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Activated Notch1 Maintains the Phenotype of Radial Glial Cells and Promotes Their Adhesion to Laminin by Upregulating Nidogen

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brain development; neural stem cell; progenitor; laminin; A2B5

ABSTRACT
Radial glia are neural stem cells that exist only transiently during central nervous system (CNS) development, where they serve as scaffolds for neuronal migration. Their instability makes them difficult to study, and therefore we have isolated stabilized radial glial clones from E14.5 cortical progenitors (e.g., L2.3) after expression of v-myc. Activated Notch1 intracellular region (actNotch1) promotes radial glia in the embryonic mouse forebrain (Gaiano et al., 2000), and when it was introduced into E14.5 cortical progenitors or radial glial clone L2.3, the cells exhibited enhanced radial morphology and increased expression of the radial glial marker BLBP. A representative clone of L2.3 cells expressing actNotch1 called NL2.3-4 migrated more extensively than L2.3 cells in culture and in white matter of the adult rat spinal cord. Microarray and RT-PCR comparisons of mRNAs expressed in these closely related clones showed extensive similarities, but differed significantly for certain mRNAs including several cell adhesion molecules. Cell adhesion assays demonstrated significantly enhanced adhesion to laminin of NL2.3-4 by comparison to L2.3 cells. The laminin binding protein nidogen was the most highly induced adhesion molecule in NL2.3-4, and immunological analyses indicated that radial glia synthesize and secrete nidogen. Adhesion of NL2.3-4 cells to laminin was inhibited by anti-nidogen antibodies and required the nidogen binding region in laminin, indicating that nidogen promotes cell adhesion to laminin. The combined results indicate that persistent expression of activated Notch1 maintains the phenotype of radial glial cells, inhibits their differentiation, and promotes their adhesion and migration on a laminin/nidogen complex.

INTRODUCTION

Radial glia exhibit unique properties including serving as scaffolds for neuronal migration during neurogenesis (Hatten 1999; Rakic 1990). They lose these properties with differentiation; for example, after transplantation of radial glia into adult CNS tissues, they differentiate into astrocytes (Cao et al., 2002; Hasegawa et al., 2005). Prolonging radial glial phenotypes may be a useful strategy that takes advantage of their beneficial properties to promote functional recovery after spinal cord injury (Hasegawa et al., 2005). Recent studies indicate that radial glia in the developing CNS are neural stem cells (NSC) or neuronal progenitor cells (NPC), but very few persist in the adult brain and spinal cord (Alvarez-Buylla et al., 2002; Anthony et al., 2004; Kriegstein and Gotz, 2003).

Radial glia are NSC or NPC that are challenging to maintain and study *in vitro*, because they tend to differentiate. Although progress has been made in isolating NSC/NPC from embryonic stem cells and maintaining them in culture in defined media conditions (Conti et al., 2005), approaches are still needed to control their behavior and fate, particularly after transplantation into the CNS, where they are subjected to multiple signals that induce differentiation. Toward that goal, clones of embryonic brain cells have been isolated after introduction of v-myc (Villa et al., 2000). We isolated two clones (L2.3 and RG3.6) from embryonic rat brain cortices that share properties with NSC and radial glia (Hasegawa et al., 2005; Li et al., 2004) and RG3.6 cells promote functional recovery following transplant into the injured rat spinal cord (Hasegawa et al., 2005). Upon extended passage in culture, these cells transition to express immunological markers recognized by monoclonal antibodies A2B5 and 4D4 that are found on glial restricted precursors (Li and Grumet, 2007; Li et al., 2004; Noble et al., 2003). Developmental studies showed that radial glia appeared as early as E9-10 in the CNS, while the A2B5 and 4D4 markers for glial-restricted precursors appeared later, suggesting that glial-restricted precursors may be derived from radial glia, as they become

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restricted and develop into neurons and glia (Li et al., 2004). Other cell type specific markers including nestin and vimentin are expressed in NSC and NPC, but their expression is not restricted only to these cells. BLBP is restricted to radial glia during development, but it persists in astroglia and adult NPC (Feng et al., 1994; Hartfuss et al., 2001). NSC in culture also expresses BLBP (Conti et al., 2005). Although the function of BLBP is poorly understood, its expression is regulated by Notch1, which is transiently expressed in radial glia (Anthony et al., 2005; Gaiano et al., 2000).

Notch is a transmembrane receptor that mediates lateral inhibition by interacting with ligands on opposing cells including delta (Lai, 2004). In response to ligand binding, Notch undergoes proteolysis and the resulting Notch intracellular domain translocates into the nucleus. Notch intracellular domain acts as a transcriptional activator that turns on downstream genes including BLBP, and the transcriptional repressors Hes1 and Hes5 that suppress neurogenesis (Yoon and Gaiano, 2005). Thus, Notch signaling plays important roles in cell fate determination during CNS development. ActNotch1 promotes the radial glial phenotype *in vivo* (Gaiano et al., 2000), and maintains NPC/NSC in an undifferentiated state in the mammalian CNS (Hitoshi et al., 2002).

The morphological integrity of radial glial cells in developing forebrain has been thought to be influenced by the cortical pial basement membrane, where the endfeet of radial glial cells are anchored (Halfter et al., 2002). Basement membranes are thin layers of extracellular matrix, containing collagen IV and XVIII, laminin and a linker protein nidogen (Yurchenco and Schittny, 1990). Nidogen plays an important role in maintaining basement membranes structure. Mice with a targeted deletion of the nidogen-binding site of laminin γ1 showed disrupted pial membranes during cortical histogenesis and radial glial cells with retracted endfeet suggesting its importance in cortical development (Halfter et al., 2002). These mice also exhibited abnormal neuronal migration, which is probably due to the deformed radial glial morphology. The main source of nidogen and laminin proteins in the pial membranes is thought to be the meningeal cells (Sievers et al., 1994).

In the present study, we found that activated Notch1 promoted radial morphology in NPC in culture, maintained BLBP expression, and inhibited cell differentiation. Expression of activated Notch1 in a subclone of L2.3 called NL2.3-4 yielded cells with an elongated radial morphology in culture and after transplantation into the spinal cord that resisted differentiation. These radial glial-like cells expressed nidogen, which promoted their adhesion to laminin. In addition, primary radial glia expressed and secreted nidogen.

MATERIALS AND METHODS

Cell Culture and Differentiation

Generation and cultures of radial glial clone L2.3 have been described previously (Li et al., 2004). The same culture protocol was used for NL2.3-4 clone and primary cortical radial glia. Briefly, culture medium contained DMEM/F12 (Invitrogen) supplemented with 25 mM glucose (Sigma), 2 mM glutamine (Invitrogen), penicillin/streptomycin (Invitrogen), 10 ng/mL FGF2 (BD Biosciences), 2 μg/mL heparin (Sigma), and 1× B27 (Invitrogen). Cells were propagated as neurospheres and passaged by mild trypsinization (0.025% for 5 min) every 3 days. For differentiation, cells were cultured on laminin-coated coverslips in FGF2 containing serum-free medium for 1 day, then the medium was replaced with culture medium lacking FGF2, including 1% fetal bovine serum, for 6 days, and the cultures were fixed, and immunostained. For Brefeldin A treatment (5 μg/mL, CalBiochem), cultures were incubated for 90 min at 37°C before fixation.

Retroviral Infection and Selection of Clones

Primary NSC were isolated from E13.5 cortices and maintained in serum-free medium as described earlier. The next day, cells were mechanically dissociated by gently pipeting to yield 1–2 × 10^6 cells/mL in serum-free medium containing 8 μg/mL polybrene. ActNotch1-GFP or control GFP viruses (Yoon et al., 2004) was added at 1:1,000 dilution and incubated with cells for 1 h on a shaker at 100 rpm. After three washes in serum-free medium, the cells were plated in NSC culture medium. GFP signals were observed within 48 h after infection. Infected cells were passed and plated in culture medium at clonal density (1,000 cells/10-cm dish). After 3–4 days, individual neurospheres were picked, expanded, and characterized.

Measurement of Cellular Process Lengths

Images of undifferentiated cells stained with nestin were measured using Zeiss Laser Scanning Microscope (LSM) imaging software; cell processes were traced using the measurement tool. When there were multiple processes arising from one cell body, only the longest process was measured. Measurements were sorted by range (e.g., 0–20, 20–40, 40–60 μm, etc.) and the number/group were graphed.

Affymetrix Genechip™ Analysis and Quantitative-RT-PCR (Q-RT-PCR)

Total RNA was purified from neurosphere cultures using RNeasy kit (Qiagen). Samples (n = 3 per group) were sent to the Bionomics Research and Technology Center, Rutgers University, for Affymetrix processing. Results were normalized using RMA, fit to a linear model using the *affylmGUI* in Bioconductor (Smyth, 2004; Wettenhall and Smyth, 2004; Wettenhall et al., 2006), and tested for significance at a 5% false discovery rate (FDR) to control for multiple measurements error and at least 2-fold mean difference between groups

TABLE 1. Primers for Q-RT-PCR

Gene names	Forward	Reverse
B-actin	CGTAGCCATCCAGGCTGTGT	CCAGTGGTACGACCAGAGGC
eGFP	GCAAAGACCCCAACGAGAAG	TCACGAACTCCAGCAGGACC
Fabp7(BLBP)	GGAAGCTGACAGACAGCCAGA	CGCCCAGAGCCTTCATGTAC
Nestin	CAAGCAGCAGGGTCACTTCC	AGGTTTGTGGCTAAGGAGGTCA
Vimentin	GAGCACCCTGCAGTCATTCA	CGTGCCAGAGAAGCATTGTC
Hes5	GCACCAGCCCAACTCCAA	ACGGCCATCTCCAGGATGT
Pax6	TCTAACCGAAGGGCCAAGTG	GAGGAGACAGGTGTGGTGGG
Notch1	AGCACTGGAAAGGACTCCCA	AAGGACCCCAGCTTCCGT
Tnc	CCTTCATTAAGACCCGCTGG	TGCAGCCTCATGAAGCAGAG
Nid	AGCACCTTTCCTGGCTGACTT	TACACATTCCCCAGGCCATC
Cd44	AAGGACACGTGGTAATCCCG	TGGCTGCACAGATAGCGTTG
Alcam	ACCATTGTCATGCCTTGCAG	GCCAAACATGAGGTTCTGAGGT
NCAM	TGATGTGGTCAGCTCTCTGCC	CGGCCTTTGTGTTTCCAGAT
Itgb1	AAGTCCCAAGTGCCATGAGG	CTGCAGGCTCCACACTCAAAT

(Supplemental Table 1). Gene ontology (GO) analysis was performed using GeneSpring (Agilent, Supplemental Table 2).

For PCR analyses, 1 μg was reverse-transcribed into cDNA using oligo-dT primer and SuperScript II reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed as described previously (Li et al., 2003) using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). β-actin was used to normalize the expression levels of each sample. Primers for detecting genes are listed in Table 1.

Cell Adhesion Assay on Laminin

Dissociated single cell suspensions (10^6 cells/mL) were incubated on laminin-1 (20 μg/mL, Invitrogen) coated spots (5,000 cells per spot) for 40 min at 37°C, washed with PBS, fixed, and stained with 1 μg/mL propidium iodide (Sigma). Blocking antibody anti-nidogen is a rat IgG from Chemicon. Laminin-2 (Smirnov et al., 2002) and the laminin-2-NS with a deletion of the nidogen-binding site within laminin γ1 chain (Halfter et al., 2002) were generously provided by Dr. Peter Yurchenco and used at 20 μg/mL. Attached cells per spot were counted and average cell numbers were calculated from triplicate spots for each condition.

Neurosphere Spreading Assay on Laminin

Cultured neurospheres labeled with DAPI were incubated at 37°C on laminin-coated substrates. Pictures were taken soon after the neurosphere attachment (30 min), and when cells migrated out of neurospheres (2 h) at the same positions. DAPI images were used to quantify the neurosphere spreading area at 30 min (a1) and at 2 h (a2). Spreading area ratios [(a2-a1)/a1] were calculated.

Transplantation into the Spinal Cord and Cell Migration

NL2.3-4 and L2.3 cells were labeled with CellTracker CFDA-SE (Molecular Probes) according to manufacturer's protocol, and 200,000 cells were transplanted into adult Sprague–Dawley rat spinal cords at T9 and T13; animals were sacrificed after 3, 14, or 28 days, and spinal cords were sectioned for histological analysis as described (Hasegawa et al., 2005).

Western Blot Analysis

Cultured cells were harvested in SDS lysis buffer, and heated at 95°C for 5 min. Proteins were separated on 10% SDS-PAGE and transferred onto polyvinylidene flouride (PVDF) membranes and immunoblotted with anti-GFP (1:500, mouse IgG, Chemicon), anti-nidogen [1:200, (McKee et al., 2007)] or anti-NCAM (1:50, rabbit IgG, Grumet lab), followed by horseradish peroxidase-conjugated secondary antibodies (1:5,000, Jackson lab). The blots were developed using ECL plus detection system (GE Healthcare Amersham). Anti-actin (1:1,000, rabbit IgG, Sigma) was used to normalize the sample loading.

Immunocytochemistry and Immunohistochemistry

Cultured cells were fixed with 4% paraformaldehyde and immunostained as described (Li and Grumet, 2007). The primary antibodies used were monoclonal mouse IgMs: 4D4 (neat, a gift from Dr. Kaprielian's lab) (Liu et al., 2002), A2B5 (1:200, Chemicon), 5A5 (1:1, DSHB) and RC1 (1:5, DSHB); monoclonal mouse IgGs: anti-vimentin (1:10, DSHB), anti-nestin (1:50, DSHB), GalC (1:50, Mckinnion lab), TuJ1 (1:500, Covance); polyclonal rabbit IgGs: anti-BLBP (1:1,000, a gift from Dr. Heintz's lab), anti-nidogen (1:200), and anti-GFAP (1:200, DAKO). DAPI (10 μg/mL, Sigma) was included in the secondary antibody incubations to label nuclei.

In acquiring the fluorescence intensity of BLBP, confocal images were analyzed with the Carl Zeiss LSM 510 program. The appropriate channels were chosen (GFP chnl and BLBP chn2). The images were opened in the "histogram display mode," where both "area" and "colocalization" functions were chosen. The cells colocalizing both markers were individually selected by tightly drawing a borderline around the cells with the "close polyline drawing mode" function. The "show table" function showed all the parameters that had been measured. The "area" and "mean intensity" parameters were chosen and multiplied to finally produce the Fluorescence Intensity of BLBP depicted in the figure ($^{**}P < 0.01$).

RESULTS

Activated Notch1 (actNotch1) Promotes Radial Glia Phenotypes in Culture and Inhibits Differentiation

To determine whether actNotch1 promotes radial glia phenotype *in vitro*, we used a GFP version of the same viral construct to infect L2.3 cells (Li et al., 2004) and cortical cultures that are derived from E13.5 rat

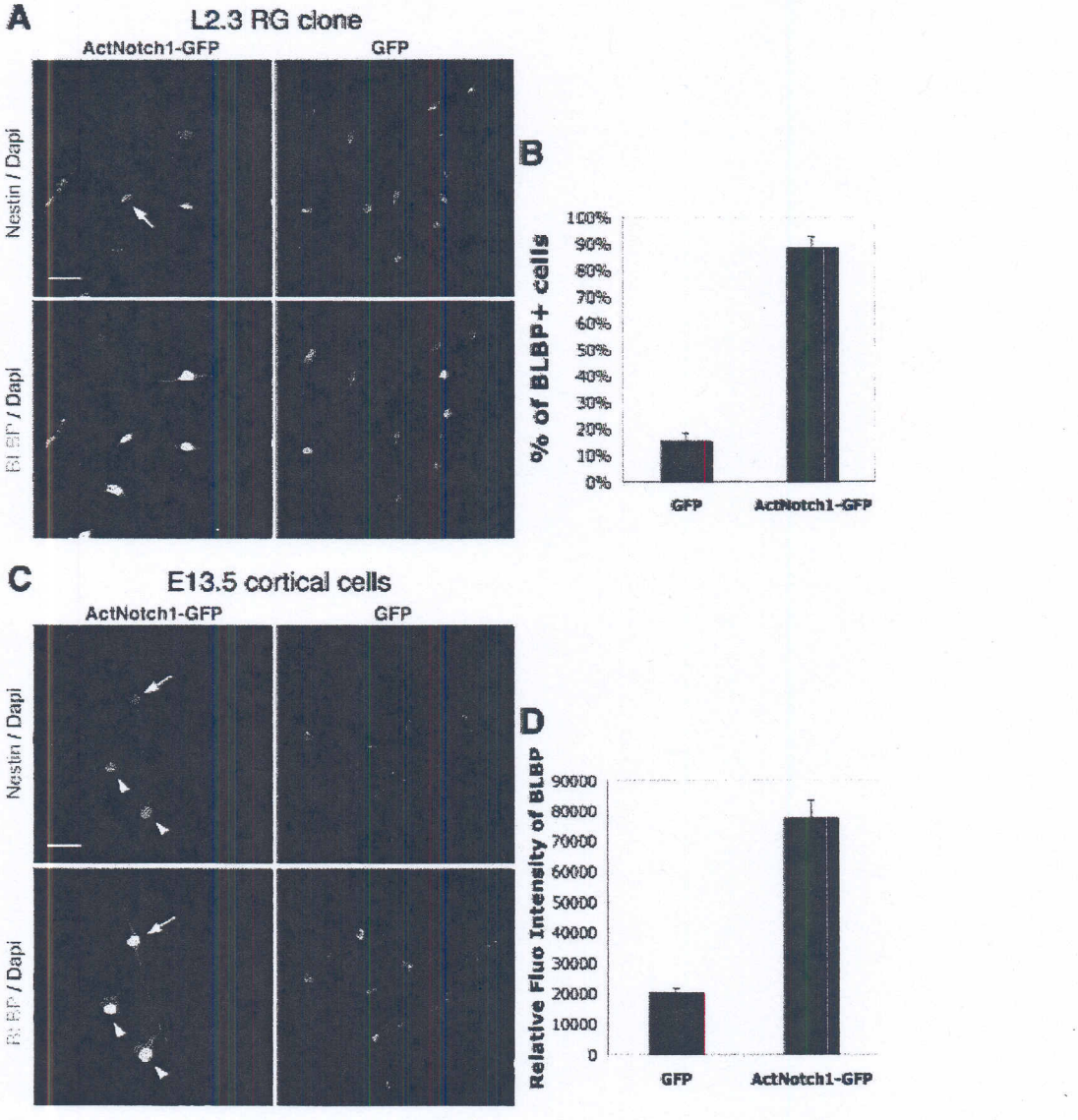

Fig. 1. Activated Notch1 promotes radial glial phenotype in cultured L2.3 clone and E13.5 cortical cells. (**A**) L2.3 cells infected with retrovirus overexpressing actNotch1(actNotch1-GFP), showed enhanced radial morphology and elevated expression of BLBP by comparison to control infection (GFP). Activated Notch1 fused with GFP translocates to the nucleus (arrow). Nestin (red) shows cellular morphology, while DAPI (blue) labels nuclei. The percentage of BLBP (purple) positive cells among infected cells were quantified (**B**) (n = 493 for GFP, n = 473 for actNotch1-GFP). (**C**) E13.5 cortical cells were infected with actNotch1-GFP retrovirus showed long bipolar morphology (arrow), while others were flat (arrowheads). The expression levels of BLBP protein were quantified by relative fluorescence intensity (**D**) (n = 76 for GFP; n = 85 for actNotch1-GFP). Scale bars, 20 μm.

forebrains. The retrovirus encodes for Notch1 intracellular domain fused with GFP, thus the Notch infected cells were easily recognized by their green nuclei. Most actNotch1 infected L2.3 cells exhibited bipolar radial morphology as shown by nestin immunostaining (Fig. 1A, arrow). Notch induces expression of BLBP (Anthony et al., 2005; Gaiano et al., 2000), and we confirmed that actNotch1 induced BLBP expression in actNotch1-GFP cells by comparison to GFP-infected controls (Fig. 1A). The percentage of BLBP+ infected cells was ~6-fold higher with actNotch1-GFP than with GFP alone (Fig. 1B). Similarly, in E13.5 cortical cultures, we found that actNotch1-GFP infected cells exhibited strong BLBP expression (Figs. 1C,D) and some of these cells had very long bipolar processes resembling radial glia (Fig. 1C, arrow). Other actNotch1-GFP cells had flat shapes (Fig. 1C, arrowheads) suggesting they may already have begun to differentiate and actNotch1 apparently did not revert them into radial glia. The expression of BLBP protein, measured by relative fluorescence intensity, was much higher after actNotch1-GFP infection than in GFP controls (Fig. 1D).

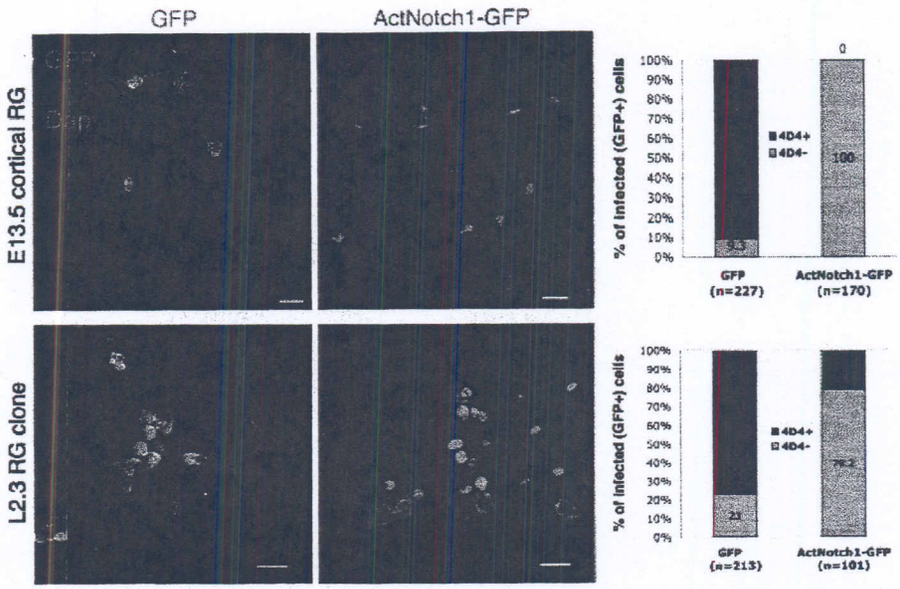

Fig. 2. Activated Notch1 suppresses expression of the glial-restricted precursors marker 4D4. E13.5 cortical radial glia and L2.3 clone were infected with either actNotch1-GFP (green nucleus) or control GFP retrovirus. The resulting cultures were fixed and stained with the glial-restricted precursor marker 4D4 (red) (Li and Grumet, 2007), which was dramatically lower in the actNotch1-GFP infected cells compared with controls. Percentages of 4D4+ and 4D4− cells were measured among infected (GFP+) cells shown in panels on right. DAPI (blue) was used to label the nuclei. Scale bars, 10 μm.

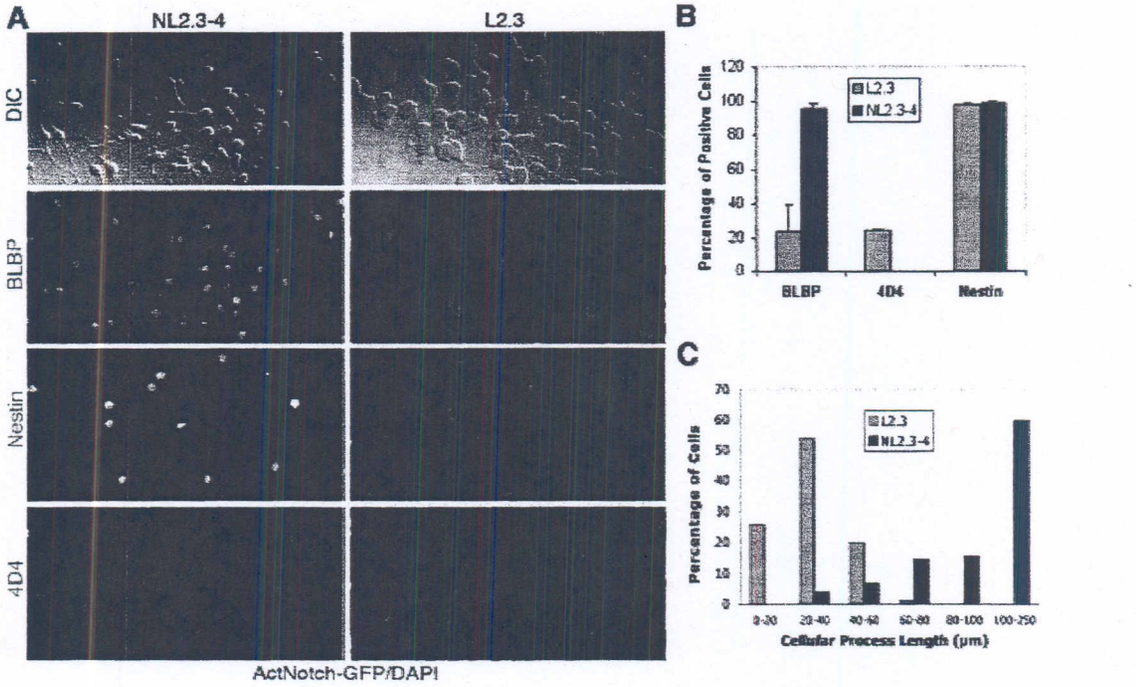

Fig. 3. Cellular characterization of an actNotch1 expressing clone, NL2.3-4. (**A**) Expression of cellular markers, BLBP, Nestin and 4D4, were compared between NL2.3-4 and its parental radial glia clone L2.3 by immunostaining. DIC shows that NL2.3-4 cells are more bipolar than the L2.3 cells. ActNotch1-GFP shows green nuclear localization, while DAPI labels nuclei blue. The percentage of marker positive cells is shown in (**B**). Lengths of cellular processes was measured based on nestin staining and categorized into groups by length (**C**). Scale bar, 50 μm.

We have shown that the fate of radial glial cells becomes restricted during forebrain development, as they acquire the glial-restricted precursor markers A2B5 and 4D4 (Li et al., 2004). To determine whether Notch signaling plays a role in radial glial cell fate restriction, we examined 4D4 expression. Compared with GFP infected cultures, where 90.7% of cells were positive for 4D4, actNotch1-GFP+ cells showed little or no expression of 4D4 in E13.5 cortical cells (see Fig. 2). Similar results were also observed in radial glial clone L2.3 but ~20% of cells continued to express 4D4 typically at very low levels (see Fig. 2), while the cells that did not express actNotch1 stained strongly for 4D4. These data suggest that Notch signaling maintains the radial glial phenotype and prevents, or at least delays, differentiation toward glial phenotypes.

Isolation and Characterization of a Notch1 Expressing Radial Glial-Like Clone, NL2.3-4, from NSC/NPC Clone L2.3

To study the effect of Notch on radial glia in more detail, we isolated clones from actNotch1 expressing L2.3 cells. Five out of eight GFP+ clones showed radial morphology on laminin substrates. Most of them (seven out of eight) showed strong BLBP expression, an indication of active Notch signaling. One clone, NL2.3-4 was characterized further because of its homogeneous BLBP expression, radial morphology, and absence of 4D4. Almost 100% of NL2.3-4 cells exhibited GFP in their nuclei and BLBP staining in their cytoplasm, whereas only ~20% of L2.3 cells expressed BLBP (Figs. 3A,B). All cells in both clones showed nestin expression as expected for NPC or NSC (Fig. 3B). However, 4D4 expression was absent in NL2.3-4 cells, while ~25% of L2.3 cells showed 4D4 immunoreactivity. Thus, persistent expression of actNotch1 blocked expression of 4D4.

Further comparison of NL2.3-4 to its parental clone L2.3 revealed differences in cell morphology and differentiation. On laminin substrate and in the presence of FGF2 (10 ng/mL), NL2.3-4 exhibited longer bipolar processes than L2.3 cells that were as much as 20 times as long as the cell body diameter, as revealed by both differential interference microscopy (DIC) and nestin immunostaining (Figs. 3A,C). Upon FGF2 withdrawal, L2.3 cells were multipotent giving rise to small numbers of neurons (TuJ1+) and oligodendrocytes (GalC+), and primarily to astrocytes (GFAP+) (Fig. 4 and Li et al., 2004). In contrast, NL2.3-4 cells responded to express GFAP, but markers for other neural cell types were not detected (Figs. 4A,B). Interestingly, the GFAP+ NL2.3-4 cells retained their bipolar elongated morphologies, suggesting that their differentiation was incomplete (Fig. 4A), however, both L2.3 and NL2.3-4 expressed another astrocytic marker S100β upon differentiation (data not shown). The combined results suggest that actNotch1 inhibits cell differentiation in neural cells.

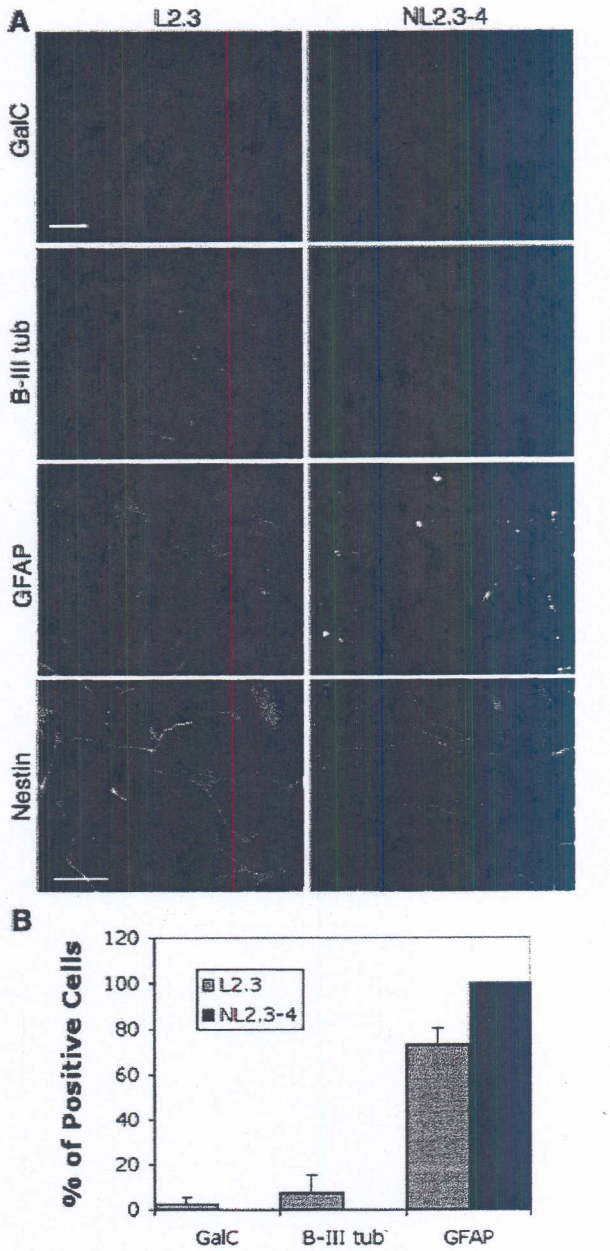

Fig. 4. NL2.3-4 cells differentiate into GFAP+ cells, but not into oligodendrocytes and neurons. (A) L2.3 and NL2.3-4 cells were cultured for 6 days after FGF2 withdrawal on laminin-coated substrate. Cells were fixed and stained with antibodies against GalC (oligodendrocytes), β-III tubulin (neurons), GFAP (astrocytes), nestin (NSC/radial glia), and DAPI (nuclei). All cells were nestin positive and the percentage of cells positive for differentiation markers is shown in (B).

NL2.3-4 Cells Migrate More Robustly Than L2.3 in Normal Rat Spinal Cord and Resist Differentiation

The migratory property of NL2.3-4 cells was evaluated in rat spinal cord (Hasegawa et al., 2005; Hormigo et al., 2001). NL2.3-4 and L2.3 cells labeled with CFDA-SE

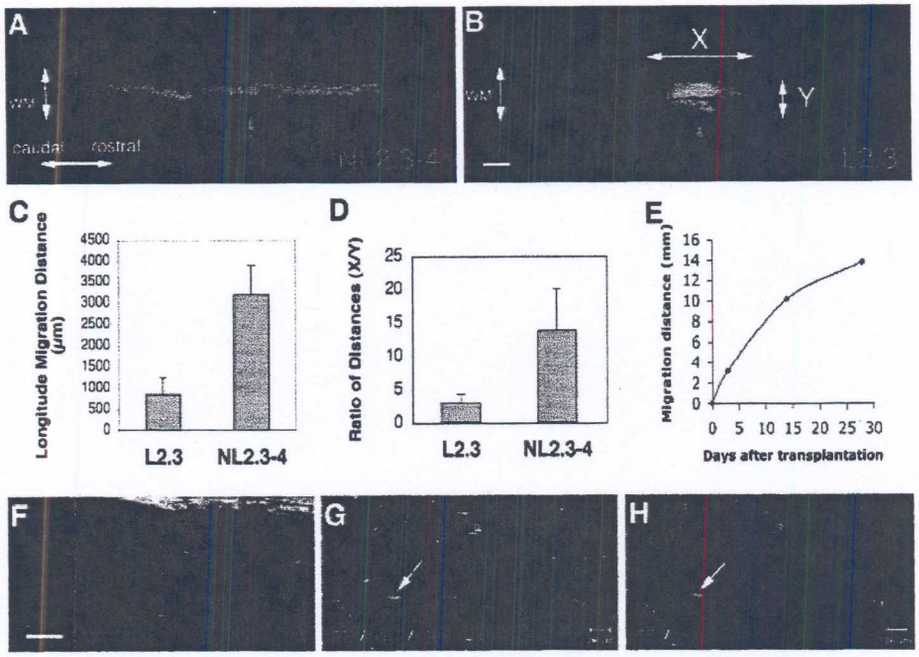

Fig. 5. ActNotch1 promoted migration of L2.3 in white matter of adult rat spinal cord. NL2.3-4 and L2.3 cells labeled with CellTracker CFDA-SE were transplanted into adult rat spinal cord white matter. After 3 days, rats were killed and histological analysis of spinal cords after sectioning revealed cell migration patterns primarily in white matter (WM). Red arrows indicate the injection sites (**A**, NL2.3-4 and **B**, L2.3). Longitudinal migration distances of the transplanted cells were measured in all sections that contained labeled cells. Rostro-caudal (X) and dorso-ventral (Y) cell migration distances are defined by the arrows in (B); results show averages with standard derivations (**C**) and ratios (X/Y) of migration distances (**D**). Total rostro-caudal migration distances of NL2.3-4 transplants in spinal cord at Day 3, 14, 28 were measured (**E**). Four weeks after transplantation in spinal cords, NL2.3-4 cells maintained bipolar morphologies with long processes as revealed by nestin staining (red in **F**). Confocal images showed nestin positive cellular processes (red) with persistent actNotch1-GFP expression in the nucleus (**G**, arrow) of bipolar cells that lacked GFAP (purple) expression (**H**). Scale bars: 200 μm in (A) and (B), 100 μm in (F), 50 μm in (G).

were transplanted into adult rat normal spinal cord and the migration pattern of the cells was examined after 3 days. NL2.3-4 cells migrated greater distances in spinal cord white matter than L2.3 cells and differences in rostro-caudal migration (X) were measured across serial sagittal sections (Figs. 5A,B; 3 mm for NL2.3-4 and 0.8 mm for L2.3). Analysis of fluorescence in serial sections yielded three-dimensional images of the transplants that had relatively similar volumetric measures for NL2.3-4 (11.67×10^6 μm^3) and L2.3 (11.31×10^6 μm^3), suggesting that at least at 3 days after transplantation, there was no dramatic difference in graft survival. In addition, the asymmetry in the migration was confirmed by calculating the ratio of longitudinal/lateral migration in the spinal cord (X/Y, Fig. 5D). The maximal migration of NL2.3-4 cells increased up to 13.8 mm at 4 weeks after transplantation in spinal cord white matter (Fig. 5E). Higher magnification revealed nestin positive bipolar NL2.3-4 cells in spinal cord white matter even 4 weeks after transplantation. Detection of green labeled nuclei in the radial glial-like cells (Figs. 5F,G) indicated the persistence of actNotch1-GFP expression but we did not detect GFAP in these cells, suggesting that they did not exhibit astroglial differentiation. In contrast, NPC without (Cao et al., 2001) or with v-myc (Hasegawa et al., 2005) showed more extensive differentiation (e.g., GFAP expression) 2–4 weeks after transplantation. The results suggest that persistence of actNotch1-GFP expression prevents differentiation of these cells *in vivo*.

Microarray Analysis Revealed Elevated Adhesion Molecules in NL2.3-4 vs. L2.3

Considering differences between NL2.3-4 and L2.3 cells in morphology and migration, we compared their patterns of gene expression using the Affymetrix rat genome chip, rat 230 2.0. Among more than 30,000 transcripts detected on the chip, 814 were significant at a 5% FDR with at least a 2-fold mean difference in expression between NL2.3-4 and L2.3 (Supplemental Table 1). Several known downstream Notch target genes were upregulated in NL2.3-4 by comparison to L2.3, including BLBP (Fabp7) and Hes5 (but not Hes1) (de la Pompa et al., 1997), confirming that Notch signaling was active in the NL2.3-4 cells. In addition, the radial glial markers BLBP, GLAST, pax6, Tenascin C, nestin, and vimentin (Bartsch et al., 1992; Gotz et al., 1998; Hartfuss et al., 2001; Heins et al., 2002) were upregulated in NL2.3-4 cells (Fig. 6A and Supplemental Table 1). CD-9, which is found in NSC (Klassen et al., 2001) was also upregulated, but prominin-1 (CD133) (Pfenninger et al., 2007)

A

Notch or RG-related Genes

2 fold higher in NL2.3-4

Fold-Change	Affymetrix ID	Symbol	Genbank	Description
48.45	1373401_at	Tnc	AI176034	Tenascin C
41.74	1393207_at	Hes5	BF554509	Transcribed locus
40.54	1370024_at	Fabp7	NM_030832	fatty acid binding protein 7, brain
5.11	1368242_at	Pax6	NM_013001	paired box gene 6
4.42	1371491_at	Notch1	BM390614	Notch gene homolog 1 (Drosophila)
4.16	1386848_at	Nes	NM_012987	nestin
2.25	1367574_at	Vim	NM_031140	vimentin

B

Cell Adhesion

2 fold higher in NL2.3-4

Fold-Change	Affymetrix ID	Symbol	Genbank	Description
59.25	1371518_at	Nid1	AI235948	Nidogen 1
20.97	1370043_at	Alcam	NM_031753	activated leukocyte cell adhesion molecule
20.35	1374432_at	Alcam	BE118251	Activated leukocyte cell adhesion molecule
16.82	1382294_at	Alcam	AI576111	Activated leukocyte cell adhesion molecule
14.04	1368921_a_at	CD44A	NM_012924	CD44 antigen
10.92	1384812_at	Ncam1	AI229409	Transcribed locus
9.97	1390659_at	Cd44	BI302830	Transcribed locus
6.95	1387934_at	ALPBRE	U37142	brevican
6.71	1368474_at	VCAM1B	NM_012889	vascular cell adhesion molecule 1
6.28	1367881_at	Bit; SHPS-1	NM_013016	protein tyrosine phosphatase, non-receptor type substrate 1
5.66	1383284_at	Ncam1	BG372598	Transcribed locus
3.84	1387541_at	neurocan	NM_031653	chondroitin sulfate proteoglycan 3
3.76	1370016_at	Nell2	NM_031070	nel-like 2 homolog (chicken)
3.71	1368472_at	Megf2	NM_031320	cadherin EGF LAG seven-pass G-type receptor 3
2.91	1388551_at	Pcdhgc3	AW434022	Protocadherin gamma subfamily C, 3
2.84	1387039_at	Gpc1	NM_030828	glypican 1

2 fold higher in L2.3

Fold-Change	Affymetrix ID	Symbol	Genbank	Description
0.46	1371036_at	Nrcam	BG671431	Neuron-glia-CAM-related cell adhesion molecule
0.06	1368541_at	Emb	NM_053719	embigin

C

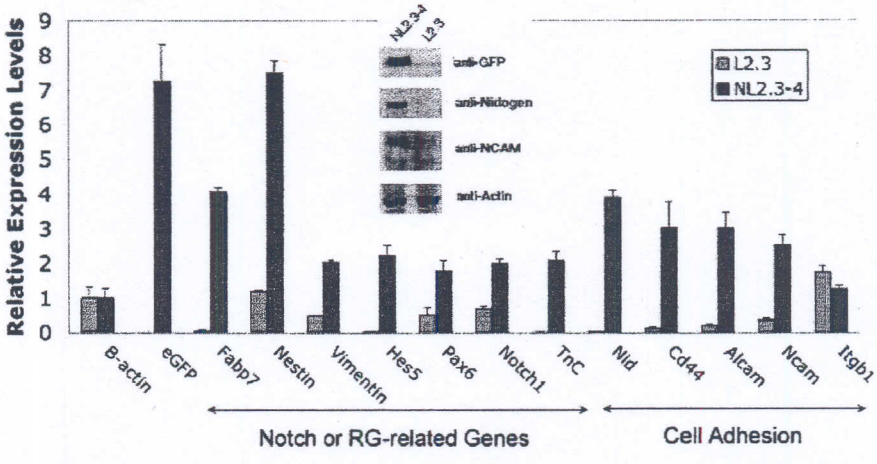

Fig. 6. Microarray analysis indicates higher expression of adhesion molecules in NL2.3-4 cells than L2.3 cells. Gene expression comparison was performed in triplicates between NL2.3-4 and L2.3 RNAs using Affymetrix Rat 230-2 microarrays. Among the 31,100 genes on the chip, 720 genes were higher in NL2.3-4 and 349 genes were higher in L2.3 by 5% FDR and two-fold cutoff. Radial glial (RG) and Notch related genes (**A**) as well as adhesion molecules are listed (**B**) along with confirmation of candidates by Q-RT-PCR (**C**) and western blotting (insert in C). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

decreased somewhat (Supplemental Table 1). These *in vitro* data suggest that actNotch1 signaling promotes Notch-related and radial glial specific genes *in vivo* (Gaiano et al., 2000).

An unbiased analysis of regulated genes was performed to identify cellular functions affected by act-Notch1 expression. The GO assignments of significantly regulated genes were compared with all genes probed on the array by selecting top-level categories having a hypergeometric *P*-value of 0.01 or less (Supplemental Table 2). All major GO groupings (biological process, cellular component, and molecular function) presented interpretable functional categories relating to nervous system development. The most significant biological process categories included systems development, nervous system development, cell adhesion, and astrocyte development, supporting our hypothesis that Notch maintains the radial glial phenotype.

We paid particular attention to cell adhesion-related genes in this comparison because of the morphological,

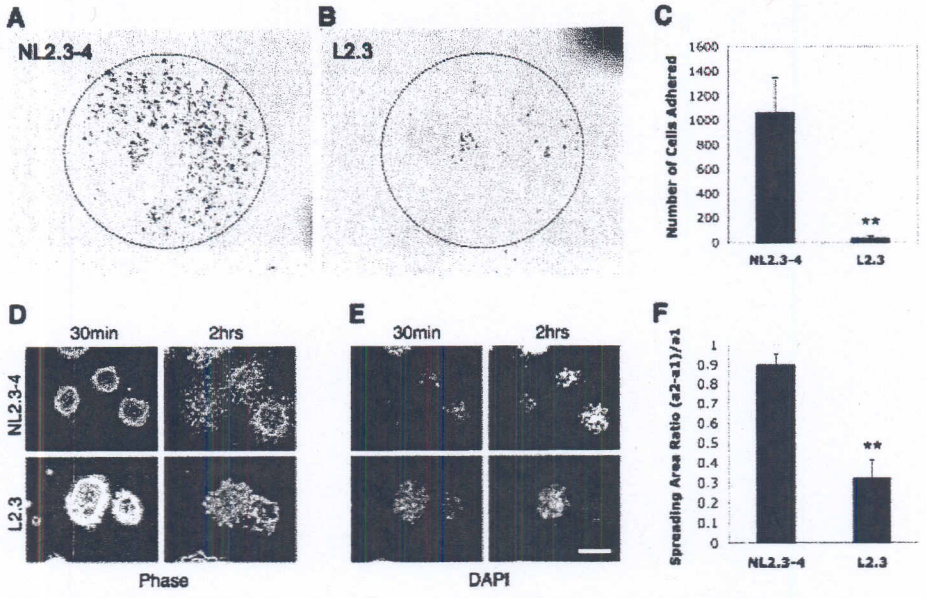

Fig. 7. NL2.3-4 cells adhered and migrated better on laminin substrate than parental L2.3 cells. Dissociated single cell suspensions of NL2.3-4 (**A**) and L2.3 (**B**) were incubated on laminin-coated spots for 40 min, and then fixed and stained with propodium iodine (PI). Inverted PI stained images are shown with dotted circles outlining the laminin-coated spots. Average numbers of attached cells per spot and standard errors of triplicates are shown (**C**). Cultured neurospheres of NL2.3-4 and L2.3 labeled with DAPI were plated on laminin-coated substrates and incubated at 37°C. Phase contrast (**D**) and DAPI (**E**) pictures were taken soon after the neurosphere attachment (30 min), and when cells migrated out of neurospheres (2 h). DAPI images (E) were used to quantify the neurosphere spreading area at 30 min (a1) and at 2 h (a2). Averages of spreading area ratios from three different experiments (a2-a1)/a1 are shown (**F**) with standard errors. $^{**}P < 0.01$ (Student's t-Test). Scale bars, 40 μm in (A and B); 20 μm in (D and E).

adhesive, and migratory properties of NL2.3-4 cells. In the cell adhesion GO category, 12 genes were found to be at least 2-fold higher in N2.3-4, while only two genes were 2-fold higher in L2.3; some of the genes appeared multiple times including Alcam, CD44, and N-CAM (Fig. 6B). The fact that more adhesion molecules were upregulated in NL2.3-4 by comparison to L2.3 suggests that NL2.3-4 cells may be more adhesive. The differential expression of several of these genes was confirmed by Q-RT-PCR analysis (Fig. 6C). In addition, upregulation of nidogen and NCAM proteins was confirmed by western blotting (Fig. 6C insert).

NL2.3-4 Cells Adhere and Spread Better on Laminin Than L2.3

NL2.3-4 cells exhibited robust adhesion to laminin and several of the genes that are most highly upregulated in response to actNotch1 expression bind to laminin including nidogen (Fox et al., 1991) and CD44 (Hibino et al., 2004). In a laminin-mediated cell adhesion assay, we detected a significant difference in cellular adhesion between NL2.3-4 and L2.3 cells within 40-min of incubation (Figs. 7A–C). The NL2.3-4 cells adhered better, and they were more migratory on laminin substrates. In a neurosphere-spreading assay, NL2.3-4 cell bodies and processes extended longer distances on laminin than L2.3 cells (Figs. 7D,E). Using DAPI fluorescence to measure the spreading area ratio, we found that NL2.3-4 cell bodies migrated significantly greater distances than L2.3 cells on laminin substrate (Fig. 7F).

Nidogen Mediates Enhanced Cell Adhesion of NL2.3-4 on Laminin

To identify adhesion molecules expressed by NL2.3-4 cells, we analyzed their potential involvement in mediating enhanced adhesion on laminin substrate. Integrins are classic receptors for laminin substrate (Reichardt and Tomaselli, 1991; Yurchenco and Schittny, 1990). The major laminin receptor, integrin β1, did not show significant differences between NL2.3-4 and L2.3 by Q-RT-PCR analysis (Fig. 6C). CD44 is a hyaluronic acid-binding protein that serves as a receptor for laminin (Halfter et al., 2002; Yurchenco and Schittny, 1990), however, anti-CD44 blocking antibody (Frank et al., 2005) did not block cell adhesion of NL2.3-4 on laminin at concentrations as high as 200 μg/mL (data not shown). Nidogen is a major component of basement membranes that plays a role in maintaining its integrity by binding to laminin γ1 subunit (Halfter et al., 2002; Yurchenco and Schittny, 1990). Anti-nidogen inhibited NL2.3-4 cell adhesion on laminin by about 50% compared with no antibody and rat IgG controls (Figs. 8A–D). Adhesion assays using recombinant laminin-2 protein (LM-2) that bears a mutation in its nidogen-binding site, called LM-2-NS (Halfter et al., 2002), showed a 5-fold reduction in cell

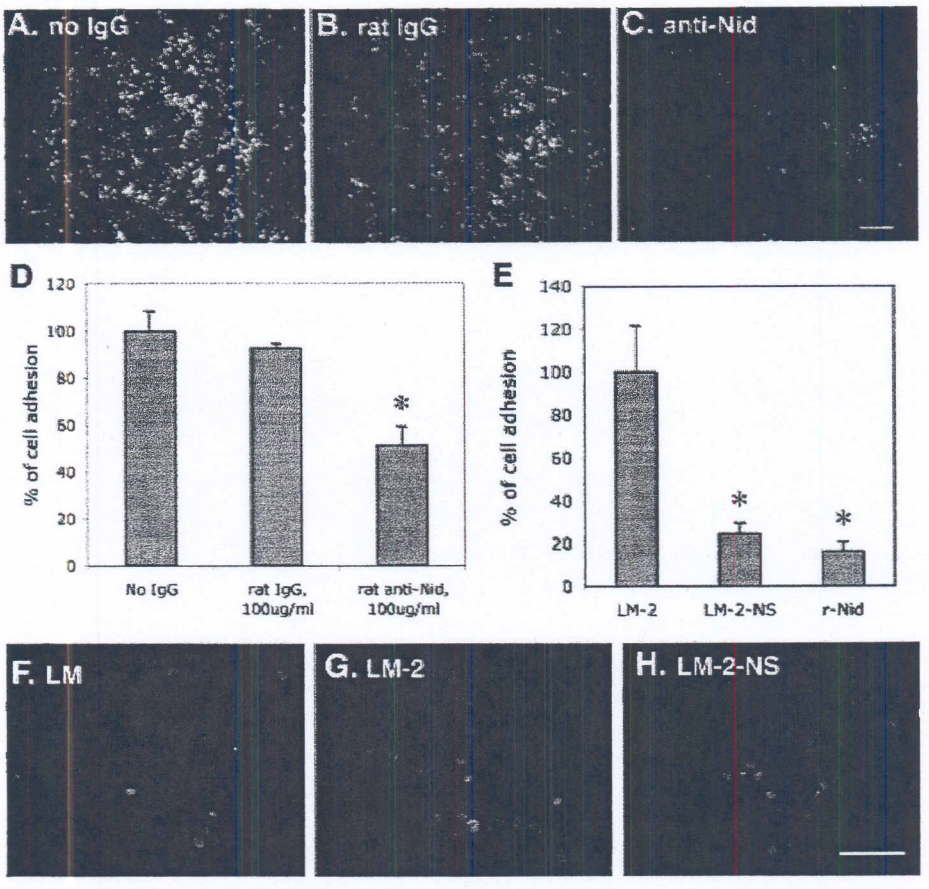

Fig. 8. Nidogen enhanced adhesion of NL2.3-4 cells on laminin. Dissociated cells were incubated on laminin for 40 min in the presence of the indicated proteins (20 μg/mL). Images of DAPI stained adherent cells are shown in (**A–C**). The percentages of cells adhered were calculated with the number of adherent cells in controls defined as 100% (**D**). The percentages of cells adhered to recombinant proteins (20 μg/mL): LM-2 (laminin-2 isoform), LM-2-NS (laminin-2 containing mutation at nidogen-binding site), and r-Nid (nidogen protein) were calculated with the number of adherent cells on LM-2 defined as 100% (**E**). Overnight cultures of NL2.3-4 cells on substrates coated with LM (**F**), LM-2 (**G**), and LM-2-NS (**H**) were fixed and stained with anti-nidogen antibody (red). Note that nidogen protein was detected (red) on the cell surface and on the substrates except with LM-2-NS (H). Scale bars, 50 μm. $*P < 0.05$ (Student's *t*-Test).

adhesion on LM-2-NS substrates compared with that on wild type LM-2 (Fig. 8E). In addition, NL2.3-4 cells adhered poorly when incubated directly on recombinant nidogen (Fig. 8E), suggesting that nidogen potentiates cell adhesion to laminin rather than acting as a ligand by itself. When the cultures were immunostained for nidogen, there was reactivity on substrates coated with laminin (EHS) and LM-2 but not on those coated with LM-2-NS, which lacks the nidogen-binding region. This effect was more robust after overnight culture (Figs. 8F–H), indicating that the secreted nidogen can bind to laminin on the culture substrate.

E14.5 Cortical Radial Glial Cells, but not Neurons, Secrete Nidogen in Culture

To explore the biological relevance of elevated nidogen expression by actNotch1, we analyzed nidogen expression in embryonic cortical radial glial cell cultures where Notch signaling is active (Gaiano et al., 2000). Using a polyclonal anti-nidogen antibody, we found that nidogen localized at the pial surface and around blood vessels as reported (Halfter et al., 2002). We also detected some very weak anti-nidogen staining along nestin 1 radial glial fibers in E14.5 spinal cord and in primary cultures (data not shown), but it was unconvincing. We considered that it might be difficult to detect nidogen in the cell because it was synthesized and released. When the cultures were treated with Brefeldin A (5 μg/mL, a Golgi apparatus blocker) for 90 min to prevent secretion of proteins, nidogen protein was found to accumulate in radial glial cells that were RC1-positive (Fig. 9A). Similarly, nidogen was found in vimentin-positive cells (insert in Fig. 9A). TuJ1-positive neurons did not show nidogen staining (Fig. 9B), suggesting that radial glia but not neuronal precursors express nidogen. Nidogen-positive cells compose a subpopulation of radial glia, defined by expression of the radial glial marker RC1, and after Brefeldin A treatment, nidogen was localized

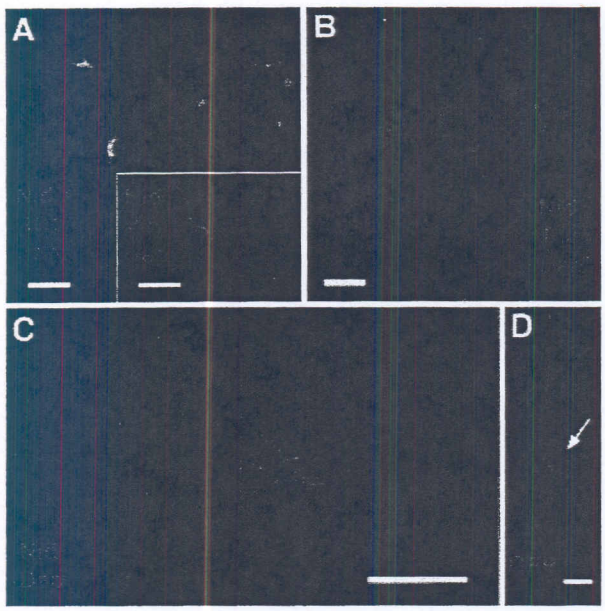

Fig. 9. Nidogen expression in primary E14.5 radial glial cells. E14.5 cortical cells were treated with the Golgi blocker, Brefeldin A (5 μg/mL) for 90 min followed by fixation with 4% paraformaldehyde (**A–C**). Immunostaining showed that some RC1+ (A) and vimentin+ (insert in A) radial glial cells express nidogen, but TuJ1+ neurons did not (B). Higher magnification of a radial glial cell showed nidogen-positive secretary vesicles inside the cell body (C) with Brefeldin A treatment. Nidogen protein was detected on the surface of NL2.3-4 cells even without Brefeldin A treatment (**D**, arrow indicates green nucleus). Scale bars, 10 μm.

in vesicular structures as expected for secreted proteins that are prevented from being released. In contrast to primary radial glia, strong anti-nidogen immunnostaining was detected in the NL2.3-4 cells even without Brefeldin A treatment confirming their robust expression of nidogen induced by actNotch1 (Fig. 9D). The results suggest that radial glia upregulate nidogen expression when Notch signaling is active.

DISCUSSION

Transient expression of Notch1 during neural development promotes survival and maintenance of radial glial NSC and suppresses or at least delays their differentiation (Androutsellis-Theotokis et al., 2006; Gaiano et al., 2000; Hitoshi et al., 2002; Tanigaki et al., 2001). In this study, we have focused on the consequences of persistent expression of actNotch1 in NPC and found that stable expressors could only be obtained when actNotch1 was co-expressed with v-myc. Transient upregulation of actNotch1 induced expression of BLBP, promoted the radial glia phenotype and suppressed cell differentiation (Anthony et al., 2005; Gaiano et al., 2000). Isolation of a NPC clone stably expressing actNotch1 (NL2.3-4) demonstrated that persistent expression maintained the radial glial phenotype both *in vitro* and *in vivo* and inhibited differentiation (Hitoshi et al., 2002).

Persistent expression of actNotch1 in NL2.3-4 inhibited differentiation induced by withdrawal of FGF2 and addition of serum (Li et al., 2004, Fig. 4). Although GFAP expression was induced in both NL2.3-4 and L2.3, the L2.3 cells changed morphology to yield cells resembling astrocytes, while the NL2.3-4 cells retained their bipolar shape indicating incomplete differentiation (Fig. 4A). Expression of GFAP itself is not sufficient criteria for determining differentiation (Bauer et al., 2007) and it is expressed by adult NSCs (Alvarez-Buylla et al., 2002). In contrast to L2.3 cells that tend with increasing passage in culture to decrease expression of BLBP and increase expression of the glial-restricted precursor markers A2B5 and 4D4 (Li et al., 2004), NL2.3-4 cells maintained robust BLBP expression and did not express these markers, indicating that actNotch1 can suppress neural cell differentiation. Interestingly, the helix-loop-helix protein Id4 was one of the most highly induced genes by expression of actNotch1 (Supplemental Table 1), and these genes may interact genetically (Liu and Harland, 2003). Id4 is required for correct timing of neural differentiation (Bedford et al., 2005) and its overexpression in oligodendrocyte progenitors prevents differentiation (Marin-Husstege et al., 2006). These properties of Id4 suggest that it may act to suppress differentiation of the NL2.3-4 cells.

Among the genes that differed most significantly between clones that do or do not express actNotch1 were several adhesion molecules with predicted interactions with laminin including nidogen. The function of nidogen is still poorly understood, although it has been known for some time that it binds to laminin (Yurchenco and Schittny, 1990). We found that radial glia secrete nidogen and that nidogen potentiated adhesion of these cells to laminin. It has been proposed that nidogen at the pial surface is deposited by meningeal cells (Halfter et al., 2002) but the present results suggest that the radial glia themselves may also contribute to its accumulation. Notch1 activation is typically transient during development suggesting that it may induce transient changes in cell adhesion and signaling. Transient nidogen expression very early during development of radial glia acting synergistically with laminin might provide functional significance to the enigmatic low levels of laminin that have been detected along radial glia (Liesi and Silver, 1988). However, *in situ* hybridization at E16 in the mouse did not show signals for nidogen in radial glia (W. Halfter, personal communication). Thus, additional studies are needed to determine whether nidogen is expressed by radial glia *in vivo* during early stages of neural development.

Several other proteins related to cell adhesion were upregulated dramatically in the cells expressing actNotch1 and they may be involved in functions of radial glia. One of these is ALCAM/DM-GRASP/SC-1/BEN, which is a member of the Ig superfamily that has been implicated in axon guidance and is expressed on Bergmann glia (Mothe and Brown, 2002); it may play a key role in nonradial cell migration during brain development (Heffron and Golden, 2000). N-CAM expression

was also upregulated by actNotch1, and it is expressed on radial glia in developing dorsal cortex (Li and Grumet, 2007). CD44 is a receptor for hyaluronic acid implicated in axon guidance across the optic chiasm, which is composed of specialized radial glial cells (Marcus and Mason, 1995; Sretavan et al., 1994). A subpopulation of astrocyte-restricted precursors express CD44 (Liu et al., 2004), consistent with our results that the actNotch1 expressing cells responded to differentiation conditions by expressing the astroglial marker GFAP. Little is known about nell2 but its mRNA has been detected in E12 ventricular zone, where cell bodies of radial glia are located (Kim et al., 2002). Thus, most of the cell adhesion genes that were identified are associated with radial glia, including several proteoglycans that contribute to brain extracellular matrix (Morgenstern et al., 2002).

Expression of Notch1 itself was also upregulated in the NL2.3-4 cells expressing actNotch1 by comparison to L2.3 cells (Supplemental Table 1). This is consistent with the known action of Notch in lateral inhibition where transient activation of Notch leads to its upregulation followed by downregulation and cell differentiation (Lai, 2004). The Notch ligands δ-like homolog and jagged1 were downregulated in NL2.3-4 vs. L2.3, consistent with the upregulation of Notch (Supplemental Table 1). Notch1 expression in the CNS is normally transient and is typically followed by differentiation into neurons and glia (Anthony et al., 2005; Gaiano et al., 2000; Temple, 2001). The resistance of NL2.3-4 cells to differentiate is also expected with the persistent activation of Notch signaling.

Notch1 behaves as a tumor suppressor in neuroepithelial cells (Lefort and Dotto, 2004), although it induces tumors in hematopoietic progenitors (Vilimas et al., 2007). Although Notch1 expression may be required for survival of gliomas, its expression correlates inversely with glioma grade (Purow et al., 2005). We were unable to obtain stable clones from NPC after inducing actNotch1 expression except in cells that stably expressed v-myc (e.g., NL2.3-4). Interestingly, although high levels of actNotch1 expression were observed after transient infection, the stable clones that were obtained only expressed moderate levels, suggesting that high levels of Notch1 may inhibit cell growth and prevent clonal expansion as suggested (Lowell et al., 2006). This is also consistent with the observation that Notch1 increased the fraction of BLBP+/RC2+/BrdU- quiescent radial glia, suggesting that it inhibits cell proliferation (Gaiano et al., 2000). ActNotch1 expression slowed cell cycle progression slightly in NL2.3-4 cells by comparison to the parental L2.3 cells (Li H, Chang Y, and Grumet M, unpublished observations), consistent with a tumor-suppressive action (Fan et al., 2004). Transplantation studies using L2.3 indicated that they form masses resembling tumors whereas the NL2.3-4 cells derived from them, which express actNotch1, migrated robustly *in vivo* and showed no indication of tumorigenicity 1 month after transplantation (see Fig. 5). Moreover, the NL2.3-4 cells continued to express nestin after transplantation into the spinal cord, in contrast to other NPC with or without v-myc that showed significant astroglial differentiation (Cao et al., 2001; Hasegawa et al., 2005). Whereas actNotch1 may be a tumor suppressor in NPC, Notch2 has the opposite effect (Fan et al., 2004).

Prolonged expression of actNotch1 in NPC maintains their radial glial properties, suggesting that they may promote recovery following spinal cord injury by modulating the environment to one that more closely resembles the developing nervous system, where regeneration even in mammals is more robust than it is in adults. This suppression of differentiation may limit reactive astrogliosis, which is not beneficial in the injured spinal cord (Silver and Miller, 2004). Moreover, the tumor-suppressive properties of actNotch1 at least in neural cells may provide an extra measure of safety that may make its expression in NSC or NPC favorable for long-term cell transplantation. Additional studies are needed to analyze this in longer-term transplants to verify that tumors do not form.

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