Genetic Modeling of Glioma Formation in Mice

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In addition to the histological features that define gliomas, mutations and other alterations in gene expression and signal transduction are classically found in these tumors. Some of these alterations are likely to be the effects of the neoplastic phenotype, while others may be causative agents essential to the etiologic origin of the disease. The determination of whether specific genetic alterations, either individually or in combination, can serve as the etiology of gliomas requires modeling in animals with the fulfillment of Koch's postulates. Animal modeling studies not only provide information on the potential causes of glioma formation, they also identify novel candidate targets for therapy and provide tumor-bearing animals for preclinical trials. Recently, remarkable strides have been made in the generation of mouse models of the diffuse gliomas that provide unparalleled opportunities for advancing our knowledge of the etiology, maintenance, and treatment of this lethal class of tumors.

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Introduction

Glial tumors are the most common primary brain tumors in humans and are traditionally categorized based on their histological features into several groups, with the majority exhibiting either astrocytic or oligodendroglial differentiation characteristics. Both classes of glioma can appear in either a high-grade (malignant) or low-grade form. In addition, mixed tumors with respect to both lineage and grade also occur.

The only proven environmental cause for glioma formation is ionizing radiation, which was demonstrated in follow-up studies of patients who received treatment for ALL, craniopharyngioma, or pituitary adenoma during childhood (121). In addition, patients with certain enzyme deficiencies are particularly susceptible to develop gliomas after exposure to certain chemicals (33, 91). There are also specific genetic syndromes, such as neurofibromatosis I and II, Li-Fraumeni and Turcot's syndrome, in which patients are predisposed to develop glioblastoma (46, 61, 67). Some familial gliomas are related to mutations in *TP53*, *CHK2*, or the $p16^{INK4A}/p14^{ARF}$ locus (9, 46, 110); others exist in the absence of a known genetic syndrome (42, 110), and presumably represent inherited mutations in genes not yet linked to glioma formation. These familial gliomas represent only a small fraction of all patients with gliomas; the vast majority of gliomas are sporadic.

The etiology of non-familial glial tumors is unknown. Over the last few years a number of somatic mutations have been identified that are common for each subtype of glioma. Of note, some of the mutations are the same as those found in the rare familial syndromes; for example, mutations in the *TP53* gene, the same gene that is altered in Li-Fraumeni syndrome, are found in some sporadic glioblastomas. Findings such as these serve to strengthen the link between glioma biology and specific genetic mutations.

Most of the genes found to be mutated in gliomas, such as *PTEN*, *TP53*, or $p16^{INK4A}/p14^{ARF}$, are also found to be mutated in other cancers and do not represent glioma-specific alterations. The association of genetic alterations with histological features in human tumors is descriptive in nature and does not prove or imply a causal nature for the mutations with respect to the tumors in which they are found. However, direct evidence for the involvement of specific genes in the formation of gliomas may be provided by experimental models in which genes are introduced into, or ablated from, animals with defined genetic backgrounds.

Description of Test Systems

Multiple methods have been employed to generate alterations in genes and gene expression that lead to the formation of brain tumors in experimental animals. The characteristics of these systems are summarized in Table 1.

Chemical carcinogenesis. Some of the first animal modeling systems for brain tumors involved treatment of test animals with DNA alkylating agents such as nitrosurea derivatives (23, 109). In many cases the histology of these tumors is comparable to that in humans. However, because identification of the critical causative genes that are mutated in these systems is nearly impos-

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Strategy	Principle	Primary genetic modification	Cell of origin	Secondary mutations
Mutagens	DNA alkylation	Unknown	Unknown	Likely
Transplantation	Xeno- or allografts, immunodeficient animals	Unknown	Unknown	Less likely
Germline genetic modification	Transgene or gene targeting	Known	Unknown	Likely
Somatic genetic modification	Replication competent retrovirus	Known	Unknown	Likely
	Replication deficient retrovirus	Known	Known	Less likely

Table 1. Summary of strategies used to model gliomas.

sible, it is difficult to derive useful genetic information from them. The development of techniques to identify and clone certain genes, as well as methods in developmental biology that allow the transfer or disruption of genes in the murine germline, have permitted the establishment of mouse tumor models.

Germline gain-of-function strategies (transgenic mice). Using DNA transfer in the male pronucleus of the zygote, transgenic mice can be generated. These mice grow up with an added gene superimposed on a clearly defined genetic background. The gene, or cDNA, is placed under the control of a tissue-specific promoter/enhancer. Thus, the mice, as well as their offspring that inherit the gene in a Mendelian fashion, will express the gene in a defined spatial-temporal pattern during development. The engineered expression of certain genes or hybrid-genes in specific cell types can be lethal during early development. However, for those animals that develop normally, the transgene will be expressed in all cells that utilize the transgene promoter.

Germline genetic disruption (knockout mice). In this model system a gene of interest is disrupted by homologous recombination in embryonic stem cells, which are then transferred into blastocysts to generate chimeric mice. Once these lines are bred true, the offspring of the chimeric mice will carry the embryonic stem cell genotype. Using this method, several mouse strains have been generated that are of particular interest to neuro-oncologists: *PTEN*, $p16^{INK4a}$-$p19^{ARF}$, *TP53*, and *RB* deficient mice. Homozygous deletions for certain genes, eg, *PTEN*, cause embryonic lethality (83, 108). Thus, adult animals with homozygous disruptions in genes that are required during embryonic development can not be obtained. Furthermore, constitutive gene disruptions in certain genes, e.g., PTEN in the heterozygous state, lead to the development of tumors in other organs, such as lymphomas or sarcomas, that limit the animal's lifespan before the development of gliomas. In addition, many genes in mammalian organisms are members of multigene families, which might provide functional redundancy. Thus, crosses designed to generate deletions in multiple genes are required in order to obtain mice with a complete absence of a certain gene function. Furthermore, all cells in the body carry the same mutation and, in the process of tumor progression, may acquire additional mutations.

The expression of genes through transgenic mice and gene disruption in mice make it difficult to study whether certain genes are sufficient for tumorigenesis for several reasons. Firstly, a certain gene might be essential for development, and high expression or knock-out might lead to embryonic lethality. Secondly, the use of transgenic mice or gene disruption in stem cells generates mice in which all cells carry the mutation of a certain gene. These mutations might therefore alter the gene expression and biology of a multitude of cells, thus making interpretation difficult. Thirdly, because all cells carry a certain mutation, new mutations may be acquired during development. The identification of secondarily acquired mutations and the determination of whether they are required for tumorigenesis is cumbersome and often problematic.

Somatic cell gene transfer using retroviral vectors. A complementary approach to germline modification has been developed. These strategies are based on the transfer of genes to cells postnataly with viral vectors. Replication-competent Moloney murine leukemia virus (119) has been used as an expression vector in mice. This virus targets a variety on replicating cell types. However, because the virus is replication-competent, it spreads throughout the tissue and infects many cells, thereby increasing the chance of generating cooperating mutations via insertional mutagenesis. Furthermore, Moloney-based vectors exhibit a broad cell type host range and tumors of various histologies may be derived

from different cells of origin. In contrast, the replication-deficient avian leukemia virus (ALV) has high selectivity. This model system exploits the entry of ALV via a defined receptor. The cDNA of the receptor has been cloned and introduced into the germline of mice under the control of tissue-specific promoters, such as the *nestin*, *GFAP*, or *β-actin* promoters. Thus, the receptor will be expressed in defined cell types; for example, either in neural progenitor cells (*nestin* promoter), astrocytes (*GFAP* promoter), or in all tissues (*β-actin* promoter). Only the cells that express the virus receptor are susceptible to infection. This is carried out either by direct injection of virus in a vector suspension (RCAS, Rous sarcoma derived replication competent cloning vector) or by injection of a chicken cell line that produces the virus into a particular tissue at a certain time of development. These injected cells are very sparse 2 days after injection and not detectable 7 days after injection (52). In this system, somatic mutations can be generated; however, the cassette size of the RCAS vector is limited to 2.5 kb. Furthermore, only a small number of cells are infected. Thus, genetic alterations that are not sufficient for transformation will not be scored because of the low probability of acquiring secondary mutations. The RCAS/*tv-a* system therefore restricts mutations to those designed in vitro. RCAS vectors carrying various oncogenes, marker genes, and recombinases have been generated (35; for a detailed discussion of the system and a complete listing of available RCAS vectors, see *http://rex.nci.nih.gov/RESEARCH/basic/varmus/tva-web/tva2.html*).

This system has been used successfully to determine that astrocytes serve as stem cells for neurons in the adult brain. Thus, RCAS vectors might be used to express genes in the neural lineage as well.

Pathways Contributing to the Formation of Gliomas: Signaling and Cell Cycle Control

Signaling molecules relevant to gliomagenesis have been identified through screening for mutations in gliomas and through basic research on the cell and developmental biology of the nervous system. Mutations occurring in gliomas frequently affect receptor tyrosine kinases (RTK) and their downstream pathways, as well as cell cycle regulatory proteins. For example, *EGFR* is amplified or mutated in 30 to 50% of human glioblastomas (126, 127). Other mutations affect *PDGF/PDGFR* (36), *IGFR* (95), *C-MYC* (116) and *PTEN/MMAC1* (68), and cell cycle regulatory pathway components such as *CDK4* (94), *CDK6* (22), *cyclin D1* (14), *MDM2* (87, 88), INK4a-ARF ($p16^{INK4A}/p14^{ARF}$) locus (78, 97), *TP53* (122,123), and *RB* (55,118).

Receptor tyrosine kinases and their pathways. Receptor tyrosine kinases (RTKs) are known to activate the RAS/MAP kinase, the AKT pathway, and protein kinase C (PKC). For example, EGFR-mediated mitogenesis requires ligand-driven dimerization of receptor monomers, tyrosine kinase activation, tyrosine phosphorylation of the receptor, and signaling through coupling and adapter molecules such as Sos, Grb2, and Shc to activate PLC-γ (phospholipase C), Ras/MAPK, or STAT (for review, see 96). The pathway is attenuated by receptor-ligand internalization and lysosomal breakdown. Mutations of Ras have not been found; however, increased activity of Ras-GTP was identified in high-grade astrocytomas (44). Activation of the Ras pathway also includes the NF1 pathway (for review, see 19). The gene product of *NF1*, neurofibromin, regulates Ras activity. Neurofibromin shares homology with the catalytic domain of the mammalian p120Ras GAP and an extended similarity with the Saccharomyces cerevisiae Ras-GAP proteins IRA1 and IRA2 (5). GAP proteins (GTPase activating proteins) inhibit low molecular weight G proteins such as Ras by stimulating their intrinsic GTPase activity through the hydrolysis of GTP to GDP (71), thus inactivating Ras. The absence of NF1 leads to increased activity of Ras. In fibroblasts, PDGF-mediated Ras/ERK activation leads to the induction of cyclin D1 and a decrease in $p27^{kip1}$, a G_1 cyclin-dependent kinase inhibitor (124). It is, however, unknown whether this pathway is active in astrocytes or gliomas.

AKT, on the other hand, is activated through another route. RTK-induced activation of PI 3-kinase (phosphoinositide-3-kinase) leads to the generation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. These membrane-bound lipids activate PDK1 and PKB (also called AKT). Whereas PI-3 kinase activates AKT, PTEN is an inhibitor of AKT. The gene encoding PTEN is deleted in a large percentage of high-grade astrocytomas as well as in other malignant tumors (68). The deletion of *PTEN* leads to increased activity of AKT in these tumors (66, 85, 129). PTEN is a protein phosphatase (68) and a 3′ phosphoinositol phosphatase (70, 76). AKT regulates many biologic events, such as transcription and translation, invasion and migration, apoptosis and survival, cell cycle control, and angiogenesis. PTEN modulates cell migration and invasion through dephosphorylation of focal adhesion kinase (FAK) (111) and hydrolysis of PtdIns(3,4,5)P_3, the product of PI 3-kinase. PtdIns

(3,4,5)P_3 is required for activation of AKT serine/threonine kinase activity, which itself targets various downstream targets. These downstream targets include the mammalian target of Rapamycin (mTOR; 2, 84), which activates ribosomal S6kinase, altering the ability of the ribosome to translate specific mRNAs, and is found to be deleted in a variety of human tumors. Other pathways of AKT include inactivation of the proapoptotic genes caspase 9 (16) and BAD (25), which promote apoptosis by dimerizing and inactivating bcl-2, and the forkhead transcription factor, which in its phosphorylated form associates with the 14-3-3 protein and is retained in the cytoplasm, but in its unphosphorylated form translocates to the nucleus and promotes apoptosis by activation of the Fas Ligand (12) and cell cycle control through $p27^{kip1}$ (73).

Other targets of AKT include NF-κB, GSK3 (glycogen synthase kinase-3), and phosphofructokinase regulating transcription, gluconeogenesis, and glycolysis. Finally, AKT has been shown to induce VEGF under hypoxic conditions (72). Glioblastoma cells, in contrast to primary human astrocytes, display high AKT activity and high levels of the PI 3-kinase products PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (45). Expression of wild-type PTEN, but not mutant forms of PTEN, reduce the levels of the 3′ phoshoinositides and inhibit AKT activity in glioblastoma cells. There is yet another route through which ATK acts: EGF, PDGF, or FGF receptor activation, leading to binding of PLC-γ through its SH2 domain to a specific phosphotyrosine in the C-terminus of the receptor tyrosine kinase. PDGF-induced activation of PLC-γ also requires PI 3-kinase products. Tyrosine phosphorylation and membrane translocation of PLC-γ is dependent upon PI 3-kinase products. The translocation of PI 3-kinase and PLC-γ to the membrane is essential for their activation, since PtdIns(4,5)P2 is the substrate of the 2 enzymes and is located in the cell membrane. PLC hydrolyzes PtdIns(4,5)P_2, generating 2 second messengers, diacylglycerol and Ins(1,4,5)P_3. Diacylglyerol is an activator of protein kinase C (PKC), and Ins(1,4,5)P_3 leads to the mobilization of intracellular Ca++ pools that is required for the activation of the conventional isoforms of PKC. The expression of various PKC isoforms has been shown in astrocytomas, and inhibitors of PKC decrease growth and increase apoptosis in human glioblastomas in cell culture and in xenografts (7, 8).

Cell cycle regulation. Multiplication of cells requires duplication of DNA, which occurs during S-phase, and cell division, which occurs during M-phase. During G_1 phase, extracellular cues determine whether the cell replicates DNA and divides or, alternatively, enters a quiescent state (G_0). The time point at which the decision is made to enter S-phase is called the "restriction point" and is usually late in G_1-phase (82). There is a second restriction point at the G_2/M transition. The ordered transition from G_1 to S-phase and from G_2 to M-phase is mediated by serine/threonine kinases called cyclin-dependent kinases (CDKs). The activity of the CDKs is regulated by cyclins. These holoenzymes contain a regulatory component (cyclin) and a catalytic (cdk) component plus other proteins that form a complex. For example, the transition through G_1 requires the activation of CDK4 and CDK6 by cyclin D, the G_1/S transition point requires CDK2 activation by cyclin E, the transition through S-phase requires CDK2 activation by cyclin A, and the G_2/M transition requires CDK1 (CDC2) activation by cyclin B.

The $p16^{INK4A}$/cyclin D/CDK4/RB/E2F pathway. There are negative regulators of the cyclins, termed cyclin-dependent kinase inhibitors (CKIs), that include the INK4 proteins (Inhibit cdk4), which inhibit the cyclin D-dependent kinases, CDK4 and CDK6, and $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, which inhibit cyclin E-CDK2 and cyclin A-CDK2. There are four INK4 proteins: $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ (for review, see 101). p16 and p15 are both located on chromosome 9, p18 is on chromosome 1, and p19 is on chromosome 19. The substrate for the cyclin D-activated kinases and CyclinE-cdk2 is the Rb protein, which is the gene product of the retinoblastoma locus. When Rb is hypophosphorylated, it forms a complex with the transcription factor E2F that is inactive. After phosphorylation of Rb, E2F is released from the complex and mediates the transcription of S-phase specific genes. E2F has also been shown to induce the expression of the anti-apoptotic genes BCL-2 and $p21^{WAF1/Cip1}$ in gliomas (41). Increased levels of $p21^{WAF1/Cip1}$ have been reported in gliomas (57), as well as high levels of BCL2 in gliomas that carry wild type *TP53* (80,1). $p21^{WAF1/Cip1}$ inhibits CDK1(CDC2) and CDK2, but activates the cyclin D/CDK4 kinase complex (65,18).

The $p19^{ARF}$/MDM2/p53 pathway. P53 acts at the G_1/S transition point as an inducer of $p21^{WAF1/Cip1}$ as well as during the G_2/M transition. In addition, p53 mediates the induction of apoptosis after DNA damage. The activity of p53 is inhibited by MDM2, which itself is inactivat-

ed by $p19^{ARF}$ (alternate reading frame). MDM2 (originally isolated from mouse double minute chromosomes) binds to the transactivation domain of p53 and activates ubiquitin ligase, thus initiating the proteosomal destruction of p53. The transcription of MDM2 is activated by p53 itself (128). Thus, there is a p53-MDM2 feedback loop. $p19^{ARF}$ inhibits MDM2. On the other hand, MDM2 is activated by Ras through the Raf/MEK/MAP kinase pathway (93). $p19^{ARF}$ binds to MDM2, sequesters MDM2 in nucleolar structures, and allows accumulation of p53 (102, 112). $p14^{ARF}$ (the human homolog of $p19^{ARF}$) is induced by E2F, myc, and Ras (6, 81, 132). Thus, the Ras/Raf/MEK/MAP kinase pathway can act indirectly through CDK4/cyclin D, and the phosphorylation of pRB and release of E2F-1 can lead to accumulation of $p14^{ARF}$ and inhibition of MDM2 (93, 101).

Thus, $p14^{ARF}$ links the pRB and p53 pathways (93, 101). Human gliomas display mutations in these pathways. Some familial gliomas, for example, have been ascribed to germline mutations of *TP53* and of the $p16^{INK4a}/p14^{ARF}$ locus (64, 77, 110). The melanoma and nervous system tumor syndrome—an autosomal dominant inherited disorder in which astrocytomas, neurofibromas, schwannomas, and meningiomas are seen in the absence of mutations of NFI—has been associated with deletions or mutations of $p16^{INK4A}$ and $p14^{ARF}$ (4), and with deletions of $p14^{ARF}$ in a setting of intact $p16^{INK4A}$ and $p15^{INK4B}$ (86). In sporadic tumors, the cyclinD/CDK4/p16/RB pathway is often affected by amplification or high level expression of *cyclin D* or *CDK4*, or deletions or mutations of $p16^{INK4A}$ or *RB* (55). On the other hand, the p19 ($p14^{ARF}$)/MDM2/p53 pathway is also often affected in gliomas by mutations of *TP53*, amplifications of *MDM2*, or mutations and deletions of $p14^{ARF}$ (54). Mutations in these pathways are found particularly in higher-grade astrocytomas (54). Some of these mutations are complementary; for example, some glioblastomas show amplification of *CDK4* without mutations of *CDKN2* ($p16^{INK2A}$ and $p15^{INK2B}$), whereas others show deletion of *CDKN2* without amplification of *CDK4*, but either mutation may lead to activation of CDK4 (97). This may result in phosphorylation of RB and the release of E2F of the RB/E2F complex. Another example is the amplification of *MDM2* in glioblastomas, which leads to the inactivation of p53 even in the absence of mutant p53 (88). *MDM2* and *CDK4* are located in the same chromosomal region, 12q13 to 12q14, in humans, and amplification in malignant gliomas (89) often affects both genes, and thus, both the cyclinD/CDK4/INK4A/RB/E2F pathway and the p53/MDM2/ARF pathway. Detailed amplicon mapping, however, has revealed 2 centers of amplification, one at the *CDK4* locus and the other at the *MDM2* locus (87), with discontinuous amplification of the genes in between suggesting independent selection.

In addition to mutations and deletions, the expression of $p16^{INK4A}$ is silenced by DNA methylation in 24% of gliomas (21, 39). Thus, genetic and epigenetic events affect the G_1-S transition pathway in gliomas and suggest that alterations at this juncture are a prerequisite for gliomagenesis. There are several mouse models with mutations in $p16^{INK4a}$, $p19^{ARF}$, or both (58, 59, 63, 98, 99; for review, see 100). The predominant tumor types in mice with inactivated $p16^{INK4a}/p19^{ARF}$ or $p19^{ARF}$ alone are sarcomas and lymphomas. Gliomas, however, were reported only in the $p19^{ARF}$ deficient mice (58). Two $p16^{INK4A}$ mutant strains have been generated (63, 99). While either strain has the capacity to develop melanomas under appropriate genetic cross and treatment with carcinogens, neither has been reported to carry central nervous system tumors (63, 99). One of them does have a low incidence of spontaneous sarcoma, lymphoma, and melanoma (99); thus, the absence of p16 alone is unlikely to yield gliomas. The G_1-S transition point has been studied in mouse astrocytes obtained from $p16^{INK4a}$-$p19^{ARF}$ deficient mice as well as by infection of *Gtv-a* astrocytes with RCAS/CDK4 (50).

The advent of microarray technology has allowed the efficient rapid study of gene expression in a wide variety of pathways, including those involved in signaling, the cell cycle and apoptosis. The expression of p53, pRB, PTEN, $p14^{ARF}$ and $p16^{INK4A}$ is lost or severely reduced in most gliomas (17). In contrast, EGFR, CDK4, and human telomerase reverse transcriptase are frequently over-expressed in gliomas, almost exclusively in glioblastomas (17). As mentioned previously, *TP53* is the gene most often mutated in Li-Fraumeni syndrome (17, 64). Recently, another gene, *CHK2*, was identified that yields the same phenotype as Li-Fraumeni, including the presence of gliomas (110). CHK2 is part of the ATM/CHK2/CDC25/CDK2 pathway (34). ATM is an inducible protein kinase that is activated by double strand DNA breaks caused by ionizing radiation (mutations in the ATM gene cause the disease ataxia telangiectasia). ATM phosphorylates CHK2, which itself is a kinase that activates CDC25. CDC25 is a phosphatase that activates CDK2, permitting transition though S-phase (60). The role of CHK2, ATM, and CDC25 in astrocytes, as well as their role in gliomagenesis, is unknown.

Gene disruption	Transgene	Somatic transfer	Cell of origin/ affected cells	Tumor	Reference
	GFAP/v-src		All GFAP-expressing cells (astrocytes)	Low-grade (early) and high-grade (late) astrocytomas	125
	GFAP/V12H-Ras		All GFAP-expressing cells (astrocytes)	Heterozygous: predominantly single low-grade (WHO II) astrocytomas Homozygous: predominantly multifocal high-grade (WHO III) astrocytomas	27
$NF1^{-/+}$; $p53^{-/+}$ in cis			All cells in animal	Astrocytomas, WHO grades II to IV (predominantly GM) Strain specific penetrance of astrocytoma phenotype	90
		Murine retrovirus/ PDGF B-chain	Mixed cell population in brain	GBM (astrocytoma, WHO grade IV); PNET	119
		K-Ras plus Akt	Infected nestin-producing CNS progenitor cells (Nt-va)	GBM (astrocytoma, WHO grade IV)	48
		K-Ras plus Akt	Infected astrocytes (Gt-va)	No tumors	48
$(p16^{INK4A}/p19^{ARF})^{+/+}$		PDGF-B	Infected nestin-producing CNS progenitor cells (Nt-va)	Low-grade oligo (WHO II)	24
$(p16^{INK4A}/p19^{ARF})^{-/-}$		PDGF-B	Infected nestin-producing CNS progenitor cells (Nt-va)	High-grade oligo (WHO III)	24
$(p16^{INK4A}/p19^{ARF})^{+/+}$ $(p16^{INK4A}/p19^{ARF})^{-/-}$		PDGF-B	Infected astrocytes (Gt-va)	Mixed oligoastrocytomas	24
$(p16^{INK4A}/p19^{ARF})^{+/+}$		Polyoma middle T	Infected astrocytes (Gt-va)	Mixed oligoastrocytomas	51
$p19^{ARF-/-}$			All cells in animal	Oligodendrogliomas	81

Table 2. Mouse glioma models with defined genetic backgrounds.

Cell of Origin for Gliomas

Identification of the cell of origin is crucial for understanding gliomagenesis. There are several different clues suggesting the possible cell of origin in gliomas.

Mutation analysis of mixed oligoastrocytomas and gliosarcomas. The presence of 2 morphologically-distinct cell types that share the same genetic profile would suggest the same cell of origin. Mixed oligoastrocytomas have been shown to exhibit loss of heterozygosity for 1p and 19q in areas of astrocytic as well as oligodendroglial differentiation, suggesting a common cell of origin (62). Furthermore, dissection of the glial and sarcomatous elements of gliosarcomas followed by sequence analysis of *TP53* revealed identical mutations in the 2 components (10,75). These data suggest that a common precursor cell has the capacity to differentiate along glial and mesenchymal lineages. Studies of rodent cortical cultures have also confirmed the broad potential of progenitor cells in the brain (117). Recently, the differentiation potential of brain-derived cells was investigated using heterotopic and heterochronic grafts (for review, see 113). These studies revealed that brain-derived cells have the capacity to differentiate into

myeloid cells after transplantation into irradiated mice (11), into skeletal muscle after transplantation into regenerating muscle (40), and into derivatives of all three germ layers after microinjection into blastocysts (20). However, the exact identity of the grafted cells has not yet been determined.

Developmental biology studies. Studies in developmental biology have identified signaling pathways that are commonly altered in gliomas. For example, PDGF is crucial during normal glial development and EGF is vital to neural stem cell proliferation and survival. During embryogenesis, PDGF is expressed by neurons and astrocytes (130), whereas glial progenitors and neurons express the PDGFα receptor (131). Mice lacking the PDGF homodimer have a reduced number of glial progenitors and oligodendrocytes compared with control mice (38). In contrast, mice carrying PDGF-AA under control of the neuron-specific enolase promoter yield an increase in the number of glial precursors that generate abnormally localized oligodendrocytes after differentiation, which undergo apoptosis before birth (15). Low-grade astrocytomas have been shown to exhibit PDGF ligand and receptor overexpression (43, 47), in addition to *TP53* mutations (120). Oligodendrogliomas also show expression of the PDGFα receptor (28) and amplifications of the *PDGFα* receptor (104). Stem cells in the ventricular zone require EGF for survival and proliferation. Mice with the hypomorphic *EGFR* allele, *waved-2*, show a decreased number of astrocytes and a smaller subventricular zone compared with those of the normal adult brain (69). In contrast, targeted deletion of *EGFR* in mice results in embryonic lethality, with cortical dysgenesis, neuronal ectopias and reduced numbers of astrocytes (74, 103, 115). Thus, EGFR may play a more complex role during CNS development. Overexpression of EGFR via retroviral vector transfer results in proliferation of stem cells as well as premature astrocytic differentiation (13) and EGF-responsive stem cells in the ventricular and subventricular zones retain the capacity to generate all 3 major cell types in vitro (92).

Neural stem cells transplanted into the lateral ventricle remain undifferentiated with simultaneous infusion of EGF, but differentiate into astrocytes without the simultaneous infusion of EGF (37). As mentioned previously, *EGFR* is often amplified and mutated in high-grade gliomas. About 40% of glioblastomas with amplification express an activated form of EGFR (called EGFRvIII, ΔEGFR, or del2-7EGFR), which lacks a portion of the extracellular ligand binding domain (53, 126) and is constitutively autophosphorylated (31, 32). This mutant form of EGFR confers enhanced tumorigenicity (79).

Somatic cell gene transfer studies. Somatic cell gene transfer into cell types of specific stages of differentiation allows identification of candidate cell types capable of giving rise to gliomas. For example, the transfer of AKT and Ras yield glioblastomas only after transfer into nestin-expressing CNS-progenitor cells (*Ntv-a*) but not after transfer into GFAP-expressing astrocytes (*Gtv-a*) (48). In contrast, the activated form of the EGFR yields lesions resembling gliomas in both *Gtv-a* and *Ntv-a* mice, but does so more commonly in *Ntv-a* mice, suggesting that gliomas arise more efficiently from immature precursors than from astrocytes (49). However, GFAP-expressing astrocytes most likely constitute a heterogeneous population, and it is unclear which cells of this particular lineage were the actual cell-of-origin in those experiments.

Glioma Models Generated in Mice Using Defined Genetic Alterations

Recently, a number of animal models have been introduced that exquisitely replicate many of the quintessential morphologic features of the different classes of human gliomas, including the diffuse infiltration of brain parenchyma that constitutes the single property most responsible for our current inability to cure these tumors. Models have been created that reproduce the morphologic features of virtually all of the specific subtypes of diffuse glioma with remarkable fidelity, including the full spectrum of diffuse astrocytoma (low-grade astrocytoma, anaplastic astrocytoma, glioblastoma), low-grade and anaplastic oligodendrogliomas, and mixed gliomas. The modeling systems that have been used to generate these tumors in mice have also identified common biologic pathways that appear capable of contributing to or causing the formation of gliomas. A summary of the different mouse models, including defined genetic alterations, cell of origin, and resultant glioma histology is provided in Table 2.

Diffuse astrocytomas. Several transgenic mouse lines have been generated that develop low-grade diffuse astrocytomas (World Health Organization grade II). In one system a transgene containing the *GFAP* promoter/enhancer and ^{V12}*H-Ras* was introduced into the murine germline (27). One line developed solitary tumors resembling low-grade astrocytomas (WHO grade II) in 80% of the animals and multiple tumors resembling anaplastic astrocytomas (WHO grade III) in 20% of ani-

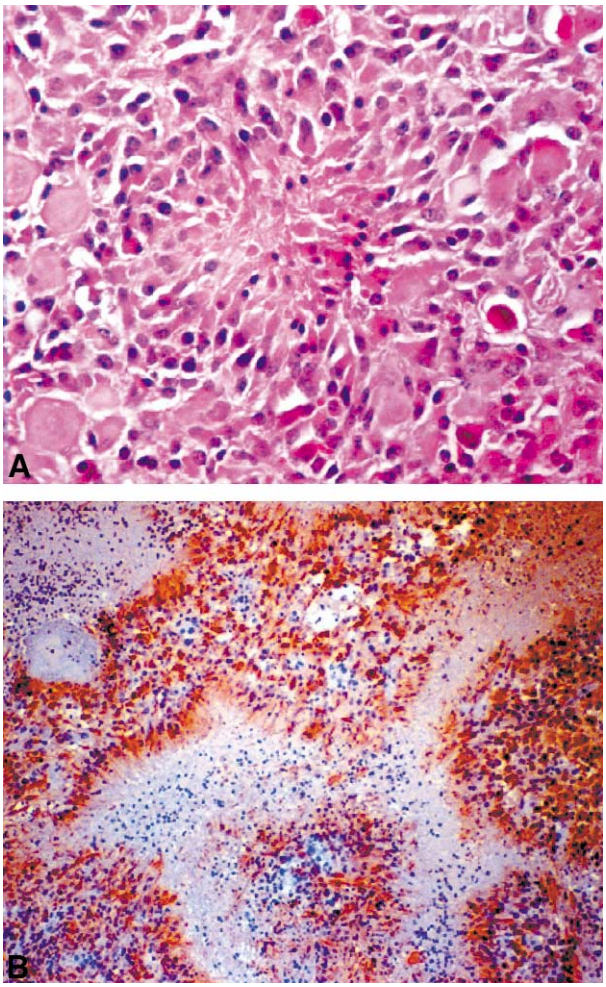

Figure 1. *Glioblastoma*. High-grade astrocytomas with morphologic features identical to those of human glioblastomas (WHO grade IV) can be generated by the combined introduction of the activated G12D mutant form of *K-Ras* together with *AKT* into *Ntv-a* transgenic mice. **A**. Like those of human glioblastomas, malignant astrocytes are characterized by pleomorphic nuclei and prominent eosinophilic cytoplasm. A central area of tumor necrosis is seen in this field. **B**. The immunohistochemical reactivities of mouse glioblastomas also recapitulate those of human tumors, as seen here with strong positivity for glial fibrillary acidic protein (GFAP) in tumor cells palisading around serpiginous zones of necrosis. (**A**, ×100, H&E; **B**, ×40, GFAP)

mals when the transgene was in the heterozygous state. With the transgene in the homozygous state, the animals developed multifocal tumors resembling anaplastic astrocytomas (WHO grade III). Consistent with the uniformly higher grade of the tumors in the homozygous mice, their survival time was shortened compared to the heterozygous mice; the median survival of heterozygous mice was 3 months compared to 4 weeks for the homozygous mice.

In another model, transgenic mice were generated using the *v-src* kinase under the control of *GFAP* regulatory elements. In 14.4% of mice, small proliferative foci as well as overt astrocytomas developed in the brain and spinal cord. Early lesions were similar to low-grade astrocytomas (WHO grade II); at later stages the tumors exhibited the histological characteristics of anaplastic astrocytoma (WHO grade III) and glioblastoma (WHO grade IV) (125).

Glioblastoma. The formation of glioblastoma (WHO grade IV) has been observed in both *GFAP/v-src* and *GFAP/^{V12}H-Ras* transgenic mice (27, 125). Cell lines established from the high-grade astrocytomas of the *GFAP/^{V12}H-Ras* transgenic mice show abnormal karyotypes, as well as expression of proteins known to be involved in apoptosis and cell cycle progression, including MDM2, p16^{INK4A}, p19^{ARF}, PTEN, and EGFR (27). From these experiments, it is unclear which of these genes in combination with *^{V12}H-Ras* is/are sufficient for gliomagenesis. The *GFAP/v-src* induced glioblastomas have not yet been studied on the genetic or karyotypic level. A spectrum of low to high grade astrocytomas has also been reported in "knockout" mice heterozygous for *NF1* and *TP53* (51). Since neurofibromin, the gene product of *NF1*, is a negative regulator of Ras, this is further evidence for the central role of Ras in gliomagenesis.

The introduction of combined activated G12D mutant form of *K-Ras* and *AKT* into *Ntv-a* transgenic mice induces glioblastoma formation (48) (Figure 1). The mice develop tumors within 9 weeks. Tumor formation is not observed with either oncogene alone, or when the combination is injected into *Gtv-a* mice. Thus, modeling of glioblastoma in mice requires the transfer of a combination of certain oncogenes on the one hand as well as transfer into specific precursor cells on the other. Transfer of *K-Ras* and *AKT* into *Ntv-a* mice carrying an inactivated allele of *INK4A-ARF* accelerates the development of glioblastoma. Interestingly, transfer of *K-Ras* into *Ntv-a* mice carrying the *INK4a-ARF* deletion yields gliosarcoma in 30% of offspring (unpublished data). This is not seen with the transfer of either *K-Ras* or *AKT* alone into *Gtv-a* mice. However, the combination of *K-Ras* and *AKT* transferred together into *Gtv-a* mice carrying an inactivated allele of *INK4a-ARF* produces spindle cell gliomas and gemistocytic astrocytomas. The production of tumors in *Gtv-a* mice by the transfer of *K-Ras/AKT* requires the absence of

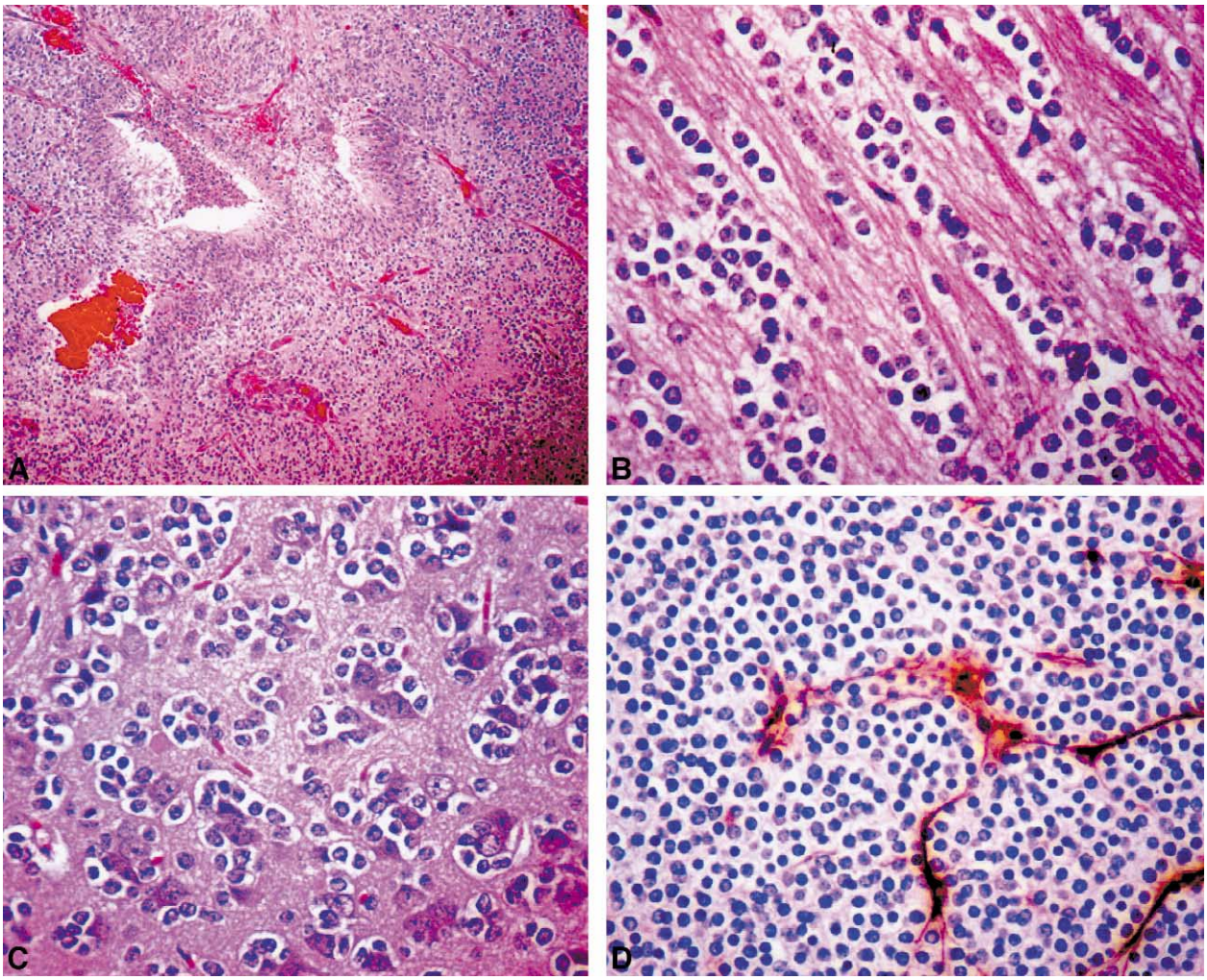

Figure 2. *Oligodendroglioma*. High-grade (anaplastic) oligodendrogliomas, such as the one illustrated here, can be generated by the transfer of the gene encoding PDGF-B into *Ntv-a* mice whose genetic background includes an inactivated allele of *INK4a-ARF*. At low power (**A**) foci of necrosis with surrounding pseudopalisading of tumor cells are seen. At higher power (**B**, **C**, **D**), the morphologic features of individual tumor cells clearly display classical oligodendroglial differentiation characteristics, with uniform, rounded nuclei surrounded by prominent cleared cytoplasm (perinuclear halos). Panel **B** shows diffuse infiltration of cerebral white matter by oligodendroglioma cells, which line up in queues between bundles of myelinated axons. Identical infiltration patterns are routinely observed with human oligodendrogliomas. Panel **C** shows perineuronal satellitosis (clustering of tumor cells around neuronal cell bodies) by oligodendroglioma cells infiltrating the gray matter of the cerebral cortex. The same secondary structures of Scherer (perineuronal satellitosis, perivascular satellitosis, subpial infiltration, intrafascicular queuing) that are seen in human oligodendrogliomas are also characteristic of the mouse tumors. The immunophenotypic characteristics of mouse oligodendrogliomas also parallel those of their human counterparts. As seen in panel **D**, just as is seen in human tumors, GFAP immunostaining labels only entrapped reactive astrocytes; neoplastic oligodendrocytes are negative. Mouse oligodendrogliomas are also negative for neuronal differentiation markers such as synaptophysin and NeuN (not illustrated). (A, ×40, H&E; B, ×100, H&E; C, ×100, H&E; D, ×100, GFAP)

$p16^{INK4a}$/$p19^{ARF}$. Glioblastomas induced by somatic transfer of *K-Ras* and *AKT* into *Ntv-a* transgenic mice do not show karyotypic abnormalities as confirmed by karyotype analysis. A separate series of experiments has demonstrated that the ectopic expression of *^{V12}H-Ras*, *hTERT* (telomerase reverse transcriptase), and the papilloma virus oncogenes *E6/7*, which inhibit the p53/RB pathway, converts human astrocytes into cells resembling those of anaplastic astrocytoma (105). With the further transfer of *AKT*, the cells acquire features of glioblastoma (106). These data support the observation that Ras can cooperate with AKT signaling to generate glial tumors of high malignancy.

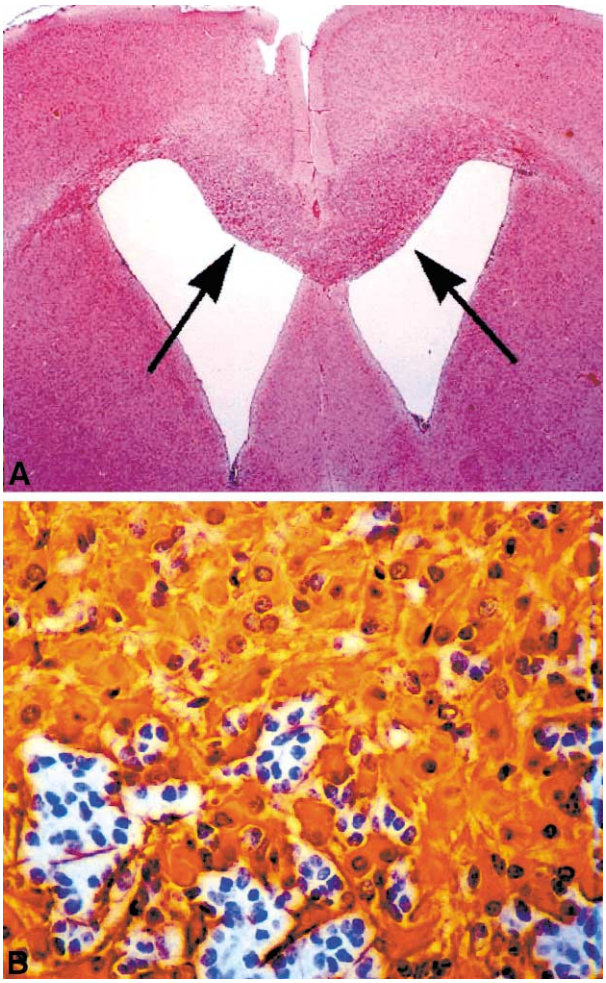

Figure 3. *Mixed oligoastrocytoma*. In addition to purely astrocytic and purely oligodendroglial tumors like those illustrated in Figures 1 and 2, respectively, mixed gliomas can also be modeled in mice. Mixed oligoastrocytomas, like the one illustrated here, can be generated by the transfer of polyoma middle T antigen or PDGF into *Gtv-a* mice. In this animal, a classical "butterfly" growth pattern is seen (arrows in panel **A**). This infiltration pattern results from invasive spread of the tumor cells through the corpus callosum. The phenotypic characteristics of mouse mixed gliomas are similar to those seen in their human equivalents. Those cells that exhibit astrocytic features (abundant eosinophilic cytoplasm and fibrillary processes) are strongly immunoreactive for GFAP, while those cells that exhibit oligodendroglial morphologic features (regular, round nuclei and perinuclear halos) are negative (**B**). Diffuse infiltration of brain parenchyma, as nicely illustrated in this mouse "butterfly" glioma of the corpus callosum, is an intrinsic feature of all human diffuse gliomas. In contrast to glioma xenografts, which typically grow as sharply circumscribed spheres similar to metastases, diffuse neuropil invasion identical to that seen in human gliomas is replicated in exquisite detail in the mouse models described in this review. (A, ×20, H&E; B, ×100, GFAP)

Oligodendrogliomas. Transfer of the gene encoding PDGF-B into *Ntv-a* mice carrying an inactivated allele of *INK4a-ARF* generates high-grade oligodendroglioma (WHO grade III) (24) (Figure 2). Features characteristic of oligodendroglioma were also found among the tumors induced by the transfer of the gene encoding the PDGF-B chain through the MoMULV into newborn mice. A second series of experiments using transgenic mice that express the viral oncogene v-erbB, an active homologue of EGFR, from the S-100b promoter (which is active in astrocytes and glial precursors) generated similar oligodendrogliomas (Weiss, unpublished). Thus, ectopic expression of activated RTK growth factors may lead to oligodendroglioma formation. Mice carrying a deletion of $p19^{ARF}$ develop oligodendrogliomas with some frequency (58), in contrast to the lack of gliomas arising in $p16^{INK4A}/p19^{ARF}$ deficient mice (98). The reasons for this discrepancy are unclear. Finally, infection of newborn mouse brains with replication-competent virus expressing the PDGF-B chain results in a variety of high-grade gliomas, including glioblastoma and oligodendroglial tumors, in addition to tumors resembling primitive neuroectodermal tumors (PNETs) (119).

Mixed oligoastrocytoma. Polyoma middle T antigen stimulates Shc (leading to Ras activation), PI 3-kinase (leading to AKT activation) and Src (3, 26, 107). The transfer of polyoma middle T antigen into *Gtv-a* mice via the RCAS vector induces mixed oligoastrocytomas (51) (Figure 3). Tumors were seen at 9 weeks in 9 of 33 mice. The histologic features of these tumors were similar to those seen in human anaplastic astrocytomas, anaplastic oligodendrogliomas and anaplastic mixed oligoastrocytomas. Features of other primary CNS neoplasms, such as PNETs or ependymomas were not seen in any of the lesions. Polyoma middle T antigen does not require additional genetic alterations, as also reported for the induction of gliomas by EGFR (49) and glioblastomas by K-Ras/AKT (48). This is consistent with the activation of multiple pathways required for tumor induction by polyoma middle T antigen alone. A second approach for the generation of mixed gliomas is the transfer of PDGF into *Gtv-a* mice (24). These tumors have a very similar histologic appearance to those generated by polyoma middle T antigen.

Unclassified gliomas. The transfer of the gene encoding constitutively-active EGFR into *Ntv-a* or *Gtv-a* mice leads to glioma production when the mice are also deficient at the *INK4a-ARF* locus (coding for $p16^{INK4A}$ and $p19^{ARF}$); tumors are more efficiently gener-

ated in *Ntv-a* mice than in *Gtv-a* mice. The somatic transfer of active EGFR into mice carrying the wild type allele of the *INK4a-ARF* locus does not result in glioma formation (49). Furthermore, mice lacking the *INK4a-ARF* locus develop lymphomas and sarcomas with a mean latency of 34 weeks (98). Thus, activated EGFR receptor requires the inactivation of the *INK4a-ARF* locus in order to yield glioma formation. Determination of which of the two gene products encoded by the *INK4a-ARF* locus, $p16^{INK4A}$, $p19^{ARF}$ (the mouse homologue of human $p14^{ARF}$), or both, needs to be inactivated to yield glioma formation in combination with EGFR requires further investigation.

Basic fibroblast growth factor introduced into normal astrocytes (*Gtv-a*) induces cell migration and proliferation without the induction of tumors (52). It is unknown which mutation(s), if any, when combined with the overexpression of bFGF might be sufficient for glioma induction in mice.

A Few Points for Discussion

Mouse models of gliomas that are derived by recapitulating the genetics of human gliomas display histologic features that resemble those seen in the human disease to a remarkable extent. It is unclear, however, whether the induction of gliomas in mice accurately reflects the human condition mechanistically. The average volume of a human tumor is significantly larger than that of a mouse at the time of symptomatic clinical presentation, which allows for the accrual of a large number of mutations. The genetic and cellular diversity of human gliomas is therefore likely to be significantly more complex than that observed in mouse models. Differences between human and mouse requirements for tumorigenesis have been observed in models of other tumors as, for example, in mutations of the retinoblastoma oncogene, which lead to retinoblastoma induction in humans but not in mice with disruption of the same gene (56). Such studies reveal differences in the requirement for certain genes for tumor development in different species. The degree to which the different animal models reflect the human condition requires further investigation.

In addition to a molecular dissection of the roles played by specific genes in glioma induction and maintenance, other important tumor-associated processes, such as angiogenesis and the immune response during gliomagenesis, can also be productively studied in mouse models. For example, mouse model studies have shown that astrocytomas arising in *GFAP/v-src* transgenic mice express VEGF even at early stages, and the endothelial cells display induction of the angiongenic receptors flt-1, flk-1 (VEGF-R2), tie-1, and tie-2 (114).

The age at which gliomas arise in humans merits special attention. Gliomas are known to occur in the newborn period, but the predominant peak is in adulthood. Mouse glioma models based on somatic transfer methodologies all involve infection with various expression vectors during the newborn period (35, 52, 119). Since in humans glioblastoma occurs most commonly in the fifth decade of life, the question arises as to which cell type(s) are giving rise to high-grade astrocytomas in later life. Studies in adult mice have shown that astrocytes in the subventricular zone can give rise to neurons (29, 30). These and/or astrocyte populations in other locations of the adult brain might serve as progenitors for gliomas; this possibilty warrants additional study. The variety of different glial populations in the adult brain also raises the question of which of them might give rise to astrocytomas; in depth characterization of the different glial subtypes in this regard is needed.

It is unclear whether continued expression of oncogenes is required for the maintenance of gliomas following their induction. This is a particularly interesting and important question in situations in which 2 oncogenes are required for glioma formation. For example, the induction of glioblastomas by the combination of AKT and K-Ras raises the question of which particular order, if any, they need to be expressed for glioma induction to occur, and whether the continued expression of either one, or both, is required for tumor maintenance. Because the 2 genes are transferred to newborn mice in this model, the question also arises as to whether gene transfer later in life would result in glioma induction or not.

Summary

Recently introduced mouse models of the diffuse gliomas are likely to yield significant insight into the complex process of gliomagenesis. They provide an unparalleled approach for precise manipulation and study of the effects of specific gene alterations that has heretofore been unavailable. The new mouse models may also provide an opportunity for improvement of glioma therapy in 2 ways. The first is through the identification of novel targets that might closely resemble those involved in human tumor pathogenesis. The second is by providing models for testing new therapeutic strategies that reliably and reproducibly recapitulate a cardinal pathophysiologic feature of human gliomas: diffuse infiltration of brain parenchyma.

Acknowledgments

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