

## SHORT COMMUNICATION

**Notch3 signaling initiates choroid plexus tumor formation**L Dang<sup>1,2,7</sup>, X Fan<sup>3,7</sup>, A Chaudhry<sup>3</sup>, M Wang<sup>4</sup>, N Gaiano<sup>1,2,5,6</sup> and CG Eberhart<sup>3,6</sup>

<sup>1</sup>Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, USA; <sup>2</sup>Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD, USA; <sup>3</sup>Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, USA; <sup>4</sup>Departments of Neurology and Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, USA; <sup>5</sup>Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD, USA and <sup>6</sup>Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD, USA

**Notch3 has been studied in the context of brain development, but whether it plays a role in the formation of brain tumors is unclear. We demonstrate that the introduction of constitutively active Notch3 into periventricular cells of embryonic day 9.5 mice causes the formation of choroid plexus tumors (CPTs). Tumors arose in the fourth ventricles in 83% of animals and were associated with hydrocephalus. They were microscopically highly similar to choroid plexus papillomas in humans, with an ongoing proliferation rate of 4–6%. Signs of Notch pathway activity were also present in human choroid plexus lesions, and receptor mRNA levels in papillomas were elevated over those in non-neoplastic choroid plexus. Notch2 was overexpressed approximately 500-fold in one case, suggesting that the role of this pathway in CPTs may not be specific to Notch3. Our findings indicate that activated Notch3 can function as an oncogene in the developing brain, and link the Notch pathway to human CPT pathogenesis.**

*Oncogene* advance online publication, 26 September 2005; doi:10.1038/sj.onc.1209074

**Keywords:** choroid plexus papilloma; Notch3

The Notch pathway is an important regulator of the developing mammalian nervous system (Gaiano and Fishell, 2002). Notch1, Notch2 and Notch3 transmembrane receptors are all expressed in ventricular zone progenitor cells of the fetal brain (Irvin *et al.*, 2001), and their roles in neural development are beginning to be elucidated. For example, an activated form of Notch1 has been shown to promote progenitor cell identity in the embryonic mouse forebrain (Yoon *et al.*, 2004). Similarly, activated Notch2 inhibits differentiation and promotes proliferation of cerebellar progenitor cells

(Solecki *et al.*, 2001). Consistent with its role in neural progenitor maintenance and proliferation, Notch signaling has been linked to cancer. Notch1 and Notch2 signaling are required for the growth of glial and embryonal brain tumors, respectively (Fan *et al.*, 2004; Purow *et al.*, 2005).

The role of Notch3 in brain development and neoplasia is not well understood. To investigate the function of Notch3 in neural progenitor regulation and neoplasia, we injected a retrovirus expressing the intracellular domain of Notch3 (N3CLE) into mouse forebrain ventricles at embryonic day 9.5 (E9.5), activating Notch3 signaling in proliferating periventricular progenitor cells. Within several weeks of birth, choroid plexus tumors (CPTs) formed in the 4th ventricle of N3CLE-injected mice, demonstrating for the first time that Notch3 can function as an oncogene in the brain. Animals injected with a control retrovirus (CLE) did not develop tumors.

Injected animals killed on E12.5 showed scattered groups of infected cells in the ventricular zone and the developing choroid plexus (data not shown). N3CLE viral infection did not appear to affect fetal viability, as postoperative survival (18 out of 34 injected) was within the normal range for this procedure (LD and NG, unpublished observations). However, of the 18 pups born from three litters injected with the N3CLE retrovirus, 15 (83%) developed enlarged heads within 10–20 days of birth. In many animals this was accompanied by ataxia, with some becoming progressively obtunded within the first month. Other less affected but still abnormal animals survived for several months prior to being killed, or, in one case, dying suddenly.

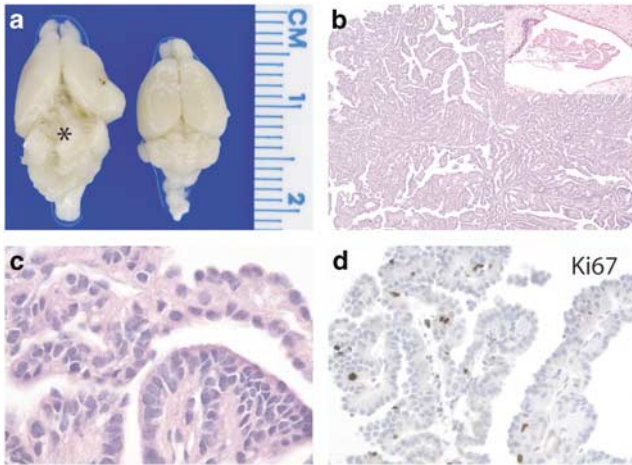
Postnatal animals were killed at ages ranging from P11 to P65. Necropsies revealed extreme enlargement and distortion of posterior fossa structures, along with moderate enlargement and flattening of the cerebral hemispheres (Figure 1a). Coronal sections showed expansion of the ventricular spaces, that is, hydrocephalus. Ill-defined masses of varying size were identified grossly in the fourth ventricle of many severely hydrocephalic animals. None of 15 animals born in two litters injected with CLE developed head enlargement, behavioral abnormalities, or hydrocephalus. Microscopic examination revealed fourth ventricular CPTs

Correspondence: Dr CG Eberhart, Department of Pathology, Johns Hopkins University School of Medicine, 720 Rutland Ave, Ross Building 558, Baltimore, MD 21205, USA.

E-mail: ceberha@jhmi.edu or Dr N Gaiano, Department of Neurology, Institute for Cell Engineering, Johns Hopkins University School of Medicine, 733 N Broadway, BRB 715, Baltimore, MD 21205, USA. E-mail: gaiano@jhmi.edu

<sup>7</sup>These two authors contributed equally to this work

Received 29 April 2005; revised 19 July 2005; accepted 19 July 2005



**Figure 1** CPTs in N3CLE-injected mice. (a) Hydrocephalus in a postnatal day 30 (P30) mouse injected with N3CLE (left), with distortion of the posterior fossa structures (asterisk) and flattening of the cerebral hemispheres. A brain lacking such changes is shown on the right. (b) A large arborizing papilloma from a P30 animal fills the fourth ventricle (original magnification  $\times 20$ ). Normal fourth ventricular choroid plexus of a P30 CLE-injected animal is shown in the inset. (c) Crowding and cytological atypia is seen focally in the nuclei of epithelial cells lining the papilloma (original magnification  $\times 260$ ). (d) Ki67 immunostaining reveals significant ongoing proliferation in a P30 papilloma (original magnification  $\times 100$ ). Preparation of the activated Notch3-CLE virus (N3CLE, amino-acids 1663–2321 of human Notch3), and CLE control virus was performed as previously described (Gaiano *et al.*, 2000). Titres of CLE and N3CLE viruses were approximately  $1 \times 10^8$  cfu/ml. Timed-pregnant CD1 mice (Charles River Laboratories, Raleigh, NC, USA) were injected with virus at E9.5 using ultrasound surgery, and animal tissues prepared by transcardial perfusion with 4% PFA followed by immersion fixation (Gaiano *et al.*, 2000). Ki67 immunostaining was performed as previously described (Fan *et al.*, 2004).

in 13/15 (87%) of mice with hydrocephalus and in 1/3 without hydrocephalus (Figure 1b). Of the two animals with hydrocephalus but no microscopically confirmed tumor, one had an enlarged ‘hyperplastic’ choroid plexus in the left lateral ventricle, and the other had a fourth ventricle tumor mass observed grossly, which was apparently lost during processing. Thus, 15 of 18 animals (83%) infected with N3CLE had grossly or microscopically identified tumors.

CPTs are divided in the current World Health Organization classification system into papillomas and carcinomas (Kleihues and Cavenee, 2000). The murine tumors we generated were histopathologically highly similar to the papillomas seen in humans, with relatively

bland epithelial cells lining papillary fronds. The lesions were generally extremely large, over 30 times the size of the normal fourth ventricular choroid plexus (Figure 1b). Several microscopic features suggest they are neoplastic, and not merely an exuberant growth of normal choroid plexus. Firstly, the cells were frequently crowded or piled up and focally showed significant cytological atypia and molding (Figure 1c). Secondly, as in human papillomas, many regions of the murine tumors lost the superficial ‘hobnail’ configuration characteristic of non-neoplastic choroid plexus (Kleihues and Cavenee, 2000). Finally, as described below, the murine papillomas proliferate at rates similar to those described in human lesions.

We used Ki-67 immunohistochemistry to evaluate the proliferative potential of the tumors (Figure 1d). The mean proliferation index in papillomas from mice killed on P11 was 18%. While the CLE-injected animals also exhibited proliferation in the choroid plexus at this time point, the rate of division was less than half that of the papillomas. By P25–30, the mean proliferation index of the murine choroid plexus papillomas had dropped to 6%. No significant choroid plexus proliferation was detected in CLE-injected animals of this age. The papillomas in animals killed between P60 and P65 continue to proliferate with 4% of cells on average positive for Ki67. In human choroid plexus papillomas, mean Ki67 proliferation indices range from 1.9 to 5.3% (Kleihues and Cavenee, 2000; Rickert *et al.*, 2002).

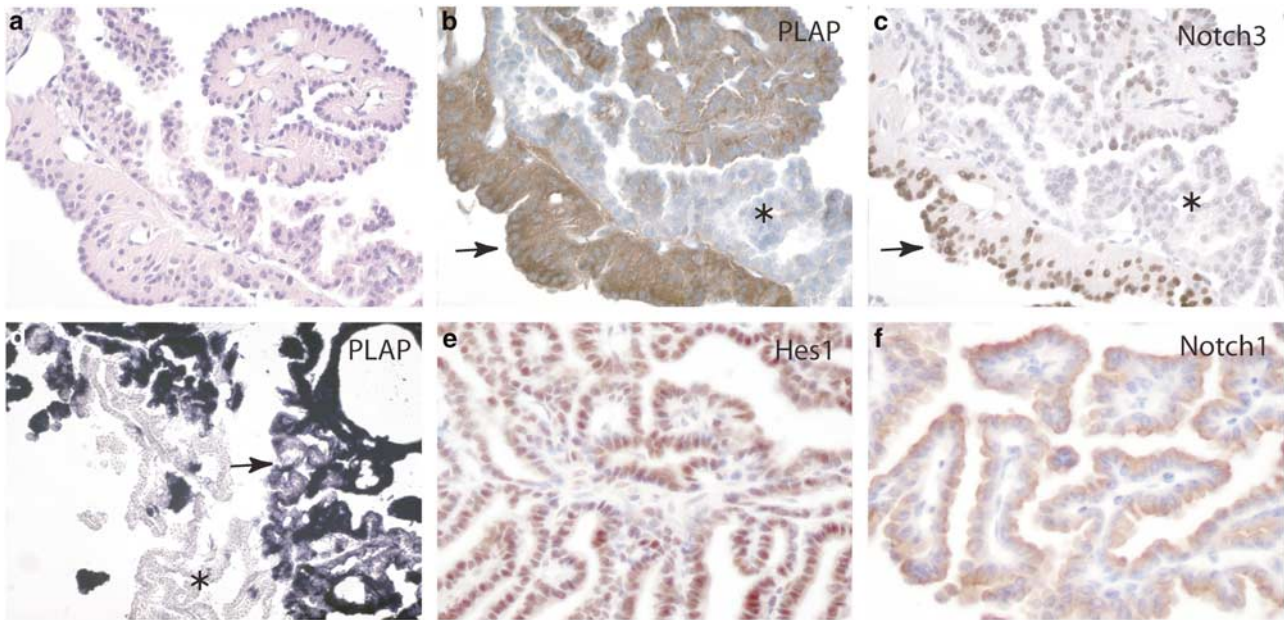
We confirmed introduction of the retrovirus into papilloma cells by immunohistochemistry using antibodies directed against human PLAP encoded by the N3CLE construct (Figure 2b). The majority of papilloma tissue was strongly PLAP immunopositive, while 10–40% of cells were weakly stained or immunonegative. Immunohistochemical analysis of Notch3 expression showed abundant nuclear staining in PLAP-positive cells, but little to none was identified in the nuclei of PLAP negative cells (Figure 2c). To further verify the presence of infected cells within papillomas, we performed histochemical staining, which sensitively detects alkaline phosphatase enzyme function. These experiments identified large regions with strong PLAP activity, but also small regions that lacked PLAP activity (Figure 2d). Cells lacking PLAP or nuclear Notch3 had Ki67 proliferation rates as high as virally infected cells, indicating they were a part of the growing tumor, and not merely entrapped non-neoplastic choroid plexus.

When Notch signaling is activated, the intracellular domain of the receptor moves into the nucleus and acts

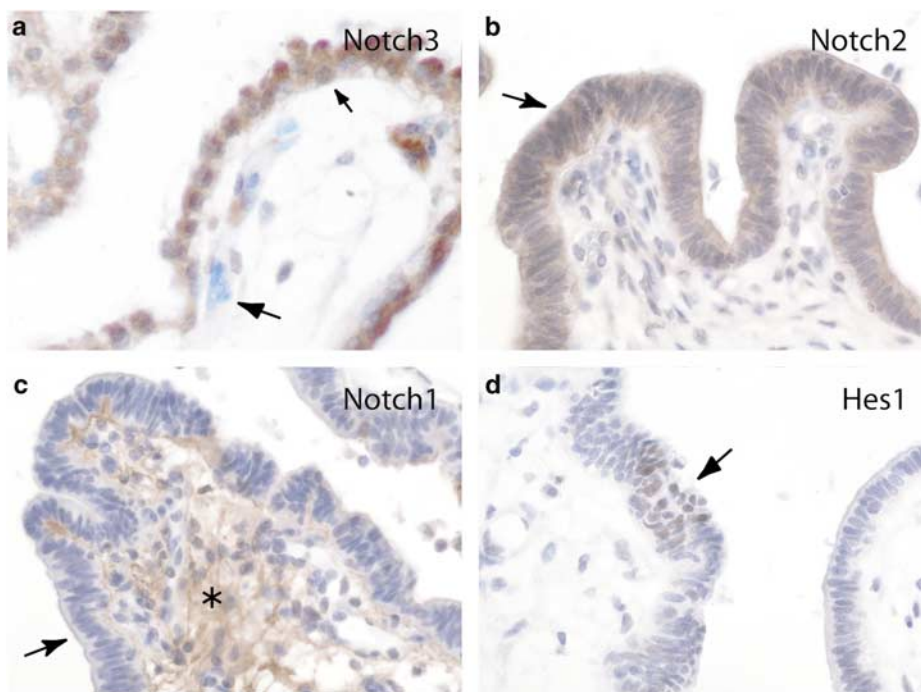
**Figure 3** Expression of Notch pathway proteins in human choroid plexus papillomas. (a) Notch3 stained the papilloma epithelium and some stromal cells. Nuclear Notch3 was seen in tumor cell cytoplasm and nuclei (small arrow), while endothelial nuclei provided an internal negative control (large arrow; original magnification  $\times 200$ ). (b) Epithelial tumor cells (arrow) are strongly positive for Notch2, and most showed some nuclear staining (original magnification  $\times 160$ ). (c) Notch1 protein is highly expressed in stromal papilloma cells (asterisk), while epithelial cells (arrow) are largely negative (original magnification  $\times 160$ ). (d) The Notch pathway target Hes1 is present in the nuclei of small groups of tumor cells (arrow; original magnification  $\times 100$ ). Surgically removed CPTs and postmortem, adult, non-neoplastic choroid plexus samples were from The Johns Hopkins Hospital. These studies were approved by the Internal Review Board of the Johns Hopkins University School of Medicine. Immunohistochemistry was performed as previously described for Notch1, Notch2, Notch3 and Hes1 (Fan *et al.*, 2004).

with cofactors to upregulate expression of target genes such as the bHLH transcription factors Hes and Hey (Iso *et al.*, 2003). We found strong nuclear

Hes1 immunostaining in all murine CPTs examined, suggesting the nuclear Notch3 was functionally active (Figure 2e). Nuclear Hes1 was also present in the



**Figure 2** Retroviral infection and Notch3 expression in murine choroid plexus papillomas. (a) Choroid plexus papilloma from a mouse killed at P30 (original magnification in a-c  $\times 100$ ). (b) Infected cells detected using immunohistochemistry for PLAP protein expressed by the viral construct, with strong staining in some regions (arrow) and weak or absent staining in others (asterisk). (c) Notch3 immunohistochemistry reveals an expression pattern similar to that seen for PLAP, with strong nuclear Notch3 staining in virus-infected cells (arrow) but not in uninfected regions (asterisk). (d) Histochemical stains for alkaline phosphatase activity also reveal both PLAP-positive (arrow) and PLAP-negative (asterisk) regions of papilloma (original magnification  $\times 40$ ). (e) Murine choroid plexus papilloma nuclei were strongly immunopositive for the Notch pathway target Hes1 (original magnification in e, f  $\times 400$ ). (f) The murine lesions contained Notch1 primarily in the cytoplasm, with only a few weakly positive nuclei. Antisera for Notch3 (SC-7424, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and placental alkaline phosphatase (A0268, DAKO, Carpinteria, CA, USA) were used at dilutions of 1:100 and 1:1000, respectively, and Notch1 and Hes1 used as previously described (Fan *et al.*, 2004). Histochemical alkaline phosphatase staining was as previously described (Gaiano *et al.*, 2000).

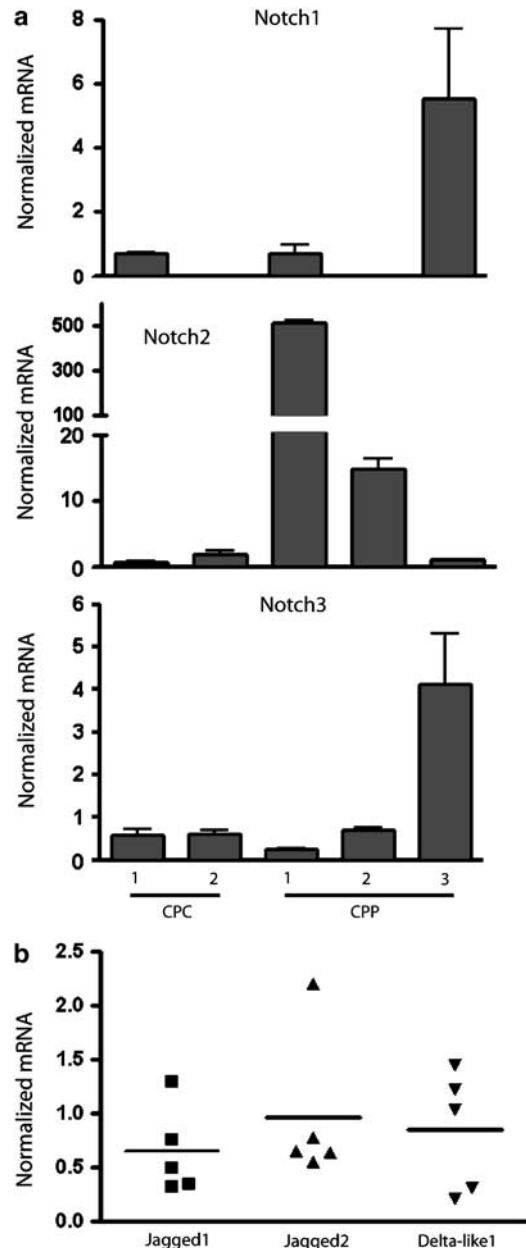


choroid plexus of CLE-injected control animals, but at lower levels and in fewer cells (data not shown). Notch1 and Notch2 receptor proteins were also detected in the murine tumors, but largely in the cytoplasm and apical membranes, suggesting they had not been activated (Figure 2f and data not shown).

We next examined human CPTs to determine if the Notch pathway might be activated. Notch3 protein was found in eight of nine cases examined, including six of seven papillomas and two carcinomas. Immunohistochemical staining was largely cytoplasmic, but in five cases varying amounts of nuclear Notch3 was detected in tumor cells, suggesting the pathway had been activated (Figure 3a). Notch2 immunoreactivity was very strong in the epithelium of all seven CPTs examined, and many cells contained nuclear protein, suggesting that Notch2 was activated (Figure 3b). Notch1 was detected in six of seven CPTs; stromal staining was particularly prominent in some tumors (Figure 3c), while epithelial staining predominated in others. The Notch pathway target Hes1 was present in scattered small groups of epithelial tumor cell nuclei in six of seven cases (Figure 3d).

In order to more precisely quantitate Notch receptor expression, mRNA prepared from three frozen human choroid plexus papillomas, two human choroid plexus carcinomas, and two non-neoplastic choroid plexus samples obtained at autopsy was analysed using quantitative RT-PCR. Notch1 mRNA was detected in three of five CPTs, while Notch2 and Notch3 mRNA were present in all five tumors examined (Figure 4a). Normalization of Notch mRNA levels to those in non-neoplastic human choroid plexus revealed relative overexpression of one or more Notch receptors in all 3 human choroid plexus papillomas. Levels were also higher than those seen in non-neoplastic human cerebellar and cerebral cortex (data not shown). Notch pathway ligands are known to be expressed in the developing and mature choroid plexus, and signaling in human CPTs might therefore be ligand driven (Irvin *et al.*, 2004). Consistent with this possibility, we detected mRNA encoding the Notch ligands Jagged1, Jagged2 and delta-like1 in human CPTs at levels similar to those in non-neoplastic choroid plexus (Figure 4b). The Notch pathway targets Hes1, Hey1 and Hey2, were also expressed in all tumors, but generally at levels similar to or lower than those in non-neoplastic choroid plexus (data not shown). The focal Hes1 protein expression observed immunohistochemically may account for the relatively low levels of Hes1 mRNA detected overall in human lesions.

We show for the first time that Notch3 can act as an initiating oncogene in solid tumors. The papillary tumors arising in our animals were highly similar to those found in humans in terms of their clinical behavior, microscopic appearance and proliferation rates. We also demonstrate that Notch receptors are overexpressed as much as 500-fold in human CPTs, linking Notch pathway abnormalities to the human disease. Notch receptors, particularly Notch2, are present at high levels during the development of fetal



**Figure 4** Notch receptor mRNAs are overexpressed in human papillomas. (a) Notch receptor mRNAs were overexpressed up to 500-fold in three snap-frozen choroid plexus papillomas (CPP) and two choroid plexus carcinomas (CPC). Expression was normalized to that in two non-neoplastic adult choroid plexus samples. Each measurement was performed in triplicate, and bars indicate s.e. (b) Notch ligands were expressed at levels similar to non-neoplastic human choroid plexus, with mean values indicated by horizontal lines. Quantitative RT-PCR was performed as previously described for Notch1, Notch2 and Hes1 (Fan *et al.*, 2004), with all reactions normalized to Actin mRNA levels. Expression of Notch3 and pathway ligands was performed using the same methods and Assays-on-Demand TaqMan probes (Applied Biosystems, Foster City, CA, USA).

choroid plexus (Irvin *et al.*, 2001). CPTs thus seem similar to medulloblastomas in that the pathways controlling normal development are associated with neoplasia when dysregulated. Notch pathway blockade

has been shown to inhibit the *in vitro* growth of embryonal brain tumors (Fan *et al.*, 2004; Hallahan *et al.*, 2004), and gliomas (Purow *et al.*, 2005), and might eventually be considered for aggressive CPTs as well.

Relatively little is known about the genetic alterations in CPTs. One large study found multiple chromosomal alterations in almost all tumors examined, but did not firmly implicate specific genes or pathways (Rickert *et al.*, 2002). CPTs have previously been generated in transgenic mice by expression of SV40 T antigen (Brinster *et al.*, 1984). Subsequent studies showed that Rb inactivation is required for tumor initiation, while p53 predominantly modulates growth of the lesions after they have formed by regulating apoptotic death of tumor cells (Symonds *et al.*, 1994). Our tumors appear similar to those described in this earlier model, but do not become as cytologically abnormal. Another difference is that all fully formed tumors in our mice arose in the fourth ventricle, while those associated with T antigen are often depicted in lateral ventricles.

Tumor tropism for the posterior fossa in our model is not due to restricted Notch pathway activation, as numerous PLAP and Notch3 expressing choroid plexus cells are detected in the lateral ventricles. Indeed, some hyperplasia was seen in these lateral regions, but no large tumor masses formed. This suggests that the cells in the fourth ventricle are either intrinsically different in their neoplastic capacity, or that the local trophic environments are different in these brain regions. It is also interesting that the CPTs described in this report do not homogeneously express Notch3, even in proliferating cells. Thus, activating Notch3 may result in noncell autonomous proliferative signals, or, alternatively, papilloma cells may continue to proliferate in the absence of Notch3 activity following retroviral silencing.

The induction of CPTs by activated Notch3 demonstrates for the first time that this receptor can drive formation of a nonhematopoietic tumor. Bellavia *et al.*

(2000) have previously shown that constitutive Notch3 activation results in T-cell leukemia/lymphoma. Other studies have documented increased expression of Notch3 in lung, renal and pancreatic carcinomas (Dang *et al.*, 2000; Rae *et al.*, 2000; Miyamoto *et al.*, 2003). As discussed in recent reviews, activation of the Notch pathway is being increasingly recognized in human cancers (Nickoloff *et al.*, 2003; Radtke and Raj, 2003; Weng and Aster, 2004). However, the effects of Notch signaling are often exquisitely dependent on cellular context. For example, Notch1 acts as an oncogene in some tissues, and as a tumor-suppressor gene in others (Radtke and Raj, 2003).

In our earlier studies, truncated Notch1 did not cause brain tumors when introduced into E9.5 fetal neural progenitors (Gaiano *et al.*, 2000). However, we previously used only about half of the Notch1 intracellular domain, while here the entire Notch3 intracellular domain was used. Thus, it will be important to determine whether the differing ability of these two constructs to generate CPTs represents a functional difference between Notch1 and Notch3, or is instead a result of the different receptor domains present. Either finding would provide valuable mechanistic insight into how Notch signaling promotes CPTs. It will also be interesting to determine whether the timing of enforced Notch activation influences the location or type of brain tumors that form.

#### Acknowledgements

We thank Dr Tetsuo Sudo for kindly providing Hes1 antibody. This work was supported by grants from the Children's Cancer Foundation and NINDS K08NS43279 to CGE, by a Kimmel Scholar Award to NG, and by NINDS K08NS041342 to MW. CGE, MW and NG are all recipients of Burroughs Wellcome Fund Career Awards in the Biomedical Sciences.

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