

Oligodendrogliomas Result from the Expression of an Activated Mutant Epidermal Growth Factor Receptor in a RAS Transgenic Mouse Astrocytoma Model¹

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ABSTRACT

A significant proportion of human malignant gliomas exhibit amplification, overexpression, or mutations of the epidermal growth factor receptor (EGFR). To define the functional role(s) of the EGFR in the pathogenesis of gliomas, we established transgenic mice that express both wild-type (wt) and mutant (EGFRvIII) EGFR molecules using the human glial fibrillary acidic protein (GFAP) promoter. Both GFAP-EGFR^{wt} and GFAP-EGFRvIII transgenic mice demonstrated increased numbers of astrocytes compared with control littermates, however, developed normally without formation of gliomas. To determine whether EGFR overexpression could modify the tumor phenotype in our previously reported GFAP-V¹²Ha-ras transgenic mouse astrocytoma model, mice expressing both activated RAS and EGFR were developed. GFAP-V¹²Ha-ras;GFAP-EGFRvIII, but not GFAP-V¹²Ha-ras;GFAP-EGFR^{wt} double transgenic mice, had decreased survival with fifty percent of the mice dead at 2–4 weeks from gliomas, compared with 12–16 weeks for the GFAP-V¹²Ha-ras mice. Furthermore, GFAP-V¹²Ha-ras;GFAP-EGFRvIII mice developed oligodendrogliomas and mixed oligoastrocytoma tumors, instead of the fibrillary astrocytomas observed in GFAP-V¹²Ha-ras mice. In addition to yielding a spontaneous model of infiltrating oligodendroglioma, this study demonstrates that astrocyte-specific expression of EGFRvIII alone is insufficient for gliomagenesis but rather contributes to glioma progression in the context of existing predisposing genetic changes.

INTRODUCTION

Diffuse gliomas are the most common primary malignancy of the human central nervous system. The WHO classifies gliomas according to their predominant cellular composition, with astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas as the most common (1). The most malignant and unfortunately the most common adult glioma is the GBM,³ with a median survival of 9–12 months (1). The glial lineage that gives rise to these heterogeneous GBMs is not known, although a large subset likely arises from type 1 fibrillary astrocytes. However, as many as half of GBMs do not express the differentiated astrocytic GFAP marker, perhaps either as a result of cell de-differentiation or a common terminal transformation phenotype of several glial lineages (1).

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³ The abbreviations used are: GBM, glioblastoma multiforme; GFAP, glial fibrillary acidic protein; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor- α ; APC, adenoma polyposis coli; FACS, fluorescence-activated cell sorting; ES, embryonic stem; IRES, internal ribosomal entry site; IHC, immunohistochemistry; HA, hemagglutinin; RA, retinoic acid; MAG, myelin-associated glycoprotein; BrdUrd, bromodeoxyuridine; MAG, myelin-associated glycoprotein; wt, wild type.

The molecular progression of GBMs involves at least two or more pathways, although the tumors are pathologically indistinguishable. One pathway, involving progression from lower grade astrocytomas to secondary GBMs, is more prevalent in younger patients and is characterized by mutations in TP53 tumor suppressor. The second and more common primary or *de novo* pathway occurs mainly in older GBM patients. These GBMs lack TP53 mutations but harbor amplifications, overexpression, or mutations of the EGFR (3). The most common EGFR mutation is the EGFRvIII variant, involving an in-frame deletion of exons 2–7 in the extracellular domain of wt EGFR molecule, resulting in a constitutively phosphorylated and activated receptor (4). Overexpression of EGFRvIII provides a growth advantage to astrocytoma cell lines (5). In addition, EGFRvIII overexpression in younger patients with GBM has been shown to represent a negative clinical prognosticator and is associated with reduced patient survival (6, 7).

In contrast to the fibrillary astrocytomas, the molecular pathogenesis of oligodendrogliomas is not as well understood, although similar molecular heterogeneity has been demonstrated (8). For example, a subset of oligodendrogliomas that typically are associated with improved survival and response to chemotherapy demonstrate loss of various regions of chromosome 1p and/or 19q (9). In contrast, another subset of oligodendrogliomas associated with poorer survival, despite radiation and chemotherapy, exhibit loss of p16 and harbor deletions in the regions of chromosome 10q and also have EGFR amplification and mutations (8). In addition, oligodendrogliomas developed in a transgenic mouse model of *v-erbB* under regulation of the S-100 promoter.⁴ This observation suggests that EGFR amplification and mutation may be a molecular marker of more aggressive oligodendrogliomas or mixed oligoastrocytomas, similar to that observed for highly malignant astrocytomas (GBMs).

To explore the functional role(s) of EGFR proteins in gliomagenesis, we used ES cell-mediated transgenesis to express wt and mutant EGFR molecules in mouse glial cells *in vivo*. We found that overexpression of EGFR^{wt} or EGFRvIII in mouse astrocytes was not sufficient to cause astrocyte transformation and result in glioma formation. However, in mice harboring an activated oncogenic Ras in astrocytes sufficient for glioma formation (10), the additional expression of EGFRvIII, but not EGFR^{wt}, led to accelerated glioma formation. In addition, the brain tumors in these mice exhibited histopathological features of oligodendroglioma and mixed oligoastrocytoma tumors. These results support the observation from human molecular clinical-epidemiological data that alterations in EGFR expression are important in the progression, rather than the initiation of gliomas. Furthermore, the finding of oligodendroglioma and mixed oligoastrocytoma lineage tumors in the mice expressing both oncogenic Ras and activated EGFR molecules supports the idea that dysregulation of multiple genetic pathways are important determinants of the histological phenotype of gliomas.

⁴ William A. Weiss, personal communication.

MATERIALS AND METHODS

Plasmid Construction. The human EGFR^{wt} and EGFRVIII cDNAs (provided by Dr. C. David James, Mayo Clinic) were ligated respectively to the human GFAP promoter (obtained from Dr. Michael Brenner, National Institutes of Neurological Disorders and Stroke) based on a cloning strategy as described previously (10). An *IRESLacZ* cassette in which *LacZ* gene was fused to a nuclear localization signal and an IRES sequence, was introduced in the above vector to form *GFAP-EGFR^{wt}* (or *EGFRvIII*)-*IRESLacZpolyA-loxP-neo-loxP* (Fig. 1A).

ES Cell-mediated Transgenesis to Establish GFAP-EGFR^{wt} and GFAP-EGFRvIII Transgenic Mice. We followed the procedure as described previously (11).

Genotyping. PCR and Southern blot analysis were applied for genotyping and analyzing transgene copy in the transgenic lines. PCR was performed with ear-punched DNA and a sense primer (5'-ACTCCTTCATAAAGCCCTCG-3') located in the GFAP promoter, and an antisense primer (5'-GTG-GAGATCGCCACTGATGGA-3') located in the EGFR cDNA. PCR was performed for 35 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1 min. Southern blot analysis was undertaken as per standard protocols.

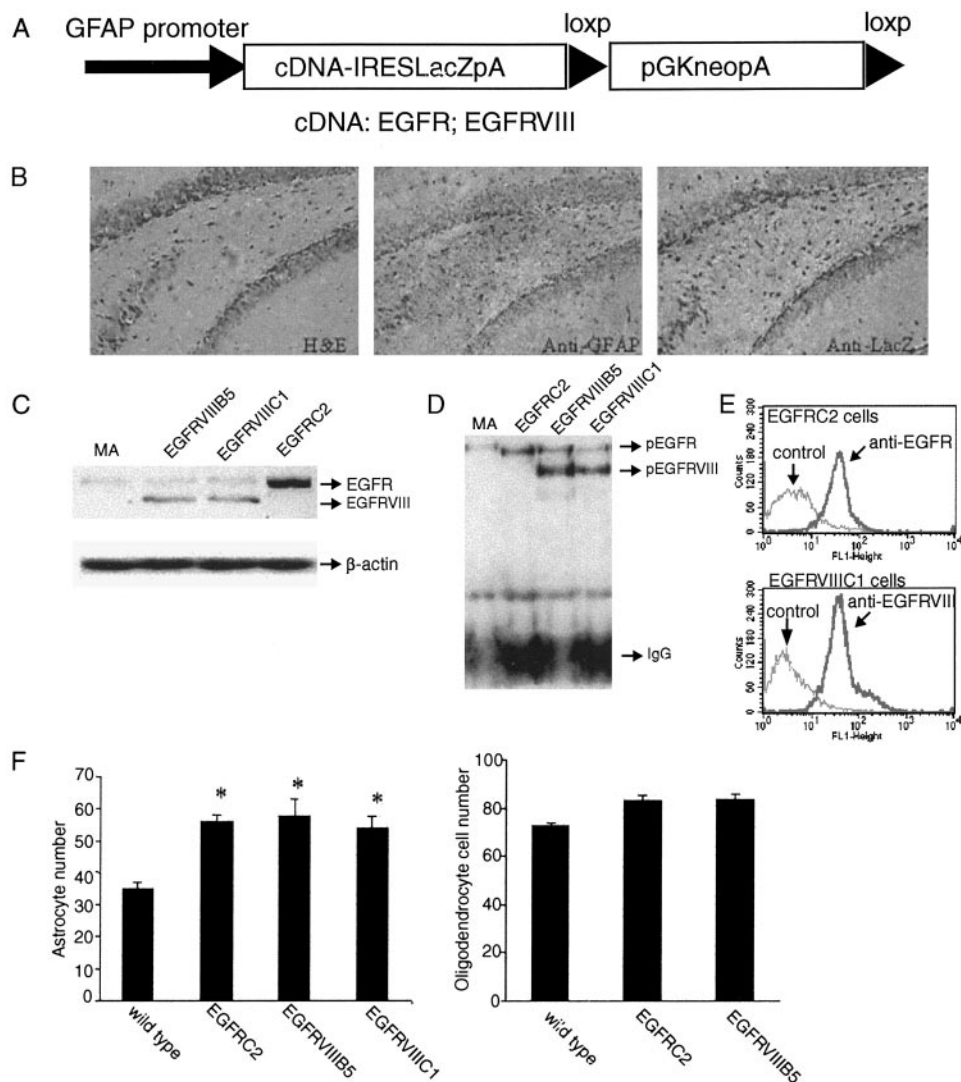
IHC and in Situ Hybridization. Preparation of tissue sections, H&E staining, and IHC analysis were performed according to standard procedures. Primary antibodies used were rabbit anti-GFAP (dilution 1:300; Dako), mouse monoclonal anti-BrdUrd (dilution 1:80; Boehringer), mouse monoclonal anti-LacZ (dilution 1:10,000; BioLab), rabbit anti-Oligo2 (dilution 1:200, devel-

oped by Dr. Takebayashi Hirohide), rabbit anti-Nestin (dilution 1:1000; Chemicon), rabbit anti-Factor VIII (dilution 1:3000; Dako), rabbit anti-PDGFR- α (dilution 1:200; UBI), mouse anti-NeuN (dilution 1:200; Chemicon), and goat anti-MAG (dilution 1:200, Santa Cruz). terminal deoxynucleotidyl transferase-mediated nick end labeling assay for detecting apoptosis was done based on the commercial kit (Boehringer). RNA *in situ* analysis of frozen sections of mouse brains were performed according to established protocols (12), with sense and antisense digoxigenin-labeled RNA probes which were *in vitro* transcribed from the full-length mouse Olig1 and Olig2 coding sequence.

Quantitation of Astrocytes and Oligodendrocytes in Transgenic Mice. GFAP and APC IHC was used to identify differentiated astrocytes and oligodendrocytes respectively in 40 μ m of free-floating sections (anti-GFAP-1: 10,000; Zymed and anti-APC (Ab-7); Oncogene Sciences). GFAP-immunoreactive astrocytes and APC-immunoreactive oligodendrocytes were counted in the CA1 region of the hippocampus of six consecutive serial sections obtained from four to six animals from each genotype, as described previously (13). The mean number of astrocytes and oligodendrocytes \pm SD was analyzed with ANOVA followed by the Bonferoni *t* test with significance set at $P < 0.05$.

Derivative Astrocytes, Cell Proliferation, Cell Transfection, Ras Activity, and Xenografts in Nod-Scid Mice. Derivative astrocytes from newborn GFAP-EGFR^{wt} and GFAP-EGFRvIII transgenics or normal ICR littermates and their proliferation measurement were performed as described previously (10). To determine the location of transgenes on cell membrane of transgenic astrocytes, FACS was performed with anti-EGFR and anti-EGFRvIII extra-

Fig. 1. ES cell mediated transgenics to develop GFAP-EGFRvIII and GFAP-EGFR^{wt} transgenic mice. A, schemata of transgenic construct containing the GFAP promoter driving expression of EGFRvIII or EGFR^{wt} cDNA and an *IRESLacZ* fragment. B, immunostaining of hippocampal sections of GFAP-EGFRvIII mice with anti-LacZ and anti-GFAP antibodies. C, Western blot analysis of EGFRvIII and EGFR^{wt} protein expression in derived astrocytes using anti-EGFR antibody. β -actin was used as internal loading control. D, Western blot analysis of the phosphorylation of EGFR^{wt} and EGFRvIII proteins in the transgenic astrocytes by immunoprecipitation and probing with anti-pTyr antibody. E, FACS of transgenic astrocytes expressing EGFR^{wt} and EGFRvIII with anti-EGFR and anti-EGFRvIII extracellular domain antibodies, demonstrating both receptors were located on the cell membrane. F, GFAP-EGFRvIII and GFAP-EGFR^{wt} mice demonstrate increased numbers of GFAP-immunoreactive astrocytes. Astrocyte and oligodendrocyte counts were determined as described in the "Materials and Methods" section for six consecutive 40- μ m sections from at least four mice/genotype. The mean and SD is shown for each genotype. The asterisks denote statistically significant increases ($P < 0.01$) using the Student's *t* test.



cellular domain (Zymed) based on standard protocol. Measurement of the activity of Akt in those established transgenic astrocytes was done by Western blot with anti-Akt and anti-phospho-Akt antibodies (Pharmagen). pCX-V¹²Ha-ras-IRESpuro in which the V¹²Ha-ras cDNA with HA at the 5' was inserted downstream of β -actin promoter with a cytomegalovirus enhancer, was transfected into these derivative astrocytes with Lipofectamine (Life Technologies, Inc.), and pooled (>100) clones selected with puromycin (2.5 μ g/ml; Sigma) selection. Expression of exogenous V¹²Ha-ras was measured using Western blot with anti-HA antibody (Sigma). Ras activity was measured as percentage of Ras-GTP/Ras-GTP + Ras-GDP, using a previously described luciferase-based enzymatic assay (14). These pooled transfectants were evaluated for tumorigenicity by stereotactic injection of 10⁵ cells into the corpus striatum of the Nod-Scid mice.

RESULTS

Establishment of GFAP-EGFRvIII and GFAP-EGFR^{wt} Transgenic Mice. The transgene constructs (Fig. 1A) were stably integrated into R1 ES cells. An *IRESLacZ* attachment 3' to the wt- and EGFRvIII-coding sequence allowed LacZ visualization of cells expressing the transgenes. Because the *GFAP* promoter is not active in ES cells, astrocytic expression of the transgene in transfected ES clones was screened by *in vitro* differentiation into astrocytic lineage using RA as described previously (15). ES clones that expressed LacZ upon RA differentiation were selected for ES cells \leftrightarrow embryo aggregation, and two chimeric lines from each transgenic construct were established. The transmitting chimeric males were crossed with 129S1 inbred and ICR outbred females to produce hemizygous-transgenic offspring.

To analyze the cell type-specific expression of the EGFRvIII or EGFR^{wt} transgenes in mice, IHC analysis demonstrated LacZ expression in subsets of GFAP-positive astrocytes (Fig. 1B) but not in adjacent neurons or brains from control littermates. Derivative astrocyte cultures from these transgenic mice brain showed increased expression of EGFR^{wt} and EGFRvIII protein by Western blot analysis, compared with nontransgenic littermates (Fig. 1C). Western blot analysis of the astrocytes expressing EGFR^{wt} and EGFRvIII confirmed that both receptors were phosphorylated (Fig. 1D). FACS assay with specific anti-EGFR and anti-EGFRvIII extracellular domain antibodies demonstrated the EGFR^{wt} and EGFRvIII protein were located on the cell membrane of transgenic astrocytes (Fig. 1E).

All established GFAP-EGFR^{wt} and GFAP-EGFRvIII transgenic mice (hemizygous and homozygous on both 129S1 and ICR genetic background) were healthy and fertile and did not demonstrate any clinical abnormalities even at 24 months of age. To determine whether these transgenic mice harbored any abnormalities in glial cell numbers, we determined both astrocyte (GFAP+) and oligodendrocyte (APC+) numbers in the CA1 region of the hippocampus from 2–3-month-old transgenic and control ICR littermates. As shown in Fig. 1D, there was a 50–60% increase in the number of GFAP-immunoreactive cells in the brains from either the GFAP-EGFR^{wt} or GFAP-EGFRvIII transgenic mice compared with control littermates. This increase in astrocyte number appears to be progressive as 5–6 week old GFAP-EGFRvIII mice exhibit 12–15% more astrocytes (data not shown). Such increased astrocytes was because of the cell proliferation, as demonstrated by an *in vitro* proliferation assay, which showed the transgenic astrocytes from GFAP-EGFR^{wt} and GFAP-EGFRvIII mice had a 1.5-fold increased cell proliferation compared with the control normal mouse astrocytes (Fig. 6C). In contrast, no significant increases in APC-immunoreactive oligodendrocytes were observed at either age (Fig. 1D).

EGFRvIII Transgene Expression Accelerated the Development of Gliomas in GFAP-V¹²Ha-ras Transgenic Mice. We previously established a GFAP-V¹²Ha-ras transgenic mouse astrocytoma model using the GFAP promoter to express an activated RAS molecule in

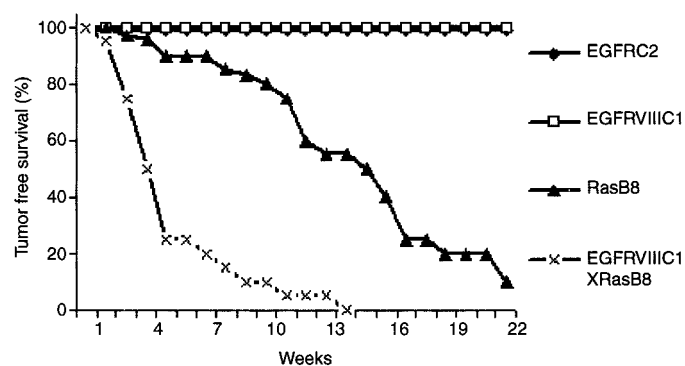


Fig. 2. Survival of single transgenic mice: GFAP-EGFRvIII, GFAP-EGFR^{wt}, and GFAP-V¹²Ha-ras (RasB8); and double transgenic mice: GFAP-EGFRvIII;GFAP-V¹²Ha-ras (EGFRvIII X RasB8). Forty offspring from each breeding were followed. As a control, wt ICR mice had 100% tumor-free survival in these experiments (data not shown).

astrocytes (10) based on previous studies demonstrating increased levels of activated Ras-GTP in human astrocytoma cell lines and tumors (16). In GFAP-V¹²Ha-ras transgenic mice harboring a single copy of the RAS transgene (RasB8), 50% of the mice developed astrocytomas of varying grades and died by 12–16 weeks (10) and (Fig. 2A), with 95% of the mice dead with astrocytomas by 24 weeks. To determine whether alterations in EGFR expression and function in combination with RAS activation could modify mouse survival or tumor histopathological phenotype, we made double transgenics by crossing the GFAP-EGFR^{wt} or GFAP-EGFRvIII mice that do not develop gliomas, with the RasB8 GFAP-V¹²Ha-ras mouse. The overall survival of the GFAP-EGFR^{wt};V¹²Ha-Ras double transgenic mice and the histopathology of the resulting astrocytomas were identical to the RasB8 mice. However, transgenic mice expressing both V¹²Ha-ras and activated EGFRvIII exhibited a dramatic reduction in survival, with 50% of these mice dying with gliomas at 2–4 weeks (Fig. 2).

GFAP-EGFRvIII;GFAP-V¹²Ha-ras Transgenic Mice Developed Oligodendroglial Lineage Tumors. In addition to an effect on tumor latency, GFAP-EGFRvIII;GFAP-V¹²Ha-ras transgenic mice developed tumors with histopathological features of oligodendrogliomas, with a small subset resembling mixed oligoastrocytomas. This pathologic appearance was different from that observed in the GFAP-V¹²Ha-Ras (B8) transgenic mouse astrocytomas, which were largely composed of GFAP and Nestin immunoreactive malignant astrocytes (Fig. 3 G–J). Instead of fibrillary astrocytomas, we found tumors in the GFAP-EGFRvIII;GFAP-V¹²Ha-ras mice exhibited oligodendroglial features (Figs. 3 and 4). The tumor cells had the typical fried egg appearance on H&E staining (Fig. 3A) with majority being GFAP and Nestin immunonegative (Fig. 3, B and C). A small percentage of the tumors observed contained a subpopulation of transformed cells that were both GFAP and Nestin immunoreactive (Fig. 3, E and F), suggesting a mixed oligoastrocytoma tumor. In addition, the infiltrative multifocal tumors had regions of intratumoral hemorrhage were often clustered in periventricular regions with the chicken wire-like vascularity seen in human oligodendrogliomas (Fig. 4, A–C; Ref. 17). Additional IHC characterization demonstrated the neoplastic cells lacked expression of markers found in mature oligodendrocytes (MAG, Fig. 4D), which are also absent in the majority of human oligodendrogliomas (18). The tumor cells were not neurons (NeuN immunonegative, Fig. 4E) but infiltrated through them. Although there is no single pathogenomic IHC marker of oligodendrogliomas, the tumor cells immunostained positively for PDGFR- α (Fig. 4F) and expressed *OLIG1* (Fig. 5, A and B) and *OLIG2* (Fig. 5, C and D) mRNA as detected by *in situ* hybridization, which collectively are highly suggestive of oligodendroglial lineage tumor cells (19, 20).

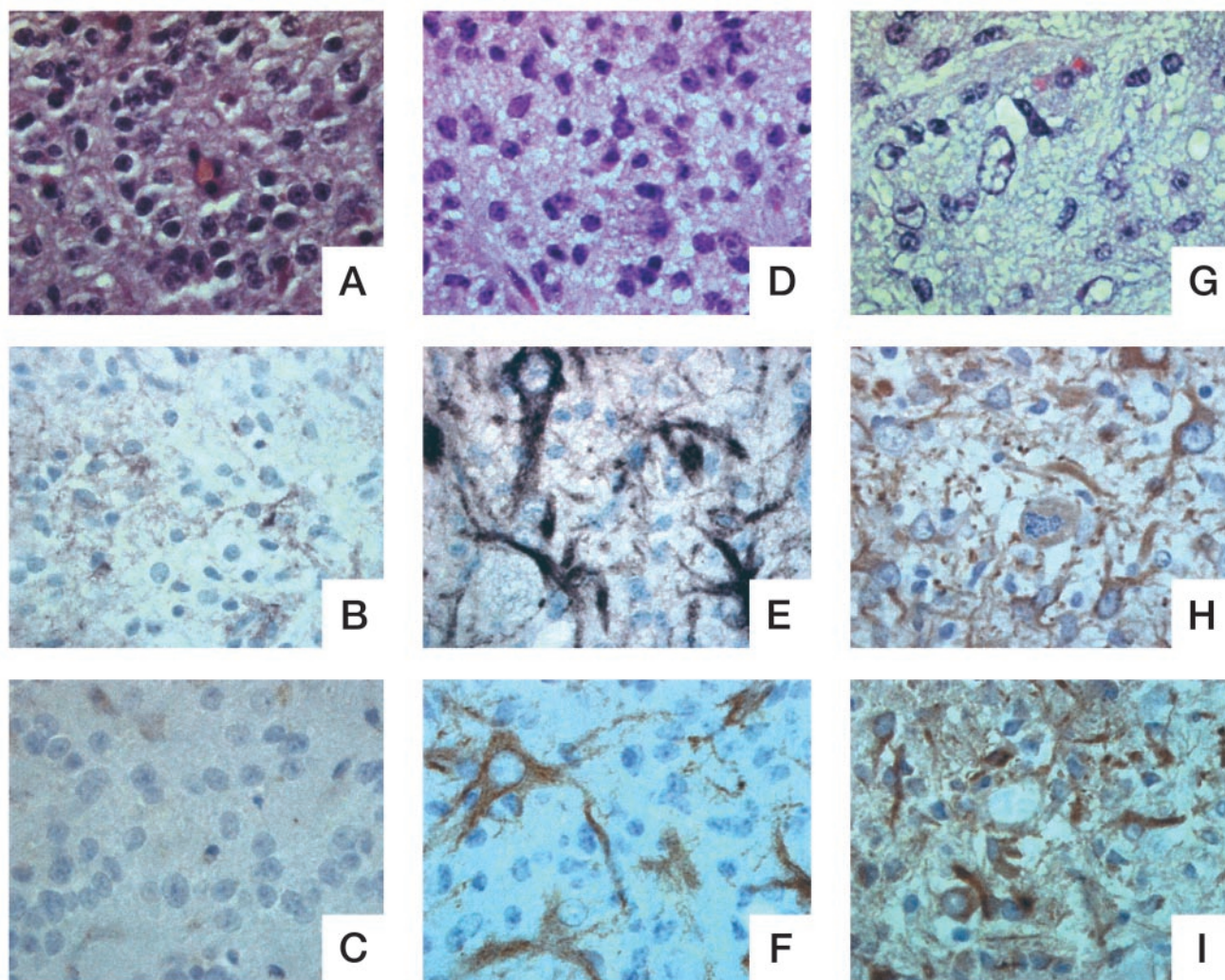


Fig. 3. Histopathological features of oligodendroglioma and oligoastrocytoma in the GFAP-EGFRvIII;GFAP-V¹²Ha-ras double transgenic mice. A–C, majority of the double transgenics were oligodendrogliomas with H&E staining (A) showing the typical fried egg appearance with homogenous round nuclei and condensed chromatin and GFAP (B) and Nestin (C) immunonegativity. D–F, a minority of the double transgenics showed oligoastrocytoma features with transformed appearing astrocytes with irregular nuclei (H&E, A) which were GFAP (E) and Nestin (F) positive, among the predominant oligodendroglioma cells. G–I, in contrast to the double transgenic mice with activated Ras and EGFRvIII, the GFAP-V¹²Ha-ras (RasB8) single transgenic mice had predominantly astrocytic differentiation with vesiculated irregular nuclei (G) with the transformed cells all being GFAP (H) and Nestin positive (I). All magnification, $\times 1000$.

To demonstrate that the cells in these tumors were neoplastic and not dysplastic cells, derivative primary low-passage glioma cell lines from GFAP-EGFRvIII;GFAP-V¹²Ha-ras transgenic mice were generated and injected intracranially in Nod-Scid mice. Tumors developed in recipient mice within 1 month, and the resulting tumor cells were GFAP immunonegative (data not shown) and expressed *OLIG2*, similar to the parental tumors (Fig. 5E).

EGFRvIII and V¹²Ha-ras Cooperate to Transform Astrocytes in Xenograft Models. To determine whether the genetic cooperativity between EGFRvIII and V¹²Ha-ras observed in the transgenic mice *in vivo* could be recapitulated in cell culture and xenograft models, derivative astrocytes from postnatal day 1 (P1) GFAP-EGFR^{wt}, GFAP-EGFRvIII, and nontransgenic littermate mice were established. P1 derivative astrocytes expressing either EGFR^{wt} or EGFRvIII had a 1.5-fold increased *in vitro* proliferation rates as wt astrocytes (up to five passages evaluated; Fig. 6C) and did not grow in Nod-Scid mice (data not shown).

GFAP-EGFR^{wt} and GFAP-EGFRvIII primary astrocytes were next transfected with pCX-HA:V¹²Ha-ras-IRESpuo and multiple clones (>100) were pooled for additional characterization. Overexpression

of V¹²Ha-ras in wt astrocytes and EGFR^{wt} transgenic mouse astrocytes resulted in cellular senescence, with complete cell death by 7 days after transfection. In contrast, EGFRvIII transgenic astrocytes expressing V¹²Ha-ras survived and exhibited more rapid *in vitro* growth rates than GFAP-EGFR^{wt}, GFAP-EGFRvIII, or wt astrocytes. These astrocytes also had an inhibition of apoptosis (<2% apoptotic cells) compared with the normal mouse astrocytes (30–40% apoptotic cells) or the transgenic astrocytes expressing EGFR^{wt} and EGFRvIII (~25% apoptotic cells; Fig. 6D). Expression of V¹²Ha-ras protein in GFAP-EGFRvIII transgenic astrocytes was verified by Western blot (Fig. 6A). Ras-GTP levels in the V¹²Ha-ras stably transfected GFAP-EGFRvIII transgenic astrocytes were 2–3-fold higher than in astrocytes from GFAP-EGFRvIII or GFAP-EGFR^{wt} transgenic or nontransgenic wt mice (Fig. 6B). These V¹²Ha-ras-expressing GFAP-EGFRvIII transgenic astrocytes exhibited a high saturation density and reduced serum requirements and anchorage-independent growth compared with parental cells (data not shown). In addition, the V¹²Ha-ras-expressing GFAP-EGFRvIII transgenic astrocytes had a high level of phosphorylation of Akt compared with the undetectable pAkt in the normal astrocytes or transgenic astrocytes only expressing

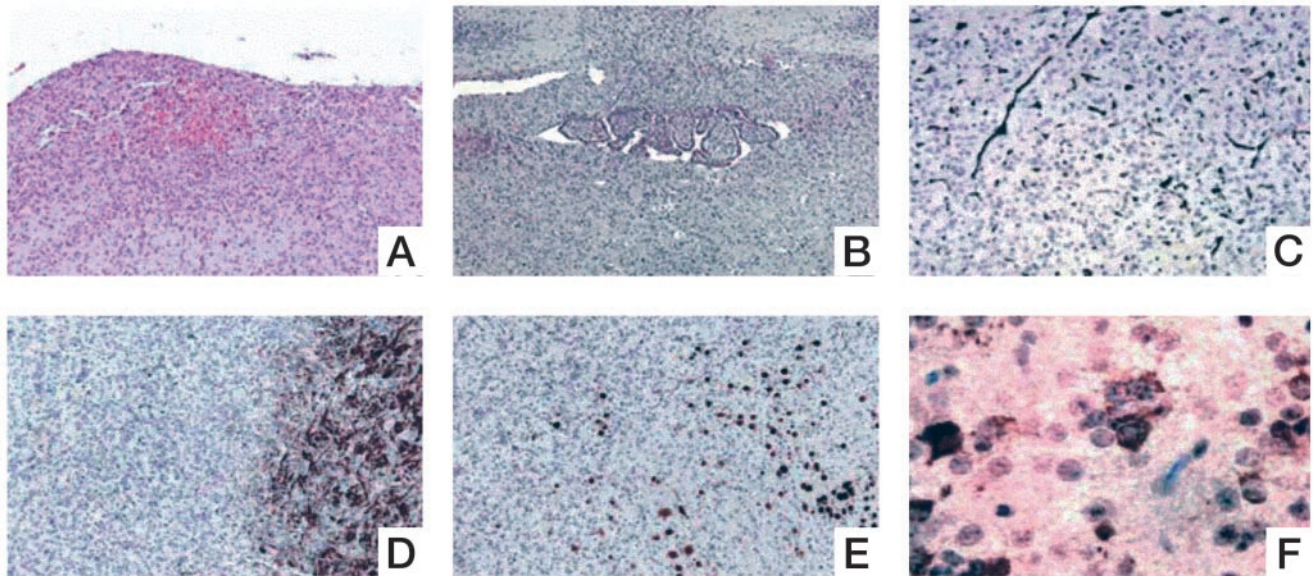


Fig. 4. Infiltrative and vascular nature of the GFAP-EGFRvIII:GFAP- V^{12} Ha-ras double transgenic mice gliomas. A and B, H&E (magnification, $\times 50$): The oligodendrogliomas were infiltrative and often had associated intratumoral hemorrhages. Periventricular location of the multifocal tumors was often observed, which sometimes resulted in occlusion of the fourth ventricle and obstructive hydrocephalus (B). C, factor VIII (magnification, $\times 200$): The tumors had a ramifying delicate vasculature that were akin to the chicken wire-like vasculature found in human oligodendrogliomas. D, MAG (magnification, $\times 100$): The glioma cells did not stain for the mature oligodendrocyte marker MAG, similar to majority of human oligodendrogliomas. E, neuronal antigen, NeuN (magnification, $\times 100$): The glioma cells did not stain the mature neuronal marker NeuN but did invade through clusters of neurons. F, PDGFR- α (magnification, $\times 630$): The glioma cells were positive for PDGFR- α , as previously reported for human oligodendrogliomas.

EGFR and EGFRvIII (Fig. 6E). Finally, the tumorigenicity of these cells was assessed by their ability to grow as s.c. explants in Nod-Scid mice. Inoculation of 1×10^6 V^{12} Ha-ras-expressing GFAP-EGFRvIII transgenic astrocytes resulted in tumor formation in Nod-Scid mice within 20 days. The resulting tumors also demonstrated features suggestive of oligodendroglioma and exhibited the fried egg appearance on H&E paraffin sections associated with increased BrdUrd labeling (Fig. 6F) and were also positive for *OLIG2* (data not shown).

DISCUSSION

Amplifications, mutations, and overexpression of EGFR are common gain of function aberrations found in GBMs but not in low-grade gliomas of any lineage (3, 21). This has led to therapeutic strategies targeting this receptor or associated downstream signaling pathways in GBMs. Amplification of EGFR is also present in a subset of oligodendrogliomas and is not observed in tumors that carry a good prognosis and exhibit loss of 1p or 19q (8). In contrast, oligodendrogliomas with EGFR amplification more often have concurrent losses of p16 and 10q (not loss of PTEN/MMAC1) and have a poorer prognosis. Our double transgenic model of oligodendrogliomas with overexpression of EGFRvIII and V^{12} Ha-ras presented is more akin to this second group of human oligodendrogliomas, with increased mortality compared with our previously reported single transgenic GFAP- V^{12} Ha-ras astrocytoma model (10). These human pathological-molecular-epidemiological data suggest two hypotheses, which are supported by mouse models. First, aberrant EGFR expression and function represent progression-associated events, rather than initiation factors in gliomagenesis. Second, aberrations in EGFR expression and function are not exclusively associated with astrocytic GBM tumors but likely lead to gliomas of both astrocytic and oligodendroglial histopathological subtypes.

The role of EGFR and its mitogenic ligands in astroglial cell proliferation has been well established (22). These mitogenic growth factors include EGF, transforming growth factor- α , and amphiregulin, which have all been shown to promote astrocyte proliferation *in vitro*.

In keeping with a growth stimulatory function for EGFR in glia, astrocytes from mice lacking EGFR expression (EGFR $^{-/-}$) exhibit slower proliferation rates *in vitro* and 30–50% fewer astrocytes *in vivo* (23, 24).

Although EGFR activation promotes astroglial cell proliferation, previous studies have suggested that EGFRvIII overexpression alone is not sufficient for glioma development. Using the RCAS/TVA retroviral approach, EGFRvIII overexpression either in mice expressing the TVA viral receptor in Nestin+ (neuroglial precursors) or GFAP+ (astrocytes) cells did not yield gliomas (25). Similarly, retroviral expression of wt or mutant (vIII) EGFR molecules into normal neural stem cells or GFAP-immunoreactive astrocytes failed to result in tumor formation when the cells were orthotopically implanted into Nod-Scid mice (2). In our experiments, we took a direct approach to evaluating the contribution of EGFR overexpression on tumor formation by establishing transgenic mice that specifically express either EGFR wt or mutant EGFRvIII in astrocytes. We observed an increase in astrocyte numbers of GFAP-EGFR wt and GFAP-EGFRvIII transgenic mice at 2–3 months of age, compared with normal littermates, but no glioma formation. In addition, primary astrocyte cultures from postnatal day 1 of the above transgenic mice had similar *in vitro* growth to normal murine astrocytes and did not grow in Nod-Scid mice. Collectively, these results suggest that overexpression of EGFR wt or EGFRvIII is not sufficient to initiate gliomagenesis.

The cooperativity observed between mutant EGFR and oncogenic RAS expression in the transgenic mice described in this study suggests that high-grade glioma formation is facilitated by additional genetic alterations associated with tumor progression. One of these progression factors is the activating mutant EGFRvIII, as we observed decreased tumor latency and increased tumor grade in mice doubly transgenic for EGFRvIII and oncogenic RAS overexpression. Similar results have been obtained using other approaches such as retroviral transgene delivery. In mice expressing the RCAS TVA receptor in Nestin+ neuroglial precursors, EGFRvIII transgene expression in

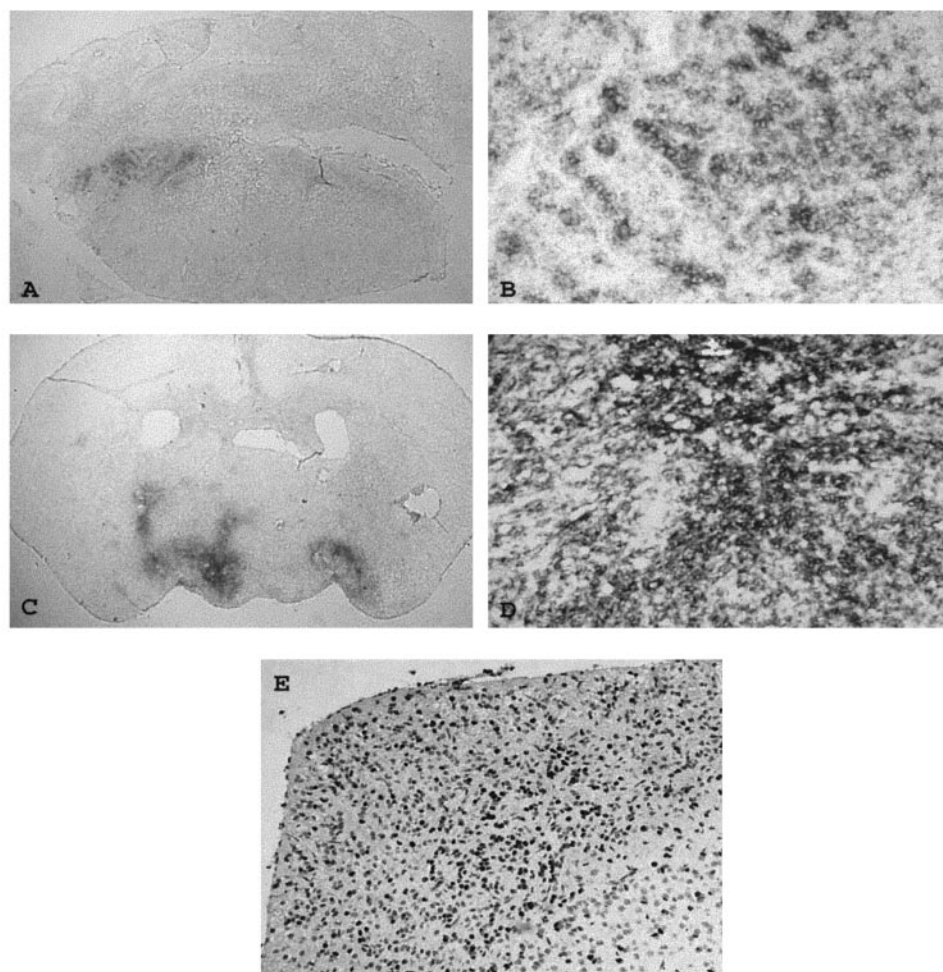


Fig. 5. Expression of OLIG1 and OLIG2 in GFAP-EGFRvIII;GFAP-V¹²Ha-ras double transgenic mice gliomas. *A* and *B*, *in situ* hybridization with antisense OLIG1 riboprobe. *C* and *D*, antisense OLIG2 riboprobe. (*A* and *C*, magnification: $\times 15$; *B* and *D*, magnification, $\times 200$). The subpial and periventricular predominant location of the multifocal gliomas are demonstrated. *E*, IHC with anti-OLIG2 antibody (magnification, $\times 200$) demonstrating the nuclear localized staining of orthotopic transplanted glioma cells, derived from the GFAP-EGFRvIII;GFAP-V¹²Ha-ras double transgenic gliomas.

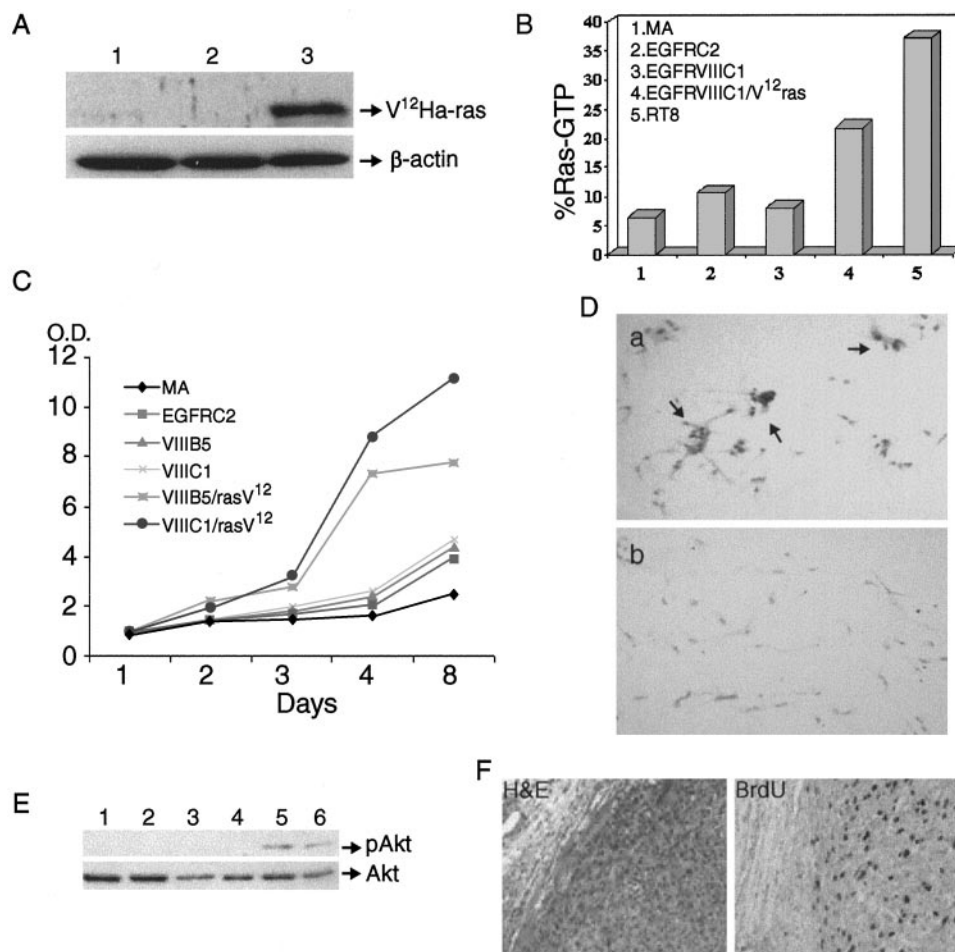
combination with *Ink4a* gene defects resulted in glioma formation (25). Cooperativity was also observed in this system with mutations in *TP53*. Using both astrocytes and neural stem cells, EGFRvIII overexpression induces high-grade glioma formation in cells lacking *Ink4a* (2). These results are consistent with our observations in the GFAP-EGFRvIII;GFAP-V¹²Ha-ras mouse. Furthermore, high-grade gliomas in the RasB8 mouse exhibit a number of additional genetic alterations, including amplification of the mouse chromosomal region containing the EGFR and overexpression of EGFR protein (10). The expression of other growth regulatory genes was also affected in the RasB8 transgenic mouse astrocytomas, including loss of p53, Rb, p16, p19, or PTEN expression and overexpression of MDM2 or cdk4 (10). In contrast, we did not observe these changes in the GFAP-EGFRvIII;GFAP-V¹²Ha-ras mouse tumors (data not shown), suggesting that expression of the activated EGFRvIII is a potent enough progression factor in mice harboring an oncogenic Ras initiating event, which additional genetic alterations are not required for gliomagenesis.

The surprising observation in this study was the development of tumors with features of oligodendroglioma in mice in which both the EGFRvIII and oncogenic Ras molecules were expressed under the GFAP promoter, with the transgenes expressed around embryonic day 14 (9). It is known that neural stem cells are capable of differentiating into both oligodendrocytes and astrocytes (26). Although O2A progenitors and glial restricted precursor cells typically lack GFAP expression, our transgenic approach might have resulted in expression of EGFRvIII in progenitor cells. In this regard, EGF-responsive progenitors are absent in the developing nervous system until after

embryonic day 14.5 (27). However, the *in vivo* administration of EGF to neuroglial progenitors results in expansion of populations of cells with immature astrocyte or oligodendrocyte phenotypes (28). Whereas oncogenic RAS expression in astroglial progenitors might have resulted in primarily type I astrocyte lineage tumors, the expression of EGFRvIII, similar to EGF administration *in vivo*, could have altered the differentiation fate toward an oligodendroglial lineage, which in combination with oncogenic RAS expression, resulted in oligodendroglial tumors.

Normally, oligodendrocyte proliferation and differentiation is regulated by another EGFR-like molecule, erbB2. erbB2-containing heterodimeric receptor complexes are responsive to neuregulin-1, which promotes the proliferation of O2A progenitor cells and maintains these cells as dividing precursors by inhibiting their progression from O4+ to O1+ oligodendroglia (29). In fact, expression of *v-erbB* under the control of S-100 promoter led to oligodendrogliomas.⁴ It is possible that aberrant expression of the EGFRvIII transgene in progenitor cells provided a permissive context for oligodendrocyte development. Inappropriate EGFR signaling could have resulted in abnormal progenitor cell survival and facilitated the development of oligodendrogliomas in the presence of a transforming oncogenic RAS molecule. In support of this notion, when a related mitogenic factor, PDGF-B, was introduced into GFAP+ or Nestin+ cells *in vitro* and *in vivo* using the RCAS/TVA system (30), oligodendroglioma tumors formed. These results suggested that aberrant PDGF autocrine stimulation was sufficient to alter astrocyte differentiation. We propose that inappropriate mitogenic growth factor signaling either conferred

Fig. 6. *In vitro* cooperativity of EGFRvIII and oncogenic 12V-Ha-ras in gliomagenesis. **A**, Western blot analysis for HA-tagged V¹²Ha-ras protein in postnatal day 1 astrocytes obtained from: 1, normal littermates; 2, GFAP-EGFRvIII mice; 3, EGFRvIII-expressing astrocytes, which have been stably transfected with the *pCX-V¹²Ha-ras-IRES-puro*. Expression of β -actin was used as an internal loading control. **B**, levels of %Ras-GTP (Ras-GTP/Ras-GTP + Ras-GDP) in different astrocytes indicated above, with RT8 murine V¹²Ha-Ras-transformed fibroblasts used as a positive control (column 4). **C**, *in vitro* proliferation assay to determine the cell proliferation of established normal mouse astrocytes (MA), the transgenic astrocytes expressing EGFR^{wt} (EGFRC2) or EGFRvIII (VIII B5; VIII C1) and the two EGFRvIII transgenic astrocyte lines transfected with V¹²Ha-ras-expressing vector (VIII B5/rasV¹²; VIII C1/rasV¹²). **D**, detection of apoptosis in mouse EGFRvIII C1 transgenic astrocytes (a) and EGFRvIII C1 astrocytes with V¹²Ha-ras expression (b). Arrows indicate the terminal deoxynucleotidyl transferase-mediated nick end labeling-positive apoptotic cells. **E**, Western blot analysis of Akt activity/phosphorylation in the normal astrocytes (Lane 1), EGFR^{wt} transgenic astrocytes (Lane 2), EGFRvIII transgenic astrocytes C1 (Lane 3), and B5 (Lane 4), and the EGFRvIII C1 cells (Lane 5) and B5 cells (Lane 6) expressing V¹²Ha-ras. **F**, tumor formation in Nod-Scid mice by EGFRvIII astrocytes C1 expressing V¹²Ha-ras. Tumorigenicity was assayed after s.c. injection of 1×10^6 cells into Nod-Scid mice. H&E (magnification, $\times 200$) demonstrating tumor cells with perinuclear halo and round and condensed nuclei, similar to the oligodendrogliomas in the double transgenic mice (Fig. 3). The cells were highly proliferative as per the high BrdUrd labeling (magnification, $\times 200$).



by PDGF or EGFRvIII overexpression results in phenotypic changes in astroglial cell differentiation and histopathological tumor appearance. Additional experiments using defined progenitor cell populations will be required to demonstrate changes in the differentiation potential of astroglial precursor cells expressing an activated EGFR.

In conclusion, we have demonstrated using transgenic mouse modeling that the mutated EGFRvIII molecule that is aberrantly activated and overexpressed in human GBMs and high-grade oligodendrogliomas is not sufficient by itself to initiate gliomagenesis. In the context of additional genetic alterations such as V¹²Ha-ras, expression of EGFRvIII potentiates glioma formation and influences glioma histological phenotype. These mice provide a reproducible and spontaneous model of oligodendroglioma, which can be used to address the genetic and cell fate conditions critical for oligodendroglioma development and progression as well as to provide a tractable preclinical model for these currently incurable tumors.

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