Notch1 Expression Is Spatiotemporally Correlated with Neurogenesis and Negatively Regulated by Notch1-Independent Hes Genes in the Developing Nervous System

In the developing nervous system, neural stem cells initially proliferate by a symmetric cell division and then undergo an asymmetric cell division, which makes one neuron (or neuronal precursor) and one progenitor. It remains to be determined how the switch from the symmetric to asymmetric cell divisions is regulated. Here, we found that Notch1 is expressed in the regions where neurogenesis occurs actively but not in the regions where neurogenesis does not yet occur. Furthermore, in Hes-mutant mice where neurogenesis is accelerated, Notch1 expression is also accelerated. Thus, Notch1 expression is negatively regulated by Hes genes and is spatiotemporally correlated with neurogenesis, suggesting that the neural stem cells that undergo asymmetric cell divisions express Notch1, whereas those that undergo symmetric cell divisions do not. We propose that initiation of Notch1 expression is one of the key features for switch from the symmetric to asymmetric cell divisions of neural stem cells and that this process is negatively regulated by Notch1-independent Hes genes.

Keywords: asymmetric cell division, delta, neural stem cell, neuroepithelial cell, radial glial cell

Introduction

In the developing nervous system, neuroepithelial cells undergo symmetric cell divisions, forming a neural plate initially and a neural tube later. As development proceeds, neuroepithelial cells become radial glial cells, which have cell bodies in the ventricular zone and radial fibers reaching the pial surface (Alvarez-Buylla and others 2001; Temple 2001; Fujita 2003; Götz and Barde 2005). Radial glial cells undergo asymmetric cell divisions, by which each radial glial cell gives rise to one neuron (or a neuronal precursor) and one radial glial cell (Malatesta and others 2000; Miyata and others 2001; Noctor and others 2001; Tamamaki and others 2001; Fishell and Kriegstein 2003). Both neuroepithelial cells and radial glial cells are considered as embryonic neural stem cells (Alvarez-Buylla and others 2001; Temple 2001; Fujita 2003; Götz and Barde 2005), but it is not known how the changes from neuroepithelial cells to radial glial cells are controlled. Particularly, it remains to be determined how the switch from the symmetric to asymmetric cell divisions is regulated in these neural stem cells.

It has been shown that multiple basic helix-loop-helix (bHLH) genes play essential roles in the maintenance and differentiation of neural stem cells (Bertrand and others 2002; Ross and others 2003; Kageyama and others 2005). The bHLH genes Hes1 and Hes5 antagonize differentiation genes, such as Mash1, and regulate maintenance of neural stem cells. Another Hes member, Hes3, also has the same activity as Hes1 and Hes5 (Hirata and others 2000). In the absence of Hes1, Hes3, and Hes5, neural stem cells are prematurely differentiated into neurons and depleted without generating the later-born cell types (Hatakeyama and others 2004). Thus, Hes genes are essential to generate neurons and glial cells in the correct numbers and full diversity by keeping neural stem cells until later stages.

During neural development, Hes1 and Hes3 are widely expressed at first in the neuroepithelium, whereas Hes5 expression occurs later and expands as Hes3 expression recedes (Hatakeyama and others 2004). Hes3 expression is gradually restricted to the dorsal neural tube and the isthmus, a boundary between the midbrain and the hindbrain, and finally confined to the isthmus only (Hirata and others 2001). At this stage, Hes1 and Hes5 expression covers almost all the developing nervous system mostly in a complementary manner (Hatakeyama and others 2004). The initial Hes1 and Hes3 expression in neuroepithelial cells occurs before the Notch ligand Delta-like1 (Dll1) is expressed, whereas the Hes5 expression occurs together with Dll1 expression. Because activation of Notch by its ligands induces the expression of Hes1 and Hes5 (but not Hes3) (Honjo 1996; Kageyama and Nakanishi 1997; Artavanis-Tsakonas and others 1999; Gaiano and Fishell 2002; Hitoshi and others 2002; Selkoe and Kopol 2003), it is likely that Hes5 expression (and maybe Hes1 expression at later stages also) depends upon Notch signaling, whereas the initial Hes1 and Hes3 expression does not (Hatakeyama and others 2004). Notch signaling is known to be involved in an asymmetric cell division, which asymmetrically distributes the Notch antagonist Numb, thereby making Numb+ and Numb− daughter cells (Cayouette and Raff 2000; Johnson 2003; Zhong 2003). It is likely that the former, in which Notch is antagonized, is differentiated into a neuron, whereas the latter, in which Notch is activated, remains undifferentiated (Ohtsuka and others 2006), although the Numb functions in mice are still controversial.

Here, we found that Notch1 is expressed in the regions where neurogenesis occurs actively but not in the regions where neurogenesis does not occur. Furthermore, in Hes-mutant mice where neurogenesis is accelerated, Notch1 expression is also accelerated. Thus, Notch1 expression is negatively regulated by Hes genes and is spatiotemporally correlated with neurogenesis. We propose that initiation of Notch1 expression is one of the key features for switch from the symmetric to asymmetric cell divisions and that this process is negatively regulated by Notch1-independent Hes genes.

Materials and Methods

Hes1−/−, Hes3−/−, and Hes5−/− Mutant Mice

All animals used in this study were maintained and handled according to protocols approved by Kyoto University. Genotypes of Hes1−/−, Hes3−/−,
and Hes5−/− embryos (Cau and others 2000; Hirata and others 2001) were determined, as described previously (Ohnuki and others 1999; Hirata and others 2001).

In Situ Hybridization
For in situ hybridization, antisense strand probes were labeled with digoxigenin, as previously described (Hirata and others 2001).

Histological Analysis
Embryos were fixed and embedded in optimal cutting temperature, as previously described (Hatakeyama and others 2001). Embedded embryos were sectioned by a cryostat at 16 μm. Immunohistochemistry was done, as previously described (Hatakeyama and others 2001) with the following antibodies: anti-TuJ1 (Babco) and anti-Hes1 (Hatakeyama and others 2006).

Results

Correlation between Notch1 Expression and Neurogenesis
We first examined the expression patterns of Notch, Delta, and Hes genes in wild-type embryos by in situ hybridization. At E7.5, Notch1 and its ligands Dll1 and Dll3 are expressed in the visceral endoderm and mesoderm but not in the neural plate (Fig. 1A,E,F, arrowheads, and data not shown). In contrast, Hes1 and Hes3 are expressed in the neural plate (Fig. 1C,D,G, arrowheads), whereas Hes5 expression is not detectable (data not shown). These results indicate that the initial Hes1 and Hes3 expression occurs independently of Notch1 and Dll1. Around E8.5, Hes3 expression is gradually down-regulated in the ventral region and restricted to the dorsal region, whereas Hes5 expression occurs in the ventral region (Hatakeyama and others 2004). This Hes5 expression is associated with the expression of Dll1 (Hatakeyama and others 2004), suggesting that Hes1 expression depends upon Delta-Notch signaling. Neurogenesis also occurs in the same regions where Hes5 and Dll1 are expressed (Hatakeyama and others 2004). At E10.5, neurons (TuJ1+) are generated widely in the nervous system but not yet in the eye regions (Fig. 1J). In the regions where neurogenesis occurs actively such as the diencephalon and mesencephalon, Notch1, Dll1, Dll3, and Hes5 are highly expressed (Fig. 1L and data not shown), whereas in the regions where neurogenesis does not occur yet, such as the eye regions, they are not expressed (Fig. 1L, arrowheads, and data not shown). These results suggest that there is a correlation between Notch1 expression and active neurogenesis. In contrast to the pattern of Hes5 expression, Hes1 mRNA is highly expressed in the regions where neurogenesis does not occur, such as the eye regions (Fig. 1Q). Hes1 protein is also expressed in a similar manner (Fig. 1N). At E10.5, Hes1 is also expressed in some regions, where neurogenesis occurs actively (Fig. 1M,N), and is co-expressed with Notch1, Dll1, and Hes5 (Fig. 1J-N). Because Hes1 is an essential effector for Notch signaling (Ohnuki and others 1999), Hes1 expression at this stage could be regulated by Notch signaling. The Hes1 expression levels in such neurogenic regions are lower than those of the nonneurogenic regions (Fig. 1M,N).

Acceleration of Neurogenesis and Notch1 Expression in Hes-Mutant Mice
In order to examine further the correlation between expression of Notch-signaling components and neurogenesis, we examined Hes-mutant mice, which exhibit premature neurogenesis (Ohnuki and others 1999; Hatakeyama and others 2004). At E8.5, before the neural tube closure, neurogenesis does not occur yet in the developing nervous system of wild-type embryos (Fig. 2A). At this stage, Notch1, Dll1, and Dll3 are not expressed (Fig. 2D,G and data not shown). In Hes1;Hes5 double-null mice, neurogenesis is somewhat accelerated in some ventral regions of the nervous system as early as E8.5 (Fig. 2B, arrow), suggesting that Hes3 alone cannot compensate sufficiently for Hes1 and Hes5 deficiency. In these mutants, Dll1 and Dll3 expression also occurs prematurely in the same ventral regions of the neural plate (Fig. 2E, arrow, and data not shown). Because Dll1 and Dll3 are expressed by postmitotic neurons, premature Dll1 and Dll3 expression agrees well with the premature neurogenesis phenotype. Interestingly, Notch1 expression also occurs prematurely in the ventral regions of Hes1;Hes5 double-null mice at E8.5 (Fig. 2H, arrow). In Hes1;Hes3;Hes5 triple-null mice, premature neurogenesis occurs widely in the nervous system as early as E8.5 (Fig. 2C). In these mutants, premature Dll1, Dll3, and Notch1 expression also occurs widely in the nervous system (Fig. 2I and data not shown). Thus, when neurogenesis is accelerated, Notch1 expression is also accelerated, indicating that it is spatiotemporally correlated with neurogenesis. These results also indicate that Hes genes inhibit not only premature onset of neurogenesis (and Dll1 and Dll3 expression) but also that of Notch1 expression.

Discussion

Correlation between Notch1 Expression and Neurogenesis
We found that Notch1 is expressed in the regions where neurogenesis occurs actively but not in the regions where neurogenesis does not yet occur. Furthermore, when neurogenesis is accelerated, Notch1 expression is also accelerated, indicating that Notch1 expression is spatiotemporally correlated with neurogenesis. It is likely that the neural stem cells that give rise to neurons by asymmetric cell divisions express Notch1, whereas those that undergo symmetric cell divisions do not (Fig. 3). We speculate that Notch1 may be required for maintenance of neural stem cells undergoing asymmetric cell divisions but not for those undergoing symmetric cell divisions, and that initiation of Notch1 expression is one of the key features for switch from the symmetric to asymmetric cell divisions. In the Notch signaling, Numb is asymmetrically distributed, generating Notch-active and Notch-inactive cells. Because neuroepithelial cells undergo symmetric cell divisions, whereas many radial glial cells undergo asymmetric cell divisions, initiation of Notch1 expression may be important for changing from neuroepithelial cells to radial glial cells.

In the wild type, Hes1 and Hes3 are initially expressed in the developing nervous system as early as E7.5 before Notch1, Dll1, and Dll3 are expressed, suggesting that the initial Hes1 and Hes3 expression is independent of Notch signaling. In agreement with this notion, Hes1 and Hes3 expression at early stages is not significantly affected in the absence of RBP-J, an essential upstream effector of Notch signaling (de la Pompa and others 1997). In contrast, the subsequent Hes1 and Hes5 expression occurs together with Notch1, Dll1, and Dll3 expression, suggesting that the later Hes1 and Hes5 expression is dependent
Thus, \textit{Hes1} could function at both \textit{Notch1}-dependent and \textit{Notch1}-independent stages, whereas \textit{Hes3} and \textit{Hes5} may be specific to \textit{Notch1}-independent and \textit{Notch1}-dependent stages, respectively (Fig. 3). The significance of the switch of \textit{Hes} members during development remains to be analyzed, but in the absence of \textit{Hes} genes, not only \textit{Dll1} and \textit{Dll3} expression but also \textit{Notch1} expression occurs prematurely. Thus, \textit{Notch1}-independent \textit{Hes} genes inhibit the premature onset of \textit{Notch1} expression, suggesting that there are at least 2 types of functionally different genes in early neural development.
Hes-expressing neural stem cells (Fig. 3). Furthermore, because neural stem cells are initially formed in the absence of Hes genes (Hatakeyama and others 2004), these results together indicate that there are 3 distinct types of neural stem cells that appear in the following order: 1) Hes- and Notch1-independent, 2) Hes-dependent but Notch1-independent, and 3) Hes- and Notch1-dependent neural stem cells (Fig. 3). Although the second type of a neural stem cell usually does not generate neurons, it has a neurogenic potential (Hatakeyama and others 2004). It remains to be determined whether or not the first type of a neural stem cell has a potential to give rise to any neurons.

**Possible Mechanisms for Different Activities of Hes Factors between Notch1-Independent and Notch1-Dependent Pathways**

Our results also suggest that Hes factors at later stages (Notch1 dependent) allow Notch1 expression and neurogenesis, whereas those at earlier stages (Notch1 independent) do not, although the molecular mechanism for such a functional difference of Hes factors remains to be analyzed. One possible mechanism is that a dimer partner could be different between Notch1-independent and Notch1-dependent stages. Hes factors act as transcriptional repressors by forming homodimers (Kageyama and Nakanishi 1997). It was previously shown that expression of Hes-related bHLH genes, Hey genes, is also induced by Notch signaling (Iso and others 2003). Interestingly, Hes and Hey can form heterodimers, and the functions of Hes homodimers and Hes–Hey heterodimers could be different (Iso and others 2003). Another possible mechanism is that Hes proteins could be differentially modified in the Notch1-dependent and Notch1-independent pathways. It has been shown that the activity of Hes1 is modified by phosphorylation (Ström and others 1997), and the phosphorylation status could be different between the earlier and later stages of neural development.

It is also possible that the expression modes of Hes factors could be different between the Notch1-dependent and Notch1-independent pathways. We previously found that Hes1 expression oscillates in many cell types with the periodicity of about 2 h after serum treatment or Notch activation (Hirata and others 2002). We also found that persistent Hes1 expression blocks neuronal differentiation (Ishibashi and others 1994; Tomita and others 1996), whereas loss of Hes1 expression inhibits self-renewal of neural stem cells (Ishibashi and others 1995; Ohtsuka and others 1999; Hatakeyama and others 2004). Thus, both persistent expression and loss of expression of Hes1 disturb the activities of neural stem cells, indicating that oscillatory expression may be important for neural stem cells to undergo both self-renewal and neuronal differentiation. Thus, an interesting possibility would be that Hes1 expression is oscillatory in neurogenic neural stem cells while being persistent in nonneurogenic neural stem cells. Further studies will be required to determine the expression modes of Hes1 in neural stem cells.
Figure 3. Changes of characteristics of neural stem cells during development. Neural stem cells/progenitors change their characteristics over time in the following order: 1) 
Hes- and Notch1-independent, 2) Hes-dependent but Notch1-independent, and 3) 
Hes- and Notch1-dependent neural stem cells. The second type of neural stem cells 
undergoes self-renewal only in wild-type embryos (symmetric cell divisions), although 
it has a potential to become neurons, whereas the third type undergoes both self-
renewal and neuronal differentiation (asymmetric cell divisions). In the second type, 
Notch1-independent Hes genes inhibit premature onset of Notch1 expression. It 
remains to be determined whether or not the first type of neural stem cells has 
a potential to give rise to any neurons. N, Neuron/neuronal precursor; P, Progenitors.

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