

c-Myc Enhances Sonic Hedgehog–Induced Medulloblastoma Formation from Nestin-Expressing Neural Progenitors in Mice¹

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Abstract

Medulloblastomas are malignant brain tumors that arise in the cerebella of children. The presumed cells-of-origin are undifferentiated precursors of granule neurons that occupy the external granule layer (EGL) of the developing cerebellum. The overexpression of proteins that normally stimulate proliferation of neural progenitor cells may initiate medulloblastoma formation. Two known mitogens for neural progenitors are the c-Myc oncoprotein and Sonic hedgehog (Shh), a crucial determinant of embryonic pattern formation in the central nervous system. We modeled the ability of c-Myc and Shh to induce medulloblastoma in mice using the RCAS/*tv-a* system, which allows postnatal gene transfer and expression in a cell type–specific manner. We targeted the expression of Shh and c-Myc to nestin-expressing neural progenitor cells by injecting replication-competent ALV splice acceptor (RCAS) vectors into the cerebella of newborn mice. Following injection with RCAS-Shh alone, 3/32 (9%) mice developed medulloblastomas and 5/32 showed multifocal hyperproliferation of the EGL, possibly a precursor stage of medulloblastoma. Following injection with RCAS-Shh plus RCAS-Myc, 9/39 (23%) mice developed medulloblastomas. We conclude that nestin-expressing neural progenitors, present in the cerebellum at birth, can act as the cells-of-origin for medulloblastoma, and that c-Myc cooperates with Shh to enhance tumorigenicity.

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layer (EGL), a germinal zone on the cortical surface of the developing cerebellum. In mice, rapid proliferation of GNPs after birth expands the EGL to a maximal thickness on postnatal day 6. Afterwards, GNPs migrate inward where they differentiate to generate the millions of neurons that make up the internal granule layer of the cerebellum (reviewed in Ref. [2]).

Overexpression of proteins that normally stimulate proliferation and/or block differentiation of neural progenitor cells may initiate medulloblastoma formation. One known mitogen for neural progenitor cells is Sonic hedgehog (Shh), a secreted protein that is a crucial determinant of embryonic pattern formation in the central nervous system. Shh binds transmembrane protein, Patched (Ptc), at the cell surface. This interaction activates a signal transduction pathway, leading to the transcription of genes that govern various aspects of neural development (reviewed in Ref. [3]). In the developing cerebellum, Shh stimulates the proliferation of GNPs and blocks their differentiation into neurons [4–6].

Mutations that activate the Ptc signaling pathway are oncogenic. Transgenic mice expressing mutant *Smoothed* in the skin develop basal cell carcinomas [7]. Inherited mutations in the human *PTC* gene segregate in families with Gorlin's Syndrome, a condition wherein affected individuals develop neural tube defects, craniofacial abnormalities, and predisposition to various neoplasms, including medulloblastomas [8]. *PTC* gene mutations occur in 3% to 14% of sporadic medulloblastomas [9–11]. Mice that are heterozygous defective for *Ptc* develop medulloblastomas spontaneously [12,13]. Expression of the wild-type *Ptc* allele in tumors from *Ptc*^{+/-} mice suggests that haploinsufficiency, not a two-hit mechanism of *Ptc* gene inactivation, promotes medulloblastoma growth [14,15]. Intrauterine injection of a Shh-expressing retrovirus into the cerebellum of mice on embryonic day 13.5 induces medulloblastoma formation [16].

Introduction

Primitive neuroectodermal tumors (PNETs) are the most common malignant brain tumors in children. Most often, these tumors arise in the cerebellum where they are called medulloblastomas. Although the precise histogenesis of medulloblastoma remains uncertain, one hypothesis maintains that the cells-of-origin are undifferentiated precursors of granule cell neurons (reviewed in Ref. [1]). Granule neuron precursors (GNPs) occupy the external granule

Abbreviations: PNET, primitive neuroectodermal tumor; GNP, granule neuron precursor; EGL, external granule layer

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Another mitogen for neural progenitors is the oncoprotein, c-Myc. We have shown that c-Myc promotes the proliferation of neural progenitor cells in mice [17]. The *c-Myc* gene is amplified in 5% to 8% of human medulloblastomas and overexpressed in 42% to 90% of cases [18,19]. Moreover, the accumulation of *c-Myc* mRNA is an unfavorable prognostic indicator for medulloblastoma patients [20,21]. Although these clinical correlations support a significant role for c-Myc in medulloblastoma, we do not know whether c-Myc expression is an early event critical for tumor initiation, or a late event involved in tumor progression.

We modeled the ability of c-Myc and Shh to induce medulloblastoma in mice using the RCAS/*tv-a* system, which allows postnatal gene transfer in a cell type-specific manner [22,23]. This system utilizes replication-competent ALV splice acceptor (RCAS) vectors, derived from the avian retrovirus, ALV (subgroup A), and a transgenic mouse line (*Ntv-a*) that produces TVA (the receptor for ALV-A) under the control of the *Nestin* gene promoter. Nestin is an intermediate filament protein expressed by neuronal and glial progenitors [24]. After infection, RCAS sequences integrate into the host cell genome where the exogenous gene is expressed from a spliced message under control of the constitutive retroviral promoter, LTR. Combinations of genes can be transferred simultaneously to individual cells by infecting them with multiple RCAS vectors carrying different genes. We transferred genes encoding Shh and c-Myc to nestin-expressing neural progenitor cells in the cerebella of newborn mice. We show here that nestin-expressing neural progenitors, present in the cerebellum at birth, can act as the cells-of-origin for medulloblastoma and that c-Myc cooperates with Shh to enhance tumorigenicity.

Materials and Methods

Mice

The production of the *Ntv-a* transgenic mouse line has been described previously [25]. *Ntv-a/Ptc^{+/−}* mice were created by breeding *Ntv-a* mice with mice containing a mutant allele (*Ptc^{neo67}*), wherein a neomycin resistance cassette replaces exons 6 and 7 of the *Ptc* gene [13]. Therefore, the mice used in these experiments are mixtures of the following strains: C57BL/6, BALB/C, FVB/N, and CD1.

Vector Constructs

RCAS-Shh was constructed by ligating full-length, chicken Shh cDNA 3' of the retroviral *env* gene into parent retroviral vector, RCASBP(A) [22]. The Shh insert contained an in-frame, carboxy-terminal epitope tag containing six repeats of the influenza virus hemagglutinin epitope (HA tag). RCAS-Myc contained full-length, human c-Myc cDNA as described previously [17].

Cell Culture

To produce live virus, we used DF-1 cells, an immortalized line of chicken fibroblasts. DF-1 cells were cultivated in

DMEM supplemented with 5% fetal bovine serum, 5% calf serum, 1% chicken serum, and 0.2% tryptose phosphate broth. We transfected plasmid versions of RCAS vectors into DF-1 cells and allowed them to replicate as viral vectors in culture.

In Vivo Infection of Transgenic Mice

DF-1 virus producer cells were harvested by trypsin digestion, collected by centrifugation, and resuspended in phosphate-buffered saline. Injections of 1 to 2 μ l (10^5 cells) were made with a gas-tight Hamilton syringe into the lateral cerebellum just posterior to the bregma of the skull, or into the right frontal lobe just anterior to the coronal suture within 48 hours after birth. The mice were sacrificed 12 weeks after injection, or sooner if they showed signs of increased intracranial pressure or became debilitated. The brains were removed and fixed in phosphate-buffered formalin (10%). After fixation, each brain was divided into quarters by parallel incisions in the coronal plane and embedded in paraffin. Tissue sections (4 μ m) were mounted on glass slides for histochemical and immunocytochemical analysis.

Immunocytochemistry

To analyze protein expression in tissue sections, we used an immunoperoxidase staining method described previously [26]. We used the following monoclonal antibodies (and dilutions) from the indicated commercial sources: 9E10 (1:50)—human c-Myc (Santa Cruz Biotechnology; Santa Cruz, CA); C19 (1:200)—mouse c-Myc (Santa Cruz Biotechnology); F7 (1:50)—HA (Santa Cruz Biotechnology); 2F11 (1:100)—70-kDa neurofilament protein (Dako, Carpinteria, CA); TuJ1 (1:400)— β III tubulin (Research Diagnostics, Flanders, NJ); 401 (1:50)—nestin (Becton Dickinson PharMingen, San Diego, CA); Mab377 (1:100)—NeuN (Chemicon, Temecula, CA). To detect GFAP expression, we used a rabbit, anticow polyclonal antibody (1:1000; Dako). For double immunofluorescence staining, we incubated formalin-fixed tissue sections with mouse monoclonal antibody 9E10 (anti-Myc), and rabbit polyclonal antibody Y11 (anti-HA; Santa Cruz Biotechnology), followed by secondary antibodies Cy5-conjugated antimouse IgG and FITC-conjugated antirabbit IgG. Cell nuclei were counterstained with propidium iodide. Digital images were generated using confocal microscopy.

Western Blot Analysis of Primary Brain Cultures Infected with RCAS-Shh

We prepared primary brain cultures from newborn *Ntv-a* mice, infected them with RCAS-Shh viral vector, and prepared whole cell lysates of the cultured cells 48 hours after infection as described previously [17]. Protein samples (60 μ g) were resolved by SDS-PAGE using 10% gels, transferred to nitrocellulose filters, and probed with monoclonal antibody F7, directed against the HA epitope tag (85 amino acids) located on the carboxy terminus of the recombinant Shh protein. Detection was carried out using alkaline phosphatase-conjugated secondary antibody (goat antimouse

IgG) and bromochloroindoyl phosphate/nitro blue tetrazolium substrate.

Results

Overexpression of Shh in Neural Progenitors Induces EGL Hyperproliferation and Medulloblastoma Formation

We modeled the ability of Shh and c-Myc, alone and in combination, to induce medulloblastoma formation by injecting RCAS vectors into the cerebella of newborn *Ntv-a* mice. To study the effect of *Ptc*, we injected multiple, different litters of mice born of *Ntv-a/Ptc^{+/-}* parents. We reported previously that RCAS-Myc produces an intact c-Myc protein that stimulates proliferation of neural progenitor cells [17]. To show that our RCAS-Shh vector was capable of producing an intact Shh protein, we performed Western blot analysis of primary brain cultures from *Ntv-a* mice before and after infection with RCAS-Shh. Two major proteins, 60 and 37 kDa in size, were detected in infected cells with an antibody directed against the carboxy-terminal HA epitope tag of RCAS-Shh (Figure 1A). These two proteins correspond to full-length Shh and a carboxy-terminal fragment resulting from Shh autoproteolysis [27].

In 5/32 mice (16%) injected with RCAS-Shh alone, the cerebella contained multiple foci where the EGL was expanded to 10 to 20 cell layers (Figure 1B). Normally, the EGL contains only none to two layers at the developmental stages of mice we examined. The cells in the thickened EGL expressed neuronal markers, β III tubulin, and NeuN, characteristic of proliferating GNPs. In 3/32 mice (9%), RCAS-Shh produced infiltrating tumors that closely resembled human medulloblastomas (Figure 1C).

c-Myc Enhances Shh-Induced Medulloblastoma Formation

Immunoperoxidase staining with an antibody against endogenous c-Myc showed abundant immunoreactivity in the cytoplasm of cells within the EGL hyperproliferations and the tumors (Figure 1D). c-Myc is normally a nuclear protein, but it localizes to the cytoplasm in cerebellar Purkinje neurons and in various tumors [28]. To determine whether the cytoplasmic localization of c-Myc in mouse tumors accurately modeled human medulloblastomas, we mapped the subcellular localization of c-Myc in 16 human medulloblastoma specimens by immunocytochemistry. Consistent with previous reports [19], c-Myc localized to the nucleus in 11 tumors. c-Myc immunoreactivity was restricted to the cytoplasm in four tumors and present in both compartments in one tumor (Figure 1E).

Immunoperoxidase staining of normal mouse cerebellum at postnatal day 3 showed expression of c-Myc throughout the EGL (not shown). Previous experiments indicate that c-Myc helps maintain neural progenitor cells in an undifferentiated and proliferating state [17]. Therefore, we hypothesized that additional c-Myc might cooperate with Shh to enhance tumorigenicity. To test this hypothesis, we injected *Ntv-a* mice with RCAS-Shh plus RCAS-Myc. With this combination, tumors developed in 9/39 mice (23%), a higher

percentage than with RCAS-Shh alone (Table 1). The tumors arose from the lateral aspect of the cerebellar hemisphere and infiltrated the adjacent brainstem (Figure 2A). In one case, the tumor filled the fourth ventricle, a growth pattern mimicking human medulloblastomas (Figure 2B). Like human medulloblastomas, these murine tumors were composed of densely packed sheets of cells with hyperchromatic nuclei and scant cytoplasm, overlying a delicate vascular network (Figure 2C). Nuclear pleomorphism and mitotic figures were present, indicating a rapid proliferative rate. To verify that cells within the observed brain lesions expressed the genes transferred through RCAS vectors, we demonstrated specific staining of most cells within tumors and EGL hyperproliferations with antibodies against epitope tags within RCAS-Shh and RCAS-Myc (Figure 2, D and E). Double immunofluorescence staining showed that 5% to 10% of cells coexpressed Shh and c-Myc (Figure 2F). All tumors showed expression of synaptophysin, a reliable histopathological marker for medulloblastomas [29] (Figure 3A). We did not see tumors or EGL hyperproliferation in mice injected with RCAS-Myc alone.

Some human medulloblastomas contain subpopulations of cells expressing antigens characteristic of differentiated neurons or glia, whereas others are composed entirely of undifferentiated cells. To assess the differentiation status of the mouse tumors, we performed an immunocytochemical analysis of seven tumors induced by Myc + Shh and two by Shh using antibodies specific for nestin, glial fibrillary acidic protein (GFAP), neurofilament protein, NeuN, and β III tubulin. Nestin was detected in 4/9 tumors where the percentage of nestin-expressing cells ranged from 5% to 20%. This low percentage indicates the lack of selective pressure for infected cells to maintain nestin expression *in vivo*. The cell type specificity of the RCAS/*tv-a* system is limited to the original gene transfer event, after which the transferred gene is expressed from the retroviral promoter, LTR.

All tumors showed abundant expression of β III tubulin in the cytoplasm and NeuN in the nucleus (Figure 3, B and C). The expression of β III tubulin by central nervous system progenitor cells is an early marker of neuronal differentiation [30]. NeuN is a nuclear protein expressed by many types of mature neurons [31]. Like β III tubulin, NeuN first appears at developmental time points corresponding to the initiation of neuronal differentiation. We did not detect the expression of neurofilament protein, a marker of terminally differentiated neurons, in any tumor. GFAP immunoreactivity was visible only in processes of entrapped astrocytes interdigitated among unstained tumor cells (Figure 3D). These immunocytochemistry results support an origin of the induced tumors from neuronal precursors.

Effect of Ptc Genotype and Gene Transfer Site on Tumor Formation

Ptc^{+/-} mice develop rhabdomyosarcomas and medulloblastomas spontaneously [12,13], but tumor incidence is highly dependent upon genetic background. In point of fact, rhabdomyosarcomas arise in 15% of CD1 *Ptc^{+/-}* mice but no such tumors arise on a C57BL/6 background [32]. To

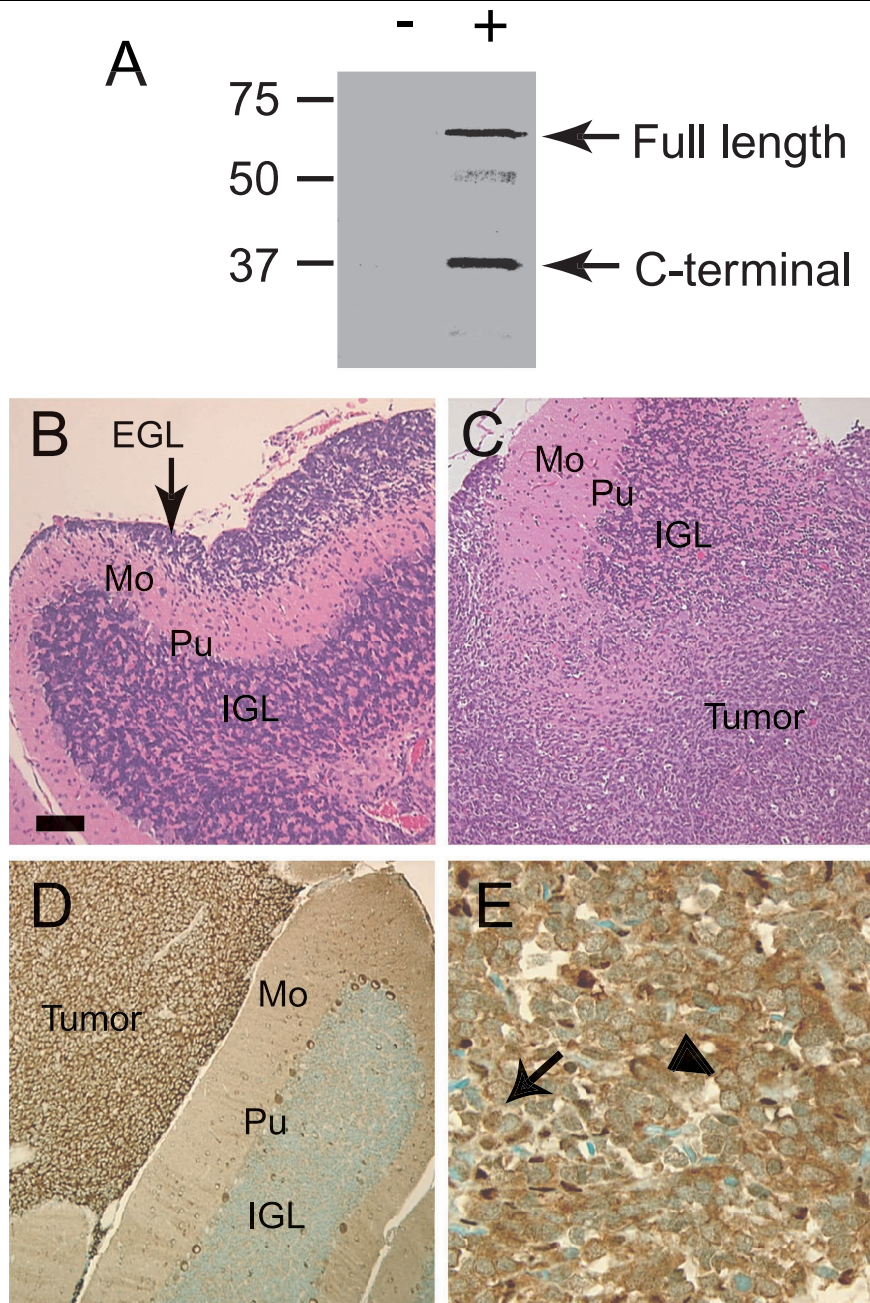


Figure 1. Histopathology of cerebellar lesions induced by Shh. (A) Western blot analysis of *Ntv-a* mouse brain cultures infected with RCAS-Shh. Two major bands (60 and 37 kDa) corresponding to full-length Shh and carboxy-terminal peptide derived from autoproteolysis were detected with anti-HA antibody in infected cells (+) but not uninfected cells (-). (B) Focal hyperproliferation of EGL induced by Shh (Mo—molecular layer; Pu—Purkinje cell layer; IGL—internal granule layer). (C) Shh-induced medulloblastoma infiltrating cerebellar cortex. (D) Cytoplasmic expression of endogenous c-Myc in tumor cells in Shh-induced medulloblastoma. (E) Human medulloblastoma specimen showing expression of c-Myc in nuclei (arrow) and cytoplasm (arrowhead) of tumor cells. Scale bar = 110 μ m (B,C), 55 μ m (D), 27 μ m (E).

determine whether medulloblastomas arise spontaneously on the mixed genetic background of our *Ntv-a/Ptc^{+/-}* mice, we examined histological sections of brains from sixteen 1-year-old mice. We found no tumors in the forebrain, brainstem, or cerebellum. Furthermore, we genotyped the 71 mice injected into the cerebellum with RCAS-Shh alone or in combination with RCAS-Myc. In this group, 53 were *Ptc^{+/-}* and 18 were *Ptc^{+/+}*. These proportions reflect the distribution of alleles among the progeny of heterozygous parents (*Ptc* nullizygotes die early in embryonic development). Medulloblastomas occurred more frequently in *Ptc^{+/+}* mice

(5/18=28%) than in *Ptc^{+/-}* mice (7/53=13%) (Table 2). Similarly, EGL hyperproliferation occurred more frequently in *Ptc^{+/+}* mice (4/18=22%) than in *Ptc^{+/-}* mice (1/53=2%). For these reasons, we cannot attribute the cerebellar lesions observed following targeted expression of Shh and/or c-Myc simply to spontaneous tumor formation.

PNETs arise in the cerebral hemispheres in children, albeit much less frequently than in the cerebellum. Nestin-expressing neural progenitors that are susceptible to infection and gene transfer with RCAS vectors are present in the cerebral hemispheres. To determine whether these

Table 1. Medulloblastoma Formation and EGL Hyperproliferation Induced by Shh and/or c-Myc.

	RCAS vector		
	Shh	Myc + Shh	Myc
Medulloblastoma	3/32	9/39	0/43
EGL hyperproliferation	5/32	0/39	0/43

cells could produce PNETs when stimulated by Shh and c-Myc, we injected RCAS-Shh plus RCAS-Myc into the right frontal lobe of newborn *Ntv-a* mice. Microscopic examination of brain sections from 29 mice sacrificed after 13.7 weeks showed that no brain tumors had formed.

Discussion

Using the RCAS/*tv-a* system, we have recapitulated in mice some of the molecular events that mediate medulloblastoma formation in humans. Expression profile analysis of human medulloblastomas using oligonucleotide microarrays has shown that transcriptional targets of Shh (Gli transcription factors and Ptc) are frequently upregulated [33]. This profile was found predominantly in a histological variant known as

desmoplastic medulloblastoma, which is characterized by dense fibrous stroma surrounding aggregates of tumor cells. The fact that our mouse tumors did not resemble desmoplastic medulloblastomas indicates that Shh/Ptc pathway activation is not unique to the desmoplastic variant and may be a more general molecular derangement in medulloblastoma.

Overexpression of the *c-Myc* oncogene occurs frequently in human medulloblastomas and correlates with poor patient prognosis [20,21]. We showed previously that c-Myc stimulates the proliferation of undifferentiated neural progenitor cells in the forebrain of mice, and we suggested that, through this mechanism, c-Myc might promote PNET formation [17]. In the present study, c-Myc was not sufficient to induce medulloblastoma formation. The enhanced tumorigenicity in mice injected with two RCAS vectors expressing c-Myc and Shh suggests that c-Myc cooperates with Shh to drive proliferating neural progenitors into malignant transformation. The fact that a minority of tumor cells coexpressed Shh and c-Myc suggests that the tumorigenic effect of Shh (a secreted protein) is mediated predominantly through a paracrine, rather than an autocrine (cell-automatous), mechanism.

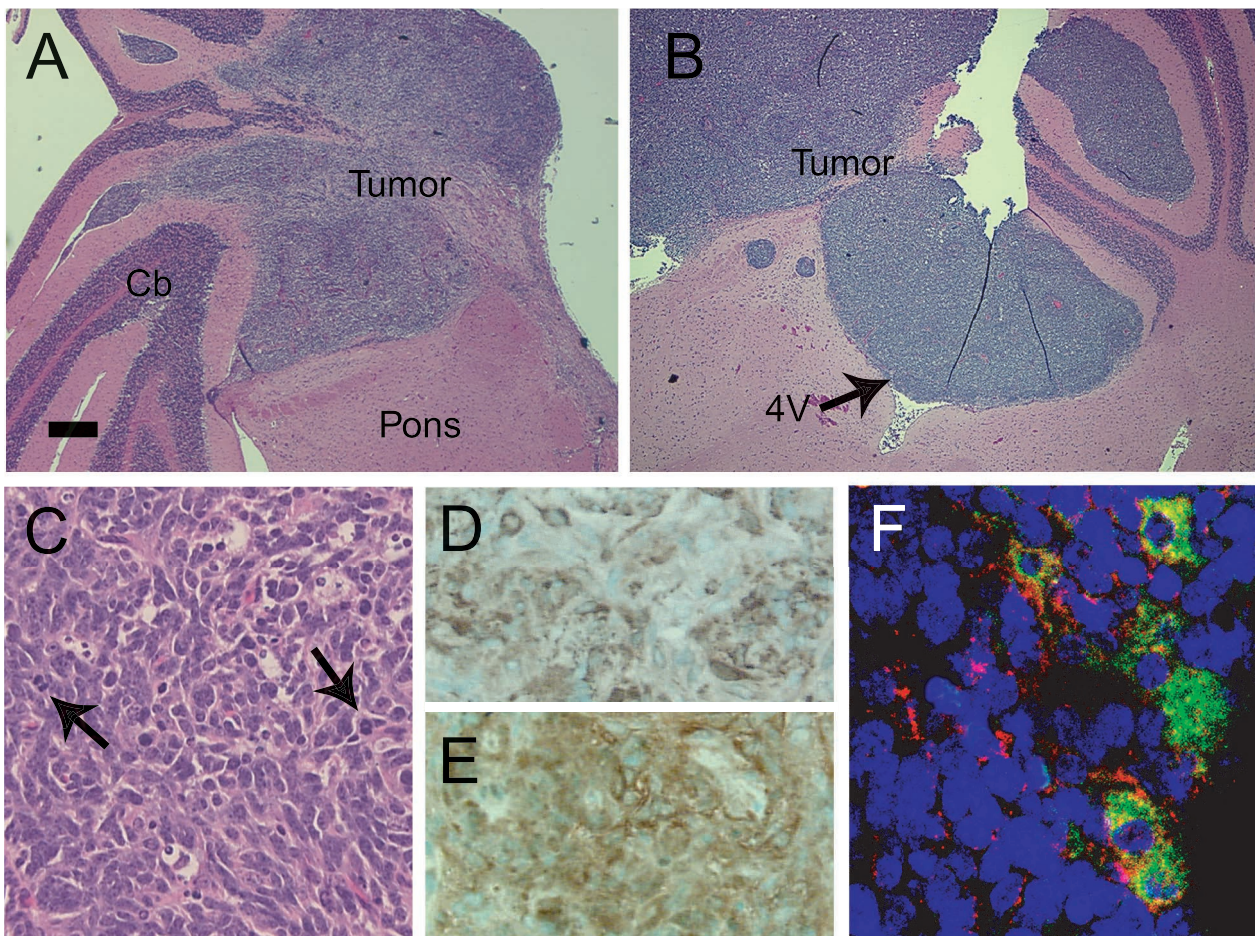


Figure 2. Histopathology of medulloblastomas induced by c-Myc plus Shh. (A,B) Medulloblastomas infiltrating the lateral pons and cerebellum (Cb) and filling the fourth ventricle (4V). (C) Cytoarchitecture of medulloblastoma induced by c-Myc plus Shh. Arrows indicate mitotic figures. (D,E) Immunoperoxidase staining of virally transduced Shh and c-Myc in tumor cells with antibodies against HA (D) and Myc (E) epitopes. (F) Double immunofluorescence staining of Shh (green) and c-Myc (red) shows colocalization (yellow) of both proteins in the cytoplasm of a subpopulation of medulloblastoma cells. Nuclei are counterstained with propidium iodide (blue). Scale bar = 474 μ m (A,B), 27 μ m (C–E), 27 μ m (F).

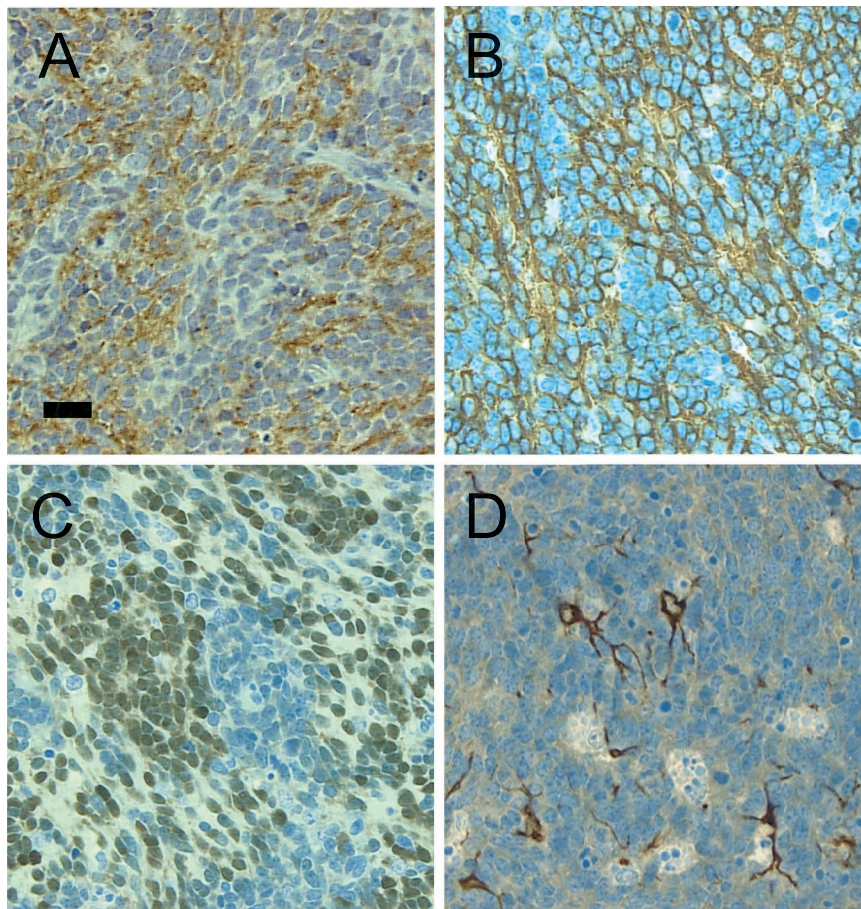


Figure 3. Expression of neural antigens in medulloblastomas induced by c-Myc plus Shh. Expression of synaptophysin (A), β III tubulin (B), and NeuN (C) in tumor cells and GFAP (D) in processes of entrapped astrocytes. Scale bar = 27 μ m.

Other mouse models of medulloblastoma have shown that these tumors can arise by subversion of cell growth pathways other than the Shh/Ptc pathway. Medulloblastomas develop in *p53*-null mice after conditional inactivation of the *Rb* gene [34], or in combination with homozygous loss of *Lig4*, which encodes a nuclear ligase critical for DNA repair [35]. Loss of p53 accelerates medulloblastoma formation in *Ptc*^{+/-} mice [36]. Transgenic mice expressing SV40 virus large T antigen, which functionally inactivates both Rb and p53, develop brainstem PNETs [37]. Considering the fact that loss-of-function mutations in either *RB* or *P53* rarely occur in human medulloblastomas, our model has the advantage of more closely paralleling the molecular pathways relevant to human tumors.

Other investigators recently reported that ectopic expression of Shh in prenatal mouse cerebellum induces EGL thickening and medulloblastomas [16]. They utilized a

murine leukemia virus vector, injected *in utero*, to infect a broad population of dividing cells. Our results confirm their observations and extend them by showing that c-Myc cooperates with Shh to enhance tumorigenicity. Furthermore, our results in *Ntv-a* mice define a more narrow population of candidate cells-of-origin for medulloblastomas, namely, nestin-expressing neural progenitors.

We cannot conclude that the tumors originated from GNP within the EGL because we did not specifically target these precursor cells. Nevertheless, several features of our model are consistent with a GNP origin for medulloblastoma. First, the EGL contains the highest concentration of mitotically active, nestin-expressing cells susceptible to infection with RCAS vectors. Second, significant numbers of cells within the EGL thickenings and in the tumors expressed β III tubulin and NeuN—markers for early neuronal differentiation. Finally, the coexistence of EGL thickening and medulloblastoma in cerebella injected with RCAS-Shh implicates hyperproliferation of GNPs as a precursor stage in the genesis of medulloblastoma.

We could *not* produce tumors in the forebrain by injecting RCAS-Shh, alone or in combination with RCAS-Myc, even though nestin-expressing progenitors are present in that part of the brain in postnatal mice. These cells are known to give rise to glioblastomas *in vivo* with specific activation of the Ras and Akt signaling pathways [38], and

Table 2. Effect of *Ptc* Genotype on Medulloblastoma Formation and EGL Hyperproliferation Induced by Shh and c-Myc + Shh.

	<i>Ptc</i> genotype	
	<i>Ptc</i> ^{+/+}	<i>Ptc</i> ^{+/-}
Medulloblastoma	5/18	7/53
EGL hyperproliferation	4/18	1/53

to oligodendrogliomas with overexpression of platelet-derived growth factor [39]. Our observation that cerebral progenitor cells are less responsive to the same molecules that are potent mitogens for cerebellar progenitors could explain why cerebral hemisphere PNETs are rare compared with cerebellar medulloblastomas.

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