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An activated *Notch* suppresses neurogenesis and myogenesis but not gliogenesis in mammalian cells

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SUMMARY

P19 cells, a mouse embryonal carcinoma line, can be induced to differentiate into neurons. After induction, however, only a small subpopulation of cells develop as neurons, suggesting that equipotent cells adopt different cell fates. In invertebrate systems, the *lin-12-Notch* family of genes is thought to control the choice of cell fate. We have therefore asked whether activation of murine *Notch* (*mNotch*) regulates neuronal differentiation in P19 cells. We demonstrate that a dominant gain-of-function mutant of *mNotch* suppresses neurogenesis, as well as myogenesis in P19 cells. Overexpression of the full-length *mNotch* protein also suppresses neurogenesis. In contrast, the differentiation of glia is not affected by an activated *mNotch* homologue. These data indicate that mNotch may play a central role in the choice of cell fate in differentiating cells in culture and suggests that mNotch may play a similar role in the choice of fate in the developing mammalian embryo.

Key words: cell fate, receptor, *Notch*, neurogenesis

INTRODUCTION

P19 cells, a mouse embryonal carcinoma line, resemble pluripotent stem cells which can be induced to form derivatives of each of the three germ layers (Rudnicki and McBurney, 1987). Exposure of P19 cells to retinoic acid results in the maturation of neuroectodermal derivatives, including neurons and glia (Jones-Villeneuve et al., 1982; McBurney et al., 1988), whereas DMSO induces the differentiation of mesodermal derivatives, including cardiac and skeletal muscle (Edwards et al., 1983). If an aggregate of P19 cells is exposed to retinoic acid for six to seven days, about 15% of the cells exhibit neuronal properties. These cells express the neuronal markers N-CAM and neurofilament and elaborate neuronal processes (Levine and Flynn, 1986; McBurney et al., 1988). Moreover, subclones of undifferentiated P19 cells exposed to RA again give rise to cultures with 15% neurons (Berg and McBurney, 1990; Fujii and Hamada, 1993; and studies within). These observations pose the question as to why cells of apparently equivalent potential adopt different cell fates. Why do only a small subpopulation of equipotent cells, rather than all cells within the culture, differentiate into neurons?

It is possible that a subpopulation of cells initiates neuronal differentiation. These cells may then suppress neuronal development in neighboring cells which then adopt alternative cell fates. In this manner, a population of equipotent P19 cells can give rise to multiple cell types in response to a single inducer. This mechanism has been proposed to explain the role of the *lin-12-Notch* family of genes during invertebrate development (Artavanis-Tsakonas and Simpson, 1991; Greenwald and Rubin, 1992). During *Drosophila* neurogenesis, for example, the generation of a subpopulation of neuroblasts from equipotent ectodermal cells is thought to activate *Notch* in neighboring cells. *Notch* signalling will suppress neuronal development, ultimately leading these cells to adopt an alternative epidermal fate (Hoppe and Greenspan, 1990; Heitzler and Simpson, 1991, 1993).

The lin-12-Notch family of proteins are thought to represent cell surface receptors, composed of an extracellular domain with multiple epidermal growth-factor-like motifs, followed by characteristic lin-12-Notch repeats (Greenwald, 1985; Wharton et al., 1985; Kidd et al., 1986; Yochem et al., 1988; Yochem and Greenwald, 1989; Austin and Kimble, 1989). The cytoplasmic domain consists of tandem CDC10/SW16 or ankyrin repeats (Breeden and Nasmyth, 1987; Lux et al., 1990), followed by a C-terminal PEST sequence. Deletions that remove the extracellular domain elicit gain-of-function phenotypes in invertebrates, consistent with constitutive Notch activation (Fortini et al., 1993; Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993). In the *Drosophila* embryo, for example, expression of the cytoplasmic domain of Notch blocks neurogenesis, whereas epidermal development proceeds normally (Struhl et al., 1993).

Notch homologues have been identified in vertebrates (Coffman et al., 1990; Weinmaster et al., 1991; Reaume et al., 1992; Stifani et al., 1992; Weinmaster et al., 1992; Franco del Amo et al., 1993; Kopan and Weintraub, 1993; Lardelli and Lendahl, 1993), but the function of these genes in cellular differentiation remains unclear. Homozygous deletions in mouse *Notch1* result in embryonic lethality (Swiatek et al.,

1994). Although neural structures are apparent in these mutants, the neuroepithelium reveals extensive cell death. Injection of RNA encoding a presumed gain-of-function allele of *Xenopus Notch* into blastomeres results in hypertrophy of mesodermal and neural structures in the *Xenopus* embryo (Coffman et al., 1993). The *tan-1* allele in humans (Ellisen et al., 1991) and the *int-3* (Jhappan et al., 1992; Robbins et al., 1992) allele in mice represent naturally arising rearrangements where a truncated Notch homologue is produced, giving rise to lymphoid and mammary tumors, respectively. These studies demonstrate the significance of Notch signalling in vertebrate development, but do not define the role of Notch in cellular differentiation.

We have therefore examined the consequences of mNotch activation on neural and mesodermal differentiation in P19 cells. We demonstrate that a dominant gain-of-function mutant of *mNotch* suppresses both neurogenesis and myogenesis in P19 cells. Glial development, however, is not suppressed by mNotch activation. These data indicate that mNotch regulates the choice of cell fate in differentiating cells in culture and suggest that mNotch may play a similar role in the developing mouse embryo.

MATERIALS AND METHODS

Cloning and construction of epitope tagged *mNotch* vectors

A 0.65 kb fragment of rat *Notch-1* DNA (Weinmaster et al., 1991) was used to screen a random-primed adult thymus cDNA library (Stratagene) at high stringency. After rescreening the library with partial *mNotch* clones, fragments spanning the entire coding region were obtained. Four overlapping cDNA clones (Fig. 1) were used to assemble a complete *mNotch* cDNA using unique restriction sites.

mNotchIC is composed of an N-terminal myc epitope (Munro and Pelham, 1986) followed by mNotch sequences encoding the entire intracellular domain, initiating at amino acid 1753 (Notch1 numbering (Weinmaster et al., 1991; Franco del Amo et al., 1993)) and terminating in 3′ untranslated sequence. A partial *Sau*I-*Eco*RI fragment fragment of *mNotch* cDNA (amino acids 1760 to C terminus) was subcloned into a pBluescriptSK(–) vector containing two adjacent oligonucleotides: 5′GGGATCCACCATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAGATC3′ encodes a *Bam*HI site, a translational start site and a myc epitope. The second oligonucleotide (5′GATCCACCATGCAGCATGGCCCAGCTCTCCTTCCCTGA3′) encodes *mNotch* amino acids 1753-1759 with a 3′ *Sau*I site complementary end. The resulting myc-tagged amino terminal sequence is MEQKLISEEDLRFTMQHGQLWFPE (underlined sequences are from mNotch). The resulting cDNA was subcloned into pcDNA1-amp (Invitrogen), a CMV promoter-containing expression vector.

mNotch-myc Construction. A full-length *mNotch* cDNA clone with a myc epitope inserted at amino acid 2295 was constructed. An *Eco*RI-*Hin*dIII fragment of *mNotch* cDNA was subcloned into pBluescriptSK(–) with an oligonucleotide (5′AGCTCCGAGCAAAAGCTCATTTCTGAAGAGGACCTA3′) encoding a myc epitope, flanked by *Hin*dIII sites. This was subcloned into pcDNA1amp along with an a *Hin*dIII-*Spe*I fragment of mNotch encloding the mNotch 3′ end. The resulting inserted sequences encode the amino acids SEQKLISEEDLS at the *Hin*dIII site (amino acid 2295).

P19 culture, transfection and differentiation

P19 cells were cultured according to Rudnicki and McBurney (1987). Stable transformants were obtained as follows: RSV-neo and the desired plasmid were cotransfected at a ratio of 1:100 using calcium precipitate-mediated cotransformation (Wigler et al., 1979). Colonies were selected on G418 (500 μg/ml), expanded and frozen in multiple vials. Positive transformants were determined by indirect immunofluorescent staining with appropriate antibody. Early passage (1-3) cells were used for all experiments.

For neurons and glia, differentiation was initiated by treating 1-200,000 cells/ml with 1 μM RA in siliconized glass dishes. Cell aggregates were washed at day 2, then washed and plated on polylysine-treated slides or tissue culture plates at day 4 without retinoic acid. For muscle cells, the same protocol was followed but 0.8% DMSO (for cardiac muscle) or 1.5% DMSO plus 0.01 μM RA (for skeletal muscle) was used to initiate differentiation. At the desired time, cells were harvested or fixed and analyzed as described below.

RNase protection analysis

Total RNA was prepared from P19 cells or transformants, or from mouse tissues using RNAzol (Tel-Test, Inc). RNA (20 μg) was hybridized to a 192-base ^{32}P-labelled antisense *mNotch* RNA probe derived from sequences encoding the intracellular domain, or a control probe derived from the human γ-actin gene. The template for antisense *Notch* RNA was derived from a *Not*I-*Sau*I fragment of *mNotch* cDNA. RNase protection analysis was performed (Zinn et al., 1983) and a protected fragment of 93 bases is predicted from *mNotch* RNA.

Immunoblot analysis of P19 transformants

Cultures of undifferentiated cells were harvested with enzyme-free cell dissociation solution (Specialty Media) and extracts were prepared in 2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris-HCl pH 6.8, EDTA 5 mM with PMSF 20 μM, antipain, soybean trypsin inhibitor, pepstatin and aprotinin (10 μg/ml). Extracts of 10^6 cells were electrophoresed through 8% SDS-PAGE and transferred to nitrocellulose. Monoclonal antibody 9E10 (anti-myc) (Evan et al., 1985) and anti-mouse F(ab)′$_2$-peroxidase (Boehringer) were used to visualize the bands.

Immunofluorescence analysis

Primary antibodies were obtained from the following sources: Mouse monoclonals, Neurofilament 160 kDa, 2H3 (Developmental Hybridoma Studies Bank); Myosin Heavy Chain, MF20 (Bader et al., 1982); CD8, OKT8 (Ortho); SSEA1 (Solter and Knowles, 1978) (480-1 from J. Dodd); Nestin, Rat401 (Hockfield and McKay, 1985); NeuN, A60 (Mullen et al., 1992); myc (Evan et al., 1985), (9E10, S. Morton), GFAP, (ICN Pharmaceuticals); Rabbit polyclonal antiserum anti-β-galactosidase (Cappel). For CD8 immunostaining, cells were stained live followed by fixation and second antibody staining. In all other staining experiments, cells were fixed with 4% paraformaldehyde for 30 minutes prior to staining by standard techniques and detection with anti-mouse or anti-rabbit fluorescent F(ab)′$_2$ conjugated antibodies (Boehringer).

For double-label immunofluorescence where detection of myc antibody and a second mouse monoclonal antibody was required, a biotin-conjugated myc antibody was used. The ascites of monoclonal antibody 9E10 was purified on Protein A resin, conjugated using NHS-XX-Biotin (Pierce) and detected in staining experiments with avidin-Texas red or FITC (Neutralite, Molecular Probes). Sequential staining was performed with first primary monoclonal, anti-mouse secondary antibody followed by biotinyl-myc antibody and fluorescent avidin. Purified IgG$_1$ (10 μg/ml) was used to block cross-reactivity of anti-mouse secondary antibody.

Single cell analysis and statistical methods

For cell counting experiments, P19 transfectant cell lines were induced to differentiate (Rudnicki and McBurney, 1987), dissociated, plated on polylysine-coated glass slides (LabTek) at various densities and fixed with paraformaldehyde at 6-7 days for neurons or 12-14 days for glia. Cells were stained with appropriate antibodies and

analyzed using a Zeiss Axioskop fluorescence microscope. A cell was scored as a neuron if there was visible staining with neurofilament antibody along with neuronal processes, glia for presence of GFAP staining and astrocytic morphology, and positive for the transfected gene myc, β-galactosidase or CD8 with appropriate antibody 9E10, anti-β-gal, or OKT8 respectively. Cell counts are expressed as a percentage of total cells or nuclei. Total cells were determined by counting nuclei stained with Hoechst 33342 or by phase-contrast microscopy. Three different control transformants were analyzed and the results presented for all lines represent the mean of two or more experiments where 400-1000 cells were counted.

To determine the presence of the transfected gene in neurons or glia, cultures were double immunofluorescence stained, as described above, for neurofilament, or GFAP and the appropriate marker with FITC- and Texas Red-conjugated secondary antibodies, respectively. Analysis was performed by counting 200-400 neurons or glia, scoring each one for the presence or absence of transfected gene immunofluorescence. In control experiments, myc or β-gal-positive cells were scored for the presence of neuronal or glial antigens and yielded essentially equivalent results. The strategy of scoring neurons or glia for the transfected gene was used because neurons and glia may be reliably identified even when they are present at low frequencies in a culture.

RESULTS

Cloning and expression of *mNotch*

In initial experiments, we isolated murine *Notch* cDNA clones to examine the expression of *mNotch* RNA in differentiating P19 cells. A full-length mouse homolog of the rat *Notch1* cDNA was obtained by screening a mouse thymus cDNA library with probes derived from rat (Weinmaster et al., 1991). A full-length *mNotch* cDNA was assembled from overlapping cDNA fragments (Fig. 1A). A fragment of the *mNotch* cDNA was transcribed in vitro and used in RNase protection analyses to determine whether *mNotch* RNA is expressed in P19 cells and whether the levels of *mNotch* RNA change upon exposure to RA (Franco del Amo et al., 1992). *mNotch* RNA is present at low levels prior to exposure to inducer (Fig. 2). A 3- to 5-fold induction is observed after 3 days in RA, and the levels continue to rise to a plateau 3 days after initiation of retinoic acid treatment. RNA blots hybridized with an mNotch probe reveal a single hybridizing band of 9.5 kb from thymus, brain and differentiated P19 cells (not shown). Thus, expression of *mNotch* RNA is induced at a time when neuroblasts begin to differentiate.

mNotch activation suppresses neurogenesis

If differential *mNotch* activity is responsible for the generation of alternative cell fates in P19 cells, we might expect that suppression of *mNotch* activity would permit neurogenesis, whereas persistent *mNotch* activity should suppress neurogenesis. Activation of *Notch* is thought to require the interaction with its ligand, *Delta* (Vaessin et al., 1987; Kopczynski et al., 1988). We therefore attempted two means to activate *mNotch* in the mouse P19 cells. First, a dominant gain-of-function mutant of *mNotch* was constructed and expressed in differentiating clones of P19 cells. Truncated derivatives of *Notch*, consisting of only sequences encoding the cytoplasmic

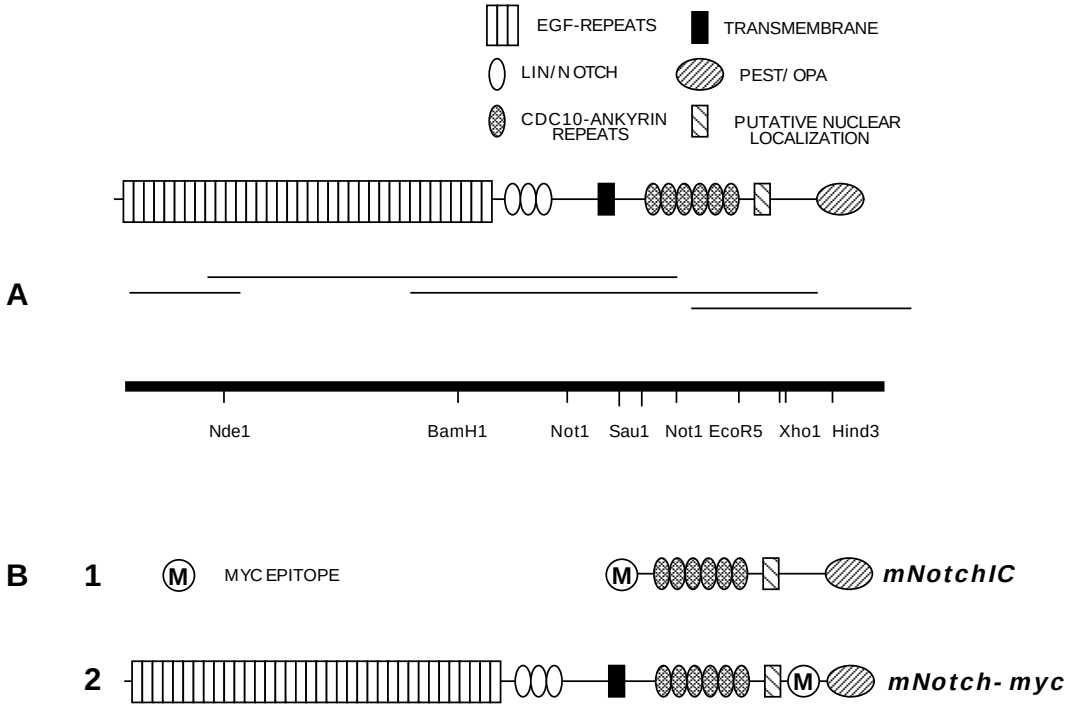

Fig. 1. Isolation of *mNotch* cDNA and the construction of epitope-tagged mutants. (A) Isolation of *mNotch* cDNA. Four overlapping cDNA clones from mouse thymus cDNA were obtained which span the entire coding region of *mNotch*. Uniques restriction sites as shown were used to assemble a complete *mNotch* cDNA. (B) Construct of epitope-tagged *mNotch* mutants (**1)** *mNotchIC.* The sequences include a fragment of *mNotch* encoding the entire cytoplasmic domain with the myc epitope (MEQKLISEEDL) tag at its N terminus. (**2)** *mNotch-myc.* A full-length clone of *mNotch* cDNA was myc-epitope tagged near its C terminus.

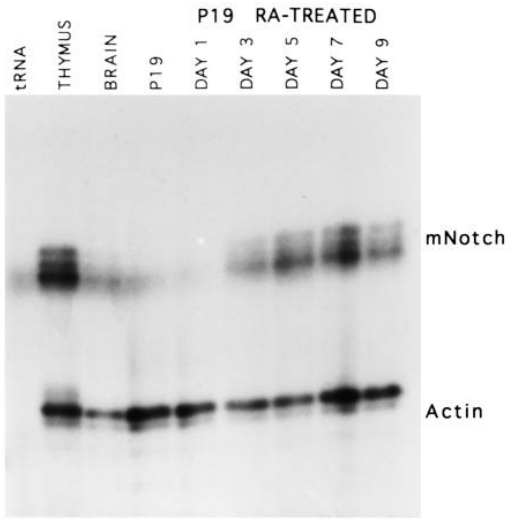

Fig. 2. Expression of *mNotch* RNA in differentiating P19 cells. RNase protection analysis was performed using a 192 bp [^{32}P]RNA probe to detect *mNotch*. This probe was hybridized to 20 μg RNA from adult mouse thymus, brain and P19 cells (prior to and during RA-induced differentiation). The predicted RNA species protected by *mNotchI* RNA is 93 bp. Differentiated P19 cell RNA induces a triplet species at 93 bp, indicating that P19 cells induce the *mNotch* gene. A probe derived from the γ-actin gene was used as a control for added RNA.

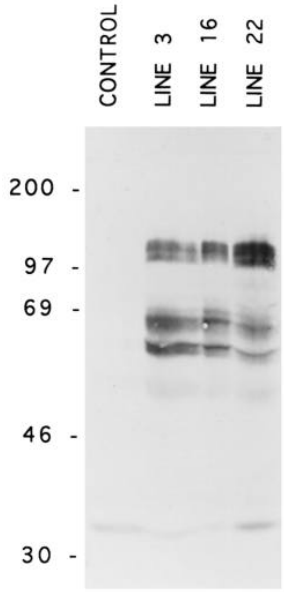

Fig. 3. Immunoblot analysis of P19 *mNotchIC* transformants. Undifferentiated P19 lines transfected by control plasmid (CONTROL) or *mNotchIC* (lines 3, 16 and 22) were obtained (see Methods) and analyzed by immunoblot using antibody to myc epitope (Evan et al., 1985). *mNotchIC*-transfected lines express the predicted sized species at M_r 100-110K as a doublet as well as multiple 60-70K species.

domain, exhibit a dominant gain-of-function phenotype in both *Drosophila* and *C. elegans* (Coffman et al., 1993; Fortini et al., 1993; Lieber et al., 1993; Rebay et al., 1993; Roehl and Kimble, 1993; Struhl et al., 1993). A plasmid encoding the cytoplasmic domain of the protein mNotch was constructed, tagged with a myc epitope (Munro and Pelham, 1986) and placed under the control of the CMV immediate early enhancer and promoter (*mNotchIC*, 'intracellular', Fig. 1B).

Undifferentiated P19 cells were co-transformed with *mNotchIC* DNA, along with a plasmid encoding neomycin-resistance marker (neo^r) to allow for the selection of cotransformants (Wigler et al., 1979). In control experiments, P19 cells were cotransformed with plasmid DNA alone or plasmids encoding a nuclear localized β-galactosidase gene and the human CD8 gene (Littman et al., 1985). Neomycin-resistant colonies expressing mNotchIC were identified by immunoreactivity with anti-myc antibodies (Evan et al., 1985). Interestingly, in cells expressing mNotchIC, myc immunoreactivity is largely restricted to the nucleus although the functional significance of nuclear localization remains obscure in the P19 cell. However, nuclear localization of mNotchIC appears to be important for its inhibition of myogenesis in fibroblasts (Kopan et al., 1994).

Representative neo^r colonies were then analyzed by immunoblot with anti-myc antibodies (Fig. 3). Transformants that were positive by immunocytochemistry express an immunoreactive 100-110×10^3 M_r doublet which approximates the predicted size for the *mNotchIC* protein product (M_r=100,000). Multiple bands of lower intensity at 65×10^3 M_r are also apparent in transformed clones. These species may arise from endogenous proteolysis or from experimental processing. Both the 110 and 65×10^3 M_r bands are absent from control neo^r colonies.

We next examined the efficiency of neural differentiation in control P19 cells and P19 cells expressing mNotchIC. Transformed clones were exposed to retinoic acid as aggregates, dissociated at day 4 and scored at day 6-7 for neuronal differentiation with antibodies directed against neurofilament (Fig. 4; Table 1). In control cultures, and cultures transformed with CD8 or β-galactosidase genes, 12-17% of the cells differentiate into neurons (Table 1). The frequency of neurons in clones expressing mNotchIC is significantly reduced and varies from 3.5-8.3%. The effect of *Notch* activation is far more dramatic than these values would suggest since the expression of

Table 1. Neuronal differentiation of P19 cells expressing *mNotchIC*

Cell line	Positive cells (% total cells)	Neurons (% total cells)	Positive neurons (% total neurons)
Control	–	14.2	–
mNotchIC			
line 9	18.2	8.3	0
line 22	38.6	6.2	0
line16	79.2	3.5	0
mNotch-myc			
line 1	17	13.3	2.4
line 2	9	13.2	1.4
CD8			
line 1	17	15.7	33
line 2	29	17.2	26
nβ-gal	17	nd	35
	7.1	12.4	16.2

P19 transformants were induced with RA and cultured for 6-7 days. Dissociated cultures were double-label immunostained for the transfected gene (*myc*, *CD8* or *β-galactosidase*) and for neurofilament. Positive cells represent the % of cells that express the tranfected gene. Neurons represent the % of cells that express neurofilament and exhibit neuritic processes. Positive neurons represent the % of neurons that express the transfected gene (*myc*, *CD8* or *β-gal*).

exogenous genes in P19 cells is heterogeneous and mNotchIC expression is only observed in a subpopulation of the transformed cells. For example, three transformed lines were obtained in which 79%, 38%, and 18% of the cells expressed the *mNotchIC* gene. The frequency of neurons in these clones is inversely correlated with the frequency of cells expressing the mNotchIC. A clone in which 79% of the cells express mNotchIC reveals only 3.5% neurons, whereas clones with 18% mNotchIC expression exhibit 8% neurons. It is possible that some cells expressing mNotchIC do not reveal visible immunofluorescence. This might explain why P19 lines expressing only 18% positive cells reduce the frequency of neurons about 40%. Most importantly, upon analysis of single cells, we have never observed a neuron that expresses mNotchIC in the 12/12 transformed clones that we have generated. No effect on neuronal differentation is observed in cells expressing the CD8 or β-galactosidase genes. These results provide clear evidence that the expression of the intracellular domain of mNotch (mNotchIC) suppresses neurogenesis.

Activated *mNotch* suppresses myogenesis

We also examined the consequences of mNotchIC on myogenesis in the P19. Notch function is not restricted to neurogenesis in invertebrates (Poulson, 1940; Shellenbarger and Mohler, 1978; Lehmann et al., 1983; Cagan and Ready, 1989); genetic analysis reveals that *lin-12* and *Notch* play significant roles in several cell fate decisions during development, including myogenesis (Corbin et al., 1991). When control P19 cells are aggregated and treated with DMSO and retinoic acid, a subpopulation of cells differentiate into skeletal myocytes which ultimately form syncytia or myotubes (Fig. 5A) (Edwards et al., 1983). However, when P19 cell lines expressing mNotchIC are subjected to the same treatment, few if any myocytes are found in the resulting aggregates (Fig. 5B) and no syncytia formation is observed. When control P19 lines are induced with DMSO alone, differentiation of cardiac myocytes is observed (Fig. 5C). These cells express myosin heavy chain (MHC) and represent up to 20% of the culture after 7 days. However, when P19 lines expressing mNotchIC are treated with DMSO, only rare cells expressing MHC are observed. Moreover, double label immunofluorescence indicates that the rare myocytes present in these lines do not express mNotchIC (Fig. 5D). Thus, expression of the mNotchIC in P19 cells suppresses DMSO-induced myogenesis. In an accompanying paper, we further demonstrate that gain-of-function mutants of *mNotch* suppress myogenesis in the vertebrate embryo as well as in differentiating cell culture systems (Kopan et al., 1994).

Overexpression of mNotch supresses neurogenesis

We next examined the consequences of overexpression of full-length mouse Notch on neuronal differentiation in P19 cells (Fig. 3C and Table 1). In *Drosophila*, mutants that contain two or three times the dosage of wild-type *Notch* show only subtle wing abnormalities, but are otherwise phenotypically normal. Mosaics have been generated in which patches of cells containing an extra copy of the *Notch* gene are adjacent to cells containing the wild-type number of *Notch* genes (Heitzler and Simpson, 1991). At this boundary, neurons only rarely arise from cells bearing the higher gene dosage of *Notch*. Rather, the choice of the neuronal fate is restricted to cells with the wild-type dosage of *Notch*. Therefore, we might expect that, in a mix of P19 cells expressing different levels of *Notch*, cells expressing the highest levels of *Notch* would show the lowest frequency of neurons.

Full-length *mNotch* cDNA was epitope tagged near its C terminus and introduced into P19 cells (*mNotch-myc*, Fig. 1B). Myc immunoreactivity was then used to identify transformants expressing the *mNotch-myc* protein. Analysis of RNA in these transformants indicates that the transfected gene is transcribed as much as 10-fold higher than the endogenous *mNotch* (data not shown). In two clones identified, only a small percentage of the cells (17% and 9%) express the *mNotch-myc* protein (Table 1). This heterogeneity of mNotch-myc expression provides a cell culture equivalent of a mosaic: cells with different dosages of *mNotch* are adjacent to one another, allowing us to examine the fate of cells bearing different dosage of *mNotch*. Upon differentiation of the transformed lines, there is no overall reduction in the frequency of neurons (Fig. 4D; Table 1). However, the percentage of neurons expressing mNotch-myc is 2.4% and 1.4% in the two cells lines, values significantly lower than the percentage of cells which express mNotch-myc (9 and 17%). In contrast, differentiation of control cell lines expressing CD8 or β-galactosidase gave rise to neurons that were positive for CD8 or β-galactosidase at frequencies approximating the frequency of positive cells (Table 1). Thus, in a population of cells expressing different levels of mNotch, the overexpressing cells have a lower probability of differentiating into neurons. Increased *mNotch* activity in these cells may be triggered by a ligand for *mNotch* expressed by P19 cells. Alternatively, expression of high levels of mNotch-myc may result in constitutive *mNotch* activity independent of ligand.

Time of action of *Notch* in P19 differentiation

Differentiation of the P19 cell line involves the ordered appearance and disappearance of cellular antigens (Jones-Villeneuve et al., 1983; McBurney et al., 1988; Berg and McBurney, 1990), allowing us to determine when in the course of differentiation of P19 cells *mNotch* may function (Table 2). Undifferentiated P19 cells express high levels of stage-specific embryonic antigen (SSEA1), a glycolipid antigen found predominantly on preimplantation embryos in the inner cell mass and primitive ectoderm (Solter and Knowles, 1978). After 72 hours of exposure to RA, SSEA1 expression on P19 cells is dramatically reduced. After 4 days, all cells within the culture express high levels of the intermediate filament protein, Nestin (Lendahl et al., 1990). At this time, a subpopulation of cells undergoes neuronal differentiation and diminishes Nestin expression. These cells (neurons) make neuronal processes, express neurofilament and the NeuN antigen, a nuclear protein observed in adult neurons (Mullen et al., 1992). Similarly, in embryonic development, all cells of the inner cell mass express SSEA1 (Solter and Knowles, 1978). This antigen is then diminished in the neural plate and neural tube, and reappears later in only selected neuronal cell types (Yamada et al., 1991; Oudega et al., 1992). Nestin expression is a characteristic of proliferating neuroepithelial precursors and myotomal cells (Hockfield and McKay, 1985; Frederiksen and McKay, 1988). Nestin expression is extinguished upon maturation of neural precursors, which express neurofilament and NeuN as markers

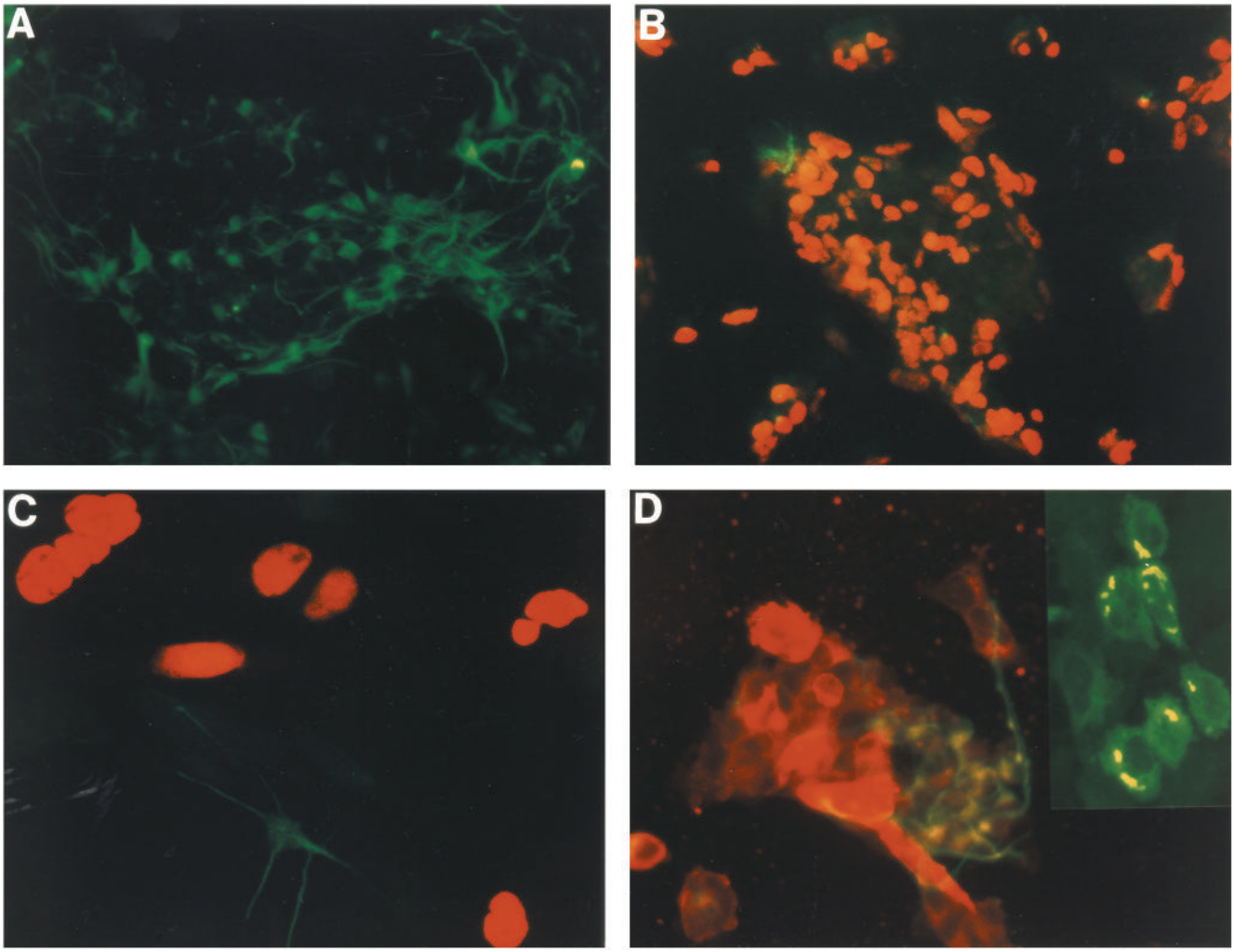

Fig. 4. Analysis of neurogenesis in P19 cells expressing mNotchIC and mNotch-myc. P19 transformants were differentiated and analyzed at day 6-7 by indirect double-label immunofluorescence staining (See Methods). (A) Aggregate of P19 cells transformed with a control plasmid (line 6) was stained with anti-neurofilament and shows many neurons as defined as neurofilament-positive cells with processes. (B) Aggregate of P19 *mNotchIC* (line 3) double stained with anti-neurofilament (green) and anti-myc (red) showing only rare neurons and many myc-positive cells per aggregate. (C) P19 *mNotchIC* (line 16) dissociated after differentiation and double stained with anti-neurofilament (green) and anti-myc (red) for counting. Note the nuclear localization of myc immunofluorescence and the absence of neurofilament-positive cells staining for myc. (D) Aggregate of P19 *mNotch-myc* (line 12) double stained for myc (red) and neurofilament (green). Note the abundant neurons arising primarily in myc-negative cells. Neurons appear yellow because of underlying red cells. INSET: *mNotch-myc* transfectant (line 12) stained for myc (green). Note the large intracellular vesicles.

of mature neurons. Thus, the ordered appearance of antigens in P19 cells is reminiscent of the pattern of antigen expression during embryogenesis and allows us to determine at which stage mNotch may be regulating neuronal differentiation.

In P19 cells expressing mNotchIC, all cells express SSEA-1 antigen prior to differentiation (Table 2). These cells extinguish SSEA-1 expression after exposure to retinoic acid and subsequently express the intermediate filament, Nestin. Thus, the initial events in neural differentiation are virtually identical in control and mNotchIC-expressing cells. mNotchIC-expressing cells differ from control P19 cells only late in development where induction of the more mature neuronal markers Neu-N and neurofilament are not observed. This suggests that RA induces a neuroepithelial pathway of differentiation even in the presence of mNotchIC expression. mNotchIC inhibits the emergence of neurons from a Nestin-positive population of neuroepithlial precursor cells.

Activated *mNotch* does not suppress the generation of glial cells

P19 cells exposed to RA give rise to glia after 12 days in culture

Fig. 6. Glial cell differentiation is not reduced by mNotchIC expression. P19 control transformants (A,C) and mNotchIC-expressing lines (B,D) were induced with RA and cultured for 12-14 days (see Methods). Glial cells were identified with anti-GFAP (A,B) and neurons with anti-neurofilament (C,D). Note the presence and increased intensity of GFAP staining in mNotchIC lines (B) versus control lines (A), and the absence of neurons in mNotchIC transformants (D) compared to control (C) at 12 days.

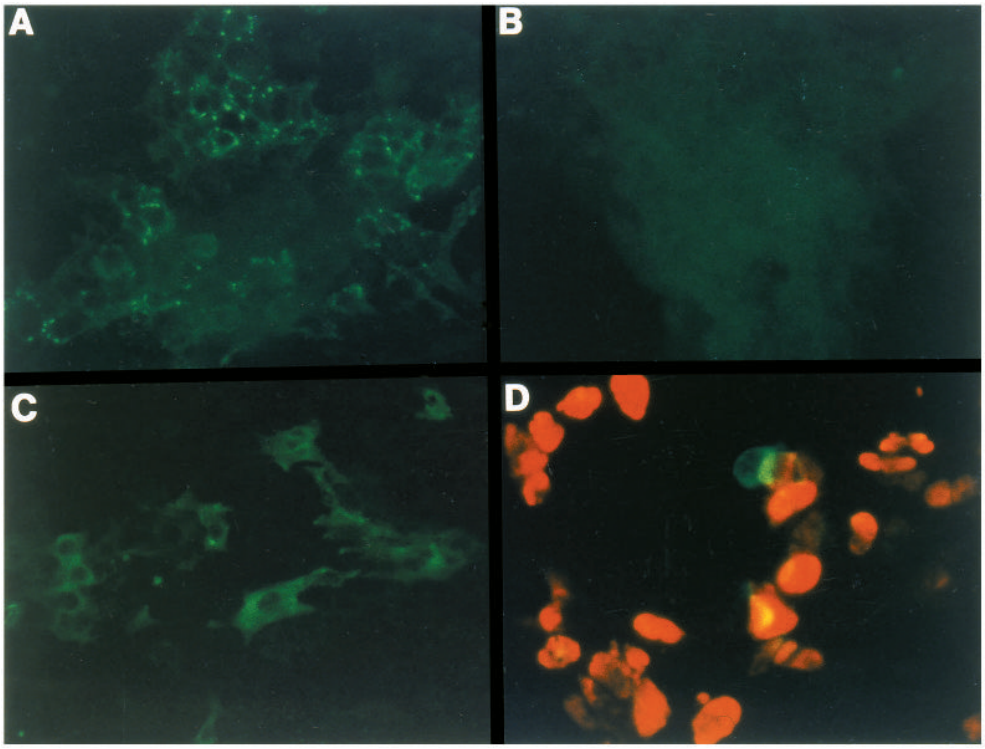

Fig. 5. Inhibition of myogenesis in P19 embryonal carcinoma by mNotchIC. P19 transformants were induced to differentiate as skeletal muscle (A,B) with DMSO (1.5%) and RA (10 nM) or cardiac muscle; (C,D) with DMSO 0.8%. Myogenesis in a control line (line 6) (A,C) was compared to P19 cells expressing mNotchIC (line 3) (B,D) by detection of myosin heavy chain (MHC) immunofluorescence and FITC-conjugated secondary antibody. Note the marked dimunition of MHC staining in P19 *mNotchIC* cells (B) compared to control (A). (D) Cardiac muscle differentiation of a *mNotchIC* line. Cells were dissociated and double stained with myc (Texas Red) and MHC (FITC). Note the absence of myc-positive myocytes.

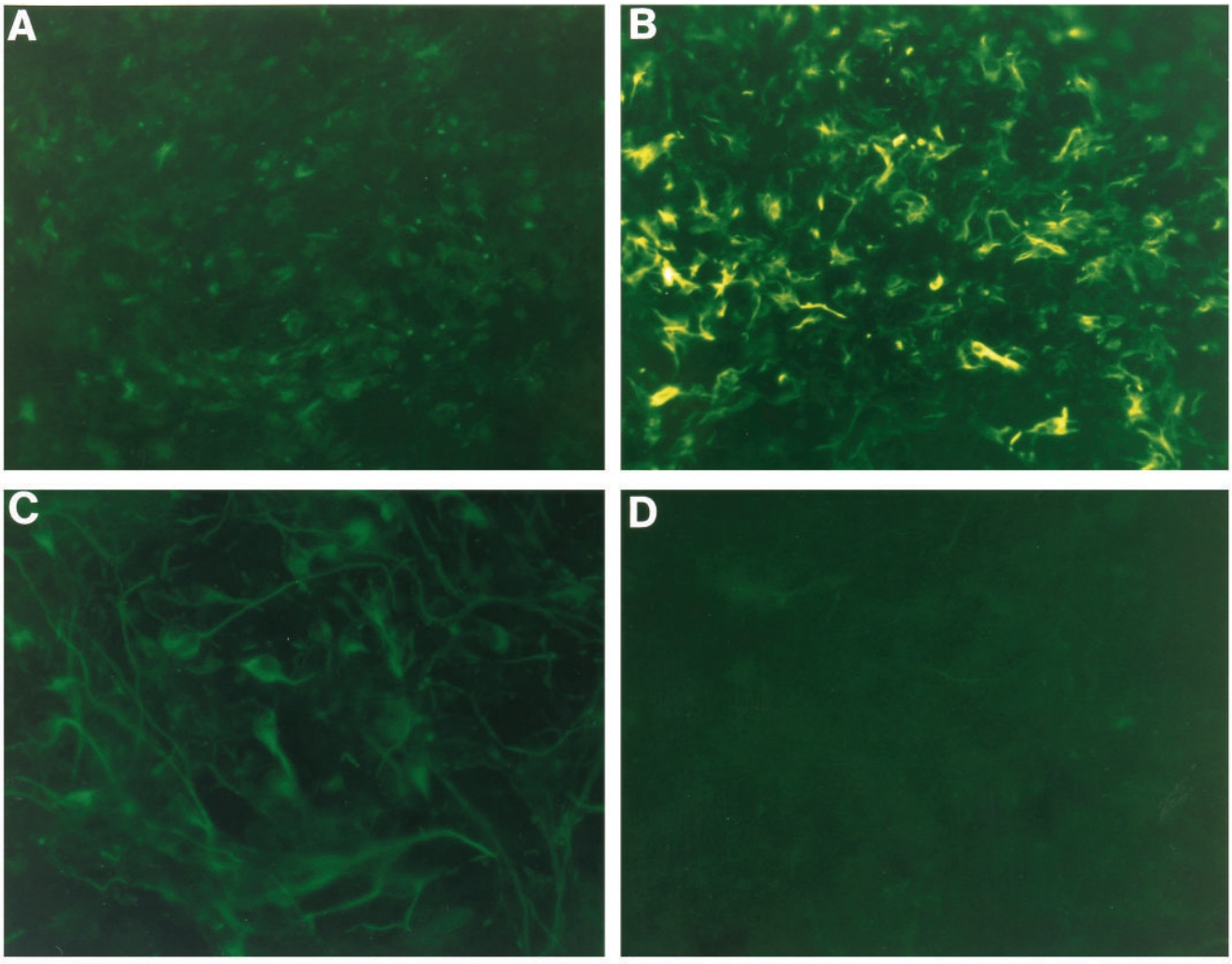

Table 2. Time course of differentiation in P19 cells expressing mNotchIC

	Pluripotent SSEA-1		Neuroepith. Nestin		Neuronal Neurofilam.		Neuronal NeuN	
	wt	mNIC	wt	mNIC	wt	mNIC	wt	mNIC
Day 0	+++	+++	–	–	+/–	–	–	–
Day 3	+/–	+/–	–	+/–	+ *	nd	–	nd
Day 4	+/–	+/–	+/–	+	++	–	+/–	nd
Day 5	+/–	+/–	+	+	+++	nd	+ *	nd
Day 6	+/–	+/–	+	+	+++	nd	+*	nd
Day 7	+/–	+/–	+	+	+++	–	++*	–

Control (wt) or *mNotchIC* (mNIC) P19 transformants were induced to differentiate (See Methods), and cells were dissociated, plated on glass slides,and fixed on the day indicated. Staining by the indicated monoclonal antibody was scored as follows: (–) no visible fluorescence, (+/–) weak staining or rare cells, (+) easily visible fluorescence, (++) strong fluorescence, (+++) bright fluorescence on positive cells. (nd, not done). Two different cell lines (lines 3 and 16) expressing >80% *mNotchIC*-positive cells were analyzed with identical results.

*Note that SSEA1 and nestin are expressed in all cells of the culture early in development, while neurofilament and NeuN-positive cells represent only a subpopulation of the culture.

Table 3. Differentiation of glia in P19 cells expressing *mNotchIC*

Cell line	Myc-positive (% total)	Glia (GFAP+) (% total ± s.e.m.)	Myc-positive. glia (% glia)
Control	–	15.2±1.7	–
myc-mNotchIC	27.1	20.2±1.7	26.7

P19 transformants were differentiated and maintained in culture until day 12-14. Total cells were counted and scored for staining with myc antibody (myc-positive) or anti-GFAP antibody (glia). These are expressed as a percentage of total cell nuclei. Double staining was performed to determine the % of glial cells expressing the transfected *mNotchIC* myc epitope.

(Rudnicki and McBurney, 1987). We therefore asked whether the inhibitory effect of mNotchIC on neurogenesis is also observed for the differentiation of glia. When P19 cells or P19 cells transfected with control plasmids are exposed to RA, 15% of the cells exhibit an astrocytic morphology and express glial fibrillary acid protein (GFAP) (Fig. 6 and Table 3). A slightly higher frequency of glia (20%) are observed in P19 cells expressing mNotchIC. However, the frequency of glia in the population of mNotchIC+ cells is the same population as in total population of P19 cells. Glial cells in cultures transformed with mNotchIC appear to express increased levels of GFAP as determined by immunofluorescence (Fig. 6). The enhanced expression of GFAP is present within mNotchIC+ and mNotchIC– glia, suggesting that the apparent increased level of GFAP reflects clonal variation, rather than an effect of mNotchIC on gliogenesis. Thus the expression of mNotchIC results in a striking suppression of neurogenesis, but does not affect the differentiation of glia in retinoic acid-treated P19 cells.

DISCUSSION

These results suggest that *mNotch* plays an important role in the differentiation of mammalian embryonal carcinoma cells into neurons and myocytes. When an aggregate of P19 cells is exposed to retinoic acid, subpopulations of cells mature into neurons and glia. Expression of a gain-of-function mutant of *mNotch* suppresses neurogenesis, but does not affect the differentiation of glia. One important implication of these data is that, during the course of development in culture, signalling by the wild-type *mNotch* may direct cells to adopt a non-neural fate. Experiments in invertebrates indicates that the neural fate is responsible for the activation of *Notch* in neighboring cells which then adopt alternative non-neural fates (Artavanis-Tsakonas and Simpson, 1991; Greenwald and Rubin, 1992). Our data suggest that this mechanism may be operative in P19 cells to assure that only a subpopulation of equipotent cells may differentiate into neurons.

The ordered appearance of distinct cell types allows us to define further the role of *mNotch* during P19 cell development. Upon exposure to RA, all cells in culture develop into apparently neuroepithelial precursors. This differentiation event is not affected by the expression of a gain-of-function allele of *mNotch*. A subpopulation of these precursors undergoes neuronal differentiation. mNotchIC inhibits the generation of neurons, but does not suppress glial development. These data are consistent with a model of P19 differentiation in which a subpopulation of neuroepithelial precursors initiate neurogenesis. This, in turn, activates *Notch* signalling in neighboring cells which then adopt alternative non-neuronal fates which are perhaps reflected by glial cells.

How does *Notch* activation direct cells to adopt alternative cell fates? Studies of *lin12-Notch* function in invertebrates have led to two models. Neuronal differentiation is thought to result in *Notch* activation in neighboring cells. In one model, *Notch* activation is thought directly to activate a non-neural pathway of differentiation (Greenwald and Rubin, 1992). Alternatively, *Notch* activation may not direct differentiation, but could maintain cells as uncommitted precursors which will respond to inductive signals present later in developmental time (Artavanis-Tsakonas and Simpson, 1991; Coffman et al., 1993). For example, as neuroblasts segregate from the ventral ectoderm of the *Drosophila* embryo, *Notch* activation is thought to be elicited in the underlying ectodermal cells. *Notch* activation is required for these neuroectodermal cells to subsequently generate the epidermis (Hoppe and Greenspan, 1990). It is not clear however whether *Notch* signalling directly elicits epidermal development or whether Notch suppresses neurogenesis such that these cells retain their competence and become epidermis only in response to subsequent inductive signals.

Support for the latter model derives from studies of *lin12/Notch* function in vertebrate and invertebrate development. In *Xenopus*, for example, injection of RNA encoding a presumed gain-of-function allele of *Xenopus Notch*, *Xotch*ΔE, into a single blastomere results in hypertrophy of neural and mesodermal tissues only on the side of the injection (Coffman et al., 1993). The authors suggest that constitutive *Notch* signalling early in embryogenesis results in the accumulation of excess numbers of uncommitted cells. Later in development, the levels of the mutant *Notch* protein decline (as a consequence of degradation of the injected RNA), allowing the differentiation of an expanded population of precursors. These studies, however, attempt to explain the role of *Notch* early in embryogenesis, but only examine the consequences of transient expression of an activated *Notch* long after expression is thought to be extinguished. Moreover, in a companion study, injection of *mNotchIC* RNA into the *Xenopus* embryo results

in the suppression of myogenesis and neurogenesis (Kopan et al., 1994). These conflicting observations could reflect differences in the activity of the mutant *Notch* RNAs injected, or differences in the half-life of the injected RNAs during embryogenesis. These differences notwithstanding, the data of Kopan et al. (1994) suggest that *Notch* activation prevents differentiation of uncommitted precursors into nerve cells and muscles.

Embryonal carcinoma cells provide a simpler cell culture system to examine the consequences of *Notch* activation in individual cell fates. Our data argues that *Notch* plays a central role in the generation of mature neurons and muscle cells in differentiating P19 cells. The suggestion that *mNotch* functions to regulate neurogenesis must be tempered by the fact that our conclusions derive largely from studies with a truncated mutant of *mNotch*. It is possible that *mNotchIC* elicits neomorphic phenotypes unrelated to the role of the wild-type gene in the normal development of P19 cells. In invertebrates, however, similar mutations in *Notch* elicit a gain-of-function phenotype consistent with constitutive activation of *Notch* (Fortini et al., 1993; Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993). In the *Drosophila* embryo, for example, expression of the cytoplasmic domain of Notch blocks neuroblast segregation without impeding epidermal development (Struhl et al., 1993). Moreover, in our studies mNotchIC does not interfere with the generation of neuroepithelial precursors, nor does it suppress the maturation of glial cells. Finally, the results with mNotchIC closely resemble those obtained by overexpression of the tagged wild-type mNotch. Thus, the gain-of-function mutation in *mNotchIC* specifically suppresses neuronal maturation and this is likely to reflect a role for *mNotch* in neurogenesis in P19 cells.

If *Notch* activation suppresses neurogenesis, then what is alternative cell fate elicited in P19 cells? Neuroepithelial precursors give rise to neurons, glia, as well as other cells of unknown identity. Cells that are fated to become neurons may activate *Notch* in neighboring cells eliciting an alternative non-neuronal fate. Glia may represent one such alternative cell fate since the glial phenotype is not affected by constitutive *mNotch* activation. In this model, a population of equipotent neuroepithelial cells may give rise to both neurons and glia. This is consistent with lineage analysis, which demonstrates that glia and neurons arise from the same population of neuroectodermal precursors (Turner and Cepko, 1987; Temple, 1989; Gallileo et al., 1990). Similarly, in the sensory organs of *Drosophila*, cells fated to become neurons elicit a glial-like fate in neighboring cells (Hartenstein and Posakony, 1990). We cannot, however, exclude the possibility that neurons and glia derive independently from non-interacting precursors.

Does *Notch* play a similar role in the choice of fate in the deveoping mammalian embryo? Notch homologues are widely expressed in developing mammalian tissues, including the neural tube, mesoderm and skin (Weinmaster et al., 1991; Franco del Amo et al., 1992; Reaume et al., 1992; Weinmaster et al., 1992; Coffman et al., 1993; Kopan and Weintraub, 1993). In mouse, *Notch* is induced in the nervous system after neural tube closure (Franco del Amo et al., 1992; Reaume et al., 1992). This suggests that *Notch* signalling is not involved in the process of neural plate induction, rather it may have a role in the maturation of a neuroepithelial cell to a neuron, as shown in the P19 system. Homozygous deletion of mouse *Notch1* results in embryonic lethality before day 11 (Swiatek et al., 1994). The extensive cell death observed in these embryos prevents any analysis of the role of *Notch1* in the fate of neuroepithelial cells.

Although we have focussed on the decision to become a neuron, there are numerous steps in mammalian neurogenesis requiring cell fate decisions where *Notch* may be operative. Committed neuroblasts are faced with a vast array of distinct neuronal cell types. By analogy to invertebrate neurogenesis, mammalian *Notch* may play a central role in the initial choice of a neuronal cell fate and may subsequently participate in the generation of more mature neurons with distinct identities.

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