

# Cyclic AMP Suppression Is Sufficient to Induce Gliomagenesis in a Mouse Model of Neurofibromatosis-1

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## Abstract

Current models of oncogenesis incorporate the contributions of chronic inflammation and aging to the patterns of tumor formation. These oncogenic pathways, involving leukocytes and fibroblasts, are not readily applicable to brain tumors (glioma), and other mechanisms must account for microenvironmental influences on central nervous system tumorigenesis. Previous studies from our laboratories have used neurofibromatosis-1 (NF1) genetically engineered mouse (GEM) models to understand the spatial restriction of glioma formation to the optic pathway of young children. Based on our initial findings, we hypothesize that brain region-specific differences in cAMP levels account for the pattern of NF1 gliomagenesis. To provide evidence that low levels of cAMP promote glioma formation in NF1, we generated foci of decreased cAMP in brain regions where gliomas rarely form in children with NF1. Focal cAMP reduction was achieved by forced expression of phosphodiesterase 4A1 (PDE4A1) in the cortex of *Nf1* GEM strains. Ectopic PDE4A1 expression produced hypercellular lesions with features of human NF1-associated glioma. Conversely, pharmacologic elevation of cAMP with the PDE4 inhibitor rolipram dramatically inhibited optic glioma growth and tumor size in *Nf1* GEM *in vivo*. Together, these results indicate that low levels of cAMP in a susceptible *Nf1* mouse strain are sufficient to promote gliomagenesis, and justify the implementation of cAMP-based stroma-targeted therapies for glioma.

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## Introduction

Carcinogenesis is a complex process frequently modeled as involving two phases, initiation and promotion (reviewed in ref. 1). During initiation, cancer cell progenitors sustain genetic alterations that result in loss of tumor suppressor and/or gain of oncogene function. Cells “initiated” in this manner possess a growth advantage through mutational activation of proliferation and/or survival pathways. These genetic changes are necessary, but not sufficient, for transformation. Susceptible tumor progenitors require additional signals like those derived from tissue stromal elements (e.g., endothelial cells, leukocytes, and fibroblasts) to complete tumor promotion (reviewed in ref. 2). This multistage oncogenesis model, which also involves the effects of chronic inflammation and aging on stromal function, has been recently

elucidated for several tumor types, including breast, gastric, and hepatocellular carcinoma. These new insights have led to the identification of the essential constituent stromal cell types as well as many of the relevant pathways that mediate the critical interactions between supportive tumor stroma and receptive preneoplastic/neoplastic cells during tumor evolution.

Although the prevailing model of cancer initiation and promotion accounts for many events in the genesis of solid tumors, it does not fully explain the unique spatial and temporal pathogenesis of brain tumors, especially those in children. Tissue stroma and inflammation differ markedly in the brain. There are no brain fibroblasts, and immune system involvement in brain cancers seems to be minimal compared with that in peripheral tissues. Moreover, the stromal signals that drive tumorigenesis in the brain are unique and likely reflect the presence of spatially and temporally regulated brain-specific cues that sculpt the developing brain during embryogenesis and early fetal life.

Astrocytoma (glioma) formation in the common inherited disorder neurofibromatosis-1 (NF1) is an excellent model system for elucidating the cellular and molecular mechanisms that underlie microenvironmental contributions to brain tumorigenesis. Similar to the initiating events in other cancers, complete loss of *Nf1* protein (neurofibromin) function is necessary, but not sufficient, for NF1-associated gliomagenesis (3). In fact, gliomagenesis in NF1 displays such exquisite sensitivity to the genetics, age, and region of the surrounding brain that the necessary stroma-derived

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promoting factors can be localized in both place and time. Greater than 90% of astrocytomas in children with NF1 occur before age 7 years, and ~70% of these tumors involve the optic nerves and optic chiasm (4, 5). These observations indicate that the optic pathway of young children with NF1 is uniquely conditioned to support gliomagenesis.

A specialized function of the optic pathway in gliomagenesis is substantiated by studies in genetically engineered mouse (GEM) models of NF1. Neither *Nf1*<sup>+/-</sup> mice nor mice with complete *Nf1* loss in glial fibrillary acidic protein (GFAP)-expressing astroglial cells develop gliomas (3, 6). Only mice with both reduced *Nf1* gene expression (*Nf1*<sup>+/-</sup> mice) in the microenvironment ("tumor microenvironment" mice) and complete loss of *Nf1* gene expression in tumor progenitors ("tumor progenitor" mice) form gliomas. Although the entire brain and the spinal cord of these mice contain *Nf1*<sup>-/-</sup> glial cells within an *Nf1*<sup>+/-</sup> environment, tumors only form within the optic pathway, involving the prechiasmatic optic nerves and optic chiasm ("OPG" mice; refs. 7, 8). Together, these GEM strains highlight a complex set of requirements for gliomagenesis involving genetic events in tumor progenitors (complete loss of *Nf1*), genetic events in the microenvironment (reduced *Nf1* expression; *Nf1* heterozygosity), and region-specific signals (the optic pathway). Furthermore, these GEM strains afford a singular opportunity to investigate the mechanism(s) by which the tumor microenvironment and tumor progenitors interact to promote gliomagenesis.

Previously, we showed a correlation between brain region-specific differences in cAMP levels and glioma formation (9), and hypothesized that low levels of cAMP constituted the region-specific condition necessary for NF1-associated gliomagenesis. In the current study, we experimentally test this

hypothesis and show that focal cAMP suppression, together with global heterozygosity for neurofibromin and complete loss of neurofibromin in tumor progenitors, is sufficient to promote gliomagenesis in *Nf1* genetically engineered mice *in vivo*.

## Materials and Methods

### Animals

All animals were used in accordance with an established Animal Studies Protocol approved by the Washington University School of Medicine Animal Studies Committee. Wild-type (*Nf1*<sup>flox/flox</sup>), tumor progenitor (*Nf1*<sup>flox/flox</sup>; GFAP-Cre), and OPG (*Nf1*<sup>flox/mut</sup>; GFAP-Cre) mice were generated as previously described (ref. 7; Table 1).

### Human tumor and brain sections

Paraffin-embedded optic pathway glioma specimens from patients with NF1 (*n* = 4) and normal human brain and optic chiasm autopsy specimens were retrieved from the archives of the Department of Pathology at the Washington University School of Medicine in accordance with an institutional review board-approved protocol for the use of human pathology specimens.

### Chemicals, reagents, and antibodies

All chemicals were obtained from Sigma unless otherwise indicated. All tissue culture reagents and media were obtained from Invitrogen unless otherwise indicated. A construct containing mCherry cDNA was a gift of Dr. Roger Y. Tsien (University of California, San Diego, CA). Murine PDE4A1 (accession no. AJ297396) was provided by Dr. James Cherry (Boston University, Boston, MA). Antibodies

**Table 1. Induced tumor frequency in *Nf1* GEM**

Experimental group	<i>Nf1</i> GEM models			Induced tumor frequency				
	Genotype	Strain	Description	PDE4A1		PDE4A1-H229Q		<i>P</i>
				Tumor	No tumor	Tumor	No tumor	
Wild-type mice	<i>Nf1</i> <sup>flox/flox</sup>	<i>Nf1</i> <sup>flox/flox</sup>	Wild-type mice. Do not develop spontaneous gliomas	1	5	0	2	n.s.*
Tumor progenitor mice	<i>Nf1</i> <sup>flox/flox</sup> × GFAP-Cre	<i>Nf1</i> <sup>GFAP</sup> CKO	Wild-type mice lacking <i>Nf1</i> expression in GFAP+ cells by E14.5. Do not develop spontaneous gliomas.	1	6	0	7	n.s.
OPG mice	<i>Nf1</i> <sup>flox/mut</sup> × GFAP-Cre	<i>Nf1</i> <sup>+/-</sup> GFAP <sup>CKO</sup>	<i>Nf1</i> <sup>+/-</sup> mice lacking <i>Nf1</i> expression in GFAP+ cells by E14.5; 95% develop prechiasmatic/chiasmal optic gliomas by 10–12 wk	8	5	0	6	0.02

Abbreviation: n.s., not significant.

\**P* values were determined by two-tailed Fisher's exact test comparing the frequency of tumor induction in response to PDE4A1 versus PDE4A1-H229Q injection.

were obtained from Peprtech (CXCL12), Clontech [Dsred (mCherry)], Invitrogen (GFAP), Abcam (PDE4A and 58 kDa Golgi marker), Pharmingen (LCA), Wako (IBA-1), Cell Signaling [phosphorylated protein kinase A (pPKA) substrate], and Sigma (CNPase). Antibody directed against phosphorylated CXCR4 was developed in our laboratory and previously described (10). Antibody directed against Olig2 (DF-308) was a gift of Dr. Charles Stiles (Dana-Farber Cancer Institute, Boston, MA).

### Lentiviruses

Full-length murine PDE4A1 or catalytically inactive PDE4A1-H229Q was cloned into a lentiviral packaging vector that also encodes mCherry fluorescent protein as previously described (11). A second packaging vector (FUW-FLG) encoding a fusion of firefly luciferase and enhanced green fluorescent protein driven by the human ubiquitin C promoter within an established lentiviral backbone (12) was also used as previously described (13, 14). Viral particles were produced from each packaging vector separately by the Viral Vectors Core Facility of The Hope Center for Neurological Diseases at Washington University School of Medicine.

### Mutagenesis

The catalytically inactive PDE4A1-H229Q (15) was generated from full-length murine PDE4A1 using a Quikchange mutagenesis kit (Stratagene) according to the manufacturer's instructions and the following mutagenesis primers:

Sense: 5'-CAT GAC GTC GAC CAG CCT GGC GTC TCC AAC-3'  
Antisense: 5'-GTT GGA GAC GCC AGG CTG GTC GAC GTC ATG-3'.

Mutagenesis was confirmed by sequencing; expression and activity of catalytically active and inactive PDE4A1 constructs were determined first in Daoy medulloblastoma cells (American Type Culture Collection). Daoy cells were infected with lentivirus encoding either catalytically active or inactive PDE4A1 as previously described (11). Cells expressing PDE4A1 constructs were sorted and collected based on mCherry expression by high-speed fluorescence-activated cell sorting (MoFlo High-Performance Cell Sorter; DAKO). As described below, expression levels were measured by Western blot, intracellular localization by immunofluorescence microscopy, and effects on cAMP levels by enzyme-linked immunosorbent assay.

### Intracranial viral injections

Five- to seven-week-old wild-type ( $Nf1^{flox/flox}$ ), tumor progenitor ( $Nf1^{flox/flox}$ ; GFAP-Cre), and OPG ( $Nf1^{flox/mut}$ ; GFAP-Cre) mice were anesthetized [intraperitoneal ketamine (87 mg/kg)/xylazine (13 mg/kg); Phoenix Pharmaceuticals], the cranium was exposed, and a small hole was made 2 mm lateral and posterior to the bregma with a size 34 inverted cone burr (Dremel). Mice were positioned in a stereotactic frame (Stoelting), and 500,000 transducing units

each of FUW-FLG and PDE4A1-Cherry or PDE4A1-H229Q viruses were injected through a 27-gauge needle over 2 minutes at 3 mm below the dura mater. The incision was closed with Vetbond (3M). A few hours after the injection and every 12 for 48 hours thereafter, all animals received subcutaneous injections of 0.5 mg/kg buprenorphine hydrochloride.

### Bioluminescence imaging

**In vitro.** Bioluminescence of Daoy cells expressing FUW-FLG and PDE4A1-Cherry or PDE4A1-H229Q constructs was evaluated at the Molecular Imaging Core facility at Washington University in St. Louis. Cells were grown in 96-well black-wall clear-bottom plates (Costar). In some experiments, cells were treated with either the PDE4 inhibitor rolipram (0–20  $\mu$ M/L) or vehicle for 18 hours. Cells were washed and exposed to a solution of 150  $\mu$ g/mL luciferin for 10 minutes. Bioluminescence was measured using a charge-coupled device camera-based bioluminescence imaging system (IVIS 50, Caliper; exposure time 1–30 seconds, binning 8, field of view 12, f/stop 1, open filter). Data were expressed as total photon flux (photons/s) using LivingImage (Caliper) and IgorPro (Wavemetrics) image analysis software packages.

**In vivo.** Bioluminescence measurements were obtained 48 hours after viral injection and every 2 weeks thereafter for the subsequent 6 weeks. Hair was removed from mouse heads with chemical depilatory agents 24 hours before imaging. Images were obtained using the following parameters: IVIS 50, Caliper; exposure time 300 seconds, binning 8, field of view 12, f/stop 1, open filter. Images were then analyzed as previously described (14, 16).

### Manganese-enhanced magnetic resonance imaging

Manganese-enhanced magnetic resonance images (MRI) of the optic nerve were acquired as previously described (17).

### Preclinical study design

**Treatment.** Ten- to 12-week-old OPG mice with MRI-confirmed optic nerve gliomas were assigned to either the vehicle (water alone) or rolipram (5 mg/kg/d in the drinking water; ref. 11) treatment group (5 animals per group). After 1 or 4 weeks of treatment, all animals were deeply anesthetized and perfused with 4% paraformaldehyde, and optic nerves were isolated.

**Response evaluation.** Response evaluations included measurements of optic nerve volumes, tumor cell proliferation, and tumor cell apoptosis (18). Optic nerve volumes were assessed by measuring optic nerve diameters at the level of the optic chiasm ( $D_0$ ), as well as 200  $\mu$ m ( $D_{200}$ ), 400  $\mu$ m ( $D_{400}$ ), and 600  $\mu$ m ( $D_{600}$ ) anterior to the chiasm. Total volume was calculated from the sum of each 200- $\mu$ m segment volume ( $V_1 = D_0$  to  $D_{200}$ ) as determined using the equation for a truncated cone:

$$V_1 = 1/12 \pi h(D_0^2 + D_0D_{200} + D_{200}^2)$$

Proliferation within normal and tumor-bearing optic nerve was determined as the number of Ki-67-positive cells per high-power field. Three high-power fields per specimen were evaluated. Apoptosis was measured using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL assay, Roche Diagnostics) according to the manufacturer's instructions and evaluated in a similar manner to Ki-67 staining.

#### Tissue sections, immunohistochemistry, and immunofluorescence

Tissue sections (20  $\mu$ m) were cut from 4% paraformaldehyde-fixed, paraffin-embedded experimental brains (10). For evaluation of mCherry, GFAP, LCA, Olig2, CNPase, pPKA substrate, and IBA-1 expression, slides underwent antigen retrieval with Target Retrieval Solution (DakoCytomation) according to the manufacturer's instructions. Primary antibody concentrations were as follows: Dsred (mCherry, 1:200), GFAP (2.5  $\mu$ g/mL), PDE4A (1:300), LCA (1:50), IBA-1 (1:1,000), 58 kDa Golgi protein (1  $\mu$ g/mL), Olig2 (1:20,000), CNPase (1:500), and pPKA substrate (1:100). With the exception of PDE4A, 58 kDa Golgi protein, and PDE4A-GFAP costains, staining was detected using biotin-conjugated secondary antibodies augmented by streptavidin-horseradish peroxidase, and visualized using either 3,3'-diaminobenzidine or with the Vector VIP Peroxidase Substrate kit (DAKO). Sections were counterstained with either hematoxylin or methyl green (Olig2/CNPase). PDE4A, 58 kDa Golgi protein, and PDE4A-GFAP staining was detected by immunofluorescence using the secondary AlexaFluor 555-conjugated and AlexaFluor 488-conjugated secondary antibodies at a concentration of 1:750 (Molecular Probes) for 90 minutes. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Parallel staining was performed on normal mouse and human brain and optic chiasm autopsy specimens. Control sections were processed identically in the absence of primary antibody.

#### cAMP measurement

cAMP was measured by competitive immunoassay using a Correlated Enzyme Immunoassay Kit (Assay Designs) according to the manufacturer's instructions and as previously described (9). cAMP values were normalized to protein for each sample individually.

#### Western blot analysis

Western blot analysis for PDE4A1 or PDE4A1-H229Q expression was performed as previously described (11). Briefly, protein extracts were obtained by lysing cells in lysis buffer [20 mmol/L Tris (pH 7.4), 137 mmol/L NaCl, 10% glycerol, and 1% Triton X-100] supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Calbiochem 524628). The proteins (25 mg) were resolved with 10% Bis-Tris gels (Invitrogen) and transferred onto Hybond ECL nitrocellulose membrane (Amersham) according to standard protocols. Blots were probed with an antibody to PDE4A, followed by incubation with IRDye-conjugated secondary antibody (LI-COR). Actin expression served as a loading control. Blots

were imaged with the Odyssey fluorescent scanning system (Li-Cor).

#### Statistical analyses

Significance of induced tumor frequency was determined with two-tailed Fisher's exact test. Comparisons were made between the rate of tumor formation in response to PDE4A1 and PDE4A1-H229Q in each strain of mice. Differences in cAMP levels in Daoy cells and the effect of rolipram on optic pathway glioma cell proliferation and apoptosis were evaluated by a two-tailed *t* test.

## Results

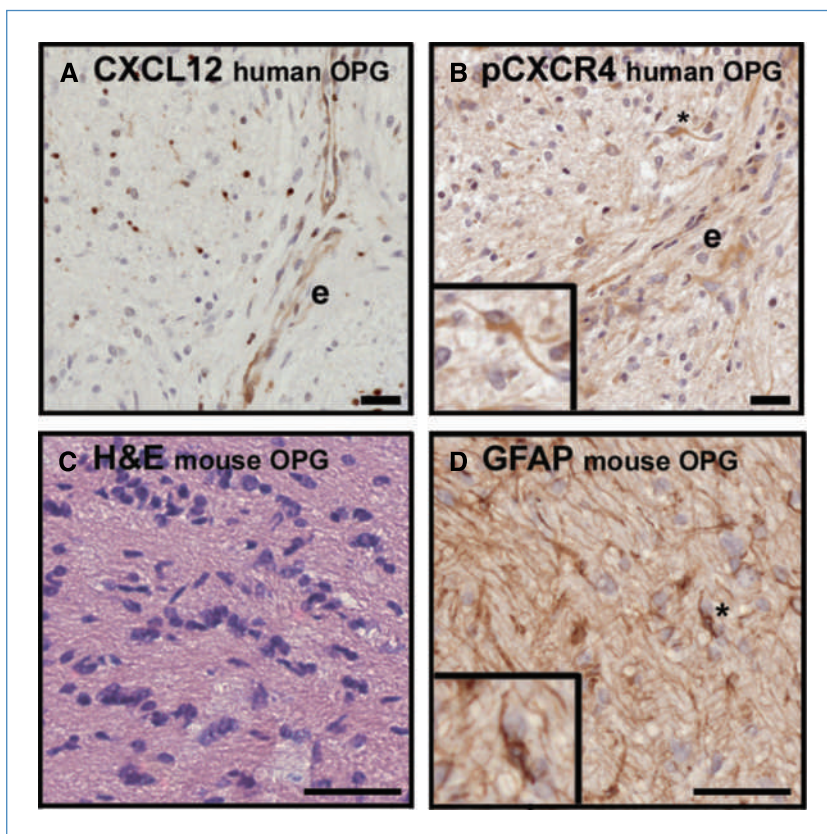
Gliomagenesis in NF1 occurs with remarkable spatial and temporal specificity, predominantly affecting the optic nerves and chiasm of young children (19). This pattern of glioma formation indicates that a necessary interaction occurs between glial *NF1* gene inactivation and signals present in the optic pathway microenvironment during NF1-associated gliomagenesis.

In prior studies involving human specimens and a GEM model of NF1-associated optic glioma (OPG mice), we identified CXCL12 as a candidate stromal signal capable of stimulating *Nf1*-deficient glial cell growth (9). Multiple brain-derived cells within optic pathway glioma express CXCL12, including endothelial cells (Fig. 1A), entrapped axons, and infiltrating microglia. The presence of a functional paracrine relationship between CXCL12-expressing stromal elements and tumor cells is shown by the frequent presence of the CXCL12 receptor, CXCR4, in a CXCL12-induced phosphorylated form within bipolar pilocytic cells (Fig. 1B, inset). The potential importance of stromal activation of CXCR4 lies in its ability to suppress intracellular levels of cAMP, which we found was necessary for the stimulation of *Nf1*<sup>-/-</sup> astroglial cell growth (9). Moreover, we found region-specific differences in cAMP levels in the mouse brain that correlated with the pattern of glioma formation in NF1. The optic pathway had the lowest, whereas the cortex had the highest cAMP levels. Based on these observations, we hypothesized that differences in cAMP levels underlie the distinct pattern of gliomagenesis in NF1.

To evaluate this hypothesis *in vivo*, we sought to alter the pattern of brain region-specific cAMP levels to determine whether this would coordinately change the pattern of tumor formation in an established GEM model of NF1 OPG. Astrocytomas in *Nf1* GEM models share many, but not all, of the features of human OPG. Gliomas within the optic nerves of OPG mice (Fig. 1C) contain bipolar GFAP-positive astrocytoma cells (Fig. 1D; refs. 20, 21) that also express the transcription factor Olig2 (Supplementary Fig. S1A). Misexpression of Olig2 was recently shown to be highly correlated with pilocytic astrocytoma (22, 23). In addition, murine OPG exhibit microglial infiltration reminiscent of human tumors (Supplementary Fig. S1B; refs. 24–26). In contrast to human pilocytic astrocytomas, murine tumors do not contain Rosenthal fibers or eosinophilic granular bodies.



**Figure 1.** Optic nerve gliomas in humans and mice. A, an optic nerve glioma specimen from a patient with NF1 shows mild hypercellularity and CXCL12 expression in the endothelium of tumor-associated blood vessels (e) as well as in scattered infiltrating cells. B, serial section from the tumor presented in A stained for the presence of a ligand-induced phosphorylated form of CXCR4 (pCXCR4) reveals a high level of receptor activation in proximity to the CXCL12-expressing endothelium. Phosphorylated CXCR4 is present in a pilocytic cell (inset). C, a hypercellular lesion with nuclear atypia is evident within the optic nerve of an OPG mouse with hematoxylin and eosin (H&E) stain. D, the tumor pictured in C contains GFAP-expressing cells with the elongated bipolar morphology characteristic of pilocytic cells. In all cases, expression appears brown. Scale bars, 20  $\mu$ m (A and B) and 50  $\mu$ m (C and D).



To generate foci of decreased cAMP, we ectopically expressed lentiviral-encoded cAMP-specific phosphodiesterase-4A1 (PDE4A1; ref. 27) through stereotactic injection into the cortex of OPG mice. PDE4A1 was chosen for these experiments based on our prior demonstration that increased PDE4A1 expression decreased intracellular cAMP levels and enhanced tumor growth in a xenograft model of malignant glioma *in vivo* (11). The cortex was selected as the site for PDE4A1 injection based on the following considerations, which suggest that it is usually not a permissive microenvironment for glioma formation in NF1: (a) patients with NF1 rarely develop cortical tumors; (b) glioma formation in *Nf1* mouse models does not occur in the cortex; and (c) the cortex exhibits the highest levels of cAMP (9).

Catalytically active and inactive (PDE4A1-H229Q; ref. 15) forms of PDE4A1 were cloned into a lentiviral packaging vector also encoding for mCherry fluorescent protein (11). In control studies, Daoy medulloblastoma cells were infected with lentivirus encoding PDE4A1 or PDE4A1-H229Q. Whereas both forms of PDE4A1 were equally expressed (Supplementary Fig. S2A) and localized to the Golgi apparatus (Supplementary Fig. S2B), only catalytically active PDE4A1 decreased intracellular cAMP levels (Supplementary Fig. S2C). To mimic the low levels of cAMP normally present within the optic pathway, we coinjected PDE4A1/mCherry lentivirus with a second lentivirus encoding firefly luciferase (14) into the cortex of wild-type, tumor progenitor, and OPG mice. Bioluminescence imaging of firefly luciferase expres-

sion was used to monitor transgene expression and served as a potential measure of neoplastic growth. In control studies, the expression of PDE4A1 or PDE4A1-H229Q in Daoy cells, or treatment with the pan-PDE4 inhibitor rolipram, had no effect on luciferase activity (Supplementary Fig. S2D and E).

Because wild-type mice do not develop cortical gliomas, tumor induction following ectopic PDE4A1 expression in wild-type mice (*Nf1<sup>fllox/fllox</sup>*) would indicate that cAMP suppression is sufficient for glioma initiation and promotion. Although tumor progenitor mice (*Nf1<sup>fllox/fllox</sup>*; GFAP-Cre) possess occasional foci of hyperproliferating astrocytes, they do not develop gliomas (3). PDE4A1-induced tumor formation in these mice would occur only if suppression of cAMP alone induced the combined tumor-promoting properties of an *Nf1*<sup>+/-</sup> microenvironment and the optic pathway. Finally, cortical glioma formation in OPG mice (*Nf1<sup>fllox/mut</sup>*; GFAP-Cre) would suggest that cAMP suppression is sufficient, within the context of an *Nf1*<sup>+/-</sup> microenvironment, to bring about the neoplastic growth of *Nf1*<sup>-/-</sup> tumor progenitors. PDE4A1-H229Q injections serve as controls for noncatalytic effects of lentiviral injection and PDE4A1 overexpression.

In 51 of 58 injected mice, we detected a bioluminescent signal, indicating successful viral transduction and transgene expression. Increased bioluminescence imaging on serial studies showed replication of virally infected cells. The brain of each animal was subjected to an extensive blinded

evaluation for the presence of neoplasia according to the algorithm outlined in Supplementary Fig. S3.

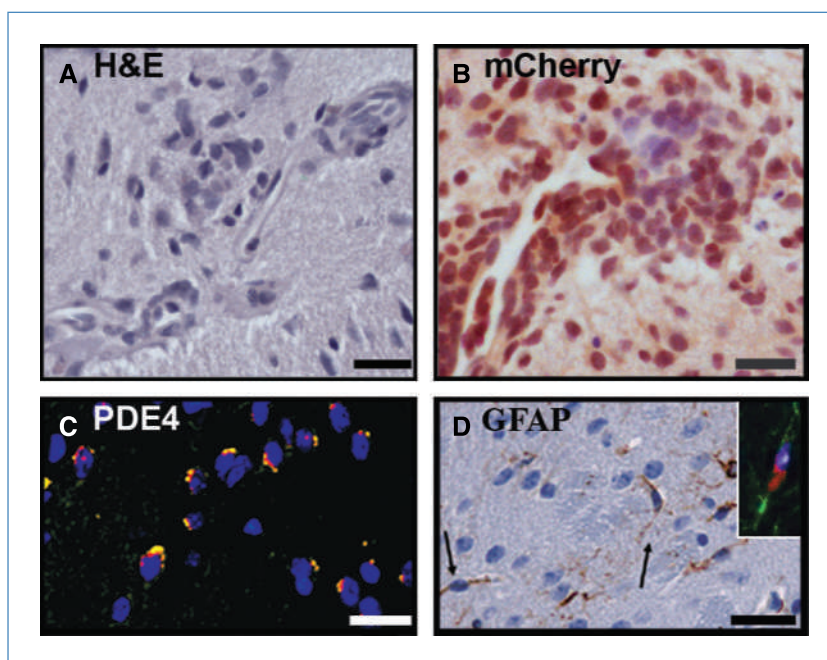
Hematoxylin and eosin–stained sections from micro-injected cortices were examined for common features of neoplasia, including hypercellularity, cellular clustering, and nuclear atypia, as well as the specific features of low-grade glioma seen in the *Nf1* OPG GEM model (Fig. 1; Supplementary Fig. S1). Twelve of 13 OPG mice injected with catalytically active PDE4A1 lentivirus showed increased bioluminescence imaging and expression of mCherry fluorescent protein/PDE4A at the injection site (Fig. 2A–C). The diagnosis of an induced glioma was made when hypercellular lesions expressing mCherry fluorescent protein and PDE4A also contained GFAP-positive cells with the bipolar morphology characteristic of tumor cells (piloid cells) commonly found in the low-grade gliomas of NF1 patients (Fig. 2D). Further indication of glioma was evident in increased Olig2 expression (Fig. 3A). In each case, induced tumor sites exhibited greater numbers of Olig2-positive nuclei per high-powered field than did the same location in the contralateral cortex. As a group, the induced tumors showed significantly increased Olig2 expression compared with the uninvolved contralateral cortex (Fig. 3B). Comparable histologic findings were not observed in the contralateral cortex, nor are they observed in normal human or mouse cortex and optic nerve (Supplementary Fig. S4).

Importantly, bipolar piloid cells expressed PDE4A in a perinuclear distribution characteristic of PDE4A1 localization (Fig. 2D, inset), suggesting a role for PDE4A1 expression in gliomagenesis. The mechanistic significance of this finding was further underscored by our finding that induced tumors had lower levels of cAMP. Because the induced tumors are small and preclude direct cAMP measurements, cAMP levels were inferred from an immunohistochemical analysis using a

surrogate marker, the intensity of PKA substrate phosphorylation. Tumor sites exhibited decreased labeling with pPKA substrate antibody, indicating decreased PKA activity (Fig. 3C and D) and suggestive of decreased levels of cAMP. These findings are consistent with a relationship between lower levels of cAMP and gliomagenesis in NF1.

In total, 41 of the 51 mice with increased bioluminescence imaging had injection sites with evidence for exogenous PDE4A1 or PDE4A1-H229Q expression and were deemed evaluable for tumor formation. Eight of the 13 OPG mice with increased bioluminescence imaging following PDE4A1 injection were considered to have induced cortical gliomas (Table 1). This was a statistically significant rate of induced tumor formation, as determined by Fisher's exact test ( $P = 0.02$ ). In contrast, only one of seven tumor progenitor mice and one of six wild-type mice had evidence of induced glioma. None of the 15 control injections of PDE4A1-H229Q resulted in a tumor. The frequency of tumor formation in control mice was not statistically significant.

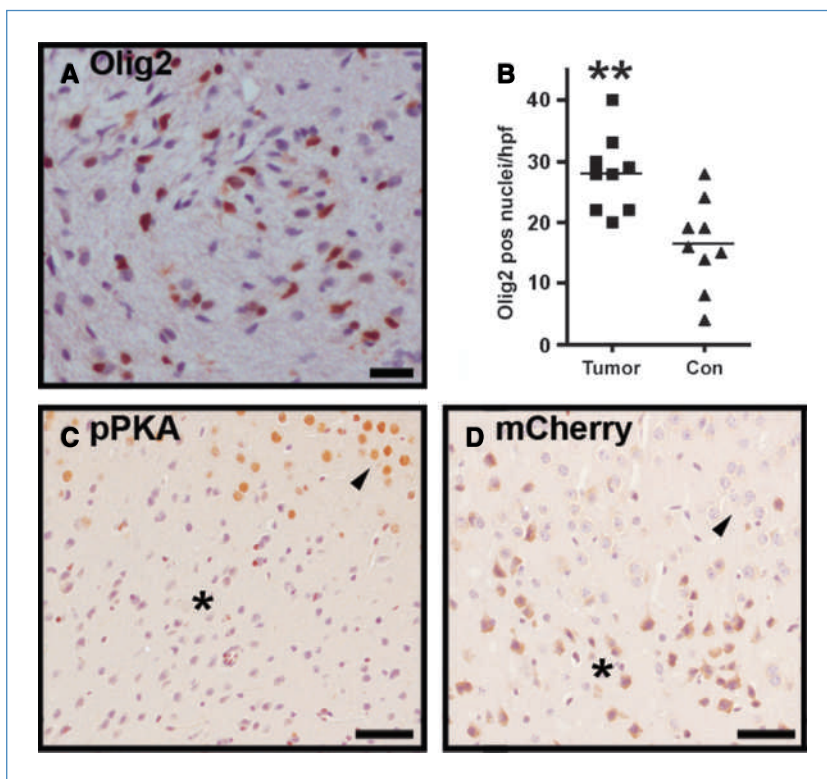
Although PDE4A (not shown) and mCherry-positive cells (Fig. 4A) were observed at injection sites in mice without tumors, these differed from induced tumors by the absence of abnormal astrocyte morphologies (Fig. 4B) and their lack of increased Olig2 expression (Fig. 4C). Disruption in tissue architecture at these injection sites was limited to inflammation, as evidenced by the presence of LCA-positive cells (Fig. 4D). LCA-positive inflammatory cells were only a minor component of the cellular constituents in the induced tumors (data not shown). Together with our prior studies, the pathologic features of PDE4A1-induced cortical tumors in OPG mice show that low cAMP levels are sufficient to promote glioma formation when *Nf1*<sup>−/−</sup> glioma progenitors arise within the context of an *Nf1*<sup>+/-</sup> micro-environment.



**Figure 2.** Induced tumors occur at PDE4A injection sites. Tumors were identified in 8 of 13 OPG mice injected with catalytically active PDE4A1. The representative images were taken of four such induced tumors. A, induced tumors had foci of cellular clusters and nuclear atypia evident on hematoxylin and eosin–stained sections. Scale bar, 25  $\mu$ m. B, injection sites were further identified as hypercellular, disorganized lesions expressing virally encoded mCherry fluorescent protein (brown; scale bar, 25  $\mu$ m). C, PDE4A in characteristic perinuclear localization (yellow). DAPI counterstains nuclei blue. Scale bar, 25  $\mu$ m. D, GFAP-expressing cells (brown) display an elongated bipolar phenotype (arrows) characteristic of piloid cells. Scale bar, 50  $\mu$ m. Inset, colabeling of a piloid cell with antibodies directed against GFAP (green) and PDE4 (red) supports the role of PDE4A1 expression and cAMP suppression in the genesis of glioma.



**Figure 3.** Induced tumors exhibit increased Olig2 expression and evidence for decreased cAMP levels. A, cells within induced tumors exhibited increased Olig2 expression. Scale bar, 25  $\mu$ m. B, the number of Olig2-positive nuclei per high-powered field (hpf) in induced tumors was greater than in the contralateral cortex. \*\*,  $P < 0.005$  as determined by two-tailed  $t$  test. C, immunolabeling of injection sites with antibody directed against the phosphorylated form of PKA substrates revealed a high level of staining in normal cortex (arrowhead) but low level of staining in tumor cells (\*). D, serial sections through the same tumor site pictured in C and labeled with antibody directed against mCherry fluorescent protein reveal the interface between tumor (\*) and cortex (arrowhead). Scale bars, 50  $\mu$ m.



To determine whether restoration of normal cAMP levels would attenuate optic glioma growth *in vivo*, we treated OPG mice for 1 or 4 weeks with rolipram or vehicle following the detection of an optic glioma by manganese-enhanced MRI (Fig. 5A). We previously showed that PDE4 inhibition with rolipram blocked *in vivo* malignant glioma growth through elevation of cAMP levels (11, 14). Using this preclinical paradigm, 4 weeks of rolipram resulted in tumor regression as measured by a restoration of optic nerve volumes to near normal values (Fig. 5B and C). Moreover, rolipram treatment suppressed tumor cell proliferation (Fig. 5D), but had no effect on tumor cell apoptosis as measured by TUNEL staining (data not shown). These observations show that low levels of cAMP are necessary for the maintenance of optic glioma proliferation *in vivo*, and further support the critical role of cAMP signaling in gliomagenesis and continued tumor growth.

## Discussion

Clinical observations and experimental data indicate that cancers frequently arise within specific tissue contexts (see ref. 2 for review). For example, gastrointestinal cancers are often associated with chronic inflammation and senescent fibroblasts can promote the transformation of preneoplastic epithelial cells (28). Prevailing models of how inflammation and aging contribute to tumor promotion focus on the actions of fibroblast and leukocyte-derived cytokines and growth factors. However, this model is not directly applicable to brain tumors, as there are no fibroblasts within the central

nervous system (CNS), and chronic CNS inflammation involving leukocytes is uncommon. Moreover, pediatric brain tumors develop in the absence of the effects of aging. Nevertheless, the unique spatial and temporal distribution of brain tumors (gliomas) in children with NF1 highlights the significant role that microenvironmental factors can play in brain tumor formation.

Based on the natural history of patients with NF1 as well as *Nf1* GEM models (7, 29), we and others have proposed that the milieu of the normally growing nervous system functions similarly to aging or inflamed peripheral tissues by providing the necessary cellular elements and growth-regulatory factors for neoplastic transformation. Thus, whereas complete loss of neurofibromin function in glial progenitors is a requisite initiating step in transformation, the formation of NF1-associated mouse gliomas also requires a permissive *Nf1* heterozygous microenvironment and region-specific effectors. It is the confluence of all three of these elements—homozygous *Nf1* gene inactivation in tumor progenitors, heterozygous *Nf1* gene inactivation in the tumor surround, and region-specific effectors—that results in NF1-associated glioma formation in the optic nerves and chiasm of young NF1 heterozygous children and mice.

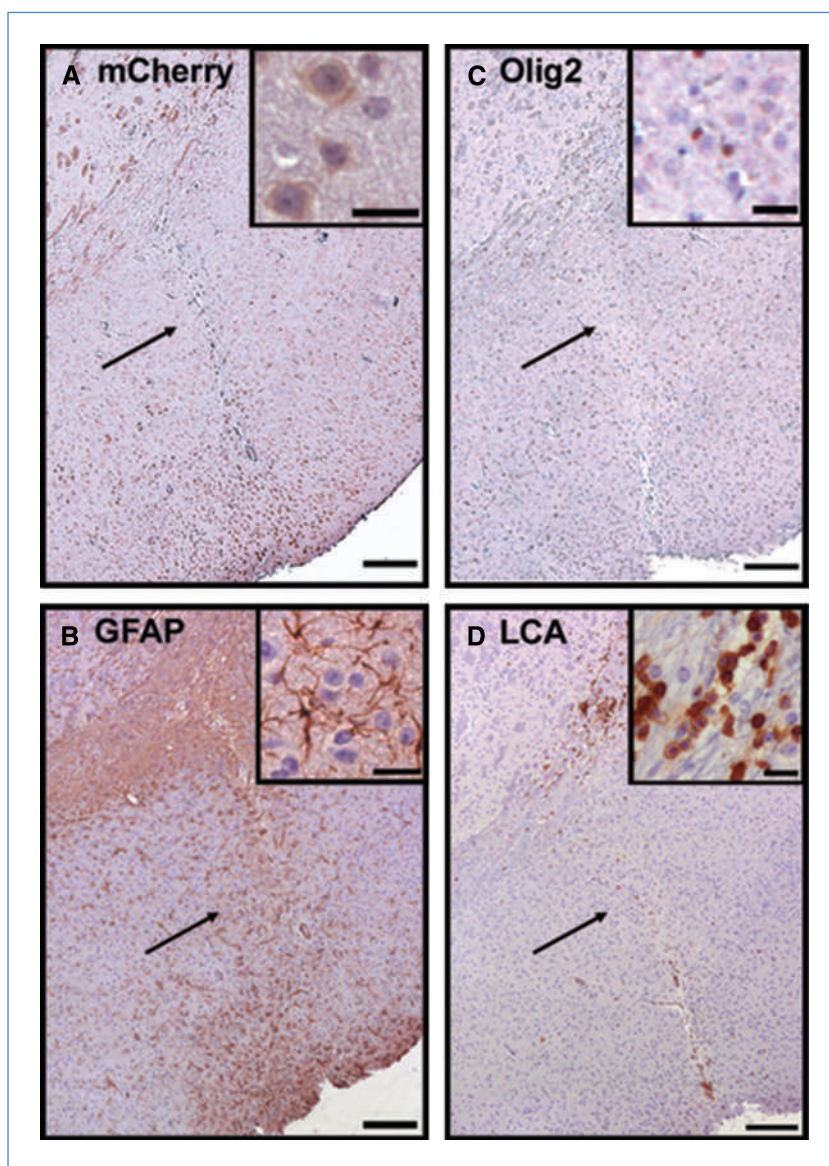
Several recent studies suggest that *Nf1* heterozygosity creates a permissive microenvironment by altering the function of specific cell types that can be co-opted to promote tumorigenesis. In the central nervous system, we showed that *Nf1* heterozygosity alters microglia function, resulting in increased proliferation and motility, and elevated

production of growth-promoting molecules such as CXCL12 and hyaluronidase (9, 25). Similarly, peripheral nerve tumor (neurofibroma) development requires *Nf1*<sup>+/-</sup> mast cells, which are released from the bone marrow in response to c-kit receptor activation (30).

Region-specific effects could also include region-specific differences in cell types that participate in gliomagenesis (31) or region-specific differences in growth-regulatory signals. Previously, we suggested CXCL12 as a candidate optic pathway-derived glioma-promoting factor and showed that complete loss of neurofibromin function alters CXCR4 signaling, such that CXCL12 induces significant sustained suppression of cAMP levels. This abnormal CXCL12-mediated cAMP suppression creates an inappropriate survival response in *Nf1*<sup>-/-</sup> astrocytes. Neither CXCL12-induced cAMP

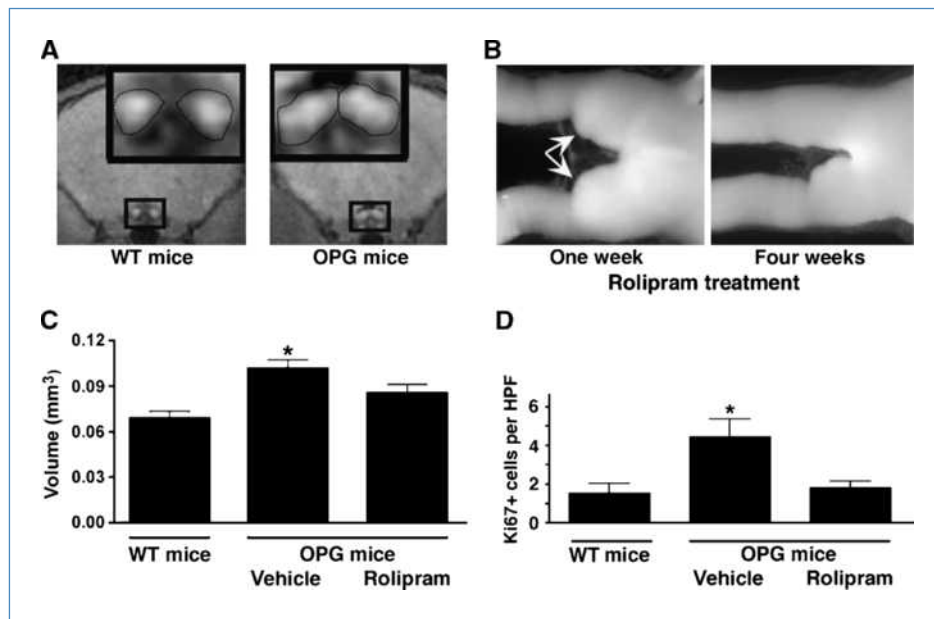
suppression nor the resultant enhanced survival response is observed in wild-type astrocytes (9). Collectively, these data provide an attractive model for tumorigenesis, in which complete loss of neurofibromin function not only initiates oncogenesis but also secondarily contributes to tumor promotion by dysregulating CXCR4-cAMP signaling. In this fashion, loss of neurofibromin results in the acquisition of two hallmark features of malignant transformation—hyperproliferation and increased survival—but only when neurofibromin loss occurs in a CXCL12-rich microenvironment, such as the optic pathway of young children and mice.

The current study provides novel *in vivo* support for the hypothesis that suppression of cAMP constitutes an important region-specific signal for gliomagenesis in *NF1*. The creation of cortical foci in which cAMP levels were suppressed



**Figure 4.** Histologic features of nontumor reactions. In 31 cases, no induced tumor was identified in GEM brains injected with PDE4A1 or PDE4A1-H229Q. Pictured is the sequential assessment of an injection site from a single animal with positive bioluminescence imaging but negative tumor evaluation. A, the injection site, which had been identified by hematoxylin and eosin stain and PDE4A expression (not shown), showed expression of mCherry fluorescent protein. B, there are numerous GFAP-positive cells at the injection site, but they display a stellate morphology typical of reactive (nonneoplastic) astrocytes. C, there is a lack of increased Olig2 staining. D, also evident at this injection site is an inflammatory response characterized by numerous LCA-positive leukocytes. In all cases, expression appears brown. Scale bars for large images, 200  $\mu$ m. Inset scale bars, 25  $\mu$ m.





**Figure 5.** Targeted inhibition of PDE4 attenuates tumor growth in OPG mice. A, optic nerve gliomas were identified by enlarged optic nerves (white areas within the boxed region and demarcated by dotted line in inset) in OPG mice by MRI and assigned to treatment (rolipram) or control (vehicle) groups. B, optic nerve volumes in animals treated for 1 or 4 weeks with rolipram were compared. The arrowheads indicate thickened optic nerves characteristic of these tumors. After 4 weeks of rolipram treatment, optic nerves returned to a normal diameter. C, quantitation of optic nerve volumes indicates that there is a statistically significant increase in optic nerve volumes in untreated mice compared with wild-type mice (WT, without tumors) and that rolipram abrogates this difference in volume ( $n = 5$  mice per group). D, the antitumor effect of rolipram was associated with inhibition of tumor cell proliferation as determined by Ki67 staining and the numbers of Ki67-positive cells per high-power field ( $n = 5$  mice per group). \*,  $P < 0.05$ , difference between untreated OPG and WT mice as determined by two-tailed  $t$  test.

rendered the cortex similar to the optic pathway and thus susceptible to glioma. Whether CXCL12 is the primary regulator of cAMP effects during spontaneous gliomagenesis in NF1 remains unknown. Further, the critical cell type(s) through which PDE4A1 affects gliomagenesis are not yet identified.

Lentiviral expression of PDE4A1 could have reduced cAMP levels in *Nf1*<sup>-/-</sup> glial cells, and/or other cells in the *Nf1*<sup>+/-</sup> cortex, including neurons, microglia, and endothelial cells. However, although cAMP might regulate tumor-promoting functions in these multiple cell types, growth-regulatory effects of cAMP in tumor cells are well described. For example, increased cAMP levels induced p38 phosphorylation and apoptosis in acute promyelocytic leukemia cells (32) and enhanced radiation-induced apoptosis of lung cancer cells through upregulation of Bak (33). Similarly, decreased cAMP inhibited the function and expression of the proapoptotic Bcl-2 family member Bim (34, 35). Consistent with cAMP suppression functioning within glioma progenitors, our previous studies indicated that suppression of cAMP within *Nf1*<sup>-/-</sup> astroglial cells endowed them with a survival advantage (9). Moreover, cAMP promotes astrocyte differentiation (36). Thus, suppression of cAMP levels in *Nf1*<sup>-/-</sup> tumor progenitors might inhibit differentiation and maintain a progenitor-like state and, in concert with tumor-promoting genetic and microenvironmental changes, facilitate tumorigenesis. The current work is focused on identifying in which cells cAMP suppression is required for gliomagenesis and

whether cAMP dysregulation in *Nf1* preneoplastic cells modulates apoptosis and/or proliferation.

Important issues also raised by these studies include the identity of the initiated tumor progenitor cell, the downstream mediators of cAMP signaling in tumor promotion, and the best way to target the cAMP pathway in the treatment of gliomas. Previous studies reported that both differentiated astrocytes and neural stem cells could serve as glioma cell of origin in a model of gliomagenesis dependent on loss of *Ink4a/Arf* and gain of epidermal growth factor receptor activation (37). In contrast, recent work on dermal neurofibromas in a GEM model of NF1 suggested that only tissue stem cells could function as tumor progenitors (38). The cortical injections in this study did not target any specific cell population. Thus, PDE4A1-induced gliomas could have developed from transformed differentiated astrocytes or neural stem cells. The known ability of stem cells to extensively migrate throughout the brain to sites of pathology suggests that this latter possibility is a tenable hypothesis (39). Future studies will be required to identify the cell of origin of low-grade gliomas in *Nf1* GEM.

Finally, as underscored by rolipram-mediated attenuation of optic glioma growth in OPG mice (this study) and by reduced *Nf1* GEM neurofibroma size following imatinib-mediated c-kit pathway inhibition (30), components of the stromal signaling pathway represent potential targets for future antineoplastic therapy. These exciting results firmly establish that the experimental tractability of

*Nf1* GEM model systems offer unprecedented opportunities to identify the cellular and molecular bases for stromal control of tumor formation. In addition, this spontaneous brain tumor model is an excellent platform for the development of stroma-directed chemotherapeutic strategies. Further, it was recently shown that 23% of high-grade glioblastoma tumors harbor inactivating *NF1* gene mutations (40). Thus, insights gained from the *NF1* model will be informative with regard to stromal contributions to and stroma-directed therapy for other brain tumors in children and adults.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Cyclic AMP Suppression Is Sufficient to Induce Gliomagenesis in a Mouse Model of Neurofibromatosis-1

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