Transgenic E2F1 Expression in the Mouse Brain Induces a Human-Like Bimodal Pattern of Tumors

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Abstract

The Rb/E2F pathway is deregulated in most human brain tumors, and the finding that loss of *E2F1* reduced pituitary tumorigenesis in $Rb^{+/-}$ mice suggests that loss of pRb induces brain tumors by activating E2F1. We therefore investigated the role of E2F1 in the development and maintenance of brain cancer using a transgenic mouse model engineered to express E2F1 specifically within glial cells (*GFAP-tgE2F1*). *GFAP-tgE2F1* mice developed a highly penetrant phenotype characterized by neurologic defects, and examination of the brains revealed the presence of brain tumors in 20% of these animals. Importantly, the distribution of tumors according to mouse age suggests the existence of a bimodal pattern of tumor development, forcing a comparison with the human disease. Mice, at an early age, with deregulated E2F1 show the formation of embryonal brain tumors such as medulloblastoma, choroid plexus carcinoma, and primary neuroectodermal tumor. Conversely, at an older age, mice escaping embryonal tumor formation present with malignant gliomas, which are typically identified in the human adult population. Thus, this study offers the first evidence for a global role of E2F1 in the formation and maintenance of multilineage brain tumors, irrefutably establishing E2F1 as an oncogene in the brain. [Cancer Res 2007;67(9):4005–9]

Introduction

Glioblastoma is the most frequent and malignant primary neoplasm of the human nervous system. Despite advances in surgery, radiotherapy, and chemotherapy, glioblastoma patients have a very poor prognosis and usually succumb to the disease within 2 years of diagnosis (1). The molecular basis of these neoplasms is poorly understood. It is known that this tumor originates from glial or glial precursor cells in the nervous system and, for this reason, is diffuse in nature. Furthermore, there are few reproducible genetic mouse models for studying this disease, making therapeutic advancements limited (2). Genetic alterations affecting proteins that govern phosphorylation of the retinoblastoma protein (Rb), inducing uncontrolled cell cycle progression, are commonly found in human brain tumors (3). Although the Rb protein interacts with more than 100 proteins (4), it exerts control over the cell cycle mainly through its ability to form protein repressor complexes with members of the E2F family of transcription factors, including E2F1 (5). However, the role of E2F1 in promoting or repressing the development of human cancers is not completely understood. Based mainly on data from the tumor-prone knockout $E2F1^{-/-}$ mouse model (6, 7) and the ability of E2F1 to induce apoptosis (8), it is hypothesized that E2F1 functions as a tumor suppressor molecule. However, there has been no consistent finding of loss-of-function mutations in E2F1 within human cancers (9). Moreover, the finding that loss of *E2F1* reduced tumorigenesis in $Rb^{+/-}$ mice suggests that loss of pRb induces brain tumors by activating E2F1 (10). Further supporting a pro-oncogenic role for E2F1 are the numerous reports of overexpression and/or gene amplification of E2F1 in human cancer (11). In gliomas, for instance, it has been reported an almost universal dysfunction of the Rb pathway (3), with the ultimate increase in E2F1 transcriptional function (12). Our group has recently reported the presence of E2F1 overexpression in malignant gliomas and its value as a prognostic of survival (13). In this study presented here, we investigated the role of E2F1 in the development and maintenance of brain cancer using a transgenic mouse model engineered to express E2F1 within glial cells and/or neural precursor cells (*GFAP-tgE2F1*). *GFAP-tgE2F1* mice developed a highly penetrant phenotype characterized by neurologic defects and a wide range of brain tumors. Mice, at an early age, with deregulated E2F1 show the formation of embryonal brain tumors such as medulloblastoma, choroid plexus carcinoma, and primary neuroectodermal tumor. Conversely, at an older age, mice escaping embryonal tumor formation present with malignant gliomas. Thus, this study offers the first evidence for a global role of E2F1 in the formation and maintenance of multilineage brain tumors, irrefutably establishing E2F1 as an oncogene in the brain.

Materials and Methods

Generation and maintenance of *GFAP-tgE2F1* mice. The *Pst*I/*Bam*HI fragment from pSI (Promega), including a chimeric intron, multiple cloning region, and SV40 late polyadenylation signal (SV40pA), was inserted into the *Pst*I/*Bam*HI site of pBluescript II KS + (Stratagene). This new construct (pBSI) was then digested with *Sma*I/*Nhe*I, and human full-length E2F1 cDNA (1.45 kb) was inserted. Finally, the human glial fibrillary acidic protein (*GFAP*) promoter, a 2.2-kb fragment (ref. 14; generous gift from A. Guha, University of Toronto, Canada), was inserted into the *Hin*dIII/*Eco*RV restriction sites directly upstream of the enhancing chimeric intron. The final construct of 4.0 kb (*Spe*I/*Hin*dIII fragment) was pronuclearly injected into FVB zygotes, and the offspring generated were regarded as the transgenic founders. For genotyping,

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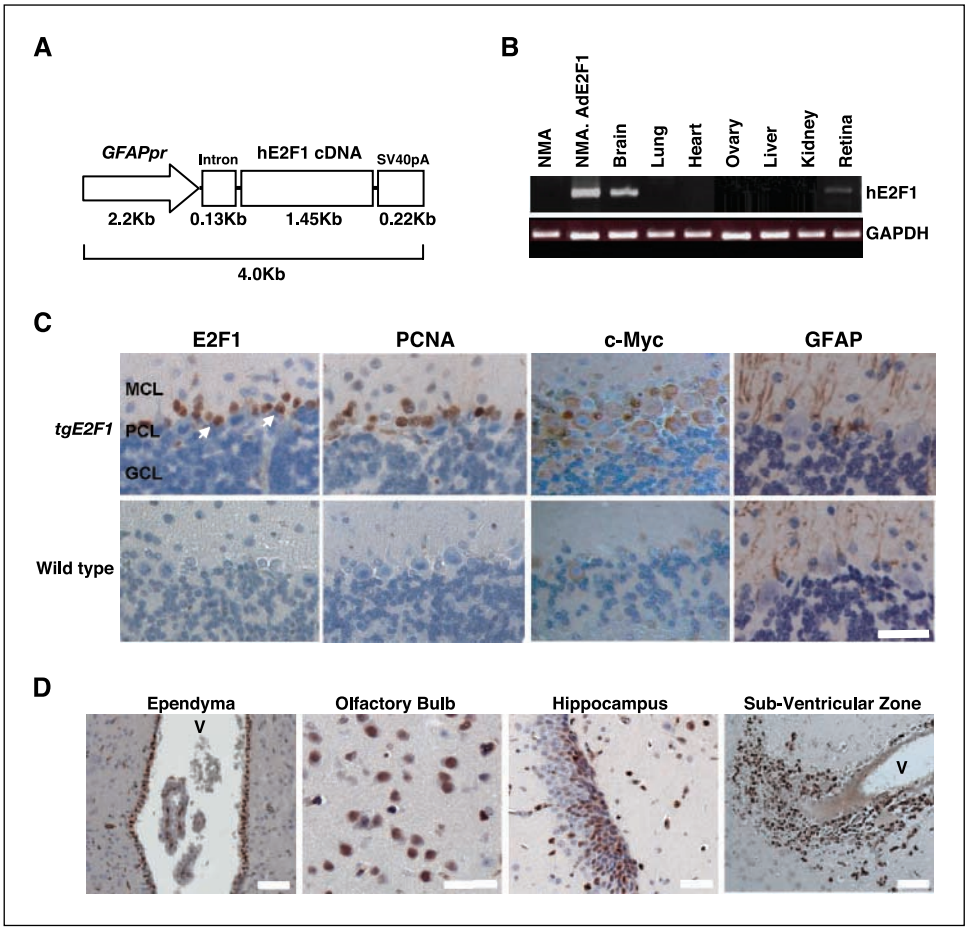


Figure 1. Molecular characterization of the *GFAP-E2F1* transgenic mouse model. *A,* schematic representation of the transgenic construct for *E2F1*, which contains the glial fibrillary acidic protein promoter (*GFAPpr*) upstream of a chimeric intron (*Intron*), human E2F1 cDNA, and the polyadenylation signal from SV40 (*SV40pA*). *B,* expression of the E2F1 transgene was analyzed by RT-PCR of total RNA extracted from the indicated organs of a 3-month-old mouse. Total RNA extracted from normal mouse astrocytes (*NMA*) or normal mouse astrocytes infected with an adenoviral vector containing an E2F1 expression cassette (NMA.AdE2F1) were used as template controls. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) amplification is shown as a loading control. *C,* expression of human E2F1 and E2F1 transcriptional targets (PCNA and c-Myc) in the cerebellum of a 3-month-old *tgE2F1* mouse was analyzed by immunohistochemical analysis. Immunostaining of brain sections from a sibling mouse (wild type) was used as control. *Arrows,* E2F1-positive Bergmann glial cells. *MCL,* molecular cell layer; *PCL,* Purkinje cell layer; *GCL,* granular cell layer. *D,* transgenic E2F1 expression was determined by immunohistochemical analysis in several regions of the mouse brain such as the ependyma plate, olfactory bulb, hippocampus, and subventricular zone. *V,* cerebral ventricle. *Bar*, 50 μm.

genomic DNA obtained from tail snips was subjected to PCR analysis with the following primers flanking exon 1: forward primer, 5′-TGTCAGGACCTTCGTAGCATTG-3′ and reverse primer, 5′-TCCTCAGGGCACAGGAAAAC-3′. This reaction amplified both human *E2F1*, as a product of 167 bp, and endogenous *E2F1*, as a 220-bp fragment. E2F1 transgene expression was determined using total cellular RNA extracted from several organ tissues and subjected to reverse transcription-PCR (RT-PCR) analysis using the following primers: forward, 5′-GACGGCTTGAGGGGTT-3′ and reverse, 5′-CACCTACGGTCTCCTCA-3′.

B6.129S2-*Trp53*^{tm1Tyj}/J mice with functionally inactivated p53 (denoted *p53*$^{+/-}$ heterozygous and *p53*$^{-/-}$ homozygous deletion) were obtained from The Jackson Laboratory and crossed with *tgE2F1* mice to generate *tgE2F1:p53*$^{+/-}$ and *tgE2F1:p53*$^{-/-}$. *p53*$^{-/-}$ mice were obtained from The Jackson Laboratory and were in the C57BL/6J strain background. The genetic background of mice in this study was, therefore, a mixture of FVB and C57BL/6J strains. All experiments with mice were done in accordance with national guidelines and regulations and approved by the Institutional Animal Care and Use Committee.

Histology and tumor analysis. Animals were sacrificed when signs of distress appeared and, in some cases, animals were selectively sacrificed. Brains were immediately removed and fixed for 24 h in 10% formalin solution, transferred to a 70% ethanol solution, and processed for paraffin embedding. Serial sections (6 μm) were prepared and stained with H&E according to standard histopathologic techniques. Stained sections were examined under light microscope, independently, by K.D.A. and G.N.F. Tumor grading was determined for astrocytomas by K.D.A. and G.N.F. based upon the WHO grading system (15).

For immunohistochemistry analysis, paraffin-embedded brain tissue specimens were cut into 6-μm-thick sections and mounted on microscope slides. Tissue sections were dried for 16 h at 60°C and then dewaxed and dehydrated by exposure to various grades of alcohols and finally distilled water. Tissues were then treated using a heat-induced epitope retrieval technique using citrate buffer (pH 6.0). Sections were then blocked against endogenous peroxide with a 3% hydrogen peroxide solution for 10 min followed by a 10% serum block for 30 min at room temperature. The primary antibodies were then added to the sections, for 16 h, in a humidified chamber at 4°C, at the following dilutions: E2F1 (KH95, 1:75; Santa Cruz), GFAP (prediluted, Venta Medical System), proliferating cell nuclear antigen (PCNA; 1:500; DakoCytomation), c-Myc (9E10, 1:50; Santa Cruz), synaptophysin (Syn88, 1:50; Biogenex), NeuN (1:1,000; Chemicon), and cytokeratin by a combination of four antibodies: AE1/AE3 (1:500; DakoCytomation), CAM 5.2 (1:50; Becton Dickinson), cytokeratin (MNF116, 1:50; DakoCytomation), and keratin (8/18 Zym52, 1:25; South). Immunodetection was done using the horseradish peroxidase system (Elite Vector Stain ABC System, Vector Laboratories), and then sections were counterstained with hematoxylin. Sections were examined under light microscopy.

Isolation and characterization of cortical astrocytes. Astrocytes were isolated from mouse cortex at postnatal day 1 or 2, according to methods previously described by McCarthy and de Vellis (16). Cells were dissociated and plated on D-lysine–coated plates. Cultures were maintained in DMEM/F12 medium (1:1) supplemented with 10% fetal bovine serum daily. At 7 days after plating, the dishes were shaken for 16 h, and adherent cells were selected for further subcultures. Confirmation of the astrocytic origin of the selected cells was done by morphologic examination and GFAP expression analysis. Immunofluorescent staining was done on monolayer culture of cortical astrocytes plated on cover glass. Forty-eight hours after seeding, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 15 min. After serum blocking, cells were incubated with anti-E2F1 (KH95, 1:75; Santa Cruz) or anti-GFAP (1:500; DakoCytomation) antibodies.

The visualization of primary antibodies was done with immunofluorescence by using either FITC- or Texas Red–conjugated secondary antibodies (1:200; Santa Cruz). Sections were mounted with 4′,6-diamidino-2-phenylindole–infused mounting media (Molecular Probes). Cultures of passages 1 to 4 were used for subsequent experiments.

For immunoblot analysis of E2F1 expression, cortical astrocyte lysates were prepared with CHAPS lysis buffer and protease inhibitor cocktail (Roche). Blots were incubated with anti-E2F1 (KH95, 1:200; Santa Cruz) and anti-GFAP (C19, 1:500; Santa Cruz) antibodies and developed according to the ECL protocol of Amersham.

The distribution of cells in the cell cycle was analyzed by measuring their DNA content with flow cytometry, as described previously (13). Cell samples were collected 72 h after being plated at a confluency of 60%.

For karyotype analysis, first-passage cortical astrocytes isolated from five pooled *tgE2F1* animals and five pooled littermate control animals (normal mouse astrocytes) were used to make chromosomal preparations. Cytologic preparations were incubated with colcemid (0.04 μg/mL) for 1 h followed by 0.075 mol/L hypotonic treatment and methanol/acetic acid fixation (3:1). Metaphases were then subjected to Giemsa staining and analyzed with oil-immersion microscopy. Thirty metaphases from each sample were analyzed for genomic instability.

Statistical analysis. Survival related with brain tumor formation (animals were considered censored when the cause of death was not related with brain tumors, or when sacrificed selectively and no brain tumors were found) was assessed by plotting survival curves by the Kaplan-Meier method, and comparisons were analyzed with the log-rank test.

Results and Discussion

We undertook this work to test the hypothesis that E2F1 plays an oncogenic role in brain tumors. To this end, we generated a transgenic *E2F1* (*tgE2F1*) animal model in which ectopic human E2F1 was under the transcriptional control of a *GFAP* promoter (ref. 14; Fig. 1*A*) to direct its expression to glial and neuroprogenitor/neural stem cells (14, 17). After the pronuclear injection of the transgenic construct into FVB zygotes, positive transgenic founders were identified, and two independent transgenic lines (tg2 and tg10) were developed that expressed the transgene in the brain. The *tgE2F1* mice were viable with no signs of embryonal lethality, as revealed by the Mendelian inheritance of the transgene in a cohort of 140 mice followed for more than 1 year. RT-PCR amplification showed that the ectopic E2F1 was expressed in neural tissues but not in other organs (Fig. 1*B*). The ectopic E2F1 protein was immunolocalized primarily, as previously reported (17), to several populations of glial cells, as the Bergmann glial in the Purkinje cell layer and the astrocytic population in the white matter of the brain (Fig. 1*C* and *D*), and it was scattered in layers that are predominantly populated by stem cells, such as the olfactory bulb and hippocampus (Fig. 1*D*). Certain neuronal populations were distinctly negative, such as Purkinje cells of the cerebellum (Fig. 1*C*) and spinal motoneurons (data not shown). We also observed in the same brain regions increased levels of E2F1 transcriptional targets such as PCNA and c-Myc (ref. 18; Fig. 1*C*), suggesting the expression of a functional E2F1 protein. Interestingly, p73, a negative regulator of cell proliferation and direct target of E2F1 (19), was not found up-regulated in E2F1-expressing glial cells (data not shown).

We initially tested whether the overexpression of E2F1 induced a transformed phenotype in cultures of astrocytic cells isolated from the brain of the transgenic mice. Cortical astrocytes isolated from *tgE2F1* mice expressed high levels of E2F1 (Fig. 2*A* and *B*). Further analysis of *tgE2F1* astrocytes revealed an aberrant cell cycle profile (Fig. 2*C*) and karyotypic abnormalities that strongly suggested genomic instability (Fig. 2*D*). Thus, >85% of tg10 astrocytes were polyploid and showed frequent aberrations such as chromosomal fractionations (>65%), fusions (>45%), and the presence of marker chromosomes. These results are in agreement with previous studies on the association of E2F1 with genomic instability (20, 21).

As mentioned above, tg2 and tg10 mice were viable; however, they exhibited a highly penetrant phenotype characterized by neurologic symptoms (Fig. 3*A*) that included ataxia, seizures/tremors, paralysis, and head tilt (Fig. 3*B*; see also Supplementary Fig. S1A and B). Because E2F1-induced apoptosis is linked to p53 function (8), we crossed tgE2F1 with mice hemizygous or nullizygous for the *p53* gene to enhance the proliferative capability of E2F1. The tgE2F1-related phenotype was not significantly modified in this cohort of animals; however, we observed a significant decreased in the overall survival due to brain tumors in tgE2F1;$p53^{+/-}$ or tgE2F1;$p53^{-/-}$ mice ($P = 0.0046$, log-rank test; Supplementary Fig. S2). Examination of the brain revealed brain tumors in 25% of symptomatic *tgE2F1* mice over 6 months of age, and the incidence was greater at 33% in

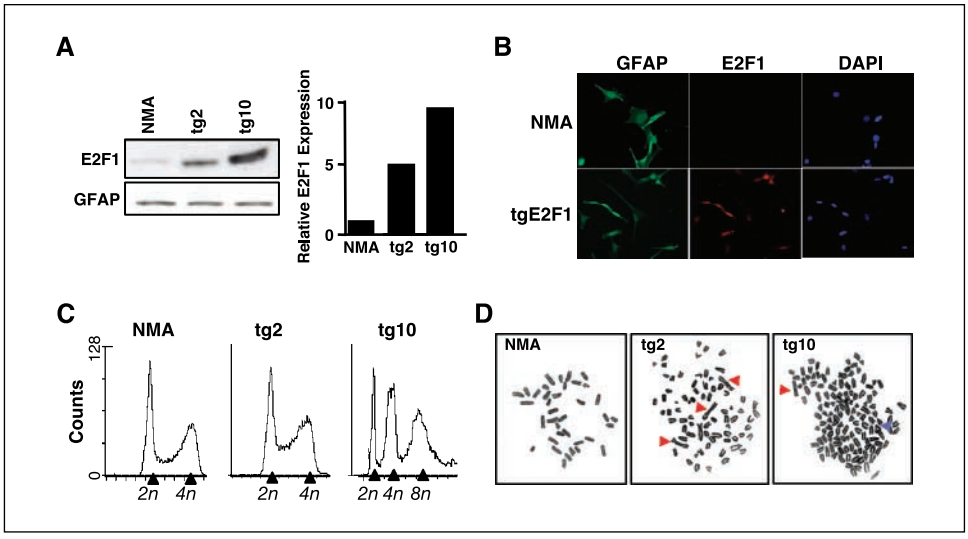
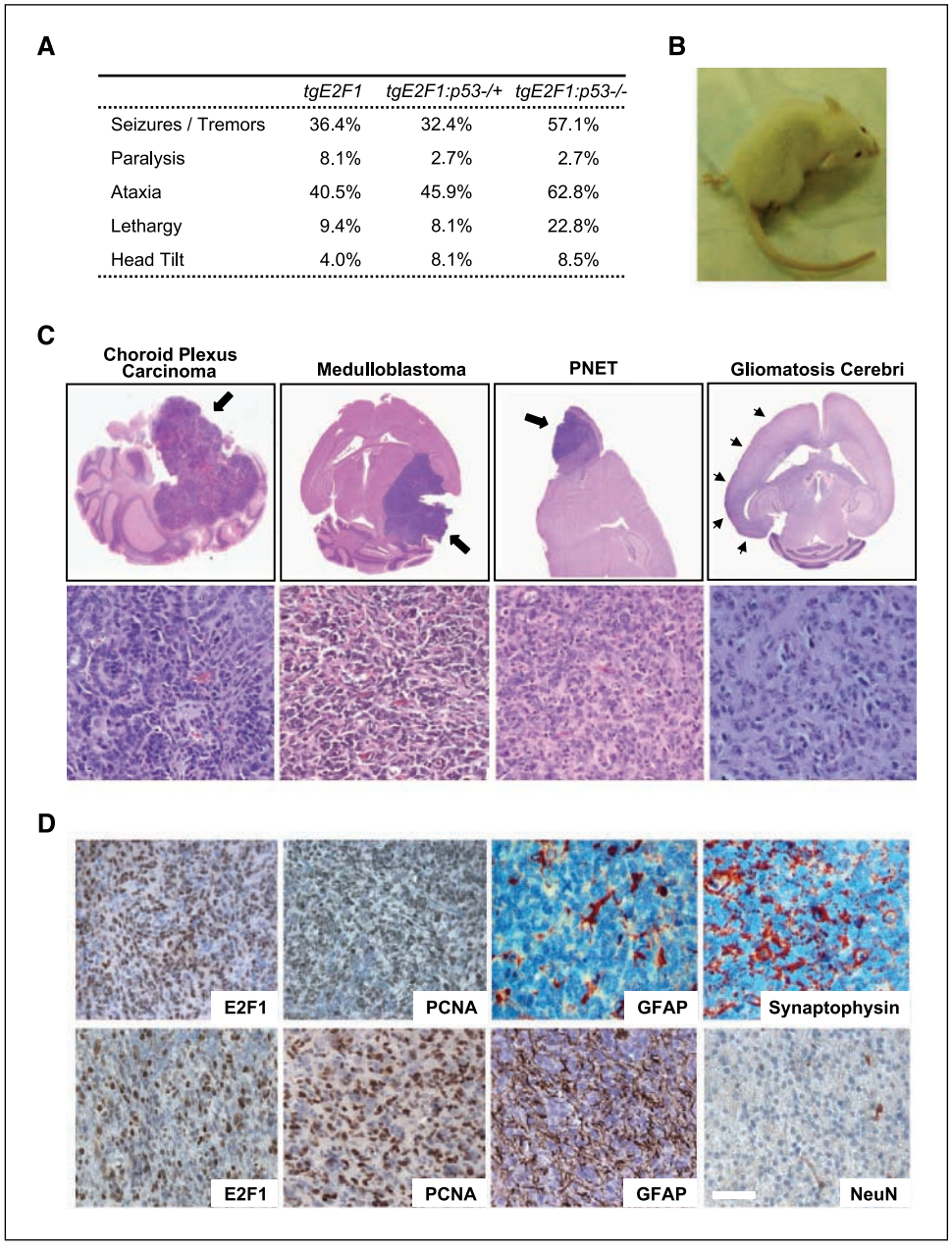


Figure 2. *In vitro* characterization of *tgE2F1* cortical astrocytes. *A,* immunoblot analysis (*left*) and densitometric quantification (*right*) of E2F1 protein levels in isolated cortical astrocytes from both transgenic lines (tg2 and tg10) and normal mouse astrocytes. E2F1/GFAP expression ratio was determined and expressed as relative to that of normal mouse astrocytes (equal to 1). *B,* immunohistochemical analysis of isolated cortical astrocytes to analyze the colocalization of GFAP expression with transgenic E2F1 expression. *C,* cell cycle profiles of normal mouse astrocytes and tg2 and tg10 cortical astrocytes 72 h after being plated at a confluency of 60%. 2n, 4n, and 8n populations (*arrowheads*). *D,* karyotypic analysis of tg2 and tg10 cortical astrocytes showing polyploidy and the presence of markers (*red arrows*) and fusions (*blue arrow*). Representative karyotype from a normal mouse astrocyte.

symptomatic *tgE2F1* mice with either the $p53^{+/-}$ or $p53^{-/-}$ genotype (Supplementary Table S1). Interestingly, the tumors originated in several regions of the brain and exhibited histologic and immunohistochemical characteristics compatible with a variety of brain tumors (Fig. 3*C* and *D*). All tumors displayed an aggressive behavior characterized by dense hypercellularity and a highly infiltrative phenotype (Fig. 3*C*). The malignant gliomas (Fig. 3*C* and *D*) showed the typical features of highly infiltrative anaplastic astrocytomas (WHO grade 3) without the microvascular proliferation and necrosis characteristic of glioblastoma (WHO grade 4; ref. 15). These tumors localized in the cerebral hemispheres and brain stem and consisted of a limited mass with ill-defined borders. The primary neuroectodermal tumor comprised a single tumoral focus that was localized to the olfactory bulb (Fig. 3*C*). The medulloblastoma and the choroid plexus tumor originated in the posterior fossa and were histologically similar to those found in mice with simultaneous inactivation of the Rb and p53 pathways (22, 23).

Analysis of the distribution of the different histologic types of tumors according to age revealed a bimodal curve analogous to that seen in human patients (ref. 15; Table 1; Supplementary Table S1). That is, tumors that formed in young mice, those less than 6 months of age, were similar observed in children (embryonal tumors), and tumors that formed in older mice consisted of astrocytic tumors, the most common primary brain tumor in adult human patients. These observations of comparative oncology suggest that activation of a single oncogene may result in different type of tumors over age, opening the possibility to speculate whether the histologic type depends on the differentiation status of the brain cells when they acquire the critical accumulation of



	tgE2F1	*tgE2F1:p53-/+*	*tgE2F1:p53-/-*
Seizures / Tremors	36.4%	32.4%	57.1%
Paralysis	8.1%	2.7%	2.7%
Ataxia	40.5%	45.9%	62.8%
Lethargy	9.4%	8.1%	22.8%
Head Tilt	4.0%	8.1%	8.5%

Figure 3. Phenotype of *tgE2F1* mice. *A,* incidence of neurologic symptoms in *tgE2F1* mice homozygous, heterozygous, or nullizygous for *p53*. *B,* representative examples of *tgE2F1* animal displaying head tilt ataxia. *C* and *D,* histologic analysis of spontaneous brain tumors in *tgE2F1* mice. *C,* macroscopic (*top*) and microscopic (*bottom*) brain sections stained with H&E showing (*left* to *right*) choroid plexus carcinoma of the fourth ventricle, cerebellar medulloblastoma, primary neuroectodermal tumor of the olfactory bulb, and malignant astrocytoma infiltrating 75% of the cerebral hemispheres. Tumors (*arrows*). *D,* representative embryonal tumor (*top*) and malignant glioma (*bottom*) sections were immunostained with anti-E2F1, anti-PCNA, anti-GFAP, anti-synaptophysin, or anti-NeuN antibodies. *Bar*, 50 μm.

Table 1. Modeling *tgE2F1* spontaneous brain tumors to the human disease

Tumor type	Mouse age (mo)	Human age of most frequent tumor incidence* (y)
Medulloblastoma	3	<15
Choroid plexus carcinoma	3	<15
PNET	5	<10
Anaplastic astrocytoma	7-18	20-69

*Based on combined clinical studies referred to the *WHO Classification of Tumors* (15).

mutations and suggesting that cancer initiation from precursors or from post-mitotic cells does not necessary result in the same phenotype.

The results presented here directly link our observations to studies on human brain tumor specimens that described a significant increase in E2F1 expression levels and activity (12, 13) and thus embracing a useful and credible operational definition of E2F1 as an oncogene in brain tumors.

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