A constitutively active epidermal growth factor receptor cooperates with disruption of G_1 cell-cycle arrest pathways to induce glioma-like lesions in mice

Eric C. Holland,[1,3] Wendy P. Hively,[1] Ronald A. DePinho,[2] and Harold E. Varmus[1]

[1]Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892 USA;
[2]Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115 USA

The epidermal growth factor receptor (*EGFR*) gene is amplified or mutated in 30%–50% of human gliobastoma multiforme (GBM). These mutations are associated usually with deletions of the *INK4a–ARF* locus, which encodes two gene products ($p16^{INK4a}$ and $p19^{ARF}$) involved in cell-cycle arrest and apoptosis. We have investigated the role of *EGFR* mutation in gliomagenesis, using avian retroviral vectors to transfer a mutant *EGFR* gene to glial precursors and astrocytes in transgenic mice expressing *tv-a*, a gene encoding the retrovirus receptor. TVA, under control of brain cell type-specific promoters. We demonstrate that expression of a constitutively active, mutant form of EGFR in cells in the glial lineage can induce lesions with many similarities to human gliomas. These lesions occur more frequently with gene transfer to mice expressing *tv-a* from the progenitor-specific nestin promoter than to mice expressing *tv-a* from the astrocyte-specific glial fibrillary acidic protein (*GFAP*) promoter, suggesting that tumors arise more efficiently from immature cells in the glial lineage. Furthermore, *EGFR*-induced gliomagenesis appears to require additional mutations in genes encoding proteins involved in cell-cycle arrest pathways. We have produced these combinations by simultaneously infecting *tv-a* transgenic mice with vectors carrying *cdk4* and *EGFR* or by infecting *tv-a* transgenic mice bearing a disrupted *INK4a–ARF* locus with the *EGFR*-carrying vector alone. Moreover, *EGFR*-induced gliomagenesis does not occur in conjunction with *p53* deficiency, unless the mice are also infected with a vector carrying *cdk4*. The gliomagenic combinations of genetic lesions required in mice are similar to those found in human gliomas.

[*Key Words*: *EGFR*; gliomagenesis; cell cycle arrest]

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Gliomas are the most common forms of primary brain tumors and are classified into four clinical grades (Kleihues et al. 1993). The most aggressive tumors, grade 4, also known as glioblastoma multiforme (GBM), appear to arise either de novo as GBMs or present initially as lower grade tumors that progress to higher grades over time (von Deimling et al. 1993). Certain mutations and other properties are highly correlated with one or the other of these two pathogenic pathways. Gliomas that are first detected as grade 4 tumors usually lack an intact *INK4a–ARF* tumor suppressor locus (Schmidt et al. 1994; He et al. 1995; Ichimura et al. 1996); and they are thus unable to make the two *INK4a–ARF* gene products, $p16^{INK4a}$ and $p19^{ARF}$, which arrest the cell cycle by different pathways in G_1 and in both G_1 and G_2, respectively (Fig. 1; Serrano et al. 1993; Quelle et al. 1995). In addition, these tumors occur frequently in older patients, contain mutations or amplification of the *EGFR* gene, tend to be more aggressive, and are generally diploid (Louis et al. 1993; He et al. 1994; Schlegel et al. 1994; Ono et al. 1996; Hayashi et al. 1997). In contrast, gliomas that progress from lower to higher grade lesions are usually wild type for *INK4a–ARF* , but have an amplified *CDK4* locus or have lost the *Rb* gene, and frequently also display either loss of p53 function or amplification of *mdm2*. Therefore, in both types of tumors the same two cell-cycle arrest pathways are disrupted by different mutations (Fig. 1). Furthermore, the tumors retaining a wild-type *INK4a–ARF* gene arise at an earlier age, are less aggressive, and are usually aneuploid (Lang et al. 1994; Rasheed et al. 1994; van Meyel et al. 1994; Watanabe et al. 1996).

[3]Corresponding author. Present address: Departments of Neurosurgery and Molecular Genetics, MD Anderson Cancer Center, Houston, Texas 77030 USA.
E-MAIL eholland@notes.mdacc.tmc.edu; FAX (713) 794-4950.

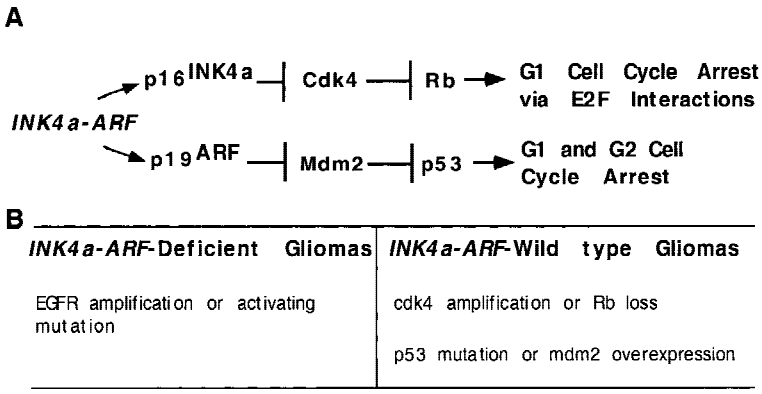

Figure 1. Proposed roles of *INK4a–ARF* in cell-cycle arrest and human gliomagenesis. (*A*) Cell-cycle arrest pathways involving the *INK4a–ARF* gene products $p16^{INK4a}$ and $p19^{ARF}$. (*B*) Subdivision of human gliomas into those with mutations in *INK4a–ARF* and those with wild-type *INK4a–ARF* loci. Mutations of other genes associated frequently with these two tumor populations are indicated. The gliomas with mutations *INK4a–ARF* are more common and associated with abnormalities in the *EGFR* gene, whereas the *INK4a–ARF* wild-type gliomas disrupt the G_1 arrest pathways downstream of the *INK4a–ARF* gene products.

We have previously designed a system for glia-specific gene transfer in vivo that allows us to investigate the effects of both individual mutations and combinations of mutations on gliomagenesis in mice (Holland and Varmus 1998). This system utilizes replication-competent ALV splice acceptor (RCAS) viral vectors, derived from the avian retrovirus, ALV subgroup A, and a transgenic mouse line (*Gtv-a*) producing TVA, the receptor for ALV-A, from the astrocyte-specific promoter for the gene encoding glial fibrillary acidic protein (*GFAP*). Astrocytes from *Gtv-a* mice are susceptible to infection and gene transfer by RCAS vectors both in vivo and in vitro. We have used this system previously to show that infection of $GFAP^+$ cells with RCAS carrying the coding sequence for basic fibroblast growth factor (bFGF) causes glial to proliferate, migrate over long distances, and assimilate into the normal brain structure without tumor formation (Holland and Varmus 1998). In this study we describe a second transgenic mouse line (*Ntv-a*), expressing *tv-a* from the *nestin* promoter, which is active in cells early in the glial lineage. We also describe the construction and use of an RCAS vector that transfers a potent oncogene encoding a constitutively active form of EGFR (RCAS–*EGFR**) to *tv-a*$^+$ cells. With these and other tools, we have compared the effects of gene transfer to *Ntv-a* mice (presumably to glial progenitors) and to *Gtv-a* mice (presumably to terminally differentiated astrocytes) and asked whether EGFR*, alone or in combination with other genetic lesions observed in human gliomas, can initiate gliomagenesis.

We show here that transfer of the *EGFR** gene into either *Gtv-a* or *Ntv-a* transgenic animals induces glioma-like lesions in mice deficient for *INK4a –ARF*, but not in transgenic mice wild type at the *INK4a –ARF* locus. The frequency of gliomagenesis is higher after infection of *Ntv-a* mice than *Gtv-a* mice, consistent with the possibility that cells earlier in the glial lineage may be more susceptible to transformation than terminally differentiated astrocytes. The cooperativity between *EGFR** and *INK4a –ARF* deficiency appears specific, as transfer of *EGFR** to $p53^{+/-}$ mice does not result in gliomatous changes. Coinfection with RCAS–*cdk4* and RCAS-*EGFR** induces lesions in mice with wild-type *INK4a –ARF* and *p53* loci at low frequency. However, this combination induces glioma-like lesions at a higher frequency in *p53*-heterozygous mice than in wild-type mice, implying that *cdk4* overexpression and *p53* deficiency also cooperate in this model system as they seem to do in human tumors.

Results

Construction of Ntv-a *transgenic mice*

The intermediate filament protein nestin is expressed in central nervous system (CNS) progenitors during embryonic development and in glial progenitors after birth (Tohyama et al. 1992). A transgene (NES 1689/*lacZ*), in which *lacZ* expression is under the control of the *nestin* promoter, produces β-galactosidase in glial and neuronal progenitors throughout the developing CNS (Lothian and Lendhal 1997). We have constructed a *nestin–tv-a* transgene (*Ntv-a*) by replacing the *lacZ* gene in NES 1689/*lacZ* with the 800-bp quail cDNA encoding the glycosylphosphatidylinositol-linked form of TVA (Fig. 2A; Bates et al. 1993; Hunter 1997). *Ntv-a* is designed to be expressed in glial progenitors and to allow gene transfer with RCAS vectors to this cell population. In contrast, the original *Gtv-a* transgenic line allows genes to be transferred primarily to terminally and near terminally differentiated astrocytes (Holland and Varmus 1998).

Three independent lines of *Ntv-a* mice transmitted the *Ntv-a* transgene in a simple Mendelian inheritance pattern and were infectable with RCAS vectors. To demonstrate appropriate gene transfer, we infected both *Gtv-a* and *Ntv-a* mice at birth with RCAS–*AP*, which carries a gene encoding the histologic marker, alkaline phosphatase (AP) (Fig. 2B). Three weeks after infection of newborn *Ntv-a* mice, AP^+ cells surrounded the needle track. The morphology of the AP^+ cells was similar to that seen with RCAS–*AP* infection of *Gtv-a* mice (Holland and Varmus 1998; Fig. 4, below; data not shown). This is expected, because the mitotically active $nestin^+$ cell population present at birth gives rise primarily to astrocytes and oligodendrocytes. Although nestin is expressed embryonically in neuronal progenitors, the majority of these are postmitotic after birth (Jacobson 1991), when the infection with RCAS vectors takes place and infection with avian retroviruses requires a round of cell di-

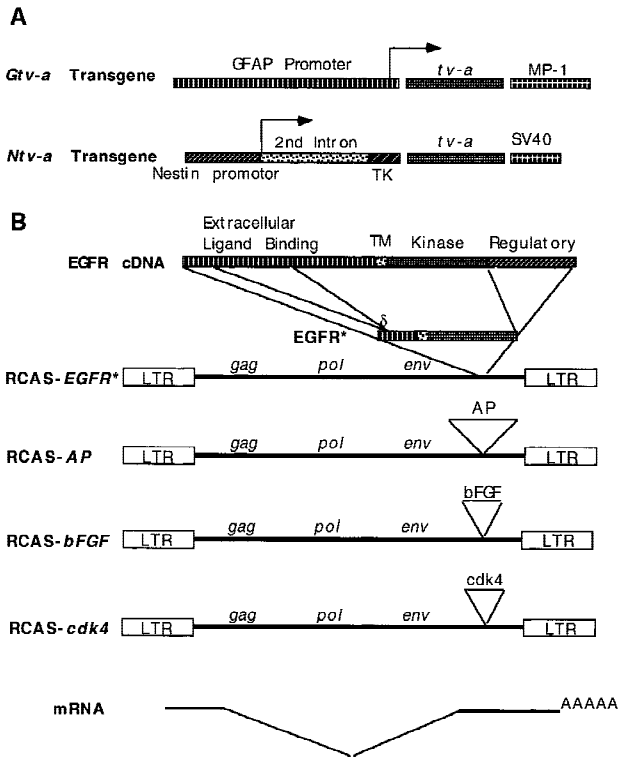

Figure 2. *tv-a* transgenes and RCAS vectors. (*A*) Transgenes expressing the ALV subgroup A receptor, TVA. The astrocyte-specific *Gtv-a* transgene was described previously (Holland and Varmus 1998). The *Ntv-a* transgene utilizes a modified *nestin* promoter including a portion of its second intron and thymidine kinase promoter sequences that have been shown to direct expression to CNS progenitor cells (Lothian and Lendahl 1997). Transcription start sites are indicated with arrows. (*B*) RCAS vectors carrying an exogenous gene 3′ of *env*. RCAS–*EGFR** expresses a constitutively active, mutant form of human *EGFR* (*top*) with deletions of intra- and extracellular sequences as indicated (*TM*). The region coding for the transmembrane domain; (δ) the position of the novel junction formed by the deletion in the coding region for the extracellular domain of EGFR. RCAS–*AP*, RCAS–*bFGF*, and RCAS–*cdk4* carry human placental alkaline phosphatase cDNA, mouse *bFGF* cDNA, and human *cdk4* cDNA, respectively. Although these vectors replicate only in avian cells, the exogenous genes are expressed in both avian and mammalian cells from a spliced message as illustrated.

vision. Consistent with these features, we have not detected AP^+ neurons in *Ntv-a* mice infected with RCAS–*AP* at birth.

*Development and characterization of a vector, RCAS–*EGFR**, for expression of a mutant constitutively active EGF receptor*

Increased EGFR kinase activity in gliomas is associated with amplification of the *EGFR* gene and three general classes of mutations that result in constitutively active gene products. The most common mutation (vIII) deletes part of the extracellular domain of EGFR, causing the receptor to signal through its protein–tyrosine kinase activity in the absence of ligand (Wong et al. 1992; Ekstrand et al. 1994). A second deletion removes an intracellular kinase-regulatory domain. A third group of alleles has both kinds of deletions (Ekstrand et al. 1992). Because efficient packaging of viral RNA genomes into RCAS vectors requires inserts of 2.6 kb or less, we constructed an RCAS vector carrying the shortest (doubly deleted, *EGFR**) form of the *EGFR* cDNA (Fig. 2B). Using a monoclonal antibody specific for the junction peptide generated by the extracellular deletion, we demonstrated with the Western blot method that the chicken fibroblast line DF-1 infected with RCAS–*EGFR** produced a shortened form of EGFR (Fig. 3A), and after repeated passage, the infected cells appeared transformed morphologically (data not shown). Anti-EGFR* immunostaining of DF-1 cells infected with RCAS–*EGFR** demonstrated that the expression of *EGFR** could be detected specifically in a distribution consistent with a plasma-membrane location (Fig. 3B). We also confirmed that the expression of *EGFR** by *Gtv-a* transgenic astrocytes infected with RCAS–*EGFR** in vitro could be detected specifically by immunohistochemistry (Fig. 3C).

EGFR* *gene transfer in vivo*

To demonstrate the effects of transferring the *EGFR** gene to glia in vivo, we injected a mixture of DF-1 cells producing RCAS–*AP* and RCAS–*EGFR** into the frontal cerebral lobes of newborn *Ntv-a* mice. Eight weeks later, cells adjacent to the needle track stained positive for AP activity and with antibodies to EGFR* and nestin (Fig. 4A,B). The nestin- and EGFR*-positive cells resembled

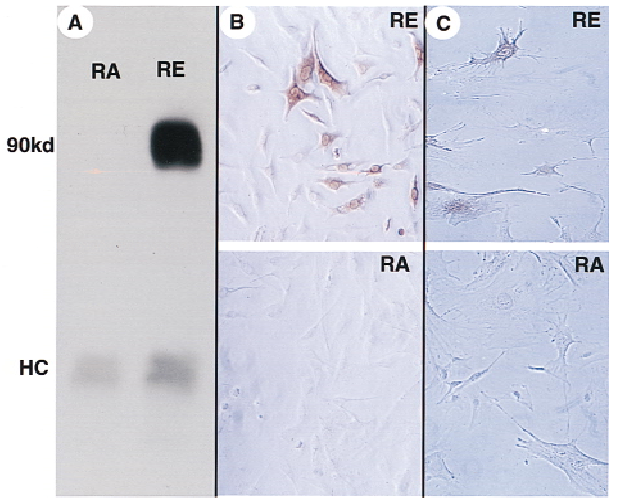

Figure 3. Characterization of an RCAS vector carrying a gene for activated EGFR (RCAS–*EGFR**). (*A*) Immunoprecipitation and Western blot analysis of extracts of the chicken cell line DF-1 infected with and producing either RCAS–*EGFR** (*RE*) or RCAS–*AP* (*RA*). A band is detected migrating at the position expected for a glycosylated protein with the sequence predicted from the *EGRF** cDNA (molecular mass ~90 kD). (*HC*) The position of the immunoglobulin heavy chain. Immunochemical staining with antibodies specific for EGFR* in DF-1 cells (*B*) or *Gtv-a* astrocytes (*C*) infected with either RCAS–*AP* (*RA*) or RCAS–*EGFR** (*RE*).

mature astrocytes with small nuclei and long processes. Such cells were detected occasionally adjacent to the ventricles (Fig. 4C,D), presumably a result of infection via cerebrospinal fluid. These *nestin*- and *EGFR**-expressing cells in *Ntv-a*; *INK4a–ARF*$^{+/+}$ mice appeared normal histologically by hematoxylin and eosin (H&E) staining in five such animals. As documented in Table 1, no structural abnormalities were seen by H&E staining in 21 *Gtv-a*and 24 *Ntv-a* mice infected with a mixture of RCAS–*AP* and RCAS–*EGFR**. Cells near the needle track or ventricles of nontransgenic littermates infected with these vectors did not stain for AP, nestin, or *EGFR** (data not shown) as anticipated from earlier findings (Holland and Varmus 1998).

Features of gliomas are induced by EGFR* *gene transfer in INK4a –ARF*-deficient mice

Otherwise normal *tv-a* transgenic mice infected with RCAS–*EGFR** did not develop histologic changes characteristic of gliomas. In contrast, *tv-a* transgenic mice carrying targeted deletions in the *INK4a –ARF* locus frequently displayed glioma-like lesions after infection with RCAS–*EGFR**. The properties of these lesions were varied but included several characteristics also observed in human gliomas: (1) structural abnormalities seen histologically after H&E staining (distortion of normal structures by cell mass or hydrocephalus); (2) increased cell density; (3) immunopositivity for expression of the mutant form of EGFR; and (4) immunopositivity for GFAP (identifying astrocytic character) and nestin (indicating a more primitive expression pattern). For the purpose of this study lesions were defined as gliomas if they displayed all four characteristics.

One such lesion in the hippocampus of an *Ntv-a* mouse infected with RCAS–*AP* and RCAS–*EGFR** is illustrated in Figure 5 (A–C). This lesion demonstrates increased cell density, immunohistochemical staining for both EGFR* and GFAP, increased vascularity and tumor cells surrounding blood vessels at a distance from the tumor mass. A second lesion generated by *EGFR** gene transfer into a *Gtv-a* mouse homozygous for the mutant *INK4a–ARF* allele (Fig. 5D–I) shows evidence of previous hemorrhages, increased vascularity and cell density, as well as invasion of adjacent normal tissue. This lesion was sufficiently large to obtain numerous sections for immunohistochemical staining for other gene products. Similar to what has been reported for high-grade human gliomas (Takahashi et al. 1990; Plate et al. 1994), cells in the implicated region overexpress endogenous bFGF and vascular endothelial growth factor (VEGF). The cells comprising this lesion also stain with antibodies to GFAP and nestin, indicating an immature astrocytic cell type.

Another histopathological feature is illustrated in sections from a *Gtv-a*; *INK4a –ARF*$^{+/-}$ mouse infected with RCAS–*AP* and RCAS–*EGFR** (Fig. 6). This mouse developed hydrocephalus at 6 weeks of age (recognized clinically by macrocephaly, lethargy, and dehydration) and was sacrificed. The lesion was comprised of EGFR*- and GFAP-positive cells spread diffusely throughout the brain stem and thalamus, leading to an increased cell density in these regions. Areas of intraparenchymal hemorrhage in brains with similar lesions were adjacent to astrocytic cells staining for EGFR* and nestin, but a distinct tumor mass was usually absent (data not shown).

EGFR**-induced gliomagenesis requires disruption of cell-cycle arrest pathways and occurs more frequently after infection of* Ntv-a *than* Gtv-a *mice*

As described earlier, we did not detect any gliomas after

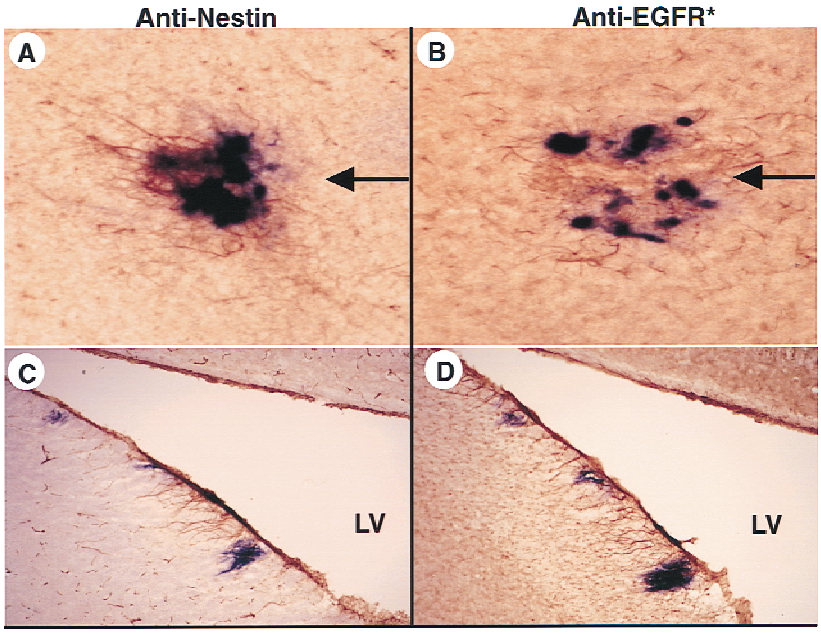

Figure 4. Intracerebral infection of *Ntv-a* mice with RCAS–*AP* and RCAS–*EGFR**. (*A*) Simultaneous transfer of *AP* and *EGFR** genes to *Ntv-a* astrocytes in vivo. Newborn *Ntv-a* mice were injected in the right frontal lobe with a mixture of DF-1 cells producing RCAS–*AP* or RCAS–*EGFR** and sacrificed at 8 weeks of age. Sequential brain sections (frozen, 40 µm) were stained for AP activity and with either anti-nestin (*A*,*C*) or anti-EGFR* antibodies (*B*,*D*). *A* and *B* include the injection track used to introduce producer cells (arrows); *C* and *D* represent the margin of the lateral ventricle (LV). Cells with a strocytic morphology stain for nestin and EGFR* in the vicinity of AP^{+} cells.

Table 1. *Induction of gliomas by RCAS vectors in* tv-a *transgenic mice*

	Genes introduced with RCAS vectors[a]			Genetic background[b]			
tv-a transgenes	*EGFR**	*cdk4*	*bFGF*	wild type	*INK4a*$^{+/-}$	*INK4a*$^{-/-}$	*p53*$^{+/-}$
Gtv-a	+	−	−	0/21	1/17	2/6	
Ntv-a	+	−	−	0/24	13/25	8/19	0/16
Ntv-a	+	+	−	1/8	5/19	4/10	3/8
Ntv-a	+	+	+	3/29	4/7	6/16	17/39

Glioma formation, as measured by either histologic inspection of frozen brain sections or by clinical appearance of hydrocephalus, is tabulated for *Gtv-a* and *Ntv-a* transgenic mice from four different genetic backgrounds with respect to the tumor suppressors *INK4a–ARF* and *p53*, after infection of newborns with RCAS vectors carrying the indicated genes.

[a]*EGFR** (RCAS–*EGFR**); *cdk4* (RCAS–*cdk4*); *bFGF* (RCAS–*bFGF*). Tumors induced by infections with RCAS–*EGFR** alone were scored at 8–10 weeks by histologic analysis of fixed frozen sections. Mice infected with combinations of RCAS–*EGFR** and RCAS–*cdk4* were scored for hydrocephalus between 3 and 8 weeks of age, after determining in >15 mice that early hydrocephalus is highly correlated with histologic features of gliomas.

[b]*INK4a*$^{+/-}$ and *INK4a*$^{-/-}$ are heterozygous and homozygous for a targeted deletion of the *INK4a–ARF* tumor suppressor locus; and *p53*$^{+/-}$ is heterozygous for a targeted deletion of the *p53* tumor suppressor gene.

RCAS–*EGFR** infection of *Gtv-a* or *Ntv-a* transgenic mice wild type for the *INK4a –ARF* locus. Only one glioma was observed after infection of 17 *Gtv-a* transgenic; *INK4a –ARF*$^{+/-}$ mice with RCAS–*EGFR**, and two mice developed gliomas after infection of six *Gtv-a*; *INK4a –ARF*$^{-/-}$ mice (see Table 1). Gliomas were induced at a much higher frequency in *Ntv-a*; *INK4a –ARF*-heterozygous and -nullizygous animals (13 of 25 and 8 of 19, respectively). Furthermore, infection of *Ntv-a* and *Gtv-a* mice with RCAS–AP indicate that the efficiency of viral uptake and expression of these two mouse lines are similar (data not shown). These results suggest two signficant conclusions: (1) induction of gliomagenesis by those producing a *EGFR** requires additional genetic lesions, such as deficiency of p16^{INK4a}, p19^{ARF}, or both; and (2) gliomas arise with greater efficiency when *EGFR** is introduced into *Ntv-a* mice, presumably into cells relatively early in the glial lineage. The second conclusion may imply that either glial progenitors are generally more prone to malignant transformation or that the specific interaction between *EGFR* signaling and G_1 and cell-cycle arrest is more efficient in cells earlier in the glial lineage.

The data summarized in Table 1 suggest that *INK4a –ARF*$^{+/-}$ mice are as susceptible to RCAS–*EGFR**-induced gliomas as are *INK4a –ARF*$^{-/-}$ mice. The most obvious explanation for this is that either the remaining *INK4a –ARF* allele in tumors arising in *INK4a –ARF*$^{+/-}$ mice is inactivated at high frequency or that haploinsufficiency of the *INK4a –ARF* locus is sufficient for glioma

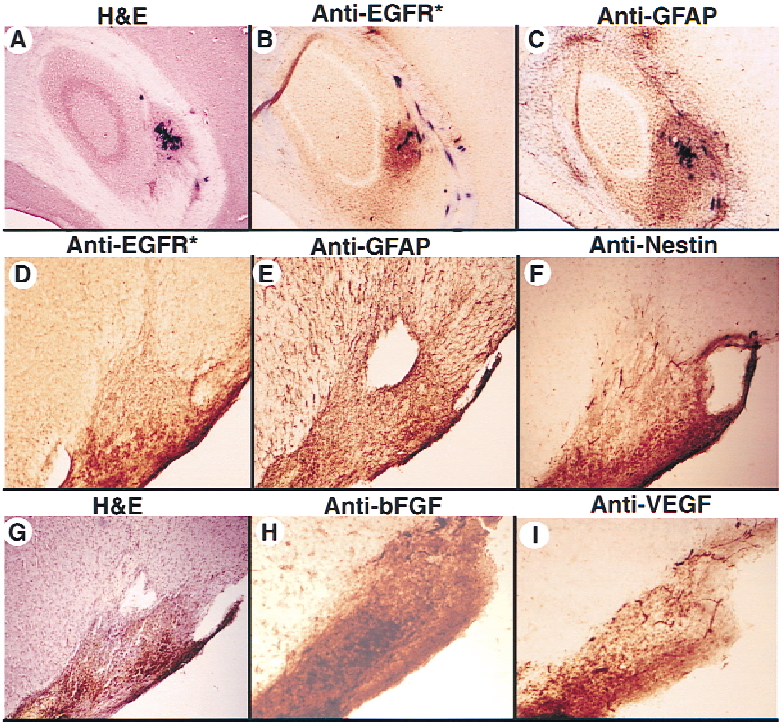

Figure 5. *EGFR**-induced lesions in *INK4a–ARF*$^{+/-}$ and *INK4a–ARF*$^{-/-}$ mice. (*A–C*) Eight weeks after injection with RCAS–*AP* and RCAS–*EGFR**, the brain from a *Ntv-a*; *INK4*$^{+/-}$ mouse was sectioned (frozen, 40 µm), stained for AP activity, and then stained with either H&E (*A*), or monoclonal antibodies recognizing EGFR* (*B*) or GFAP (*C*), as indicated. (*A–C*) Magnification, 40×. This tumor, located in the hippocampus, is a mixture of AP$^+$ and AP$^-$ cells that express EGFR* and GFAP, indicating an astrocytic cell origin. (*D–I*) Brain sections from a 10-week-old *Gtv-a*; *INK4a–ARF*$^{-/-}$ mouse infected with RCAS–*EGFR**. The brain was fixed, and frozen sections (40 µm) were either stained with H&E, illustrating high cell density and previous hemorrhage (*G*) or stained with polyclonal antibodies to EGFR* (*D*), GFAP (*E*), nestin (*F*), bFGF (*H*), or VEGF (*I*). Magnification, 40× (*A–C*); 100× (*D–I*).

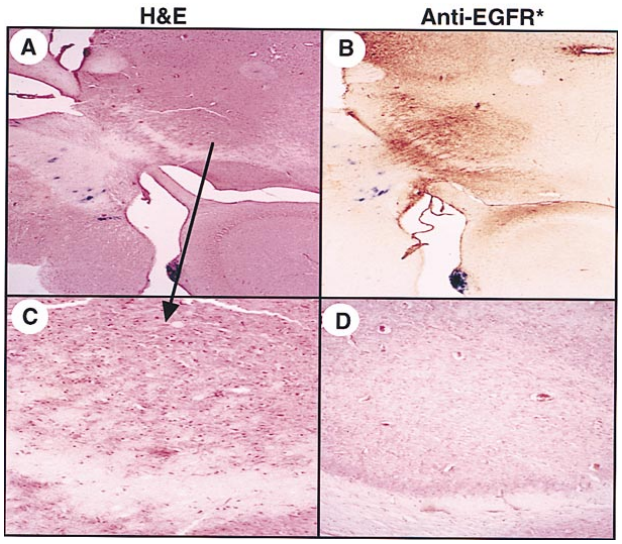

Figure 6. Diffuse thalamic glioma associated with hydrocephalus. Frozen brain sectcions (40 µm) from a *Gtv-a*;*INK4a–ARF*$^{+/-}$ mouse infected at birth with both RCAS–*AP* and RCAS–*EGFR** and analyzed after the development of hydrocephalus at 6 weeks of age. Brain sections were stained for alkaline phosphatase activity, and then stained with either H&E (*A*) or monoclonal antibodies to EGFR* (*B*). (*C*) A higher magnification of the H&E section in *A* and compared to the same anatomic region of an infected littermate that had not developed hydrocephalus (*D*). Magnification, 40× (*A*,*B*); 100× (*C*,*D*).

formation. We attempted to demonstrate the loss of p16^{INK4a} expression with an anti-p16 monoclonal antibody in a histochemical assay; unfortunately, we found the level of p16^{INK4a} in the normal adult mouse brain below the level of detection. Therefore, we were unable to measure loss of expression. The small size and diffuse character of the gliomas prevented dissection of tumor from normal tissue in most of the mice heterozygous for *INK4a –ARF*. In one sample large enough to permit dissection, PCR analysis demonstrated maintenance of the heterozygous *INK4a –ARF* genotype in the tumor tissue (data not shown). This single finding is difficult to interpret, but could indicate either contamination of the tumor tissue with normal cells or inactivation of the remaining wild-type allele in some other way, such as methylation.

bFGF *gene transfer does not enhance* EGFR*-*induced glioma formation*

bFGF is overexpressed in most high-grade gliomas (Takahashi et al. 1990), and we have shown previously that RCAS–*bFGF* induces proliferation and migration of astrocytes without tumor formation in *Gtv-a* mice (Holland and Varmus 1998). The addition of RCAS–*bFGF* to the combination of RCAS–*EGFR** and RCAS–*cdk4* for infection of *Ntv-a* mice did not change either the type of lesion or the age at onset of symptoms appreciably in any of the genetic backgrounds tested (Table 1; data not shown). One explanation for this finding is that sufficient expression of *bFGF* may be induced in gliomas secondary to *EGFR** gene expression, as in the tumor illustrated in Fig. 4. If *bFGF* expression is essential for gliomagenesis in mice, its induction appears not to be rate-limiting because the addition of virally transduced *bFGF* did not appear to affect glioma formation.

CDK4 *overexpression can cooperate with* EGFR* and is required for EGFR**-induced gliomagenesis in* p53$^{+/-}$ *mice*

In human gliomas, mutations and amplification of the *EGFR* gene are found more frequently in gliomas that lack *INK4a –ARF* (and hence cannot make p16^{INK4a} or p19^{ARF}) than in tumors with abnormalities of *cdk4* or *Rb* (Hayashi et al. 1997). Furthermore, *EGFR* and *p53* mutations appear to be mutually exclusive in these tumors (Watanabe et al. 1996). These observations are consistent with recent data demonstrating that loss of *p19*^{ARF} and *p53* mutations appear to be mutually exclusive in mouse fibroblast culture (Kamijo et al. 1997), and that RAS-induced *INK4a –ARF*-deficient melanomas remain consistently wild type for *p53* (Chin et al. 1997). Gliomas with amplification of the *CDK4* gene most frequently harbor *p53* mutations, implying synergy between the effects of these two genetic alterations in gliomagenesis. One interpretation of these findings might be that the combination of *CDK4* amplification and *p53* loss (or *mdm2* amplification) inactivates both the Rb and p53 cell-cycle arrest pathways, similar to the effect of *INK4a –ARF* loss (see Fig. 1), and that inactivation of both pathways promotes gliomagenesis strongly (see Discussion).

In human gliomas, *EGFR* and *p53* mutations are found in the same tumor rarely, implying a lack of cooperation between the effects of these two abnormalities. To investigate the interaction of *EGFR* mutations and *p53* loss in our model, we infected *Ntv-a*; *p53*$^{+/-}$ mice with RCAS–*EGFR**. No glioma-like lesions or other intracranial structural abnormalities appeared in 16 *p53*$^{+/-}$; *Ntv-a* mice after infection with RCAS–*EGFR**.

Multiple genes can be transferred to specific cell types in *tv-a* transgenic mice. Therefore, we used mixtures of RCAS vectors (RCAS–*cdk4* and RCAS–*EGFR** or RCAS–*cdk4*, RCAS–*EGFR**, and RCAS–*bFGF*) to infect *tv-a* transgenic mice, including mice that also carry mutations in tumor suppressor genes. As demonstrated above, RCAS–*EGFR** alone was unable to generate gliomas in wild-type mice; however, the addition of RCAS–*cdk4* to RCAS–*EGFR** produced hydrocephalus in ~10% of *Ntv-a* mice with an otherwise wild-type genetic background. We examined two of these hydrocephalic brains from *Ntv-a* mice infected with RCAS–*cdk4* and RCAS–*EGFR** and found histologic features of diffuse gliomas. These gliomas depend presumably on the cooperative effects of excessive *EGFR* signaling and disruption of the G_1 arrest pathway caused by overexpression of *CDK4*. However, we did not determine the status of p53, Mdm2, and p16^{ARF} in these tumors.

Strikingly, although RCAS–*EGFR** alone caused no abnormalities in *Ntv-a*; *p53*$^{+/-}$ mice, the addition of

RCAS–*cdk4* to RCAS–*EGFR** caused hydrocephalus and death within the first 3–5 weeks of life in 40% of *p53* heterozygous mice. In the hydrocephalic mouse brain, the cerebrospinal fluid (CSF) was frequently frankly bloody and associated with subdural hematomas, intraparenchymal hemorrhage, vascular proliferation, and increased cell density and gliotic changes (Fig. 7). A periventricular proliferation of glia was also observed, with astrocytic cells that expressed *GFAP*, *nestin*, and *EGFR** diffusely infiltrating the thalamus and striatum. Thus, in our mouse model, overexpression of *CDK4* appears to be required for either efficient induction or progression of gliomas by *EGFR** in *p53*-deficient mice. Although it is probable that gliomas arising in $p53^{+/-}$ mice have lost the remaining wild-type *p53* allele, similar to what has been reported for other tumor models arising in $p53^{+/-}$ mice (Donehower 1995), because of the diffuse nature of the tumors and heavy contamination with normal cells we were unable to determine the status of the *p53* locus in these lesions by PCR or Southern blot analysis.

Discussion

We have generated an animal model for gliomagenesis that recapitulates the genetic abnormalities found in human gliomas. The lesions generated by this model have many characteristics similar to those found in human gliomas, including expression of glial markers and induction of angiogenic growth factors such as bFGF and VEGF. Human gliomas have a number of histologic characteristics; we find many of these histologic characteristics in the lesions induced in our mouse model.

All the tumors we have generated thus far require expression of the activated form of EGFR (EGFR*) as well as combinations of lesions affecting cell-cycle regulation. EGFR* induces gliomas in conjunction with overexpression of *CDK4* only occasionally, whereas *INK4a–ARF* loss, which eliminates both the Rb- and p53-mediated pathways for cell-cycle arrest, cooperates efficiently with EGFR* in gliomagenesis. In addition, EGFR* appears incapable of generating gliomas in a *p53*-deficient background unless CDK4 is also overproduced. These patterns of mutations—especially the cooperation between EGFR* and *INK4a–ARF* deficiency and the apparent inability of EGFR* alone to stimulate gliomagenesis in *p53*-deficient mice—resemble those seen in human gliomas.

Infection of tv-a *transgenic mice with RCAS vectors as a method for modeling human cancer*

There are various methods for modeling the contribution that combinations of mutations make to oncogenesis. Cell-type-specific gene transfer using RCAS vectors has a number of advantages over previously reported strategies. First, with the use of transgenic mouse lines expressing *tv-a* in specific cell types, combinations of genes can be tested by the use of easily constructed or previously existing viral vectors. Therefore, the cost and time required to test complex combinations of genetic lesions are significantly less than for strategies in which each genetic alteration requires a separate line of mice, and multiple matings are necessary to observe interactions between mutations (e.g., Kwan et al. 1992; Donehower 1995). The *tv-a* system can also combine gain-of-function (by infection with RCAS vectors carrying oncogenes) and loss-of-tumor-suppressor-gene function, either as shown here by breeding to mice with targeted germ-line deletions or in the future by deleting alleles that contain *lox* recombination sites by infecting with an RCAS vector carrying *cre*, encoding a *lox*-specific recombinase. Second, classic transgenic strategies express oncogenes in all cells utilizing the transgenic promoter. Cells giving rise to tumors from such a population have acquired further (usually unknown) mutations resulting in tumor formation. In contrast, when injected into the

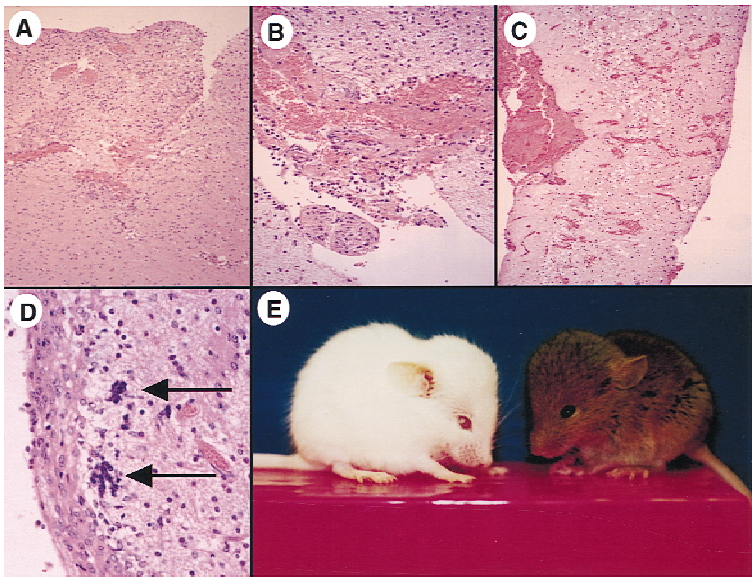

Figure 7. Characteristics of brains harboring lesions induced by multiple mutations. (*A–D*) Microscopic findings after RCAS–*EGFR** and RCAS–*cdk4* infection of *Ntv-a; INK4a–ARF*$^{-/-}$ mice. (*A*) Increased cell density and vascularity relative to adjacent brain parenchyma (40×). (*B*) Vascular endothelial hyperproliferation (100×). (*C*) Intraparenchymal hemorrhage throughout the cortex (40×). (*D*) Multinucleated cells (arrows) and nuclear pleomorphism (200×). (*A–D*) Paraffin-embedded sections stained with H&E. (*A*,*D*) The same mouse sacrificed at 5 weeks of age; (*B*,*C*) Two other mice sacrificed at 4 and 5 weeks, respectively. (*E*) The appearance of two hydrocephalic *Ntv-a* mice infected with RCAS vectors at birth. The white mouse was *Ntv-a; p53*$^{+/-}$, infected with RCAS–*cdk4*, RCAS–*bFGF*, and RCAS–*EGFR** and photographed at 4 weeks of age; the brown mouse was *Ntv-a*; *INK4a–ARF*$^{-/-}$, infected with RCAS–*bFGF* and RCAS–*EGFR**, and photographed at 10 weeks of age.

brain, RCAS vectors infect only a few hundred cells in the vicinity of the injection track (Holland and Varmus 1998). Therefore, any observed phenotypes are more likely to be direct effects of the transferred genes, since a relatively small number of cells would be at risk initially of secondary mutations. Finally, retroviral insertional mutagenesis has been used to identify genes that cooperate with oncogenic transgenes (e.g., van Lohuizen et al. 1991; Shackleford et al. 1993). This strategy can detect novel genes, but it is likely to recognize the combinations that result in the most rapidly growing cell populatios. In contrast, because of the limited number of cells infected initially by RCAS vectors, the *tv-a* system will be informative mainly about known genes carried by RCAS vectors, but it can be used to assess weakly oncogenic combinations.

Gliomagenesis requires multiple mutations

Combinations of mutations appear to be essential for the induction of gliomas in mice, because in our study single mutations were incapable of generating gliomas with any measurable frequency. We have shown previously that neither *bFGF* (Holland and Varmus 1998) nor *CDK4* gene transfer alone (Holland et al. 1998) results in glioma formation in mice, and *p53*-deficient mice have not been reported to develop gliomas (Donehower et al. 1992). In this report we show that *EGFR** gene transfer alone does not induce gliomagenesis either. Furthermore, *INK4a–ARF*-deficient mice develop normally without glioma formation (Serrano et al. 1996); therefore loss of both $p16^{INK4a}$ and $p19^{ARF}$ also appears insufficient to induce gliomagenesis.

Similar to the experience of others with *INK4a–ARF*-deficient human gliomas (Louis et al. 1993; Lang et al. 1994; Rasheed et al. 1994; Schlegel et al. 1994; He et al. 1995; Hayashi et al. 1997), we find cooperation between loss of *INK4a–ARF* and expression of mutant *EGFR* during gliomagenesis. In our study the percent of mice developing EGFR*-induced gliomas was similar in *INK4a–ARF*$^{+/-}$ and *INK4a–ARF*$^{-/-}$ genetic backgrounds. We were unable to test conclusively whether tumors arising in *INK4a–ARF*$^{+/-}$ mice retained a functional *INK4a–ARF* allele. It is possible either that haploinsufficiency of the *INK4a–ARF* gene allows EGFR* to induce gliomas in these mice or that the tumorigenic cells are nullizygous physically or functionally. In one published series of 120 human glioblastomas, 38% of the tumors demonstrated deletion of both copies of *INK4a–ARF*, whereas 33% maintained one wild-type allele of *INK4a–ARF*. Furthermore, the majority of gliomas in this series with hemizygous deletion of *INK4a–ARF* did not have mutations in the remaining *INK4a–ARF* allele (Ichimura et al. 1996). Methylation of the *INK4a–ARF* locus in human gliomas is one possible mechanism for loss of $p16^{INK4a}$ protein production in gliomas with hemizygous deletions of *INK4a–ARF*, although in most tumors in this series there was evidence for neither methylation nor mutation of the remaining *INK4a–ARF* locus (Schmidt et al. 1994).

INK4a–ARF appears to be normal in ~30%–50% of gliomas in adults. These gliomas usually contain *p53* mutations and display *EGFR* mutations rarely; by the time they reach grade 4 status, they frequently overexpress *cdk4* or lose *Rb* (von Deimling et al. 1993). In Holland et al. (1998), we show that *CDK4*-immortalized astrocyte cultures sometimes acquire *p53* mutations. Although the *p53* mutations are not required to escape senescence or destabilize the genome—a phenomenon that we attribute to the varied consequences of expressing high levels of CDK4—they may enhance growth potential. These observations in cell culture are further evidence that overexpression of *CDK4* and loss of *p53* may cooperate in some way to achieve more rapid proliferation of astrocytes in vivo or in vitro.

Our data indicate that RCAS–*EGFR** can efficiently induce glioma-like changes either in *INK4a–ARF*$^{-/-}$ animals or in animals carrying an inactive *p53* gene and coinfected with RCAS–*cdk4*. These results are consistent with the view that *INK4a–ARF* governs both the RB and p53 pathways and that the inactivation of both pathways is essential for efficient EGFR*-induced gliomagenesis. In this sense, the mutations that promote gliomagenesis by EGFR* resemble the action of SV40 T antigen, which is known to achieve its oncogenic effect by disrupting both p53 and RB function (Fanning 1992).

In the accompanying paper, we show that either loss of *INK4a–ARF* or overexpression of *CDK4* can lead to immortalization of astrocytes in culture (Holland et al. 1998). Clearly, there must be additional constraints on proliferation in vivo to prevent cells with inactivated G_1 arrest pathways from proliferating abnormally, as neither infection withRCAS–*cdk4* n or the *INK4a–ARF*$^{-/-}$ genotype is sufficient to induce gliomas. In this study we show that if cells with aberrant G_1 arrest pathways also express a constitutively active EGFR, they are capable of escaping these apparent constraints. Many signaling pathways function downstream of EGFR, and some involve proteins governing progression through the G_1 phase of the cell cycle. However, it is not clear which pathways are involved in gliomagenesis or how they cooperate with the inactivation of G_1 arrest mechanisms.

Previous work has indicated that although *bFGF* and *VEGF* are overexpressed in most high-grade human gliomas, these genes are not mutated or amplified. These findings have been interpreted to suggest that augmented activity of these genes is secondary to tumor initiation and progression. The mouse glioma illustrated in Figure 5 was initiated by infection with RCAS–*EGFR**, but also produces high levels of endogenous bFGF and VEGF. These findings imply that overexpression of bFGF and VEGF can be induced secondarily and support the notion that regulation of growth factors in human gliomas can be regulated in response to other genetic alterations. For example, an active form of Ras, a signaling protein also activated by EGFR, has been shown to enhance VEGF expression in glial cell culture (Okata et al. 1998).

Thus far, our gene transfer model for gliomagenesis reflects the patterns of mutations seen in human gliomas

and, therefore, represents a useful tool for understanding the molecular basis of gliomagenesis. Using glioma formation as an assay, it is possible to identify the tumorigenic components of signaling pathways downstream of EGFR or implicated in G_1 arrest. These components may prove to be targets suitable for interventions directed against human gliomas.

Materials and methods

Constructs

The *Gtv-a* transgene (with a 2.2-kb fragment of the GFAP promoter driving expression of the quail *tv-a* cDNA and a fragment from the mouse protamine gene (*MP-1*) supplying an intron and signal for polyadenylation), RCAS–*AP*, RCAS–*bFGF*, and RCAS–*cdk4* have been described (Holland and Varmus 1998; Holland et al. 1998). RCAS–*puro* was obtained from Steve Hughes (National Cancer Institute). The *Ntv-a* transgene utilizes a modified *nestin* promoter, including portions of its second intron and thymidine kinase promoter sequences that have been shown to direct expression to CNS progenitor cells; polyadenylation sequences are provided by SV40 DNA. This transgene was constructed by *Not*I digestion of the NES 1689/lacZ (Lothian and Lendhal 1997) to release the *lacZ* gene, followed by incubation with Klenow fragment of DNA polymerase and ligation to the *tv-a* cDNA from pSPKE 0.8 (Bates et al. 1993), which had been digested with *Eco*RV and *Sma*I to achieve blunt, compatible ends. RCAS–*EGFR** was constructed by *Cla*I digestion of RCAS–*puro* to remove the puromycin-resistance gene and replacement with a fragment from pIND5′3′*EGFR* (Ekstrand et al. 1992; gift of David James, Mayo Clinic Rochester, and Chris Thomas, Mayo Clinic Jacksonville). This fragment contains a human *EGFR* cDNA lacking sequences corresponding to exons 2–7 (bases 275–1075) and sequences 3′ of base 3133.

Mice

Production of the *Gtv-a* mouse line has been described (Holland and Varmus 1998). The *Gtv-a* mouse line was generated originally from an FVB/N crossed with a C57B6 X BALB/C F_1. The *Gtv-a* founder was then bred to an FVB/N to generate F_1 progeny which have been interbred subsequently to maintain the transgenic line. Production of the FVB/N *Ntv-a* mouse line was by pronuclear injection of the *Ntv-a* transgene, which had been released from the plasmid by digestion with *Hin*DIII. Mice with mixed C57BL/6 and 129 genetic background and with targeted, inactivated mutations of *INK4a–ARF* and *p53* have been described previously (Donehower et al. 1992; Serrano et al. 1993). The genetic backgrounds of the *tv-a* transgenic mice used for infection were therefore mixes of FVB/N, 129, and C57BL6.

Cell culture

Primary brain cell cultures from newborn transgenic mice were obtained by mechanical dissociation of the whole brain, followed by digestion with 0.25% trypsin for 15 min at 37°C. Large debris was allowed to settle, and single cells were plated and grown in DMEM with 10% fetal calf serum (GIBCO–BRL). DF-1 cells, an immortalized line of chicken cells were a gift from Doug Foster, University of Minnesota, and were grown in DMEM with 5% fetal calf serum, 5% calf serum, 1% chicken serum, and 10% tryptose phosphate broth (GIBCO–BRL).

Infection in cell culture

The supernatant from DF-1 cells infected with and producing RCAS vectors was filtered through a 0.45-μm filter and plated directly onto primary brain cells cultures from *Gtv-a* mice. These primary brain cultures were infected with filtered medium from RCAS–*puro*-producing cells and then selected in 4 μg/ml puromycin.

Immunoprecipitation, Western blot analysis, and immunoperoxidase staining of cultured cells

Proteins were isolated from cultured cells using Tris-buffered saline (pH 8.0) with 1.0% Tween (TBST) and precipitated overnight at 4°C with 0.4 μg of mouse monoclonal anti-mutant EGFR (gift of Darrel Bigner, Duke University, Durham, NC; Wikstrand et al. 1995) using anti-mouse immunoglobulin–Sepharose (Sigma). The products were separated by gel electrophoresis and transferred to nictrocellulose. The blot was incubated in TBST with the same anti-mutant EGFR antibody, washed extensively in TBST, incubated with a peroxidase-conjugated goat anti-mouse antibody (Boehringer), and then detected with ECL (Amersham).

For immunoperoxidase analysis, cultured cells were fixed in 100% methanol and blocked in TBST with 1% goat serum. They were then incubated for 1 hr at room temperature after the addition of 0.5 μg of anti-mutant EGFR antibody, washed extensively with TBST, and detected with peroxidase-conjugated anti-mouse antibody (ABC, Vector).

Infection of transgenic mice

DF-1 cells infected with and producing RCAS vectors were harvested by trypsin digestion and pelleted by centrifugation, the cell pellets were resuspended in ~ 50 μl of medium and placed on ice. Using a 10-μl gas-tight Hamilton syringe, a single intracranial injection of 1 μl containing 10^4 cells was made in the right frontal region of newborn mice, just anterior to the striatum, with the tip of the needle just touching the skull base.

Brain sectioning and immuno- and histochemical staining

Animals were sacrificed at 4–10 weeks of age, the brains fixed in 4% formaldehyde, 0.4% glutaraldehyde, 1× PBS for 36 hr, and then dehydrated in 20% sucrose, 2% glycerol, 1× PBS. Frozen sections (40 μm) were obtained using a sledge microtome (Zeiss). The sections were then stained in solution for alkaline phosphatase activity using 5-bromo-4-chloro-indolyl-phosphate and 4-nitro-blue-tetrazolium-chloride (Boehringer) after treatment at 65°C (pH 9.5) for 3 min to remove endogenous alkaline phosphatase activity. The sections were then mounted on glass slides and counterstained with hematoxylin and eosin. For immunostaining, fixed frozen sections (40 μm) were stained in solution for AP as described. The sections were then treated with 10% hydrogen peroxidase/70% methanol for 15 min to inactivate endogenous peroxidases. The sections were then blocked with 1% goat serum in Tris-buffered saline (pH 8.0), with 0.1% Tween (TBST) solution for 20 min followed by a 1-hr incubation at room temperature after the addition of mouse monoclonal antibodies to human GFAP (Boehringer), rat nestin (Pharmingen), bovine bFGF (Calbiochem), mouse VEGF (Upstate Biochemicals), or to the vIII mutant form of human EGFR (gift of Darell Bigner; Wiksrand et al. 1995) or rabbit polyclonal antibodies to the human vIII mutant EGFR (gift of Albert Wong,

Thomas Jefferson University, Philadelphia, PA). The sections were washed extensively with TBST and antibody staining was then visualized with peroximase-conjugated anti-mouse antibody (ABC, Vector) and sections then mounted on glass slides.

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Note added in proof

We have detected aberrant transcripts that could encode the amino-terminal portion of $p19^{ARF}$ from the *INK4a–ARF* exon 1β in astrocytes derived from the mice used in this study having targeted deletions in the *INK4a–ARF* locus (W.P. Hively, E.C. Holland, R.A. DePinho, H.E. Varmus, and M. Serrano, unpubl.). However, cells from these mice and from mice with a mutation that specifically eliminates exon 1β (Kamijo et al. 1997) have very similar phenotypic properties with respect to $p19^{ARF}$ functions in apoptosis, transformation, and ploidy (Serrano et al. 1996; Zindy et al. 1998; W.P. Hively, E.C. Holland, R.A. DePinho, H.E. Varmus, and M. Serrano, unpubl.). Therefore, the INK4a–ARF mutants we have used in this paper and in Holland et al. (1998) are likely to be severely hypomorphic or null with respect to $p19^{ARF}$ function.

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