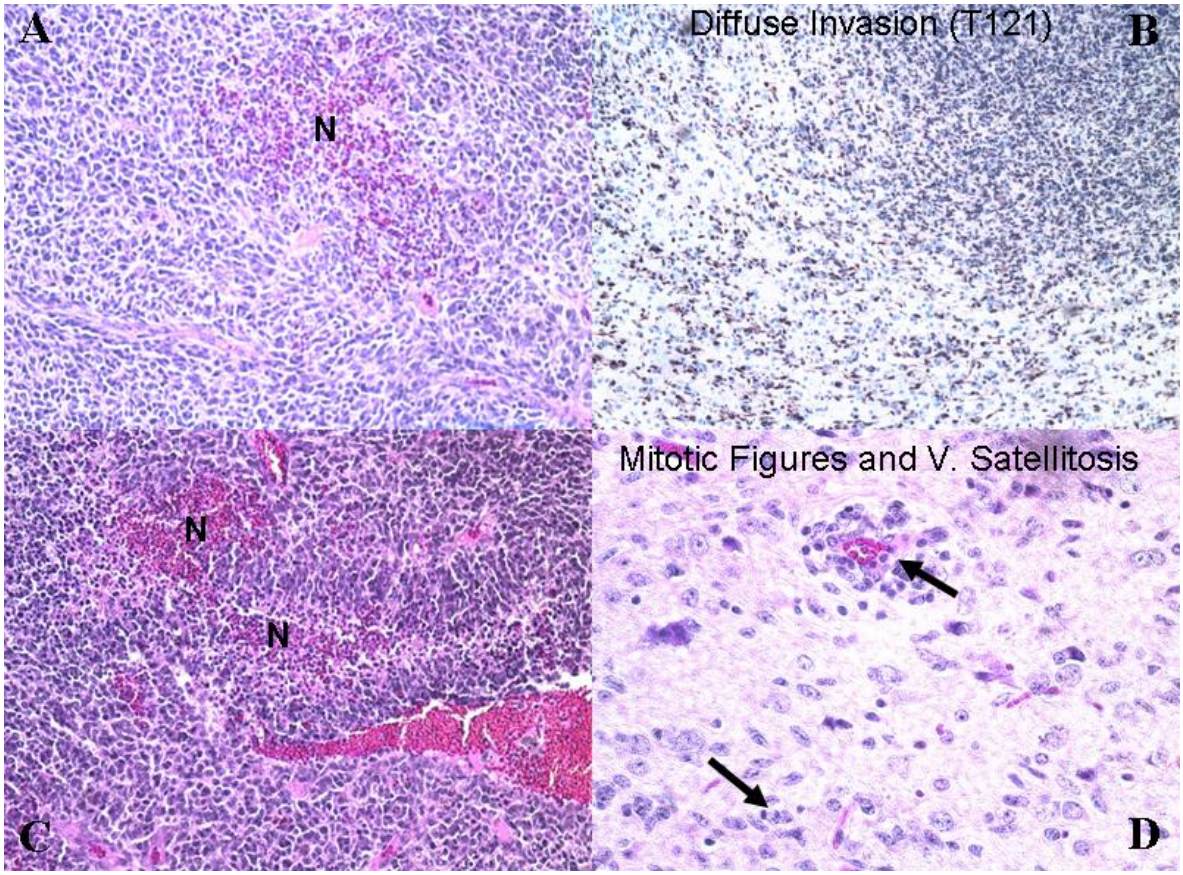


Figure 2.9 Features of GBM observed in terminal stage TRP Animals
Panels **A** and **C** show regions of necrosis within the tumor mass. Panel **B** shows the diffusely infiltrating nature of these tumors, T_{121} positive cells in brown can be seen outside the main tumor mass. Panel **D** shows mitosis and perivascular satellitosis occurring outside the main tumor mass.

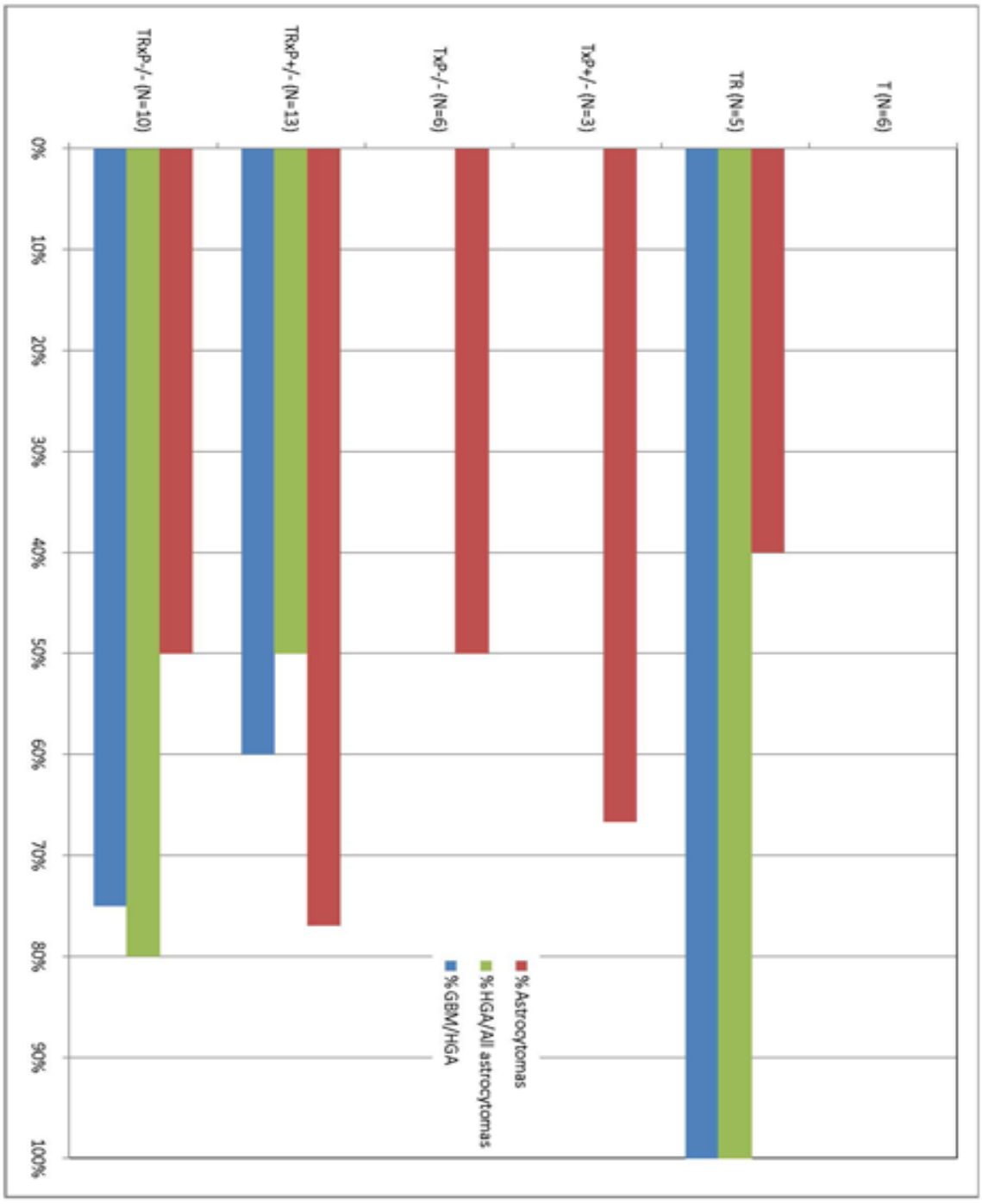


Figure 2.10 Tumor Incidence
Tumor frequency in all genotypes. Red bars denote percentage of astrocytomas in all treated animals. Green bars denote percentage of High-grade astrocytomas out of all astrocytomas. Blue bars denote percentage of GBM's out of all High-grade astrocytomas.

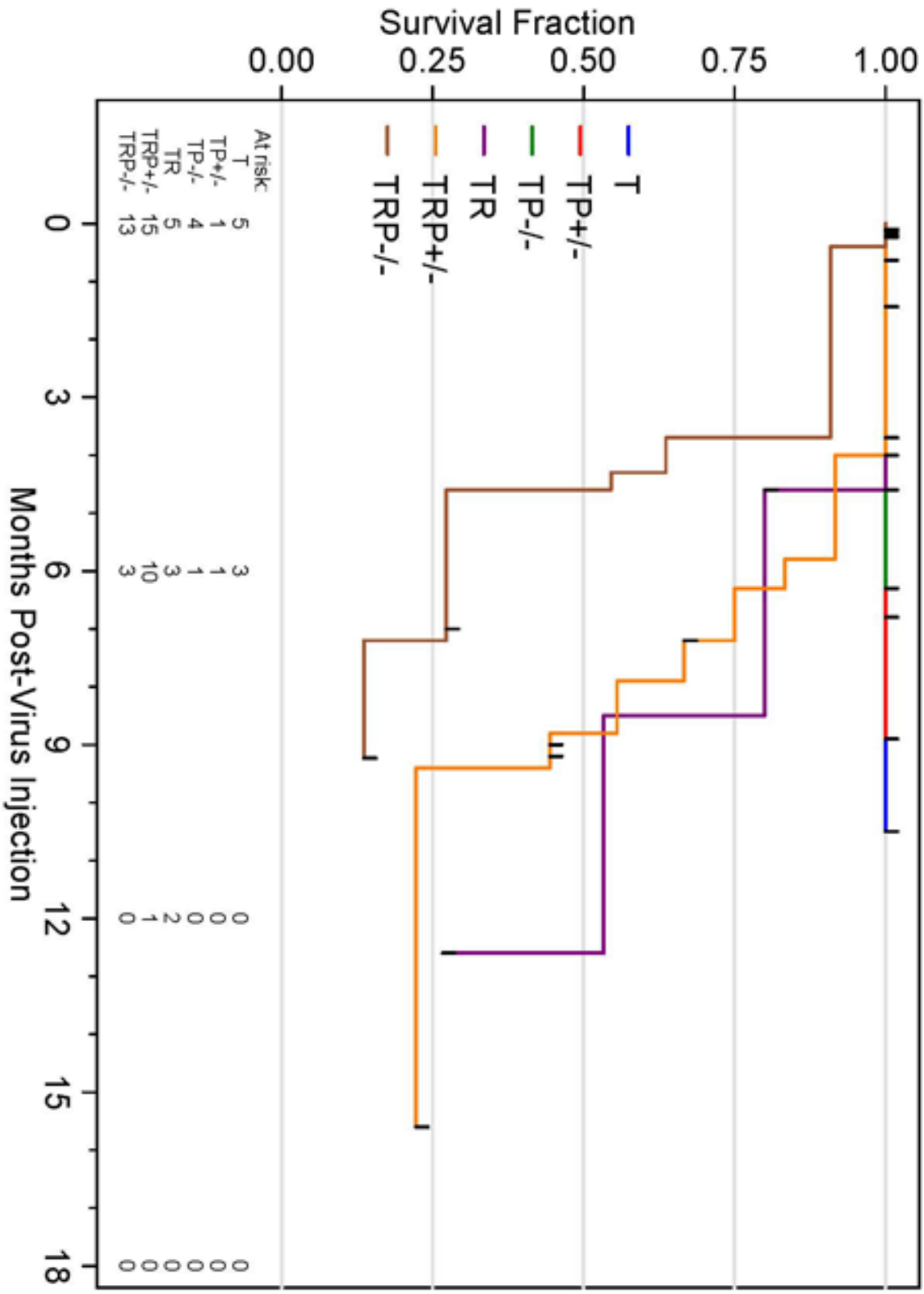

Survival Fraction
0.00
0.25
0.50
0.75
1.00
T
TP+/-
TP-/-
TR
TRP+/-
TRP-/-
At risk:
T 5 3 0 0
TP+/- 1 1 0 0
TP-/- 4 1 0 0
TR 5 1 2 0
TRP+/- 15 3 1 0
TRP-/- 13 3 0 0
0
3
6
9
12
15
18
Months Post-Virus Injection

Figure 2.11 Tumor Free Survival
Kaplan-Meier survival curves of all relevant GEM. Survival steadily decreases with the addition of oncogenic events. Notably T and TP animals did not develop terminal disease over the course of the study. TR animals required nearly a year to develop GBM. The addition of PTEN heterozygosity reduced latency to 6.5 months. PTEN knockout further decreased survival to 4.5 months.

Table 2.2. Somatic induction of astrocytomas in conditional GEM

Genotype	Mice (N)		Incidence (%)	HGA (%)	GBM (%)	Overall Survival With HGA (mean ± SEM)
	Injected	Developed Astrocytomas				
T	6	0	0	0	0	N/A
$TP^{+/-}$	3	2	67	0	0	N/A
$TP^{-/-}$	6	3	50	0	0	N/A
TR	5	2	40	2 (100)	2 (100)	322 ± NA
$TRP^{+/-}$	13	10	77	5 (50)	3 (60)	201 ± 31
$TRP^{-/-}$	11	7	64	5 (71)	3 (60)	131 ± 25

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