

Notch1 and Notch2 Have Opposite Effects on Embryonal Brain Tumor Growth

Xing Fan,¹ Irina Mikolaenko,¹ Ihab Elhassan,¹ XingZhi Ni,¹ Yunyue Wang,² Douglas Ball,^{2,3} Daniel J. Brat,⁴ Arie Perry,⁵ and Charles G. Eberhart¹

Departments of ¹Pathology, ²Medicine, and ³Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland; ⁴Department of Pathology, Emory University, Atlanta, Georgia; and ⁵Department of Pathology, Washington University School of Medicine, St. Louis, Missouri

ABSTRACT

The role of Notch signaling in tumorigenesis can vary; *Notch1* acts as an oncogene in some neoplasms, and a tumor suppressor in others. Here, we show that different Notch receptors can have opposite effects in a single tumor type. Expression of truncated, constitutively active Notch1 or Notch2 in embryonal brain tumor cell lines caused antagonistic effects on tumor growth. Cell proliferation, soft agar colony formation, and xenograft growth were all promoted by Notch2 and inhibited by Notch1. We also found that Notch2 receptor transcripts are highly expressed in progenitor cell-derived brain tumors such as medulloblastomas, whereas Notch1 is scarce or undetectable. This parallels normal cerebellar development, during which Notch2 is predominantly expressed in proliferating progenitors and Notch1 in postmitotic differentiating cells. Given the oncogenic effects of *Notch2*, we analyzed its gene dosage in 40 embryonal brain tumors, detecting an increased copy number in 15% of cases. *Notch2* gene amplification was confirmed by fluorescence *in situ* hybridization in one case with extremely high Notch2 mRNA levels. In addition, expression of the Notch pathway target gene *Hes1* in medulloblastomas was associated with significantly shorter patient survival ($P = 0.01$). Finally, pharmacological inhibition of Notch signaling suppresses growth of medulloblastoma cells. Our data indicate that *Notch1* and *Notch2* can have opposite effects on the growth of a single tumor type, and show that *Notch2* can be overexpressed after gene amplification in human tumors.

INTRODUCTION

Embryonal brain tumors, known as medulloblastomas in the cerebellum and primitive neuroectodermal tumors (PNETs) elsewhere in the central nervous system (CNS), are among the most frequent causes of cancer death in children (1). Because of their primitive appearance and potential for divergent differentiation, these malignant lesions have long been thought to arise from progenitor cells in germinal epithelia situated periventricularly and on the cerebellar surface (2, 3). Neural stem-like cells have recently been isolated from human medulloblastoma (4, 5), which supports this proposed histogenesis and which suggests that pathways affecting CNS progenitors might play a role in embryonal brain tumor pathobiology.

The Wnt (6), Hedgehog (7, 8), and Notch (9–11) developmental signaling cascades all regulate CNS stem cell dynamics, and both the Wnt and Hedgehog pathways are mutationally activated in a subset of medulloblastomas (1, 12). The role played by Hedgehog signaling is particularly significant, with aberrant activation of this pathway driving proliferation of cerebellar granule cell precursors *in vitro* and causing tumors in transgenic mice (8, 13). In contrast to Hedgehog and Wnt, the role of Notch signaling in medulloblastoma pathogenesis

is unknown. However, *in situ* hybridization (14, 15), murine reporter knockin (16), and reverse transcription-PCR (RT-PCR; ref. 15) analyses have all shown that Notch2 is expressed in rodent cerebellar granule cell precursors. In addition, activated Notch2 acts as a mitogen for cerebellar granule cell precursors (15). Taken together, these data suggest Notch2 signaling may be involved in cerebellar neoplasia. Interestingly, Notch1 is not expressed in proliferating cerebellar precursors (14, 15) but is, instead, found in differentiated internal granule layer neurons (17).

Although *Notch* was initially described as a gene regulating epidermal and neuronal cell fate decisions in *Drosophila* (18), over time it has become clear that the effects of Notch signaling are dependent on cellular context (19). This is particularly true for vertebrates, in which the presence of four Notch receptors and multiple ligands in the Jagged and Delta families leads to a complex array of possible receptor-ligand combinations (20–22). Signaling is initiated when ligands bind a Notch receptor and permit the γ -secretase-mediated proteolytic release of the Notch intracellular domain (NICD). NICD then translocates into the nucleus, in which it interacts with the transcriptional cofactor CBF1 and transactivates gene targets such as those in the *Hes* and *Hey* families (22).

Notch signaling has been implicated in several neoplasms arising outside the CNS. Dysregulated expression of Notch receptors or other pathway components has been demonstrated in both hematopoietic tumors and in cervical, pancreatic, and colon carcinomas (19, 21, 23). In human T-cell lymphoblastic leukemias, Notch1 can be activated by 9;7 chromosomal translocations resulting in expression of a truncated, constitutively active receptor (24). Bone marrow cells transduced with activated Notch1 cause similar leukemias when transplanted into lethally irradiated mice (25). Another pathway member, MAML2, is activated by translocations in mucoepidermoid carcinoma (26). Whereas genetic alterations activating Notch2 have not yet been identified in human neoplasms, truncated forms of the Notch2 receptor induce thymic lymphomas in cats (27). Notch2 can also transform rat kidney cells *in vitro* in combination with E1A (28). Given this oncogenic potential, and the mitogenic effects of Notch2 in cerebellar granule cells progenitors, we investigated whether Notch2 signaling regulates the growth of medulloblastomas and other CNS embryonal tumors, hypothesizing it would act as an oncogene.

MATERIALS AND METHODS

Clinical Material and Immunohistochemistry. 3,3'-diaminobenzidine (DAB)-immunoperoxidase staining was performed with the DAKO Vectastain Elite system with Notch2 (M20; 1:200) from Santa Cruz Biotechnology (Santa Cruz, CA) Hes1 (1:3,000) from Dr. Tesuo Sudo (Toray Industries, Kanagawa, Japan), and Ki67 (1:1,000) from DAKO (Carpinteria, CA). Peptide competition eliminated immunoreactivity for Notch2. Medulloblastoma/PNET surgical specimens were from The Johns Hopkins Hospital Department of Pathology. Frozen tissue from normal fetal cerebella was obtained from the Brain and Tissue Bank for Developmental Disorders (University of Maryland, Baltimore). The medulloblastoma tissue array was constructed as described previously (29). These studies were approved by the Internal Review Board of the Johns Hopkins University School of Medicine. Immunocytochemistry on DAOY cells was performed with standard techniques. In brief, cells were seeded in 6-well tissue culture plate and were allowed to adhere overnight.

Received 4/23/04; revised 9/9/04; accepted 9/10/04.

Grant support: Supported by grants from the Children's Brain Tumor Foundation and NINDS K08NS43279 (C. Eberhart) and National Cancer Institute R01CA70244 (D. Ball). C. Eberhart is the recipient of a Burroughs Wellcome Career Award.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: X. Ni is currently in the Department of Surgery, Shanghai Second Medical University, Shanghai, People's Republic of China.

Requests for reprints: Charles Eberhart, Johns Hopkins University School of Medicine, Department of Pathology, 720 Rutland Avenue, Ross Building 558, Baltimore, MD 21205. Phone: (410) 502-5185; Fax: (410) 955-9777; E-mail: ceberha@jhmi.edu.

©2004 American Association for Cancer Research.

After infection with AdNICD2 or Ad β gal for 48 hours, cells were fixed with 4% paraformaldehyde, permeabilized with 0.4% Triton X-100, blocked in PBS/5% bovine serum albumin, then incubated with anti-Notch2 antibody [C651.6DbHN originally developed by Dr. Artavanis-Tsakonas (30) and obtained from the University of Iowa Developmental Hybridoma Studies Bank, (1:10 dilution)] for 2 hours. Cy3-conjugated secondary antibody incubations and 4',6-diamidino-2-phenylindole (DAPI) counterstains were performed with appropriate wash steps before mounting and visualization with a fluorescence microscope (Zeiss, Germany).

Polymerase Chain Reaction. Quantitative RT-PCR was performed as described previously (31), with all reactions normalized to actin (Applied Biosystems, Foster City, CA). The primers and probes for Notch1, Notch2, and Hes1 are: Notch1 forward (F) 5'-CCGCGATTGTGCTCCTGAA-3', Notch1 reverse (R) 5'-ACCTTGGCGGTCTCCTAGCT-3', Notch1 probe 5'-AACAAAGATATG-CAGAACAACAGGGAGGAGACA-3'; Notch2 F 5'-GGCATTAAATCGTA-CAGTTGTGCT-3', Notch2 R 5'-GGAGGCACACTCATCAATGTCA-3', Notch2 probe 5'-CACCAGGATTCACAGGGCAGAGATGTAACA-3'; Hes1F 5'-AGCGGGCGCAGATGAC-3', Hes1R 5'-CGTTCATGCACTCGCTGAA-3', Hes1 probe 5'-CTGCGCTGACACAGACCAAGTGT-3'. Assays-on-Demand TaqMan probes (Applied Biosystems) were used to measure Hey1, Hey2, PTCH, Gli1, and Gli2 expression. In tumor material, Notch and Hes1 expression was normalized to the median levels in three fetal cerebella of gestational ages 19 to 20 weeks. Differential PCR quantitation of *Notch2* gene dosage was performed as described previously (31) with the following primers for three *Notch2* exons and a 1q sequence-tagged site (STS) reference sequence: Ex12 F 5'-GGGTTAATTGTGAAATTAATT-3', Ex12 R 5'-CGATTAATGCCATCCATACAGA-3'; Ex26 F 5'-ATGACAAATACTGTGCAGACCA-3', Ex26 R 5'-AGCATCCTG-GAGCAGTTGTT-3'; Ex30 F 5'-TGAAGCTGCAGACATCCGTA-3' Ex30 R: 5'-AACACATCCACCTCTGCTC-3' 1qSTS F 5'-ATTACTGCTTTC-CCCAGTTC-3', 1qSTS R 5'-CTTGTTTGTTCACACACCG-3'. DNA extracted from eight nonneoplastic brain samples was used to calibrate the assay and calculate a threshold for significant copy number gains (2.7 fold increase). Cases in which the mean ratio of Notch2 to 1qSTS exceeded this threshold for all three exons, were considered to be amplified.

Fluorescence *In situ* Hybridization. Fluorescence *in situ* hybridization (FISH) with probes generated by using bacterial artificial chromosomes (BACs) CTD-2574B15 (Notch2), and 260123 [1p32 reference probe (a reference sequence on 1p32); Research Genetics, Pasadena, CA] was performed as described previously (32). The cytogenetic localization of the Notch2 BAC clone (1p11-13) was confirmed on metaphase preparations.

Cell Culture and Xenograft Experiments. DAOY and PFSK cell lines were obtained from the American Type Culture Collection and maintained in Richter's zinc option media supplemented with 10% fetal bovine serum unless otherwise noted; UW228 cells were the kind gift of Dr. John Silber (Department of Neurological Surgery, University of Washington, Seattle, WA) and were grown in DMEM/F-12 with 10% fetal bovine serum. The Notch intracellular domain (NICD) adenovirus construction and procedures for infection have been described previously (33). Stable transfectants were established by selection with G418 with standard techniques. Flow cytometric analysis of S-phase fraction and cell cycle kinetics was performed after fixation and staining with propidium iodide with a FACSCalibur (Becton Dickinson, San Jose, CA) with CELL Quest, version 3.3 software. Small interfering RNAs (siRNAs) for Notch1 (sequence available from L. Miele, Department of Pharmaceutical Sciences and Cancer Center, University of Illinois, Chicago, IL), Notch2 (5'-GUCUCAGAAGCUAACCUAAtt-3'), Hes1 (5'-AGACGAA-GAGCAAGAAUAAtt-3'), or scrambled control siRNA were transfected with siPORT amine (Ambion, Austin, TX). Cell growth was measured by using celltiter 96 cell proliferation assay (Promega, Madison, WI), and plotted either as growth curves or as relative growth [$(A_{490 \text{ nmDay}3} \text{ to } A_{490 \text{ nmDay}1})/A_{490 \text{ nmDay}1}$]. Soft agar clonogenicity assays were performed in triplicate as previously described (34) in 2% fetal bovine serum. Subcutaneous xenografts were established in athymic (nude) mice by using three million cells in 100 μ L of culture medium mixed with 100 μ L of Matrigel (BD Bioscience, Bedford, MA). Mitoses were counted by a neuropathologist (I. M.) blinded with respect to xenograft line. Western blots contained 10 or 20 μ g of protein per lane, and were hybridized overnight with the following primary antibodies: for Notch1, Santa Cruz Biotechnology C20, 1:200; for Notch2, Santa Cruz Biotechnology M20, 1:200; or with the monoclonal antibody C651.6DbHN, 1:10, and Hes1

(polyclonal from T. Sudo) 1:2000. DFK-167 (Enzyme Systems, Livermore, CA) was diluted in DMSO.

RESULTS

Differential Expression of Notch1 and Notch2 in Normal and Neoplastic Cerebellar Cells. We first sought to define the expression pattern of Notch transcripts in embryonal brain tumors derived from the external germinal layer (EGL) and ventricular zone, predicting lower expression of Notch1 than of Notch2 in cerebellar tumors. To obtain quantitative data on gene expression, we used real-time RT-PCR to measure Notch1 and Notch2 levels in mRNA extracted from snap-frozen brain tumors. We compared Notch expression in tumors to that in rapidly proliferating fetal cerebellum in which the pathway should be active, based on developmental studies in rodents. Notch2 mRNA was present in all 30 medulloblastomas examined, and 25 expressed higher levels than did fetal cerebellum (Fig. 1A). In parallel with its absence from the proliferating outer EGL in normal development, Notch1 was undetectable in 9 of 22 medulloblastomas, and only 3 of the 13 positive cases had expression equal to or higher than that in fetal cerebellum. Hes1 message was also detected in all cases, although only five had levels higher than fetal cerebellum (data not shown). In medulloblastoma, Notch2 and Hes1 expression (Fig. 1B) showed a significant positive correlation ($P = 0.04$), whereas Notch1 and Hes1 did not, which suggests that Notch2 may regulate Hes1

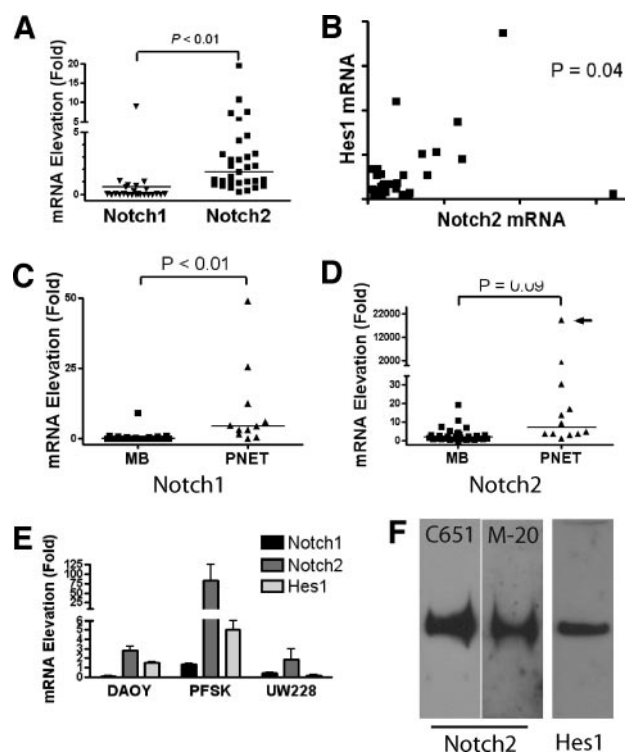


Fig. 1. Notch mRNA expression in medulloblastoma/PNET. In A, Notch1 mRNA expression, measured by quantitative RT-PCR and expressed as fold change in relation to levels in nonneoplastic fetal cerebella ($n = 3$), was low or undetectable in 19 of 22 medulloblastomas. In contrast, Notch2 was detected in all 30 medulloblastomas examined and, in 25, was more highly expressed than in normal cerebellum. In B, Notch2 mRNA expression correlated significantly with that of Hes1 in medulloblastomas. In C, Significantly higher expression of Notch1 was present in extra-cerebellar PNET as compared with medulloblastoma (MB). In D, a trend toward higher Notch2 expression in PNET was seen as well. A PNET shown by FISH to be amplified at the *Notch2* locus had especially high mRNA levels (arrow). In E, expression of Notch receptors and Hes1 was also detected in medulloblastoma (DAOY, UW228) and PNET (PFSK) cell lines. In F, the goat polyclonal Notch2 antibody used in our immunohistochemical studies identified the same M_r 120,000 transmembrane Notch2 band in DAOY protein extracts as the anti-Notch2 monoclonal C651. An appropriately sized M_r ~31,000 Hes1 band was also identified in these extracts.

expression in EGL-derived tumors. We extended our analysis to 12 supratentorial PNETs. Interestingly, these extracerebellar lesions had significantly higher levels of Notch1 (Fig. 1C) and Hes1 mRNA (data not shown) than did medulloblastoma, which supported an earlier study showing that these microscopically similar tumors can have different gene expression profiles (35). Although Notch2 expression was not statistically different in medulloblastomas and PNETs, it was higher in the latter tumors, including one PNET with Notch2 mRNA levels >20,000-fold higher than in fetal cerebellum (Fig. 1D). The Notch receptors and Hes1 were also expressed in two medulloblastoma cell lines (DAOY, UW228) and in the supratentorial PNET cell line PFSK (Fig. 1E). Notch pathway proteins were also detected in these cells. The $M_r \sim 120,000$ transmembrane Notch2 receptor was detected in DAOY protein extracts by using either polyclonal antisera or a previously described (30) Notch2-specific monoclonal antibody (Fig. 1F). The $M_r 31,000$ Hes-1 protein was also abundant in DAOY cells. Consistent with its low mRNA level, the transmembrane Notch1 protein band was only faintly detected in DAOY cells by using a monoclonal antibody (data not shown), and was undetectable with the polyclonal antibody unless overexpressed (Fig. 3B).

Activation of Notch Signaling in Medulloblastomas Is Associated with Worse Clinical Outcomes. Because Notch2 signaling is linked to proliferation, and highly proliferative tumors are more aggressive clinically, we investigated the relationship between Notch pathway activity and patient survival. Of the 41 medulloblastoma and PNET patients with Notch2 mRNA expression data and clinical follow-up available, only 14% with below-median Notch2 levels died as compared with 45% of those with above-median Notch2 expression. However, these survival differences were not statistically significant, perhaps because of the limited follow-up available for these relatively recent cases in which frozen material was collected. We, therefore, also analyzed Notch pathway activity immunohistochemically by using a previously described medulloblastoma tissue array (29) containing paraffin-embedded specimens from patients with longer follow-up times. Expression of the Notch target Hes1, which, as described above, is associated with Notch2 activity, was graded semiquantitatively in 35 medulloblastomas remaining on the array (Fig. 2A). Notch2 could not be analyzed on the array because the tissue block was depleted by earlier analyses. The 11 patients with Hes1 immunopositive tumors (+ to +++) had a higher mortality rate (64%) than the 24 with Hes1-immunonegative tumors (25%). Log rank analysis of Kaplan–Meier survival curves confirmed the statistical significance ($P = 0.01$) of the shorter survival in patients with Hes1-immunopositive tumors (Fig. 2B). In addition, of the 11 positive cases, the 4 tumors with more intense Hes1 staining (++ or +++) all came from patients who did not survive. Thus, components of the Notch pathway involved in normal development are expressed in embryonal brain tumors, and expression of the Notch pathway target Hes1 is prognostic of worse clinical outcomes.

Increased Notch2 Gene Dosage in Central Nervous System Embryonal Tumors. We were curious about the mechanisms by which Notch signaling might be activated in medulloblastoma/PNET. In some embryonal brain tumors, amplification of oncogenes can greatly increase their mRNA levels (31). Because of the extremely high level of Notch2 transcript in several PNET cases, we examined Notch2 gene dosage to determine whether chromosomal alterations might account for this overexpression. Differential PCR analysis comparing three separate regions of the Notch 2 gene to a STS reference probe on 1q revealed that 6 of 40 (15%) medulloblastoma/PNET had increased Notch2 gene copy number. FISH analysis of the Notch2 locus was successful in three of these six cases and confirmed increased gene dosage. One PNET contained >12 copies of Notch2 in ~10% of neoplastic cells (Fig. 2C). No amplification of a 1p32

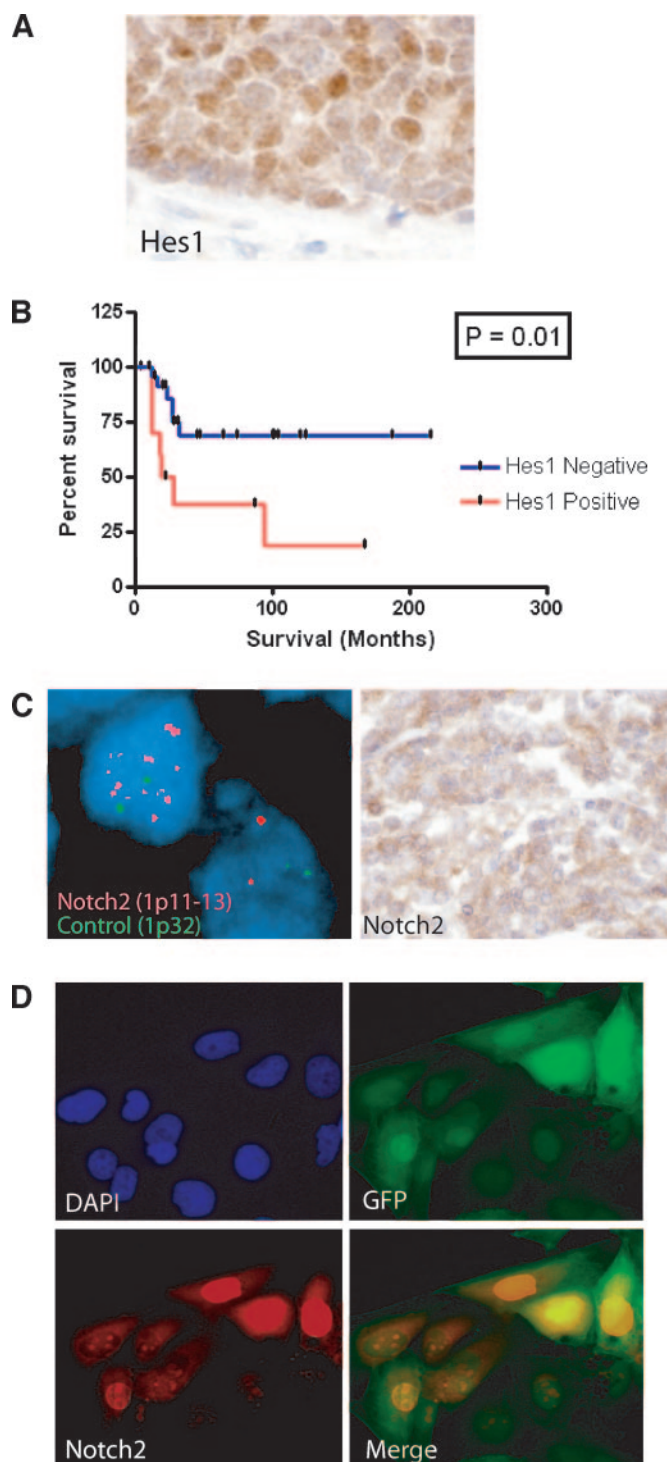


Fig. 2. Hes1 protein expression and Notch2 chromosomal alteration in embryonal brain tumors. In A, nuclear expression of the Notch pathway target Hes1 was detected immunohistochemically in 11 of 35 medulloblastomas examined ($\times 600$). Small nonneoplastic stromal cell nuclei at the bottom of the image lack Hes1 and serve as an internal negative control. In B, patients with Hes1-positive medulloblastoma had significantly ($P = 0.01$) shorter survival (log rank analysis of Kaplan–Meier survival curves). C, Notch2 gene amplification was confirmed in a PNET with FISH probes hybridizing to Notch2 (red) and a reference sequence on 1p32 (green). Notch2 protein was expressed in the Notch2-amplified tumor, with strong cytoplasmic staining and scattered positive nuclei. In D, DAOY cells, infected with AdNICD2, were identified by green fluorescent protein (GFP) expression. Immunocytochemical analysis with the C651 monoclonal antibody verified expression of Notch2, with abundant nuclear protein in most of the infected cells. (DAPI, 4',6-diamidino-2-phenylindole; Merge, GFP merged with Notch2.)

reference probe was seen in this case, which indicated that the amplicon encompassed only a fraction of the 1p chromosomal arm. Gene amplification in this tumor was associated with a Notch2 mRNA level 20,000-fold higher than in fetal cerebellum (arrow, Fig. 1D), as well as with high Notch2 (Fig. 2C) and Hes1 protein levels, strongly suggesting that the chromosomal alteration affected Notch signaling. In the other two tumors successfully analyzed by FISH, all or most of chromosomal arm 1p was gained, with 4 to 16 copies of both the 1p11–13 and 1p32 sequences present in the majority of tumor cells (data not shown). Therefore, although there was not evidence of *Notch2* amplification in these two cases in comparison with the 1p control, there was nevertheless a significant gain in copy numbers.

Notch1 and Notch2 Have Opposite Effects on the Growth of Medulloblastoma/PNET Cells. To test whether Notch1 and Notch2 activity alters the growth of embryonal tumors *in vitro*, we expressed truncated, constitutively active forms of the receptors in established human medulloblastoma and PNET cell lines. Infection of DAOY cells with adenovirus encoding the intracellular domain of Notch2 (NICD2) resulted in abundant nuclear Notch2 protein in >50% of tumor cells (Fig. 2D). Uninfected DAOY cells contained lower levels of Notch2 protein, and it was less commonly present in the nucleus. Infection with the NICD2 adenoviral constructs also led to a 3-fold or greater increase in respective mRNA levels after 48 hours (Fig. 3A), appearance of a truncated Notch2 band on Western blots (Fig. 3B), and induction of Notch pathway targets (Fig. 3A and Fig. 5A). Consistent with the low Notch1 mRNA level in DAOY cells, Notch1 protein was detected in these cells only after infection with AdNICD1 (Fig. 3B).

Interestingly, activation of the two Notch receptors revealed opposing effects on proliferation. Using fluorescent activated cell sorting to analyze tumor cell cycle changes, we found that NICD1 inhibited proliferation by 47%, whereas NICD2 stimulated proliferation by 27%, as determined by the percentage of DAOY cells in S phase (Fig. 3C). The G₁-G₀ fraction rose from 37% to 70% in AdNICD1-infected DAOY cells compared with AdβGal-infected cells, consistent with a G₁ arrest of the cell cycle. NICD2 expression was accompanied by a decrease in G₁-G₀ fraction. These experiments were repeated four times with comparable results. Similar cell cycle changes in two additional embryonal brain tumor lines (PFSK, UW228) suggest that the antithetical proliferative effects are a general property of Notch in at least a subset of embryonal tumors. The disparate effects of Notch1 and Notch2 in embryonal tumors also extended to *in vitro* assays of tumorigenicity, with NICD1 inhibiting, and NICD2 stimulating, clone formation in soft agar several-fold (Fig. 3D).

Suppression of Notch1 or Notch2 expression by RNA interference resulted in changes opposite those seen in NICD1- and NICD2-expressing cells. Transfection of siRNA-targeting Notch2 resulted in a 90% or greater knockdown of Notch2 mRNA (Fig. 4A) or protein (Fig. 4B) in DAOY cells. *Notch2* siRNA also reduced levels of three Notch target genes, evidence of functional inhibition of Notch2 signaling (Fig. 4C). *Notch1* siRNA effectively reduced Notch1 mRNA levels after transfection into PFSK cells (Fig. 4A), but protein level reductions were less pronounced (Fig. 4B). Finally, although Notch1 knockdown promoted cell proliferation, reduction of Notch2 expression decreased the fraction of cells in S phase (Fig. 4D). These loss-of-function studies strongly suggest that the inhibitory effects of NICD1 are not the result of nonspecific toxicity, and that opposing activities of Notch1 and Notch2 stem from unique functions of each receptor in these embryonal brain tumor cells.

To facilitate longer-term assays, we constructed DAOY cell lines stably transfected with the intracellular domains of *Notch1* (D: NICD1) or *Notch2* (D: NICD2). Growth alterations in these cells paralleled those seen after NICD delivery by adenoviral infection,

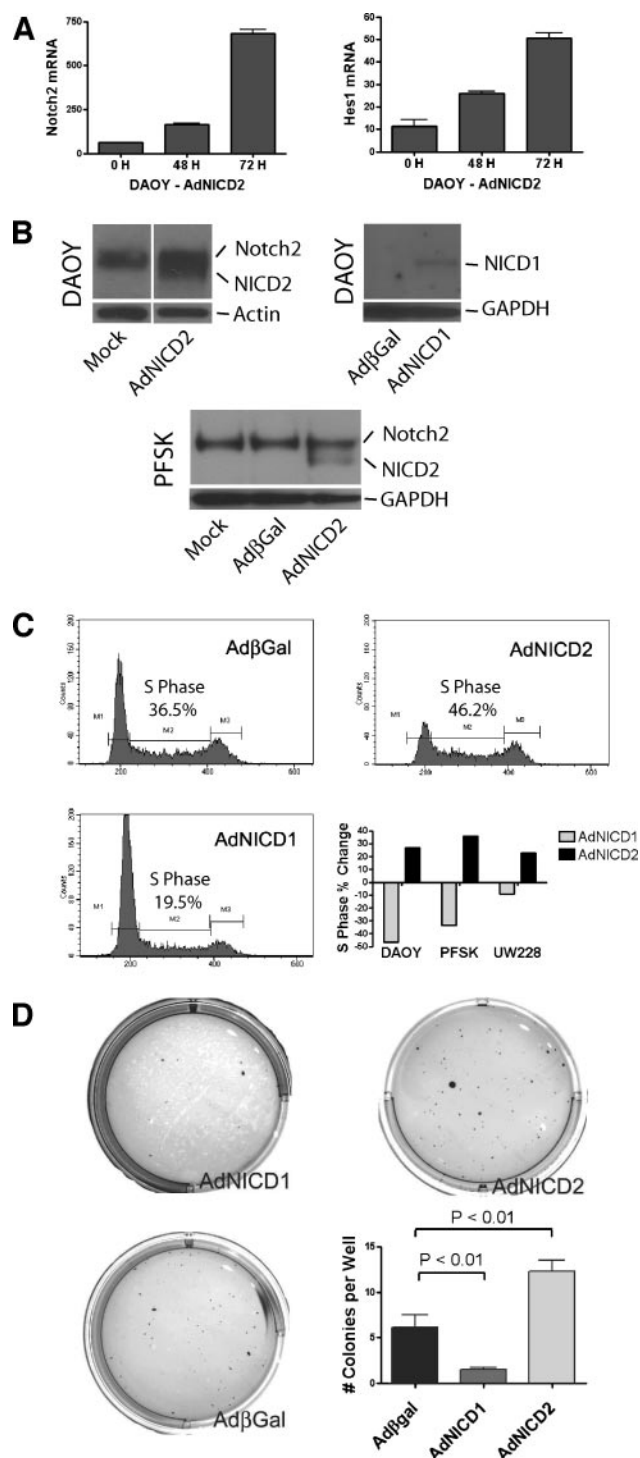


Fig. 3. Opposing effects of Notch1 and Notch2 *in vitro*. In A, transduction of NICD2 into DAOY cells resulted in a several-fold induction of Notch2 and Hes1 mRNA levels over 72 hours. In B, a truncated Notch2 band was identified on Western blots of protein extracts from DAOY and PFSK cells infected with AdNICD2 by using C651 monoclonal antibody (Mock, uninfected cells). No Notch1 protein was detected in DAOY cells using the M20 goat polyclonal antisera unless they were infected with AdNICD1. In C, flow cytometric analysis of S-phase fraction 48 hours after infection with adenovirus encoding truncated Notch1 (AdNICD1) or Notch2 (AdNICD2) revealed opposing effects on DAOY proliferation, as compared with AdβGal infected cells (2% fetal bovine serum). Similar effects were seen after infection of PFSK and UW228 cells and are depicted as percentage change relative to AdβGal infected controls. In D, soft agar colony formation at 2 weeks was inhibited 3-fold by AdNICD1 ($P < 0.01$) and was stimulated 2-fold by AdNICD2 ($P < 0.01$).

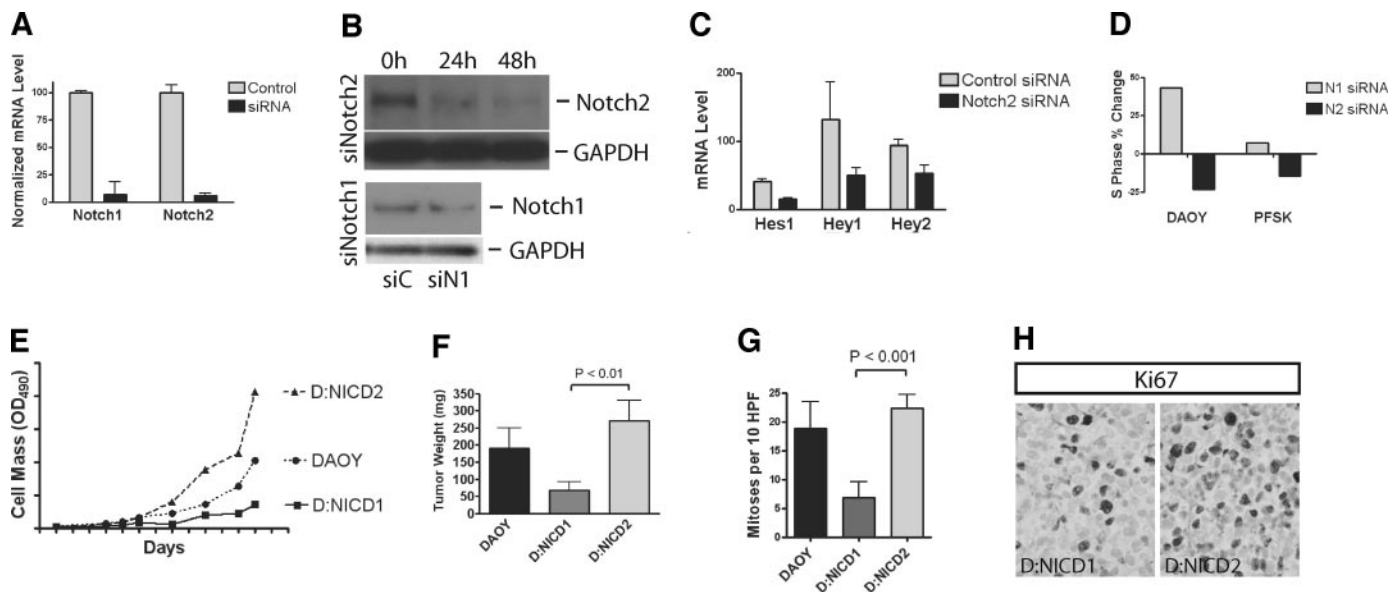


Fig. 4. Effects of Notch siRNA knockdown and stable NICD expression on proliferation and growth. In *A*, quantitative RT-PCR analysis showed that siRNAs, targeting Notch1 or Notch2, reduced their respective mRNA levels by 90% or more, 48 hours after transfection into PFSK or DAOY cells. In *B*, Notch2 protein expression also decreased >80% in DAOY cells 48 hours after siRNA treatment. Notch1 protein levels in PFSK cells decreased less significantly after *Notch1* siRNA treatment (*siN1*) compared with control siRNA (*siC*). In *C*, Notch2 knockdown was accompanied by decreased mRNA levels in several Notch targets. In *D*, loss of Notch1 (*N1 siRNA*) and Notch2 (*N2 siRNA*) caused effects on DAOY cells proliferation that were the opposite of the effects seen after their activation. In *E*, stably transfected DAOY clones, expressing truncated Notch1 (D:NICD1) or Notch2 (D:NICD2), had disparate long-term growth rates in low serum (0.5% fetal bovine serum) as measured by MTS assay. In *F*, D:NICD2- and D:NICD1-flanked xenografts in nude mice also displayed significant differences in weight when removed after 7 weeks ($n = 8$ per group). In *G*, the frequency of mitotic figures was >2-fold lower in NICD1-expressing cells compared with untransfected DAOY cells. In *H*, Ki67 immunostains highlight the higher proliferation rates associated with Notch2 activity.

with a representative D:NICD1 subclone growing 2.8-fold slower and a D:NICD2 subclone growing 2-fold faster than the progenitor cell line (Fig. 4E). Injecting these cells subcutaneously in nude mice provided additional evidence for the growth-suppressing activity of Notch1, with significantly decreased xenograft weights after 7 weeks (Fig. 4F). Mitotic cell counts confirmed a statistically significant decrease in the proliferation of D:NICD1 xenografts compared with D:NICD2 ($P < 0.001$; Fig. 4G). Microscopic examination of D:NICD1 xenografts revealed mainly sparse tumor cells admixed with residual Matrigel, and even the most cellular regions of these lesions had low proliferation indices measured by Ki67 staining (Fig. 4H). In contrast, D:NICD2 xenografts were densely cellular with abundant Ki67 positive cells. Immunohistochemical staining of xenografts with antibodies specific for cleaved caspase 3 revealed no significant differences in apoptosis between NICD1- and NICD2-expressing cells (data not shown).

Notch1 and Notch2 Have Different Effects on the Expression of Hes1 and Hedgehog Pathway Components. The canonical Notch signaling pathway regulates expression of downstream basic helix-loop-helix (bHLH) transcription factors, including, among others, Hes1 and Hey1 (22). We wondered whether such factors might be differentially regulated by Notch1 and Notch2 in embryonal tumors. Interestingly, although Hes1 and Hey1 were both up-regulated in NICD2-expressing DAOY cells, NICD1 activity was associated with increased expression of only Hey1 (Fig. 5A). Similar expression changes were seen in PFSK cells (data not shown). Thus, differential activation of direct Notch targets may account for some of the opposing tumorigenic effects we observe. The Hedgehog pathway is a second possible mediator of the disparate effects of Notch on medulloblastoma growth, because it is known to regulate medulloblastoma proliferation, and suppression of Hedgehog signaling by Notch1 was recently reported in the skin (36). We indeed found profound suppression of three targets of the Hedgehog pathway (PTCH, Gli1, and Gli2) after adenovirus-mediated delivery of NICD1 (Fig. 5B). In contrast, AdNICD2 infection increased expression of these genes.

Similar results were seen in DAOY cells stably transfected with NICD1 and NICD2. Thus, although the Notch1 and Notch2 receptors are structurally similar, their activation can induce opposing transcriptional programs. Loss of Notch receptor expression also affected Hedgehog activity, but siRNAs for both Notch1 and Notch2 increased expression of Hedgehog pathway targets, suggesting that endogenous Notch activity in DAOY cells may act mainly to suppress Hedgehog (Fig. 5C).

Because Hes1 was significantly associated with poor clinical outcomes in embryonal brain tumor patients and was also positively regulated by Notch2, we examined the ongoing requirement for Hes1 in the growth of medulloblastoma/PNET cells. siRNA sequences targeting Hes1 reduced its mRNA levels by >60% in DAOY and PFSK cell lines 48 hours after transfection. Decreased Hes1 expression was accompanied by a significant slowing in DAOY and PFSK cell growth (Fig. 5E). Thus, Hes1 may mediate some or all of the growth-promoting properties of Notch2.

Pharmacological Inhibition of Notch Signaling Arrests Medulloblastoma Growth. Current therapies for embryonal brain tumors cure only ~60% of patients and are associated with significant long-term side effects (1). Our RNA interference data indicate an ongoing requirement for Notch2 activity for medulloblastoma growth and suggest that the inhibition of Notch signaling might significantly reduce the growth of embryonal brain tumors in which Notch2 activity predominates over that of Notch1. γ -secretase inhibitors prevent Notch activation by blocking the cleavage event releasing the active intracellular domain (37, 38). One such compound, DFK-167, reduced Hes1 expression in DAOY cells in a dose-dependent fashion (Fig. 5F). The apparent IC_{50} was similar to the 20-to-30- μ mol/L value reported in Chinese hamster ovarian cells (39), and cell growth was inhibited 82% by a 50- μ mol/L concentration of the compound (Fig. 5F). Importantly, growth of D:NICD2 cells, which express already truncated receptor and do not require γ -secretase for Notch2 activity, was not inhibited by 50 μ mol/L DFK-167, which indicated that DFK-167 suppresses growth through specific effects on Notch sig-

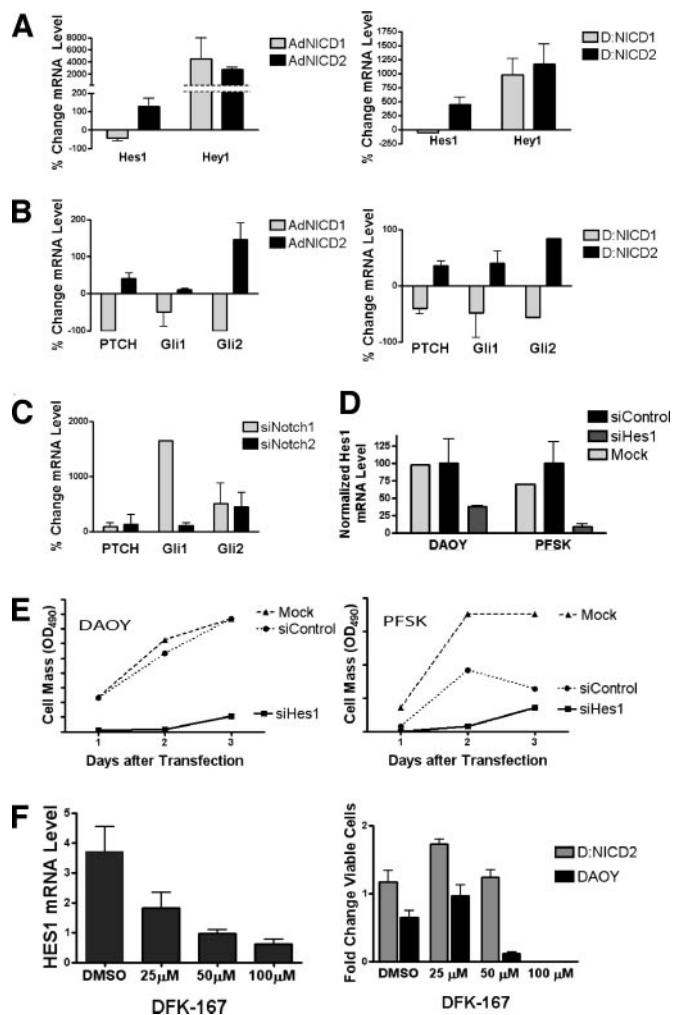


Fig. 5. Pharmacological inhibition of Notch signaling blocks medulloblastoma growth. In *A*, although Hey1 expression is increased by both NICD1 and NICD2, Hes1 is induced only by the latter. In *B*, differential responses of Hedgehog (Hh) pathway targets to Notch1 and Notch2 were also seen in medulloblastoma cell lines. PTCH, Gli1, and Gli2 transcript levels were measured in DAOY cells 48 hours after adenoviral infection and in stably transfected lines (mRNA percentage change compared with levels in Ad β Gal-infected and progenitor DAOY cells, respectively). Notable are Hh pathway suppression by Notch1 and activation by Notch2. In *C*, siRNA transfection reducing either Notch1 or Notch2 resulted in increased expression of Hh pathway targets. In *D*, siRNA-targeting Hes1 result in significantly decreased Hes1 mRNA expression 48 hours after transfection. In *E*, decreased Hes-1 expression is associated with growth inhibition of DAOY and PFSK cells. In *F*, treatment of DAOY cells with the DFK-167 γ -secretase inhibitor resulted in a dose-dependent inhibition of Hes1 mRNA levels after 48 hours, evidence of decreased Notch signaling. Growth of DAOY cells was inhibited by 50 μ mol/L DFK-167, and this inhibition could be rescued by expression of truncated Notch2. (μ M, μ mol/L.)

naling and is not generally cytotoxic at moderate doses. The more profound growth suppression at higher DFK-167 doses could not be rescued by Notch2 activity and may be caused by the inhibition of other Notch receptors, or effects on unrelated pathways also regulated by γ -secretase activity (40).

DISCUSSION

In this study, we demonstrate that the Notch signaling pathway plays a significant role in the growth of embryonal brain tumors such as cerebellar medulloblastoma. Notch activity has been previously shown to regulate the granule cell progenitors from which medulloblastomas form. Solecki *et al.* (15) reported that Notch2 and Hes1 promote the proliferation of murine cerebellar granule cell progenitors *in vitro* and in cerebellar slice cultures. Interestingly, they detected

Notch1 in postmitotic cells rather than in proliferating progenitors, suggesting it may promote cell cycle exit and neuronal differentiation in this developmental context.

The effects of *Notch1* and *Notch2* on medulloblastoma growth recapitulate these apparent developmental differences. We demonstrate that Notch1 activity inhibits proliferation of medulloblastoma cells, whereas Notch2 promotes their growth *in vitro*. *Notch1* was initially thought to be oncogenic, but is now known to suppress tumor formation in some contexts. The Notch1 intracellular domain can inhibit the proliferation of small-cell lung cancer (41), and down-regulation of Notch1 expression is likely involved in the progression of some cervical cancers (42). Nicolas *et al.* (36) have recently inactivated *Notch1* in murine epidermis and have shown that it acts as a tumor suppressor gene in the skin. Although these earlier studies highlight the potential of *Notch1* to act as an oncogene or tumor suppressor gene in different neoplasms, we demonstrate for the first time that *Notch1* and *Notch2* can have antagonistic effects on the growth of a single tumor type.

This suggests that a deeper understanding of the various Notch receptors and their downstream targets will be required for effective clinical interventions. Interestingly, in skin, as in the developing cerebellum, Hedgehog activity plays a key role in the proliferation of progenitor cells, and regulation of Hedgehog signaling seems to be one mechanism by which Notch1 suppresses tumor formation in keratinocytes (36). We demonstrate that, in cerebellar tumor cells, Notch1 activity suppresses expression of Hedgehog targets, whereas Notch2 activation up-regulates the same genes. Notch1 and Notch2 also have opposing effects on the expression of Hes1, a direct effector of Notch signaling, which suggests that differential regulation of both direct and indirect Notch targets may underlie the opposing growth phenotypes that we observe.

The expression profiles of Notch1 and Notch2 in primary medulloblastomas are consistent with their opposite effects on tumor growth *in vitro*. Using quantitative RT-PCR, we found that Notch1 transcripts were undetectable or scarce in 22 medulloblastoma specimens, whereas Notch2 levels were generally higher than those in rapidly growing fetal cerebellum. Colleagues in another laboratory have found Notch1 mRNA levels to be somewhat higher in medulloblastomas than we did.⁶ The cause of this discrepancy is not clear, but the alterations in mRNA level that we detect after Notch overexpression or siRNA knockdown suggest that our primers and probes are measuring the appropriate transcripts.

Notch2 expression correlated with that of Hes1, a well-characterized pathway target. Significantly, expression of Hes1 protein in medulloblastoma specimens was associated with worse clinical outcomes, directly implicating the pathway in the pathobiology of primary human lesions. Additional evidence for the role of Notch2 in human tumors comes from our analysis of gene dosage. *Notch2* gene copy number was increased in 15% of the 40 embryonal tumor specimens examined, and in one PNET, amplification of the *Notch2* locus was associated with mRNA levels 20,000-fold higher than those in fetal cerebellum. Gene amplification, thus, seems to cause Notch2 overexpression in a small subset of human brain tumors. The mechanisms for increased Notch2 expression in tumors without chromosomal alterations, and whether signaling in these tumors is ligand dependent, remain to be determined.

In summary, we demonstrate that, in embryonal brain tumors, Notch1 and Notch2 have opposing effects on growth that parallel their functions in normal development. These strikingly antagonistic changes are highly unusual, and further characterization of the imme-

⁶ J. Olson, personal communication.

diate effectors and downstream targets of Notch1 and Notch2 in these tumors should provide insight into the growth control functions of the Notch pathway. Finally, using RNA interference and inhibition of γ -secretase, we document an ongoing requirement for Notch2 and Hes1 in medulloblastoma growth, suggesting that modulation of Notch signaling may be therapeutically useful in one of the most common malignant solid tumors of childhood.

ACKNOWLEDGMENTS

We thank A. Chaudhry, L. Khaki, S. Karhadkar, and R. Abounader for technical assistance and advice; L. Miele for providing siRNA sequences for Notch1; and T. Sudo for Hes1 antibody. The C651 monoclonal antibody developed by Spyros Artavanis-Tsakonas was obtained from the University of Iowa Developmental Hybridoma Studies Bank, under the auspices of the National Institute of Child Health and Human Development.

REFERENCES

- Kleihues P, Cavenee W. Tumors of the nervous system. Lyon, France: IARC press, 2000.
- Bailey P, Cushing H. Medulloblastoma cerebelli: a common type of mid-cerebellar glioma of childhood. *Arch Neurol Psychiatry* 1925;14:192–224.
- Rorke LB. The cerebellar medulloblastoma and its relationship to primitive neuroectodermal tumors. *J Neuropathol Exp Neurol* 1983;42:1–15.
- Hemmati HD, Nakano I, Lazareff JA, et al. Cancerous stem cells can arise from pediatric brain tumors. *Proc Natl Acad Sci USA* 2003;100:15178–83.
- Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003;63:5821–8.
- Chenn A, Walsh CA. Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science (Wash DC)* 2002;297:365–9.
- Macholn R, Hayashi S, Rutlin M, et al. Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. *Neuron* 2003;39:937–50.
- Wechsler-Reya RJ, Scott MP. Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron* 1999;22:103–14.
- Ohtsuka T, Sakamoto M, Guillemot F, Kagayama R. Roles of the basic helix-loop-helix genes Hes1 and Hes5 in expansion of neural stem cells of the developing brain. *J Biol Chem* 2001;276:30467–74.
- Gaiano N, Fishell G. The role of notch in promoting glial and neural stem cell fates. *Annu Rev Neurosci* 2002;25:471–90.
- Kabos P, Kabosova A, Neuman T. Blocking HES1 expression initiates GABAergic differentiation and induces the expression of p21(CIP1/WAF1) in human neural stem cells. *J Biol Chem* 2002;277:8763–6.
- Gilbertson R. Paediatric embryonic brain tumours. Biological and clinical relevance of molecular genetic abnormalities. *Eur J Cancer* 2002;38:675–85.
- Goodrich LV, Milenkovic L, Higgins KM, Scott MP. Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science (Wash DC)* 1997;277:1109–13.
- Irvin DK, Zurcher SD, Nguyen T, Weinmaster G, Kornblum HI. Expression patterns of Notch1, Notch2, and Notch3 suggest multiple functional roles for the Notch-DSL signaling system during brain development. *J Comp Neurol* 2001;436:167–81.
- Solecki DJ, Liu XL, Tomoda T, Fang Y, Hatten ME. Activated Notch2 signaling inhibits differentiation of cerebellar granule neuron precursors by maintaining proliferation. *Neuron* 2001;31:557–68.
- Tanaka M, Kadokawa Y, Hamada Y, Marunouchi T. Notch2 expression negatively correlates with glial differentiation in the postnatal mouse brain. *J Neurobiol* 1999;41:524–39.
- Stump G, Durrer A, Klein AL, Lutolf S, Suter U, Taylor V. Notch1 and its ligands Delta-like and Jagged are expressed and active in distinct cell populations in the postnatal mouse brain. *Mech Dev* 2002;114:153–9.
- Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science (Wash DC)* 1999;284:770–6.
- Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat Rev Cancer* 2003;3:756–67.
- Gray GE, Mann RS, Mitsiadis E, et al. Human ligands of the Notch receptor. *Am J Pathol.* 1999;154:785–94.
- Allenspach EJ, Maillard I, Aster JC, Pear WS. Notch signaling in cancer. *Cancer Biol Ther* 2002;1:466–76.
- Iso T, Kedes L, Hamamori Y. HES and HERP families: multiple effectors of the Notch signaling pathway. *J Cell Physiol* 2003;194:237–55.
- Nickoloff BJ, Osborne BA, Miele L. Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. *Oncogene* 2003;22:6598–608.
- Ellisen LW, Bird J, West DC, et al. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* 1991;66:649–61.
- Pear WS, Aster JC, Scott ML, et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J Exp Med* 1996;183:2283–91.
- Tonon G, Modi S, Wu L, et al. t(11;19)(q21;p13) translocation in mucoepidermoid carcinoma creates a novel fusion product that disrupts a Notch signaling pathway. *Nat Genet* 2003;33:208–13.
- Rohn JL, Lauring AS, Linenberger ML, Overbaugh J. Transduction of Notch2 in feline leukemia virus-induced thymic lymphoma. *J Virol* 1996;70:8071–80.
- Capobianco AJ, Zagouras P, Blaumueller CM, Artavanis-Tsakonas S, Bishop JM. Neoplastic transformation by truncated alleles of human NOTCH1/TAN1 and NOTCH2. *Mol Cell Biol* 1997;17:6265–73.
- Eberhart CG, Kratz J, Wang Y, et al. Histopathological and molecular prognostic markers in medulloblastoma: c-myc, N-myc, TrkC, and anaplasia. *J Neuropathol Exp Neurol* 2004;63:441–9.
- Zagouras P, Stifani S, Blaumueller CM, Carcangiu ML, Artavanis-Tsakonas S. Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proc Natl Acad Sci USA* 1995;92:6414–8.
- Fan X, Wang Y, Kratz J, et al. hTERT gene amplification and increased mRNA expression in central nervous system embryonal tumors. *Am J Pathol.* 2003;162:1763–9.
- Perry A, Fuller CE, Banerjee R, Brat DJ, Scheithauer BW. Ancillary FISH analysis for 1p and 19q status: preliminary observations in 287 gliomas and oligodendroglioma mimics. *Front Biosci* 2003;8:a1–9.
- Sriuranpong V, Borges MW, Ravi RK, et al. Notch signaling induces cell cycle arrest in small cell lung cancer cells. *Cancer Res* 2001;61:3200–5.
- Abounader R, Ranganathan S, Lal B, et al. Reversion of human glioblastoma malignancy by U1 small nuclear RNA/ribozyme targeting of scatter factor/hepatocyte growth factor and c-met expression. *J Natl Cancer Inst (Bethesda)* 1999;91:1548–56.
- Pomeroy SL, Tamayo P, Gaasenbeek M, et al. Prediction of central nervous system embryonal tumour outcome based on gene expression. *Nature (Lond)* 2002;415:436–42.
- Nicolas M, Wolfer A, Raj K, et al. Notch1 functions as a tumor suppressor in mouse skin. *Nat Genet* 2003;33:416–21.
- Esler WP, Wolfe MS. A portrait of Alzheimer secretases—new features and familiar faces. *Science (Wash DC)* 2001;293:1449–54.
- Mizutani T, Taniguchi Y, Aoki T, Hashimoto N, Honjo T. Conservation of the biochemical mechanisms of signal transduction among mammalian Notch family members. *Proc Natl Acad Sci USA* 2001;98:9026–31.
- Berezovska O, Jack C, McLean P, et al. Aspartate mutations in presenilin and gamma-secretase inhibitors both impair notch1 proteolysis and nuclear translocation with relative preservation of notch1 signaling. *J Neurochem* 2000;75:583–93.
- Lee HJ, Jung KM, Huang YZ, et al. Presenilin-dependent gamma-secretase-like intramembrane cleavage of ErbB4. *J Biol Chem* 2002;277:6318–23.
- Gaiano N, Nye JS, Fishell G. Radial glial identity is promoted by Notch1 signaling in the murine forebrain. *Neuron* 2000;26:395–404.
- Talora C, Sgroi DC, Crum CP, Dotto GP. Specific down-modulation of Notch1 signaling in cervical cancer cells is required for sustained HPV-E6/E7 expression and late steps of malignant transformation. *Genes Dev* 2002;16:2252–63.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Notch1 and *Notch2* Have Opposite Effects on Embryonal Brain Tumor Growth

Xing Fan, Irina Mikolaenko, Ihab Elhassan, et al.

Cancer Res 2004;64:7787-7793.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/64/21/7787>

Cited articles This article cites 40 articles, 16 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/64/21/7787.full#ref-list-1>

Citing articles This article has been cited by 56 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/64/21/7787.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerres.aacrjournals.org/content/64/21/7787>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.