



Visualization of spatiotemporal activation of Notch signaling: Live monitoring and significance in neural development

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Abstract

Notch signaling plays various key roles in cell fate determination during CNS development in a context-dependent fashion. However, its precise physiological role and the localization of its target cells remain unclear. To address this issue, we developed a new reporter system for assessing the RBP-J-mediated activation of Notch signaling target genes in living cells and tissues using a fluorescent protein Venus. Our reporter system revealed that Notch signaling is selectively activated in neurosphere-initiating multipotent neural stem cells *in vitro* and in radial glia in the embryonic forebrain *in vivo*. Furthermore, the activation of Notch signaling occurs during gliogenesis and is required in the early stage of astroglial development. Consistent with these findings, the persistent activation of Notch signaling inhibits the differentiation of GFAP-positive astrocytes. Thus, the development of our RBP-J-dependent live reporter system, which is activated upon Notch activation, together with a stage-dependent gain-of-function analysis allowed us to gain further insight into the complexity of Notch signaling in mammalian CNS development.

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Introduction

Notch signaling plays a pivotal role in the organogenesis of many developing tissues in both vertebrates and invertebrates and controls cell fates through local cellular interactions; cells expressing Notch ligands communicate with neighboring cells that express Notch receptors (Artavanis-Tsakonas et al., 1999). Following the binding of the Notch receptor extracellular domain of Notch to its ligand, Delta/Serrate/lag-2 (DSL), the Notch receptor intracellular domain (NICD) (Weinmaster et al., 1991) is

cleaved by presenilin/ γ -secretase (Selkoe and Kopan, 2003). NICD is translocated into the nucleus and assembled into a complex with the DNA binding transcription factor, CSL (CBF1/RBP-J in mammals, suppressor of hairless in *Drosophila* and Lag-1 in *Caenorhabditis elegans*) (Kato et al., 1997), and the co-activator Lag3/Mastermind (Petcherski and Kimble, 2000). This complex then binds to specific *cis*-regulatory DNA sequences via CSL and induces the transcriptional activation of the target genes of the Notch signaling pathway, probably by recruiting p300 and other proteins into the transcriptional activation complex (Wu et al., 2000; Fryer et al., 2002; Wu et al., 2002; Maillard et al., 2003). In the absence of NICD, CSL can recruit repressor complexes to the *cis*-regulatory sequences of Notch target genes. The activation of Notch therefore acts as a switch that reverses the

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transcriptional repression of its target genes (Barolo et al., 2002). In mammals, the basic helix–loop–helix (bHLH) genes *Hes1* and *Hes5* are considered to be primary targets of Notch because Notch activation induces the transcription of *Hes1* and *Hes5* (Kageyama and Nakanishi, 1997). Consistent with this hypothesis, the regulatory regions of these genes contain several RBP-J binding sites (Jarriault et al., 1995).

Several lines of evidence now indicate that Notch1-mediated signaling pathways play crucial roles in mammalian CNS development, including the maintenance of neural stem cell/progenitor states, the inhibition of neuronal cell commitment (Nye et al., 1994; Nakamura et al., 2000), and the promotion of astroglial fates (Gaiano and Fishell, 2002; Grandbarbe et al., 2003). However, the reported findings on the role of Notch signaling in neural development, especially in gliogenesis, remain controversial (Tanigaki et al., 2001; Hitoshi et al., 2002; Grandbarbe et al., 2003). The conflicting conclusions of previous reports can likely be partly attributed to the following facts: (i) functional redundancies in Notch-signal-related molecules and the early embryonic lethality of models carrying mutant forms (Ishibashi et al., 1995; de la Pompa et al., 1997; Ohtsuka et al., 1999) have prevented definitive conclusions in loss-of-function studies, and (ii) gain-of-function studies using an activated form of Notch sometimes have different outcomes, depending on the experimental conditions, because of the context-dependent actions of Notch-signaling.

As a first step towards addressing the complex role of Notch signaling during CNS development, we recently examined the *in situ* mapping of Notch1 activation using a specific antibody that recognizes the processed form of the intracellular domain of Notch1 after it has been cleaved by the activity of presenilin/ γ -secretase (Tokunaga et al., 2004). However, this experimental system requires the cells to be fixed for the immunohistochemical analysis and cannot, therefore, be used to detect Notch1 activation in living tissue or to analyze the fate decision of cells in which Notch signaling had been activated in a prospective fashion.

To overcome these limitations, the initial goal of the present study was to generate a versatile live reporter system to detect the activation of Notch targets that are mediated by RBP-J. To establish such a reporter system, both a *cis*-regulatory element of the Notch target gene and a fluorescent protein are needed. For the *cis*-regulatory element of the Notch target gene, *Hes1* and *Hes5* are the strongest candidates because they are known targets of Notch signaling and they are expressed in the central nervous system. To exclude the possibility that the reporter gene transactivation was regulated by a signaling pathway other than RBP-J-mediated Notch activation, we utilized an RBP-J-dependent regulatory element in the endogenous target and we also employed a mutant promoter lacking the RBP-J recognition motif. We took

advantage of the 195-bp promoter region of the *Hes1* gene (one of the endogenous target genes of Notch) that includes two RBP-J binding sites and several other elements known as E and N boxes (Sasai et al., 1992; Takebayashi et al., 1994; Jarriault et al., 1995). Since other *cis*-regulatory element may exist in this region, we also utilized a mutated form of the *Hes1* promoter (*Hes1*-pAmBm), in which two RBP-J binding sites are disrupted, to evaluate RBP-J-dependent *Hes1* promoter transactivation. We also developed a reporter system using an artificial promoter, TP-1, that includes 12 RBP-J binding sites and a minimum promoter (Kato et al., 1997). For live monitoring, a recently reported fluorescent protein Venus (and its destabilized form dVenus) (Nagai et al., 2002; Nagai et al., unpublished results) was utilized; this protein is an enhanced yellow fluorescent protein (EYFP) variant that exhibits fast and efficient maturation, a strong fluorescence intensity, and a tolerance to acidosis and Cl^- exposure (see the first paragraph of the Results section for further details).

In the present study, we clearly showed that our reporter system was sensitive enough to monitor the activated status of Notch signaling in living cells; the present reporter system could also be utilized in studies on the developing CNS as well as studies on the maintenance and differentiation of neural stem cells. Importantly, the new reporter system, in combination with a conventional stage-dependent gain-of-function study, enabled the dynamics of Notch signaling in cell-fate decisions during CNS development to be examined in detail.

Materials and methods

Gene construction of the reporter system

Venus and dVenus cDNA inserts (Nagai et al., 2002; Nagai et al., unpublished results) were substituted with pEGFP-1 and pEGFP-N1 (Clontech Laboratories) to generate pVenus-1/pdVenus-1 and pVenus/dVenus-N1, respectively. The 195-bp promoter region of *Hes1*-luciferase (Jarriault et al., 1995) was subcloned into pVenus/dVenus-N1 and pVenus-1/pdVenus-1 to generate *Hes1*p-Venus/dVenus, respectively. We introduced mutations into both of the two RBP-J binding sites present within the 195-bp *Hes1* promoter region using site-directed mutagenesis to eliminate RBP-J binding activity, based on a strategy used in a previous study (Jarriault et al., 1995), and then constructed a *Hes1*pAmBm-dVenus/Venus variant. TP-1-Venus/dVenus and rBG-Venus/dVenus were generated by inserting the promoter region of TP-1 luciferase (Kato et al., 1997) and the minimal promoter region of TP-1 luciferase into pVenus/dVenus-1. To generate the mouse GFAP promoter-EGFP construct, a 2.5-kb fragment of the mouse GFAP promoter (Miura et al., 1990) was subcloned into pEGFP-1 (Clontech

Laboratories). To construct the lentivirus vectors, each *cis*-regulatory element and dVenus/Venus fragment was subcloned into a self-inactivating (SIN) vector construct (pCS-CG-PRE) (Miyoshi et al., 1999; Tahara-Hanaoka et al., 2002). In addition, the Notch1 Δ E insert (Yamamoto et al., 2001b) was subcloned into the pEF-BOS expression vector (Mizushima and Nagata, 1990). The expression vector containing mouse full-length Notch1 was kindly provided by Dr. J. Nye. The expression vector containing a dominant-negative form of RBP-J has been described previously (Chung et al., 1994). The pMX-IRES-EGFP (IE) and NotchIC-pMXIE plasmids have also been reported (Hitoshi et al., 2002). Both EGFP and monomeric red fluorescent protein (mRFP) were subcloned into the mammalian expression vector pCXN2 (Niwa et al., 1991).

Cell culture

Telencephalons were dissected from ICR E14 mice, and after dissociation the cells were cultured via the neurosphere method as previously described (Reynolds et al., 1992; Kawaguchi et al., 2001; Shimazaki et al., 2001). For lentivirus transduction, a virus solution (with an MOI of 50) was added to the medium 3 h after the seeding of the cultures. For neurosphere formation, cells were cultured for 7–10 days. For γ -secretase inhibitor treatment of the primary neurospheres, a stock of L-685458 (Bachem) in DMSO was added to the medium at a concentration of 1 μ M. Control treatments were performed using equivalent volumes of DMSO alone. The HEK293T cell line and NIH3T3 cell line were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum at 37°C in 5% CO₂.

Transient expression assay

The HEK293T cells were transfected with both 1 μ g of the reporter constructs and 2 μ g of the cDNA inserts in a pEF-BOS vector using Lipofectamine Plus reagent (Gibco-BRL). As for combined transfection of Notch Δ E and RBP-J dn, we mixed each of these constructs at a ratio of 1:2 (2 μ g of DNA in total). The cells were incubated for a further 24 h after transfection, followed by cell lysis and the determination of fluorescence intensities using a CytoFluor 4000 (PerSeptive Biosystems). The neurospheres were transfected with either pMXIE or NotchIC-pMXIE using a Nucleofector device (Amaxa), following the manufacturer's guidelines for the transfection of neural stem cells.

Cell preparation for flow cytometry and cell sorting

Flow cytometric analysis and cell sorting were performed using either a triple laser FACSVantage SE (Becton-Dickinson) or a MOFLO (DakoCytomation), as described previously (Kawaguchi et al., 2001). The cells in the viable

gate were sorted into MHM medium and counted. A 50:49:1 cocktail of the resulting cell suspension, neurosphere-conditioned medium and B27 supplement (Gibco-BRL) was plated into each well of a 96-well plate at a density of 5 cells/ μ l (1000 cells/well), and the number of resulting neurospheres was counted approximately 14 days later. After the mechanical dissociation of each neurosphere into single cells, each pool of cells derived from a single neurosphere was cultured again for secondary neurosphere formation. For the differentiation assays, neurospheres that had been cultured for 10–14 days *in vitro* were plated onto poly-L-ornithine (PO)-coated coverslips and cultured for another 5–7 days in MHM medium.

In vivo electroporation

Pregnant ICR mice were purchased from Charles River, Japan; all animals were handled in accordance with the guidelines of Keio University. Both embryonic *ex utero* surgery and electroporation methods were performed as previously described (Muneoka et al., 1986; Saito and Nakatsuji, 2001). For the reporter analysis, DNA solutions (5 mg/ml in PBS containing FAST Green, Reporter: pCXN2-mRFP; 5:1) were injected into the lateral ventricle of E14 telencephalons. Electronic pulses of 25 V were charged eight times at 950-ms intervals using a square-pulse electroporator (CUY21EDIT; Nepa Gene Company). For the electroporation experiments in postnatal mice, P0 mice were anesthetized on ice and the injection and electroporation procedures were performed as described above except that an electric pulse setting of 50 V was used for the postnatal mice.

Lentivirus production

Lentiviral vectors, pseudotyped with the vesicular stomatitis virus G glycoprotein (VSV-G), were generated as previously described (Miyoshi et al., 1999).

Immunocytochemistry and immunohistochemistry

Immunocytochemical analysis of the cultured cells was performed as previously described (Kawaguchi et al., 2001). The primary antibodies used in this study were mouse monoclonal anti-MAP2 (mouse IgG, 1:500; Sigma), anti- β -III-tubulin (mouse IgG, 1:500; Sigma), anti-GFAP (rabbit IgG, 1:400; DAKO and mouse IgG, 1:400; Sigma), anti-O4 (mouse IgM, 1:2000; Chemicon), anti-GFP (rabbit IgG, 1:500; MBL), and rabbit anti-cleaved-Notch1 (actN1) (rabbit IgG, 1:500; Cell Signaling). For double staining, the cells were also incubated for 1 h with mixtures containing the secondary antibodies: Alexa Fluor 350-, 488-, 568, or 633-conjugated goat anti-mouse and anti-rabbit IgG (Molecular Probes). Samples were observed under a fluorescence microscope (Zeiss Axiophoto) equipped with the appropriate epifluorescence filters. Immuno-

histochemistry was performed as previously described (Kawaguchi et al., 2001; Tokunaga et al., 2004). Optical sections were viewed using a scanning laser confocal imaging system (Zeiss LSM510). To quantify the immunohistochemical data, we counted the cells using a Z-slice function.

Preparation of cell lysates and immunoblotting

Cells were centrifuged at $100 \times g$ for 5 min, and the resulting cell pellets were lysed in $1 \times$ SDS–PAGE sample buffer. The subsequent immunoblotting procedure was performed as previously described (Tokunaga et al., 2004).

Results

Generation of a gene reporter system for activated Notch signaling

To visualize the activated status of Notch signaling, we developed a live reporter system based on the promoter region of a Notch signaling target and the Venus fluorescent protein (Fig. 1A). To detect the status of Notch signaling with a high time resolution, we incorporated a PEST sequence (Li et al., 1998), which is correlated with protein degradation, at the C terminus of Venus to create the fusion protein, dVenus (Nagai et al., unpublished). Our reporter

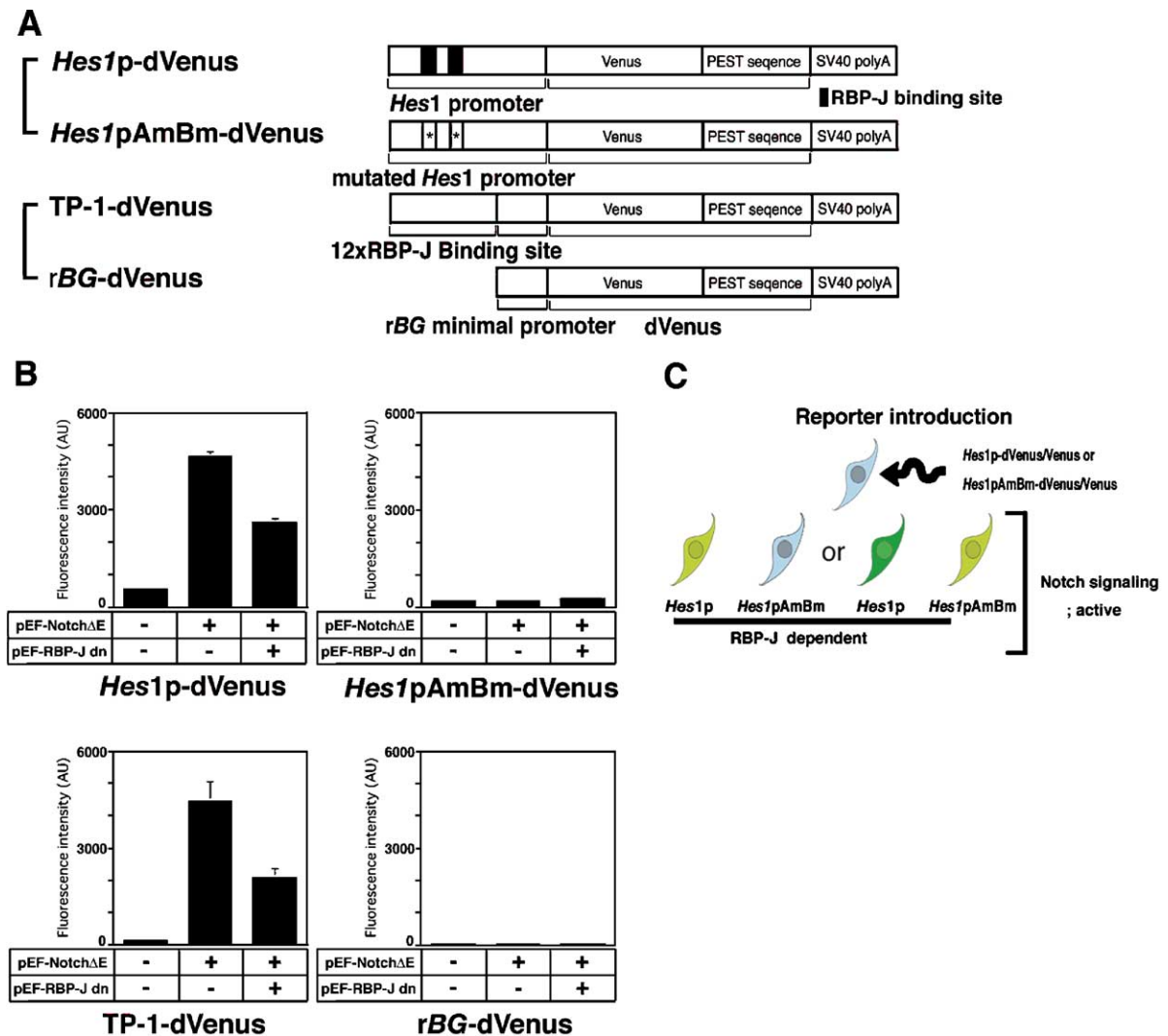


Fig. 1. Reporter systems for the detection of Notch activation. (A) Schematic representation of the reporter system (*represents disrupted RBP-J binding sites). (B) Notch signaling-dependent reporter activity according to fluorescent intensities assayed by transient transfection studies. (C) Basis for the detection of the RBP-J-dependent activation of Notch signaling in our analysis. Wild-type and mutated *Hes1* promoter-driven Venus fluorescence reactivity was compared. In the presence of Notch activation, the activity of the wild-type *Hes1* promoter should be stronger than that of the mutant promoter. Hence, the RBP-J-dependent activation of the Notch signaling target genes should be detectable. This was also the case for the functional analysis of Venus-positive cells. When the Venus-positive cell fractions driven by each promoter showed different neural cell characteristics, we regarded the differences between the Venus-positive cell fractions to reflect differences in the RBP-J-dependent Notch activity and function in those cell fractions. When no difference in the selectivity or reactivity of the two reporter-positive cell fractions was observed, we concluded that significant RBP-J-dependent activation was not involved in the context or cell-fate decision.

system consisted of two reporter sets based on both the 195-bp *Hes1* promoter region (Jarriault et al., 1995) (*Hes1p*-dVenus/Venus and *Hes1pAmBm*-dVenus/Venus) and an artificial promoter including 12 tandem RBP-J binding sequences plus a minimum promoter or a minimum promoter alone (TP-1-dVenus/Venus and rBG-dVenus/Venus) (Kato et al., 1997).

We first confirmed the reporter's sensitivity to the activation of Notch signaling in transient experiments using the combination of expression vectors shown in Fig. 1B. The intensity of the *Hes1p*-dVenus- and TP-1-dVenus-driven fluorescent signals was stronger (>8-fold) in the presence of Notch1 Δ E than in the presence of PEF-BOS, whereas the reporter experiments using either *Hes1pAmBm*-dVenus or rBG-dVenus constructs showed no response to Notch1 Δ E. When both pEF-Notch1 Δ E and the dominant-negative form of RBP-J (pEF-RBP-J dn) were co-expressed, the reporter fluorescence was less intense in cells expressing *Hes1p*-dVenus or TP-1 dVenus. Hence, our reporter system was strongly correlated with Notch signaling activity levels. Furthermore, *Hes1p*-dVenus and TP-1-Venus also showed Notch activity-dependent response *in vitro*; we could detect significant reporter activity in the presence of activated Notch1, not full-length Notch1 (Supplementary Fig. 1).

Next, we attempted to characterize the spatial and temporal profiles of RBP-J-dependent Notch activation to compare the differences between reporter sensitivities in the cells and tissue types analyzed in this study. We compared the Venus-fluorescence reactivity driven by the wild-type and mutated *Hes1* promoters, because the main purpose of this study was to examine the endogenous activation of Notch signaling during CNS development. Consistently, we could observe a relationship between active Notch1 immunoreactivity and reporter activity driven by *Hes1* promoter *in vivo* (Supplementary Fig. 2). Thus, the *cis*-regulatory element should be derived from the promoter region of endogenous Notch target genes.

The criteria for the detection of Notch activation in the experiment described below is described in Fig. 1C. Wild-type and mutated *Hes1* promoter-driven Venus fluorescence reactivity was compared. We need to compare this promoter set because the 195-bp *Hes1* promoter may contain target sequences for other signaling pathways. Thus, we can estimate the occurrence of RBP-J-dependent transactivation by observing the difference in reporter transactivation between wild-type and an RBP-J binding site-mutated promoter. In the presence of Notch activation, the activity of the wild-type *Hes1* promoter should be stronger than that of the mutant promoter. Hence, RBP-J-dependent activation of the Notch signaling target genes should be detectable (Fig. 1C). Regarding the functional assay, the Venus-positive cell fractions driven by the wild-type and mutant *Hes1* promoters exhibited different characteristics from those of neural cells; thus, the difference for each Venus-positive cell fraction reflects RBP-J-dependent Notch

activity and function. We also examined the reporter activity of TP-1-dVenus/Venus and its control rBG-dVenus/Venus (Kato et al., 1997) with regard to their regulatory elements to confirm the occurrence of RBP-J-dependent Notch activation.

Enrichment of neurosphere-initiating cells based on Notch signaling activity

Previous studies have shown that Notch signaling activity is essential for the self-renewal of neural stem cells (Ishibashi et al., 1994; Ohtsuka et al., 1999; Nakamura et al., 2000; Hitoshi et al., 2002). Hence, we further investigated this relationship by monitoring embryonic neural cells using our reporter system. For this purpose, lentiviral vector constructs carrying each of our reporter gene inserts were generated, since lentiviral vectors are known to facilitate highly efficient and stable transduction into stem cells (Miyoshi et al., 1999). To directly determine the correlation between the reporter transactivation and the cell properties, we subjected embryonic brain cells that had been transduced with reporter lentivirus to fluorescence-activated cell sorting (FACS). We could observe the dVenus-derived reporter activity on embryonic cortical cells transduced by the lentiviral vector under physiological conditions (Supplementary Fig. 3A). To confirm the specificity of the response of reporter system, we treated reporter-carrying cells with a γ -secretase inhibitor, which was known to block Notch activation via suppression of the cleavage of the intracellular domain of Notch (Martys-Zage et al., 2000) (Supplementary Figs. 3B and C). We could see a reduction in the number of Venus-positive cell fraction in wild-type *Hes1* promoter Venus-transduced cells by FACS analysis, indicating that wild-type 195-bp *Hes1* promoter faithfully mimic Notch activity (Supplementary Figs. 3B and C, left panel, respectively). Therefore, our lentiviral reporter system was also effective for detecting the activation of Notch signaling. Regarding to the results of our experiments using mutant *Hes1* promoter, we could not detect any significant response to Notch activation in the *Hes1AmBm* reporter-transduced cells. Although there appeared to be a tendency towards increase in the activity of the *Hes1AmBm* reporter in response to a dose of γ -secretase inhibitor, we could not detect any statistically significant difference (Supplementary Figs. 3B and C, right panel, respectively). However, it is noteworthy that the activity of the *Hes1pAmBm* reporter activity was insensitive to the Notch activity. In this sense, we could not rule out the possibility that either non-RBP-J-mediated or non-Notch signaling are reported by our reporter constructs. Therefore, to unveil the circumstances of Notch activation, it is important to compare the response or distribution of wild-type and mutant *Hes1* promoter transactivation.

Cortical cells, derived from E14 embryos, were cultured to produce neurospheres using a selective culture method in which the neural precursors selectively survived and

proliferated in response to EGF and FGF-2 (Reynolds et al., 1992). We then introduced pSIN-*Hes1p*-dVenus into these primary neurospheres, which were subsequently transferred to pre-coated cover slips and cultured in the presence of growth factors. In combination with an immunocytochemical analysis, selective *Hes1p*-dVenus-derived fluorescence was observed in Nestin-positive neural progenitors (Fig. 2A, upper panel) but rarely detected in β -III-tubulin-positive neurons (Fig. 2A, lower panel). Next, to further characterize the correlation between Notch signaling activation and stem cell maintenance, we performed a neurosphere assay and determined the number of neurosphere-initiating cells using a method described in our previous report that utilized the *Nestin*-EGFP reporter gene (Kawaguchi et al., 2001). Each

reporter construct was introduced into dissociated E14 cortical cells, and the fluorescent cells were sorted by flow cytometry after 24 h. The sorted cells were then cultured at a cell density of 5 cells/ μ l, which is below the cell density at which virtually all neurospheres are clonal cells (Hulspas et al., 1997). At 10–14 days, the sphere numbers were counted per 1000 reporter-gene-expressing cells and were found to be 28.9 ± 6.8 and 8.5 ± 1.50 in *Hes1p*-dVenus-expressing and *Hes1pAmBm*-dVenus-expressing cells, respectively ($P < 0.05$). In addition, the efficacy of neurosphere formation from *Hes1pAmBm*-dVenus-expressing cells did not differ from that of control EF-dVenus-expressing cells ($P > 0.05$). The number of sphere-initiating cells was therefore significantly higher among the *Hes1p*-dVenus-expressing population, compared to EF-

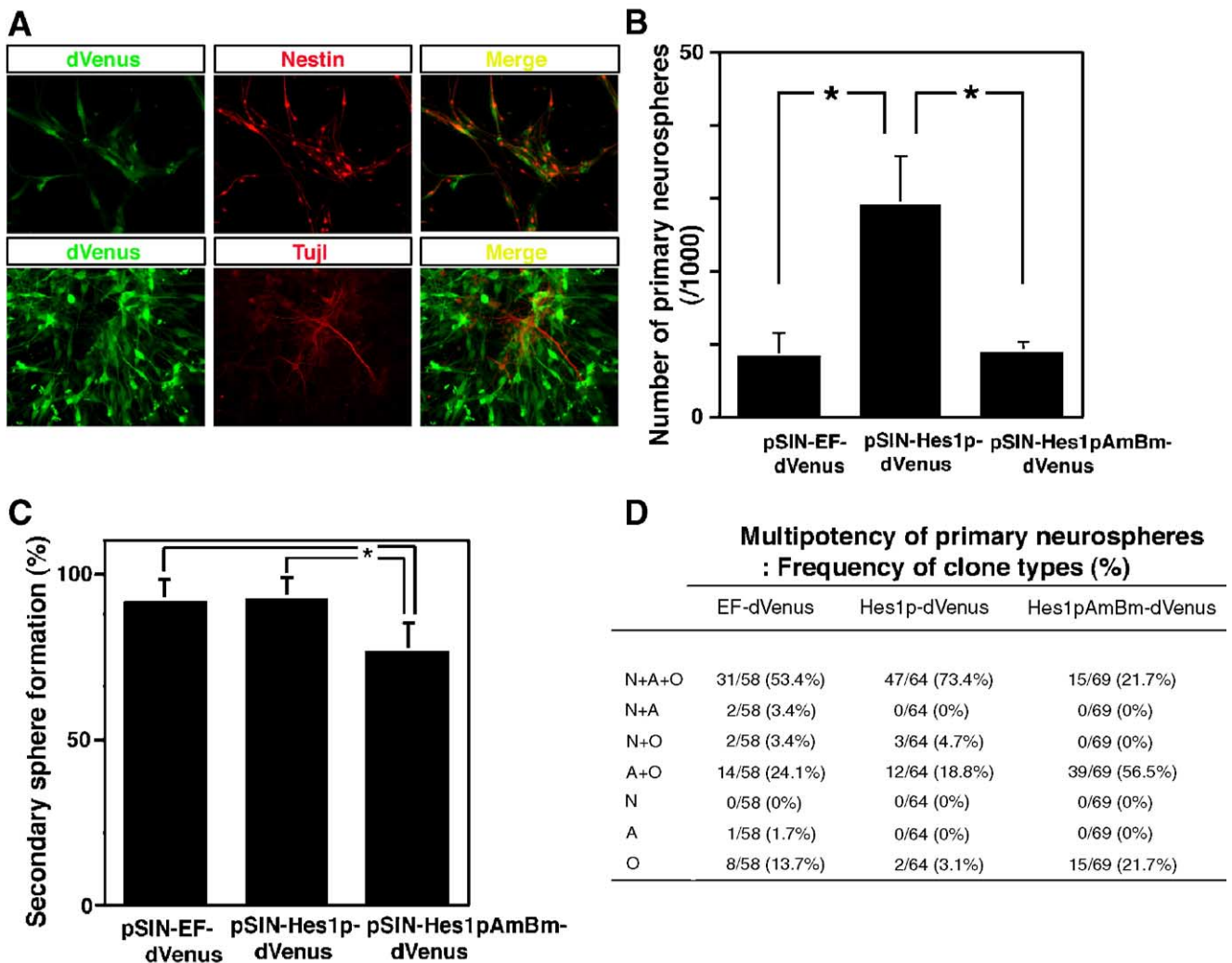


Fig. 2. Role of neuronal progenitors maintenance during mouse embryonic CNS development. (A) *Hes1p*-driven signals were selectively observed in mouse neural progenitors. Most of the Venus-positive cells were Nestin-positive neural progenitors (upper panel), whereas Venus-fluorescence was not observed in β -III-tubulin-positive neurons (lower panel). (B) Comparison of the efficiency of the neurosphere-formation potency of reporter-responsive cells. Neurosphere-initiating efficiency was significantly increased in *Hes1p*-dVenus-positive cells, compared to that in either *Hes1pAmBm*- or EF-dVenus-positive cells ($n = 4$ in EF, $n = 7$ in *Hes1p* and *Hes1pAmBm*) ($*P < 0.05$). (C) Secondary neurosphere formation efficacy. A reduction in secondary sphere formation was observed in primary neurospheres derived from *Hes1pAmBm*-dVenus-expressing cells ($*P < 0.05$). (D) Multilineage potentials were confirmed by immunocytochemical analysis of the neurons (anti- β -III-tubulin), astrocytes (anti-GFAP), and oligodendrocytes (anti-O4). The number of clones consisting of neurons (N), astrocytes (A), or oligodendrocytes (O) is shown as a percentage of the total number of clones.

dVenus- and *Hes1pAmBm*-dVenus-expressing cells. To examine the capacity of the sorted Venus-positive cells for self-renewal, the secondary neurosphere-forming efficiency of each primary neurosphere was compared to estimate the frequency of self-renewing cell division arising from the original neurosphere-producing cell (Nakamura et al., 2000; Kawaguchi et al., 2001) (Fig. 2C). We subsequently observed a reduction in the number of secondary neurospheres derived from *Hes1pAmBm*-dVenus-expressing cells compared to those from *Hes1p*-dVenus-expressing cells ($P < 0.05$).

We next examined whether the neurospheres generated from the reporter-responsive cells showed any disruption in their multipotent properties, since a certain percentage of neurospheres derived from committed progenitor cells was previously shown to differentiate only into specific lineages (Reynolds and Weiss, 1996). Primary neurospheres, generated after FACS-sorting, were clonally transferred onto pre-coated cover slips to obtain one neurosphere culture per well. These cells were then cultured without growth factors and processed for triple-labeled indirect immunocytochemistry after 5 days to detect the three major cell types, neurons, astrocytes, and oligodendrocytes, using anti- β -III-tubulin, anti-GFAP, and anti-O4 antibodies, respectively (Fig. 2D). More than 70% of the *Hes1p*-dVenus-derived neurospheres were multipotent and differentiated into clones containing neurons, astrocytes, and oligodendrocytes (NAO). However, about 20% of the neurospheres were bipotent, generating either neurons and oligodendrocytes (NO) or astrocytes and oligodendrocytes (AO). Furthermore, committed progenitors, which are capable of generating only restricted lineages, were rare in the neurospheres derived from *Hes1p*-Venus-positive cells (3.1%), but the differentiation capacity of the neurospheres derived from *Hes1pAmBm*-dVenus-positive cells showed a reduction in tripotent neurosphere formation and an increase in bipotent or monopotent neurosphere formation. The above results suggest that Notch signaling activity is positively correlated with both self-renewal and the multipotency of neural progenitors in an RBP-J-dependent manner, as shown by our Notch-dependent reporter system (Fig. 1C).

RBP-J-dependent Notch activation is observed in radial glia

We examined the in vivo distribution of reporter activity using an electroporation co-transfection method in the cortical ventricular zone (VZ) of E14 mice (Fig. 3A). The efficiency of co-transfection was confirmed using EGFP and monomeric red fluorescent protein (mRFP) constructs, driven by a pCXN2 promoter (>95%) (data not shown). Therefore, we examined the cellular distribution of positive reporter activity by analyzing the localization of mRFP-positive cells at 48 h post-transfection, when most of the transfected cells were located in the VZ intermediate zone (IZ) region. Very few positive cells were observed using the

Hes1p-dVenus reporter gene. Thus, to substantially increase the number of fluorescence-positive cells, a Venus reporter gene, instead of dVenus, was used in the series of experiments shown in Fig. 3. Venus-derived signals, from the wild-type *Hes1* promoter, were mostly observed in the VZ regions and were morphologically characterized by the extension of radial fibers from the ventricular to the pial surface (Figs. 3B and F). These *Hes1p*-Venus-positive cells located in the VZ were also positive for Nestin (Supplementary Fig. 4A).

On the other hand, *Hes1pAmBm* reporter activity was also observed in the VZ, SVZ, or IZ. Most of these *Hes1pAmBm*-Venus-positive cells were located in the SVZ-IZ (Figs. 3C and F; Supplementary Figs. 4C and D). However, these cells did not possess apparent radial fibers. In the same series of experiments, another reporter set, utilizing TP-1, was introduced by electroporation (Figs. 3D and E). The reporter activity of TP-1 was also observed in cells exhibiting radial fibers (Fig. 3D), which were also positive for Nestin (Supplementary Fig. 4E); these results are consistent with those obtained using *Hes1p*-Venus (Fig. 3B). On the other hand, a reporter carrying only the rat β -globin minimal promoter (rBG-Venus) did not exhibit fluorescence (Fig. 3E). Taken together, it is likely that the wild-type *Hes1p* and TP-1 reporters are activated in the radial glia, which could be relevant to the results of our recent report showing the selective localization of active Notch1 immunostaining in the radial glia in this context (Tokunaga et al., 2004).

Notch signaling functions in astroglial commitment, but not in astroglial maturation

In view of our previous results showing a negative correlation between immunoreactivity to GFAP and the activated form of Notch1 (Tokunaga et al., 2004), we hypothesized that Notch signaling may be down-regulated during the terminal differentiation of astroglial cells expressing GFAP. However, the exact role of Notch signaling in astroglial development remains to be elucidated. To address the role of Notch signaling in astroglial development during the early postnatal stage, transgenes expressing the intracellular domain of Notch1 (Notch1C)-IRES-GFP (Notch1C-pMXIE) or EGFP (pMXIE) were introduced by electroporation into the VZ of P0 mice brains and their effects on the generation of astroglial cell lineages were analyzed at P8. We examined the expression of the astroglial marker GFAP in the EGFP-expressing cells in the periventricular area (Fig. 4). An experiment using control EGFP vector (pMXIE) revealed that some of the EGFP-positive cells were GFAP-positive astrocytes (Figs. 4A–C). In contrast, fewer Notch1C-IRES-EGFP-expressing cells were found to be GFAP-positive astrocytes (Figs. 4D–F). These results indicate that the ectopic expression of activated Notch1 resulted in a decrease in the number of GFAP-positive astrocytes (Fig. 4G) and that the persistent

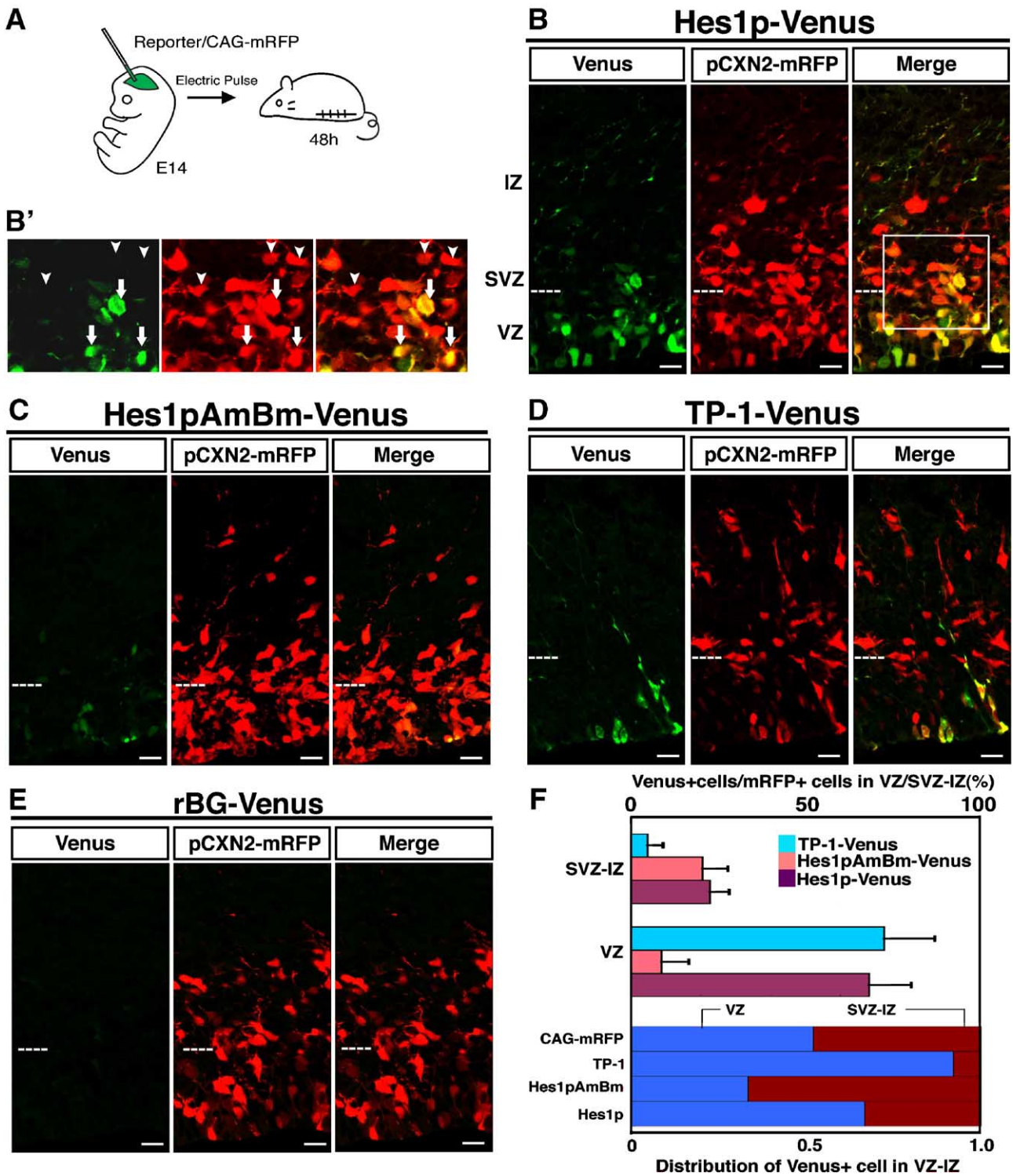


Fig. 3. Distribution of reporter activity in the developing cerebral cortex. (A) Schematic representation of the adopted in vivo electroporation procedure. (B) The distribution of *Hes1p-Venus* was mostly confined to cells in the VZ region, characterized morphologically by the presence of radial fibers. Some Venus-positive cells in the SVZ-IZ exhibited a multipolar morphology. (B') Shows a higher magnification of the boxed region of panel B. (C) Venus fluorescence, driven by *Hes1pAmBm*, was also observed in the VZ and SVZ-IZ. (D) TP-1 Venus-driven signals were observed selectively in cells of the VZ, characterized by the presence of radial fibers. Cells in the SVZ-IZ only exhibited mRFP-signals. (E) rBG-Venus-driven signals were not observed. Scale bars: 10 μ m. (F) Quantification of the distribution of cells exhibiting the activity of each reporter construct in the VZ and SVZ-IZ. We performed three independent experiments. VZ: ventricular zone; SVZ: subventricular zone; IZ; intermediate zone.

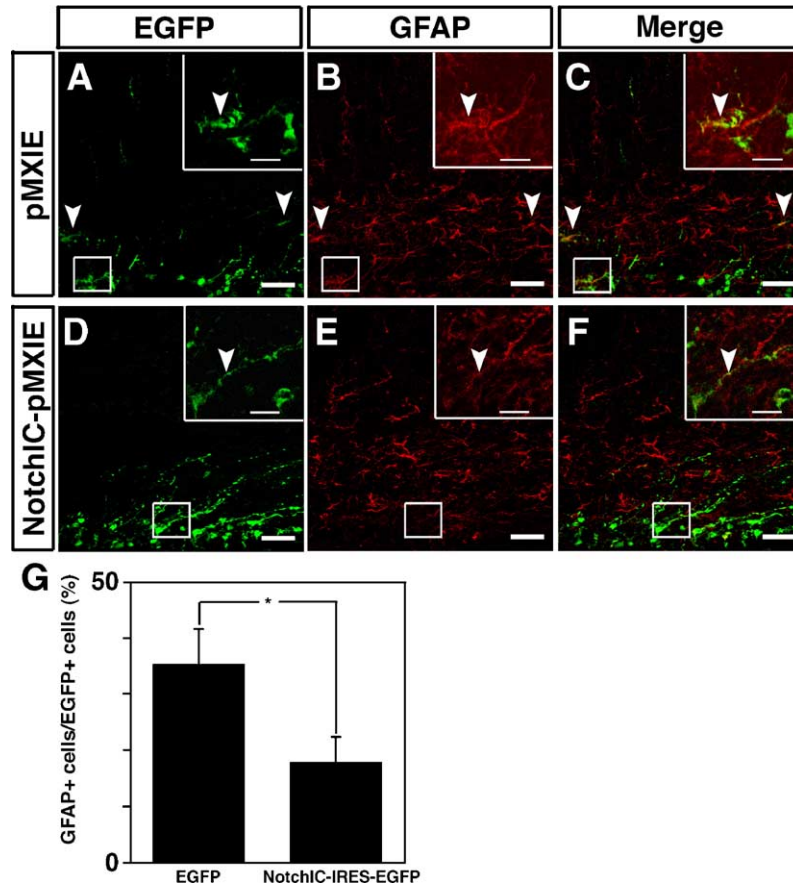


Fig. 4. Ectopic expression of the cleaved form of Notch1 in postnatal brain. The effect of Notch activation on astroglial differentiation was examined by introducing the intracellular domain of Notch1 (NotchIC)-IRES-EGFP- or control EGFP-expressing vectors in the P0 brain, and analyzing astroglial differentiation was analyzed in the P8 brain. Cells located around the VZ were examined for the expression of GFAP. (A–C) Characterization of control EGFP-vector-carrying cells. The insets represent a higher magnification of the boxed regions in each corresponding figure. (D–F) Characterization of NotchIC-IRES-EGFP-construct-expressing cells. The insets represent a higher magnification of the boxed region in each corresponding figure. (G) Quantitative analysis of the signals in these experiments (* $P < 0.05$, $n = 3$, respectively). Scale bars: A–F: 50 μm ; inset box: 20 μm .

activation of Notch signaling reduced the number of GFAP-positive astrocytes at an early postnatal stage.

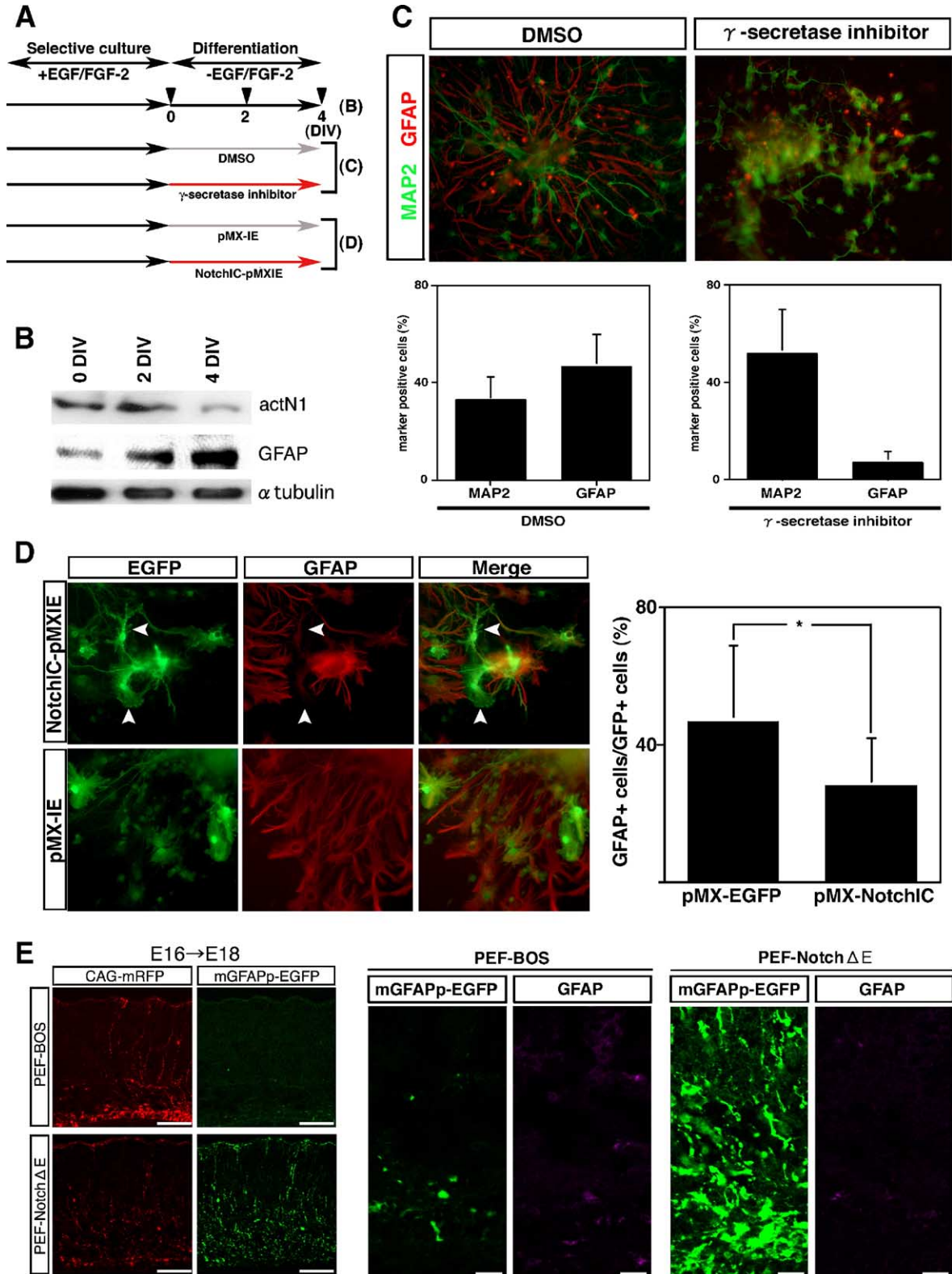
However, whether this observed phenotype occurs at the commitment or maturation of astroglial cells remains uncertain. To address this issue, we investigated the role of Notch signaling activation on astroglial development using primary neurospheres derived from E14 cortex under differentiation conditions, in which the neurospheres were cultured without growth factors on pre-coated cover slips (Fig. 5A). First, we examined the expression level of the cleaved form of Notch1 and GFAP during the differentiation of neurospheres using immunoblot studies (Fig. 5B). We found that the expression of the cleaved form of Notch1 was down-regulated, while GFAP expression was up-regulated, during the differentiation of the neurospheres. Thus, the amount of the cleaved form of Notch1 was negatively correlated with the GFAP expression level during the differentiation phase, indicating that Notch signaling may be selectively activated during astroglial commitment rather than at astroglial maturation. The significance of early stage Notch activation in astroglia generation was further confirmed by γ -secretase inhibitor treatment under differentiation condi-

tions (Fig. 5C). We treated primary neurospheres with the γ -secretase inhibitor from the beginning of the differentiation phase. The temporal expression patterns of the markers that were employed were then examined using immunocytochemistry: MAP2 for neurons, and GFAP for astrocytes. Neurospheres treated with DMSO as a negative control spontaneously differentiated into these two lineages (MAP2 positive: $33.1 \pm 9.2\%$; GFAP positive: $41.5 \pm 13.4\%$) (Fig. 5C, upper left and lower left panel). On the other hand, neurospheres treated with γ -secretase inhibitor induced an increase in MAP2-expressing cells ($52.0 \pm 18.0\%$) and a reduction in GFAP-expressing cells ($7.0 \pm 4.5\%$) (Fig. 5C, upper right and lower right panel). When γ -secretase inhibitor was added during the late phase of differentiation, i.e., 3–5 days after culturing the primary neurospheres on coated coverslips in MHM without growth factors, the number of resulting GFAP-positive astrocytes was not altered (data not shown). Thus, Notch activation is required for astroglial generation in the early phase of the differentiation of neurosphere-derived cells.

We subsequently examined whether the persistent activation of Notch signaling could suppress the generation

of GFAP-positive astrocytes in a neurosphere differentiation assay. The intracellular domain of Notch1 (NotchIC) was introduced into primary neurospheres followed by plating on pre-coated coverslips in medium without growth factors (Figs. 5A and D). We analyzed the cell type of NotchIC or

control vector-carrying cells after 4 days. A reduction in the number of GFAP-positive astrocytes generated from NotchIC-expressing cells was observed (Fig. 5D), possibly corresponding with the Notch1-persistent activation results observed during the early neonatal stage in vivo (Figs. 4D–



F). However, whether the effects of the persistent activation of Notch signaling, leading to a reduction in the number of GFAP-positive astrocytes, are active during the early phase of differentiation (e.g., commitment) or the later maturation phase remains uncertain.

Presently, a definitive, specific marker for committed astroglial progenitor cells that enables these cells to be distinguished from neural stem cells is not available (Kaneko et al., 2000; Tokunaga et al., 2004). However, to examine the effects of Notch activation during early astroglial differentiation, we took advantage of the fact that the transcriptional activation of GFAP starts before GFAP immunoreactivity becomes detectable in astroglial lineages (Riol et al., 1992; Morita et al., 1997). Interestingly, recent studies have suggested that the GFAP promoter contains an RBP-J-binding sequence and may therefore be a primary target of either Notch signaling (Ge et al., 2002) and/or RBP-J/N-CoR-mediated repression (Hermanson et al., 2002). We investigated this possibility in more detail by examining the role of Notch activation in the transcriptional regulation of the GFAP gene by constructing an EGFP reporter gene under the control of a 2.5-kb mouse GFAP promoter sequence (mGFAPp) (Miura et al., 1990) (Fig. 5E). We co-transfected the mGFAPp-EGFP reporter plasmid with either pCXN2-mRFP and pEF-BOS or pEF-Notch1 Δ E plasmids into E16 mouse brain and analyzed the expression of EGFP at E18. No obvious EGFP fluorescence, derived from mGFAPp, was subsequently detected in the control plasmid-electroporated brain tissue (Fig. 5E, upper left panel). On the other hand, EGFP fluorescence was enhanced by ectopically induced Notch activation (Fig. 5E, lower left panel). The transcriptional activation of mGFAP, however, was not accompanied by the expression of GFAP protein (Fig. 5E, right panel). Considering the findings of previous studies indicating that the transcriptional activation of the GFAP gene occurs in astrocytic precursors (Morita et al., 1997) prior to protein expression in mature cells, Notch signaling is likely to play an important role in either astroglial commitment or the early phase of astrocytic differentiation. Consistent with this hypothesis, we observed the ectopic expression of the immature astroglial marker glutamine synthetase (GS) (Akimoto et al., 1993; Tokunaga et al., 2004) in Notch1 Δ E-expressing cells in embryonic brains (data not shown).

Discussion

Generation of a reporter system for detecting the spatial and temporal activation of Notch signaling

We report here, for the first time, a method of monitoring the activation of Notch signaling in living cells. The Venus protein is an ideal fluorescent marker in reporter analyses as it overcomes the potential limitation of a relatively slow rate of fluorescence acquisition caused by the chromophore formation of fluorescent proteins (Reid and Flynn, 1997). Furthermore, to explore the transient activation of Notch signaling, we also developed a dVenus fusion protein. Regarding the *cis*-regulatory elements, a 195-bp promoter sequence of the endogenous Notch target gene, *Hes1*, was used; this sequence contains two RBP-J binding sites. Previous reports, however, have indicated that the regulation of *Hes* genes is complicated. An *in vivo* study of Notch1-deficient mice suggested that *Hes1* is regulated by a pathway other than Notch signaling, since the level of *Hes1* expression was not significantly changed in Notch1 mutant mice (de la Pompa et al., 1997). In contrast, the expression of another *Hes* family gene, *Hes5*, was reduced in Notch1 mutants, indicating that *Hes5* was responsive to Notch signaling (de la Pompa et al., 1997). Nevertheless, *Hes5* also seems to be regulated by a pathway other than Notch signaling because *Hes5* transactivation is a key step in the BMP-induced astroglial switch in neural precursors (Nakashima et al., 2001). Consistent with this contention, a Smad recognition motif has been recognized to exist in the promoter region of *Hes5* (Nakashima et al., 2001).

To overcome the potential limitations of using *Hes* genes to detect RBP-J-dependent Notch activation, we utilized the minimal length promoter region, which should respond to Notch activity, and a mutant promoter to estimate RBP-J-dependent transactivation. Thus, we employed a 195-bp promoter sequence of *Hes1* and a mutant *Hes1* promoter (*Hes1pAmBm*) carrying two disrupted RBP-J sites (Fig. 1).

In regard to Notch family members, in addition to Notch1, Notch2 and Notch3 are also expressed in neural precursor cells in the ventricular/subventricular zone of developing CNS (Higuchi et al., 1995; Prakash et al., 2002). However, in contrast to Notch1 (de la Pompa et al., 1997),

Fig. 5. Notch signaling functions in astroglial commitment, but not in astroglial maturation. (A) Scheme of experiments using primary neurospheres. E14 cortical cells were cultured in the presence of EGF and bFGF (selective culture) and primary neurospheres were generated and applied for further analysis under differentiation conditions, in which the primary neurospheres were cultured in growth factor-free medium on pre-coated coverslips. (B) Immunoblotting analysis of neurospheres cultured under differentiation conditions. (C) Requirement of Notch activation in astrocyte generation. Primary neurospheres spontaneously differentiated into MAP2-positive neurons and GFAP-positive astrocytes (upper left panel). In the presence of γ -secretase inhibitors (1 μ M), the proportion of GFAP-positive astrocytes decreased (upper right panel). Quantified data are shown in the lower panel ($n = 5$, respectively). (D) Effect of the persistent activation of Notch1 on astroglial differentiation. We transfect Notch1C carrying or control vector on differentiating neurospheres. The effect of transgene was analyzed by the GFAP expression in vector-carrying EGFP-positive cells. The repression of GFAP expression was observed in morphologically astrocytic cells (arrowhead). Quantification is shown in the right panel ($n = 3$, respectively, $*P < 0.05$). (E) *In vivo* reporter analysis using EGFP driven by the mouse GFAP promoter. GFAP immunoreactivity did not show any difference between pEF-BOS- and pEF-Notch1 Δ E-transduced brains (middle and right panel, respectively). Scale bars: 50 μ m for left panel, 10 μ m for middle and right panels.

the function of Notch2 and Notch3 in the CNS still remains largely unclear. In contrast to *Notch1*-deficient mice (de la Pompa et al., 1997), *Notch2*-deficient mutant mice did not exhibit disorganized somitogenesis, nor did they fail to properly regulate the expression of neurogenic genes, such as *Hes5* or *Mash1* (Hamada et al., 1999). Furthermore, the *Notch3*-deficient mice developed normally and the homozygous mutant adults were viable and fertile (Krebs et al., 2003), indicating that the *Notch3* gene is not essential for embryonic development. Notch1 therefore seems to be the major player of Notch signaling in developing CNS. Two pathways have been reported previously as a downstream of Notch signaling; RBP-J-dependent and RBP-J-independent pathways (Yamamoto et al., 2001a). The *RBP-J*-deficient mice showed severe phenotypes that resemble those of *Notch1*-deficient mice, indicating that the major Notch signaling pathway is mediated through RBP-J-dependent mechanisms (Swiatek et al., 1994; Oka et al., 1995). In the present study, we designed two reporter sets carrying RBP-J binding sequences. This series of studies provide a rationale for the reporter constructs used in the present study. However, we cannot rule out the possibility that Notch receptors other than Notch1 contributed to our reporter *trans*-activation.

Role of Notch signaling in the maintenance of neural progenitors

Notch signaling has been reported to play a role in the self-renewal capacity and maintenance of neural stem/progenitor cells (Gaiano et al., 2000; Nakamura et al., 2000; Ohtsuka et al., 2001; Hitoshi et al., 2002). To further investigate the underlying mechanisms of these processes, we confirmed that Notch signaling functions in the self-renewal of neural progenitors using an immunocytochemical analysis of neurospheres generated from E14 cortex tissue (Fig. 2A) and neurosphere-forming assays using FACS-sorted dVenus-derived fluorescence-positive neural cells (Fig. 2B). Within the neurospheres, Venus fluorescent protein expression, derived from the *Hes1p*-dVenus reporter, was selectively observed in Nestin-positive neural progenitors, but not in β -III-tubulin-positive neurons (Fig. 2A). Neurosphere-forming assays of FACS-sorted Venus-positive cells enabled the characteristics of cells in which Notch signaling had been activated at that particular time point to be determined (Fig. 2B). The neural cells with *Hes1* promoter activities also showed the highest level of neurosphere-forming activity, compared with cells expressing fluorescent signals under the control of either the EF or *Hes1pAmBm* promoter. Primary neurospheres derived from *Hes1p*-dVenus-positive cells also showed higher self-renewal and multipotent capacities, compared with neurospheres derived from *Hes1pAmBm*-dVenus-positive cells, suggesting that the RBP-J-dependent signal input is highly correlated with the maintenance of neural stem/progenitor cell states in the mouse CNS.

To the best of our knowledge, this is the first report documenting the live monitoring of the contribution of endogenous Notch activation to the self-renewal of neural progenitors. Our present data showing RBP-J-dependent reporter activation in the radial glia (Fig. 3 and Supplementary Fig. 4) as well as our previous report showing active Notch1 immunostaining in these cells (Tokunaga et al., 2004) lend support to the idea, because radial glia in the embryonic brain are considered to represent self-renewing neural progenitors (Malatesta et al., 2000).

Data showing the properties of cells that have been sorted according to their reporter activity and differentiation potential are shown in Fig. 2D, and the proportion of tripotent primary neurospheres generated from *Hes1pAmBm*-Venus-expressing cells was found to decrease, suggesting the selective activation of the mutant promoter in committed progenitors. However, we cannot rule out the possibility of non-RBP-J-mediated or non-Notch signaling on our mutant reporter transactivation.

Activation of Notch signaling in gliogenesis

In the present study, we addressed the astroglial function of Notch signaling by visualizing endogenous Notch activity, in a cellular context where astroglialogenesis is dominant, using several techniques, including our new reporter system, a gain-of-function analysis (Figs. 4 and 5D), and loss-of-function studies with a γ -secretase inhibitor (Fig. 5C).

Considering the results of our previous study using an antibody against activated Notch1, which showed a correlation between activated Notch1 and an astroglial progenitor marker but not the mature astrocytic marker GFAP (Tokunaga et al., 2004), we propose that Notch signaling may be selectively activated during the commitment, but not the maturation phase of astroglial differentiation. Consistent with this hypothesis, the immunoblot analysis in the present study suggested that GFAP expression was negatively correlated with the amount of the cleaved form of Notch1 (Fig. 5B). To further investigate the role of Notch signaling in astroglial development, we performed gain-of-function studies in various contexts. The ectopic expression of activated Notch1 in postnatal brain did not promote astroglial maturation (Fig. 4), but the activation of Notch signaling during the late embryonic phase promoted astroglial commitment and/or early differentiation (Fig. 5E). Consistently, neurospheres cultured with a γ -secretase inhibitor under differentiation conditions exhibited a reduction in the generation of astrocytes and a concomitant increase in the generation of neurons (Fig. 5C). As for the validity of using γ -secretase inhibitor to inhibit Notch activation, the literature is consistent in fly, zebrafish, and mice, including an *in vivo* demonstration (Pan et al., 2004). However, other signaling molecules have been reported to act as targets for γ -secretase inhibitor (Kopan and Ilagan, 2004). Therefore, strictly, we could not

rule out the possibility that inhibition of other signaling pathways contributed to the cell fate determination in our experiment.

At this point, how the down-regulation of Notch signaling is involved in the maturation of GFAP-positive astrocytes remains uncertain. However, the transient activation of Notch signaling that occurs during the commitment and/or early phase of astroglial differentiation might be sufficient to induce the transcriptional activation of the mouse GFAP gene by excluding the co-repressor N-CoR from the RBP-J complex that binds to the mouse GFAP gene promoter (Hermanson et al., 2002). Although the precise mechanism responsible for the suppressed maturation of astroglia as a result of the persistent activation of Notch signaling must be further clarified, the activation of signaling pathways other than Notch signaling (e.g., the JAK/STAT pathway) is likely to play an important role in the maturation of GFAP-positive astrocytes (Bonni et al., 1997; Nakashima et al., 1999).

Conclusions and Perspectives: the complexity of the role of Notch signaling in CNS development

In our present study, we developed new reporter systems using wild-type and RBP-J binding site-disrupted *Hes1* promoters and the fluorescent protein Venus/dVenus. Using these reporter genes, we examined the live monitoring of RBP-J-mediated Notch activation in developing mouse brain and neural stem cells. Notch activation selectively occurred in the radial glia during forebrain development, in neurosphere-initiating cells. Thus, the combination of our reporter system and gain-of/loss-of function studies clearly demonstrated the complexity of Notch signaling in CNS development, and our reporter system may be a potentially useful tool for future studies designed to elucidate the physiological roles and activation mechanisms of Notch signaling. Naturally, the *Hes1*-promoter-dependent reporter system developed in the present study would be a versatile tool for the live monitoring of in situ Notch signaling during development, stem cell maintenance, and the oncogenesis of various organs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ydbio.2005.08.003](https://doi.org/10.1016/j.ydbio.2005.08.003).

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