**Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation**

Jun Hatakeyama¹, Yasumasa Bessho¹*, Kazuo Katoh², Shigeo Ookawara², Makio Fujioka³, François Guillemot⁴ and Ryoichiro Kageyama¹†

¹Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan
²Department of Anatomy, Jichi Medical School, Tochigi 329-0498, Japan
³Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan
⁴National Institute for Medical Research, Mill Hill, London, NW7 1AA, UK

*Present address: Nara Institute of Science and Technology, Ikoma 630-0101, Japan
†Author for correspondence (e-mail: rkageyam@virus.kyoto-u.ac.jp)

Accepted 9 September 2004

Development 131, 5539-5550
Published by The Company of Biologists 2004
doi:10.1242/dev.01436

Summary

Radial glial cells derive from neuroepithelial cells, and both cell types are identified as neural stem cells. Neural stem cells are known to change their competency over time during development: they initially undergo self-renewal only and then give rise to neurons and glial cells later. Maintenance of neural stem cells until late stages is thus believed to be essential for generation of cells in correct numbers and diverse types, but little is known about how the timing of cell differentiation is regulated and how its deregulation influences brain organogenesis. Here, we report that inactivation of Hes1 and Hes5, known Notch effectors, and additional inactivation of Hes3 extensively accelerate cell differentiation and cause a wide range of defects in brain formation. In Hes-deficient embryos, initially formed neuroepithelial cells are not properly maintained, and radial glial cells are prematurely differentiated into neurons and depleted without generation of late-born cells. Furthermore, loss of radial glia disrupts the inner and outer barriers of the neural tube, disorganizing the histogenesis. In addition, the forebrain lacks the optic vesicles and the ganglionic eminences. Thus, Hes genes are essential for generation of brain structures of appropriate size, shape and cell arrangement by controlling the timing of cell differentiation. Our data also indicate that embryonic neural stem cells change their characters over time in the following order: Hes-independent neuroepithelial cells, transitory Hes-dependent neuroepithelial cells and Hes-dependent radial glial cells.

Key words: Adherens junction, Basal lamina, bHLH, Neuroepithelium, Radial glia, Tight junction

Introduction

Organogenesis involves the initial proliferation process of stem cells and the subsequent differentiation process coupled to withdrawal from the cell cycle. The timing of cell differentiation is thought to be crucial for the size, shape and histogenesis of many tissues, including the brain. In the developing nervous system, neural stem cells (previously known as matrix cells) are initially present as neuroepithelial cells, which proliferate extensively by a symmetric cell division. Around embryonic day (E) 8.5 in mouse, neuroepithelial cells become radial glia by acquiring astroglial features. As neural stem cells, radial glia give rise to neurons first and glial cells later (Fujita, 1964; McConnell, 1995; Temple, 2001). During neurogenesis, radial glial cells are known to undergo an asymmetric cell division, by which each radial glial cell forms one neuron and one radial glial cell.

The size and shape of the nervous system largely depend on how many times neural stem cells re-enter the cell cycle (Ohnuma and Harris, 2003). For example, the brain becomes enlarged when cell differentiation is delayed by null mutation for the cyclin-dependent kinase (CDK) inhibitor p27Kip1, which inhibits cell cycle progression (Fero et al., 1996; Nakayama et al., 1996), or by misexpression of a stabilized β-catenin, an effector for Wnt signalling, which induces cell proliferation (Chenn and Walsh, 2002). The timing of cell differentiation may also influence cell fate choice, because neural stem cells change their competency over time during development (McConnell, 1995; Qian et al., 2000; Temple, 2001). However, although delayed cell differentiation by misexpression of a stabilized β-catenin increases neurons as well as neural stem cells, the enlarged brain with massive expansion of the cortex has a normal spatial pattern of neuronal differentiation (Chenn and Walsh, 2002). Thus, it is still obscure how the timing of cell differentiation influences cell fate choice and the histogenesis of the nervous system. Conversely, acceleration of cell differentiation was also attempted by disrupting cyclin D and cyclin E genes, which are believed to play an essential role in cell cycle progression (Sherr and Roberts, 1999). However, unexpectedly the defects of these cyclin-null mice are mild and occur only at late stages (Fantl et al., 1995; Huard et al., 1999; Sicinski et al., 1995; Geng et al., 2003). Thus, it remains to be determined whether premature cell differentiation simply generates small tissues...
with mostly normal shapes and histology or significantly affects the structures.

The basic helix-loop-helix genes \( \text{Hes}1 \) and \( \text{Hes}5 \) are essential effectors for Notch signalling, which regulates the maintenance of undifferentiated cells (Artavanis-Tsakonas et al., 1999; Gaiano and Fishell, 2002; Hitoshi et al., 2002; Honjo, 1996; Kagayama and Nakaniishi, 1997; Selkoe and Kopan, 2003). \( \text{Hes}1 \) represses expression of the CDK inhibitor p21Cip1 (Castella et al., 2000; Kabos et al., 2002) and functionally antagonizes differentiation genes such as \( \text{Mash}1 \), thus controlling both cell cycle and differentiation. We previously reported that \( \text{Hes}1 \) and \( \text{Hes}5 \) play an important role in maintenance of neural stem cells (Tomita et al., 1996; Nakamura et al., 2000; Ohtsuka et al., 2001; Cau et al., 2000). Here, we examined the defects of the nervous system of embryos lacking \( \text{Hes}1 \) and \( \text{Hes}5 \) in more detail. We also examined embryos additionally lacking \( \text{Hes}3 \), which has a similar activity to that of \( \text{Hes}1 \) and \( \text{Hes}5 \) (Hirata et al., 2000). In these mutants, virtually all neural stem cells prematurely differentiate into neurons only without generating the later-born cell types, and the brain structures are severely destroyed. These results indicate that control of the timing of cell differentiation by \( \text{Hes} \) genes is essential not only for the size and shape, but also for the structural integrity, of the nervous system.

Materials and methods

\( \text{Hes}1^{-/-} \), \( \text{Hes}3^{-/-} \) and \( \text{Hes}5^{-/-} \) mutant mice

All animals used in this study were maintained and handled according to protocols approved by Kyoto University. Genotypes of \( \text{Hes}1^{-/-} \), \( \text{Hes}3^{-/-} \), and \( \text{Hes}5^{-/-} \) embryos (Cau et al., 2000; Hirata et al., 2001) were determined, as described previously (Hirata et al., 2001; Ohtsuka et al., 1999).

Scanning electron microscopic analysis

Embryos were perfusion-fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. The embryos were cut transversely into small pieces, and the samples including upper limb and spinal cord were further fixed in fresh fixative for 3 hours at room temperature and then overnight at 4°C. After being washed in 0.1 M sodium cacodylate buffer, the specimens were post-fixed with 1% OsO4 for 2 hours on ice, stained en bloc with 0.5% uranyl acetate for 2 hours, and then dehydrated and embedded in Epon 812. Ultrathin sections (80 nm) were cut and examined using a JEOL JEM 2000 EX-type electron microscope operated at 80 kV.

In-situ hybridization

For in-situ hybridization, antisense strand probes were labelled with digoxigenin, as previously described (Hirata et al., 2001).

Histochemical analysis

Embryos were fixed, as previously described (Hatakeyama et al., 2001). Alternatively, embryos were fixed at 4°C in 10% trichloroacetic acid solution for 1 hour, washed in PBS, and embedded in OCT (Hayashi et al., 1999). Embedded embryos were sectioned by a cryostat at 16 μm.

Immunochemistry was done, as previously described (Hatakeyama et al., 2001), with the following antibodies: anti-N-cadherin (BD Transduction Laboratories), anti-RC2 (Hybridoma Bank), anti-nestin (Pharmingen), anti-TuJ1 (Babco), anti-Ki67 (Pharmingen), anti-laminin-α1 (Chemicon), anti-ZO-1 and anti-claudin-10, -15 and -18, which were kindly provided by Dr Shoichiro Tsukita (Kiuchi-Saishin et al., 2002). TdT-mediated DUTP nick-end labelling (TUNEL) assay was performed with a detection kit (Roche).

Toxin-induced cell ablation

For pNes-DT-A and pNes-GFP, cDNAs for diphtheria toxin A subunit (DT-A) (Yamaizumi et al., 1978) and EGF, respectively, were inserted between the nestin promoter and intron II, a CNS-specific enhancer. Electroporation was performed, as previously described (Ohtsuka et al., 2001). Square electroporator CUY21 EDIT (TR Tech) was used to deliver five 50-ms pulses of 30 V with 950-ms intervals.

Results

Compensatory expression of \( \text{Hes}1 \) and \( \text{Hes}5 \)

We first examined the expression patterns of \( \text{Hes}1 \) and \( \text{Hes}5 \) in mouse embryos. These two genes were widely expressed in the developing nervous system, but their expression patterns were rather complementary to each other (Fig. 1A-E,G,I). For example, \( \text{Hes}5 \) was strongly expressed in the midbrain and hindbrain but not in the isthmus, while \( \text{Hes}1 \) was expressed in the isthmus (Fig. 1A,B, arrowheads). In the optic vesicle, \( \text{Hes}1 \) but not \( \text{Hes}5 \) was expressed (Fig. 1C-E, arrows). Interestingly, in the absence of either \( \text{Hes}1 \) or \( \text{Hes}5 \), expression of the remaining \( \text{Hes} \) gene was upregulated in many regions. For example, \( \text{Hes}5 \) expression was observed in the optic vesicles of \( \text{Hes}1 \)-null embryos (Fig. 1F, arrow). Similarly, \( \text{Hes}1 \) and \( \text{Hes}5 \) expression was expanded in the spinal cord of \( \text{Hes}5 \)-null (Fig. 1H, arrows) and \( \text{Hes}1 \)-null (Fig. 1J, arrow) embryos, respectively. This compensatory expression may explain weak or no apparent defects in \( \text{Hes}1 \)-null or \( \text{Hes}5 \)-null embryos, in contrast to those lacking both \( \text{Hes}1 \) and \( \text{Hes}5 \) (Fig. 2A-D). We thus examined the double-mutant mice in more detail to understand the significance of the timing of cell differentiation. \( \text{Hes}1;\text{Hes}5 \) double-mutant embryos survived until E10.5, and their defects were more specific to the nervous system than those of \( \text{Notch} \)-null mutant mice (Swiatek et al., 1994; Conlon et al., 1995; Hamada et al., 1999).

Disorganization of the neural tube morphology in \( \text{Hes}1;\text{Hes}5 \) double-mutant mice

Scanning electron microscopic (SEM) analysis revealed that at E10.5, cells were radially aligned throughout the wall of the wild-type neural tube (Fig. 2E,F). The endfeet of these cells were tightly connected each other, forming a smooth inner surface (Fig. 2I). By contrast, in \( \text{Hes}1;\text{Hes}5 \) double mutants, cell arrangement was severely disorganized, with many cells scattered into the lumen of the neural tube (Fig. 2G,H). These cells had lost radial morphology but had become round in shape (Fig. 2J, compare with Fig. 2I). Furthermore, the outer boundary of the neural tube was also irregular and ambiguous in the double mutant (Fig. 2G). Thus, \( \text{Hes}1 \) and \( \text{Hes}5 \) are essential for the structural integrity of the embryonal nervous system.
system. We observed this defect along the entire anterior-posterior axis of the spinal cord all the way to the diencephalon but not in the telencephalon at E10.5 (see below), which may reflect slower development in the telencephalon than in other regions. Because almost all the double-mutant embryos died by E11, we were not able to examine the defects of the telencephalon at later stages.

To investigate whether cell death is involved in the disorganized cell arrangement in Hes1;Hes5 double mutants, we next performed TUNEL assay. During E8.5-E10.5, there was no significant increase of TUNEL+ cells in the double-mutant spinal cord (Fig. 3A-F), indicating that the structural disorganization was not due to cell death. In addition, the floor plate (Shh+), V3 interneurons (Sim1+), V0 interneurons (Evx1+), D1A interneurons (Lhx2+), and the roof plate (Wnt1+) were generated in the double-mutant spinal cord as in the wild type (Fig. 3G-P), suggesting that the dorsal-ventral patterning is mostly normal in the absence of Hes1 and Hes5, despite the severe morphological abnormalities.

Premature neuronal differentiation and concomitant loss of radial glia in Hes-mutant mice

To further characterize the defects of Hes1;Hes5 double-mutant embryos, we performed immunohistochemistry. At E8.5, the neural plate consisted of nestin+ neuroepithelial cells in Hes1;Hes5 double-mutant embryos as in the wild type (Fig. 4A,D). At E9.5 and 10.5, when neuroepithelial cells became radial glia and neurogenesis actively occurred, nestin+ radial glial cells were radially aligned throughout the wall of the spinal cord in the wild type (Fig. 4B,C). By contrast, in the double mutant, nestin+ radial glial cells were prematurely lost from the ventral region (Fig. 4E,F). Furthermore, they were also decreased in the dorsal region at E10.5 (Fig. 4F). These
results indicate that in the double mutant, although neuroepithelial cells are normally formed, radial glial cells are prematurely lost. Agreeing with this notion, expression of the radial glial marker RC2 was also lost from the ventral region at E9.5 and 10.5 (Fig. 4K,L).

We next examined the differentiation status in the double mutant. In the wild type, radial glial cells, which were mitotically active (Ki67+), were present throughout the wall of the spinal cord, while neurons (TuJ1+) resided in the outer layer at E10.5 (Fig. 5A). By contrast, in the double mutant, Ki67+ radial glial cells were depleted from the ventral region and, instead, this region was occupied by prematurely differentiated TuJ1+ neurons (Fig. 5E). Expression of the Notch ligand gene Dll1 and the proneural bHLH genes Mash1 and Math3, all of which are expressed by differentiating neurons, was highly upregulated in the double mutant (Fig. 5F-H). Expression of the Notch ligand gene Dll1 and the proneural bHLH genes Mash1 and Math3, all of which are expressed by differentiating neurons, was highly upregulated in the double mutant (Fig. 5F-H). Expression of the Notch ligand gene Dll1 and the proneural bHLH genes Mash1 and Math3, all of which are expressed by differentiating neurons, was highly upregulated in the double mutant (Fig. 5F-H). Expression of the Notch ligand gene Dll1 and the proneural bHLH genes Mash1 and Math3, all of which are expressed by differentiating neurons, was highly upregulated in the double mutant (Fig. 5F-H). Expression of the Notch ligand gene Dll1 and the proneural bHLH genes Mash1 and Math3, all of which are expressed by differentiating neurons, was highly upregulated in the double mutant (Fig. 5F-H). Expression of the Notch ligand gene Dll1 and the proneural bHLH genes Mash1 and Math3, all of which are expressed by differentiating neurons, was highly upregulated in the double mutant (Fig. 5F-H).

It is likely that this upregulation of the proneural bHLH genes may be responsible for accelerated neurogenesis in the double mutant. In these mutants, the later-born cells, such as oligodendrocytes, astrocytes and ependymal cells, were not generated (data not shown). Because there was no significant increase of TUNEL+ cells in the mutants (Fig. 3D-F), the lack of the later-born cell types was not due to cell death. It is likely that, in the absence of Hes genes, radial glial cells are prematurely differentiated into neurons at the expense of late-born cells, indicating that Hes genes are essential for generation of a full range of cell types.

**Fig. 3.** Cell death and dorso-ventral patterning of the spinal cord. (A-F) TUNEL assay indicates that cell death (green) is not increased in Hes1−/−Hes5−/− spinal cord (D-F). (G-P) In-situ hybridization analysis of Shh for the floor plate (G,L), Sim1 for V3 interneurons (H,M), Evx1 for V0 interneurons (I,N), Lhx2 for D1A interneurons (J,O), and Wnt1 for the roof plate (K,P) at E10.5. Except for the hypomorphic roof plate, the dorso-ventral patterning is mostly normal in Hes1−/−Hes5−/− spinal cord (L-P). Scale bars: 100 μm.

**Fig. 4.** Premature loss of radial glia in Hes-mutant spinal cord. Histological analysis was done with the wild-type (A-C,G-I) and Hes1;Hes5 double-mutant (D-F,J-L). The dorsal is up in all panels. At E8.5, the neural plate consists of nestin+ neuroepithelial cells in Hes1;Hes5 double-mutant (D) as in the wild type (A). At E9.5 and E10.5, nestin+RC2+ radial glial cells are present throughout the wall of the spinal cord in the wild type (B,C,H,I). By contrast, in the double mutant, nestin+RC2+ radial glial cells are prematurely lost from the ventral region (E,F,K,L). Scale bars: 100 μm.

**Loss of the junctional complex in Hes1-Hes5 double-mutant spinal cord**

To determine how the structural defects occur in Hes1;Hes5 double mutants, we examined the junctional complex. Transmission electron microscopy (TEM) studies showed that the adherens junction and the tight junction were formed at the apical side of neuroepithelial cells in both the wild type and the double mutant at E8.5 (Fig. 6B,E, arrowheads). A higher magnification shows kissing points of the tight junction (Fig. 6C,F, arrows). Consistent with this observation, the tight junction molecules claudin-10, -15 and -18 (Tsukita et al., 2001), as well as the adherens junction molecule N-cadherin (Hatta and Takeichi, 1986), were expressed at the apical surface of neuroepithelial cells of both the wild type and the double mutant (Fig. 6G-L and data not shown). Thus, the structural integrity of the neuroepithium was maintained at E8.5 in Hes1;Hes5 double mutants.

At E9.5-10.5, the tight junction and the adherens junction
were observed at the apical side of radial glial cells in the wild-type spinal cord (arrows, brackets and arrows in Fig. 7E,J,K, respectively). In addition, the tight junction molecule claudin and the adherens junction molecule N-cadherin were expressed at the apical surface of radial glial cells (Fig. 7G-I,L-N and data not shown). By contrast, in Hes1;Hes5 double mutants, cells of the right and left walls of the neural tube were intermingled with each other (Fig. 7B,D) and lacked the junctional complex at E9.5 (arrowheads and arrows in Fig. 7F,O-Q, respectively). This region was occupied by prematurely differentiated neurons (TuJ1+; Fig. 7P, arrow). Thus, radial glial cells, which form the apical intercellular junctions, were prematurely differentiated into neurons in the double mutant, and this loss of the junctional complex seemed to allow neurons to scatter into the lumen.

**Loss of the basal lamina in Hes1-Hes5 double-mutant spinal cord**

Because neurons (TuJ1+) had escaped from the neural tube into the surrounding region (Fig. 8E,G,H, arrowheads), it is likely that the outer barrier was also affected in the double mutant. The outer boundary of the neural tube was demarcated by the basal lamina in the wild type (Fig. 8B, open arrows). By contrast, the basal lamina was undetectable in the ventral spinal cord of the double mutant (Fig. 8F, arrows). Furthermore, many neurons of the spinal cord and the dorsal root ganglia were intermingled with each other in the double mutant (Fig. 8D,G, arrows). In the wild type, the basal lamina glycoprotein laminin-α1 was expressed at the basal boundary (Fig. 8I-K). However, in the double mutant, laminin-α1 expression was lost or severely disorganized in the ventral region, where radial glial cells (nestin+) were depleted (Fig. 8M-O), while the expression was still intact at the dorsal boundary, where radial glial cells still remained (Fig. 8M-O). Thus, premature loss of radial glial cells led to defects of the basal lamina. In-situ hybridization analysis demonstrated that laminin-α1 mRNA was expressed in the ventricular zone of the wild type, where cell bodies of radial glial cells were present (Fig. 8L), as previously described (Thomas and Dziadek, 1993). By contrast, expression of laminin-α1 mRNA was undetectable in the ventral spinal cord of the double mutant (Fig. 8P). These results indicate that the loss of radial glia leads to disruption of the basal lamina, allowing neurons to escape into the surrounding regions. Taken together, radial glial cells are essential for the structural integrity of the nervous system by forming the apical and basal barriers.
Ablation of radial glial cells destroys the structural integrity of the neural tube

The above results show that premature loss of radial glia abolished the inner and outer barriers and allowed neurons to escape into the lumen and the surrounding regions. These results point to the importance of radial glia in the maintenance of neural tube morphology. To further confirm this notion, we next ablated radial glial cells by expressing diphtheria toxin (DT) under the control of the nestin promoter/enhancer (pNes-DT-A) (Fig. 9H). This promoter/enhancer directed specific expression in nestin+ radial glial cells (Fig. 9I-L) (Zimmerman et al., 1994). We injected pNes-DT-A into the ventricle of embryonal telencephalon at E13 and performed electroporation, which introduced the vector into the cells on the plus side. To identify the transfected region, we co-injected the vector for GFP expression under the control of the CMV promoter/enhancer (pCL-GFP) (Fig. 9D), which allowed ubiquitous GFP expression (Fig. 9A). After five days (E18), introduction of pCL-GFP alone stained both radial glia and neurons (Fig. 9O) and did not affect expression of the apical junction molecule ZO-1 (Fig. 9Q, arrowheads), the basal lamina (laminin α1) (Fig. 9N, arrowheads) or neuronal arrangement (NeuN) (Fig. 9R). By contrast, when pNes-DT-A was introduced, we found many TUNEL+ cells only in the ventricular zone after 2 days (E15) (Fig. 9E-G), indicating that the pNes-DT-A vector specifically killed nestin+ radial glial cells. Furthermore, after five days (E18), the electroporated region had lost radial glial cells (nestin+) (Fig. 9V, arrowheads) while it contained a small number of GFP+ neurons (Fig. 9T, arrowheads), which were probably nestin-negative differentiating neurons at the time of electroporation. This region lost expression of ZO-1 at the apical side (Fig. 9W, arrowheads) and laminin α1 at the basal side (Fig. 9X, between the two arrowheads). Instead of radial glial cells, the ventricular zone was occupied by ectopic neurons (NeuN+), which were scattered into the ventricle (Fig. 9Y, arrowheads). Furthermore, the cortical lamination was destroyed with rosette-like structures, and some neurons protruded into the outer region through the area that lacked laminin α1 expression (Fig. 9U, arrowheads). These abnormalities were very similar to those of Hes1;Hes5 double-mutant spinal cord, supporting the notion of the important roles of radial glia in maintenance of the structural integrity.

Eyes are not formed in Hes1;Hes5 double-mutant embryos

Similar but less severe defects were also observed in the mesencephalon and diencephalon of Hes1;Hes5 double-mutants (Fig. 10B, arrows). Although the forebrain was less severely affected, it became a rather monotonous tube, compared with the wild type (Fig. 10A,B). Strikingly, Hes1;Hes5 double-null embryos had no optic vesicles (Fig. 10B,I, compare with Fig. 10A, arrow, and H). In the wild-type optic vesicles, neuronal differentiation did not occur at
E9.5 and 10.5 (Fig. 10C,E,F), whereas in the double-mutant neurons were already differentiated at E9.5 and 10.5 in the regions that should normally become optic vesicles (Fig. 10D,G), suggesting that accelerated neuronal differentiation may inhibit optic vesicle formation. The homeodomain gene Rx, which is essential for eye formation (Mathers et al., 1997), was expressed normally in Hes1;Hes5 double-mutants, suggesting that the initial eye specification occurred in the double mutant (Fig. 10J). However, the downstream homeodomain genes Pax2, which specifies the optic stalk (Torres et al., 1996), and Chx10, which regulates retinal formation (Burmeister et al., 1996), were not expressed in the double mutant (Fig. 10K-N), suggesting that accelerated neuronal differentiation may hamper Rx-induced eye specification. Furthermore, the ganglionic eminences were not properly formed due to accelerated neuronal differentiation in Hes1;Hes5 double mutants (Fig. 10B, compare with Fig. 10A, arrowhead). Thus, the normal timing of cell differentiation is essential for organization of proper structures such as the optic vesicles and the ganglionic eminences.

**More severe defects in Hes1-Hes3-Hes5 triple-mutant spinal cord**

Although neuronal differentiation was severely accelerated in Hes1;Hes5 double mutants, neuroepithelial cells were normally formed at E8.5 and radial glial cells still remained in the dorsal region (Figs 4 and 6), suggesting that a related bHLH gene may compensate for formation of neuroepithelial and dorsal radial glial cells. To determine whether or not Hes gene activities are required for these cells, we next examined another Hes gene, Hes3, which is also expressed in the developing nervous system (Allen and Lobe, 1999). At E8.0, like Hes1, Hes3 was widely expressed in the nervous system (Fig. 11A,B,I) while Hes5 was not (Fig. 11C). At E8.5-9.5, Hes3 expression occurred widely (Fig. 11J,K) but was restricted to the medial to dorsal region (Fig. 11FL), while Hes5 and Dll1 expression started in the ventral to medial region (Fig. 11G,H). After E9.5, Hes3 expression is downregulated except for the isthmus (Hirata et al., 2001). In Hes1;Hes5 double mutants, Hes3 expression was not significantly affected (Fig. 11K-M).

Because Hes3 may compensate for Hes1 and Hes5 deficiency in the formation of the neuroepithelium and the dorsal radial glia, we thus examined Hes1;Hes3;Hes5 triple-mutant embryos. At E8.5, very few neurons (TuJ1+) were generated in both the wild-type and Hes1;Hes5 double-mutant embryos (Fig. 11N,O), whereas many neurons were prematurely differentiated in Hes1;Hes3;Hes5 triple-mutant embryos (Fig. 11P). At this stage, although there were still many neuroepithelial cells in the triple mutant (nestin"Ki67") (Fig. 11Q,R), they were not properly maintained and prematurely differentiated into neurons (Fig. 11P), suggesting that neuroepithelial cells are initially formed without Hes genes but become dependent on Hes gene activities by E8.5. At E9.5, most cells had become neurons in the ventral spinal cord of the triple mutant (Fig. 11S). Strikingly, at E10.0 virtually all radial glial cells (Ki67+) were differentiated.
into TuJ1+ neurons in Hes1;Hes3;Hes5 triple mutants (Fig. 11T-V), indicating that Hes3 is responsible for dorsal radial glia and that the three Hes genes together are essential for maintenance of virtually all radial glial cells.

Discussion

Hes genes are essential for maintenance of neural stem cells

We demonstrated here that Hes genes are essential for organization of brain structure of appropriate size, shape and cell arrangement by maintaining neural stem cells. In the absence of Hes genes, cell differentiation is severely accelerated, leading to depletion of radial glial cells and resultant disorganization of the structural integrity of the nervous system. Interestingly, Hes genes are not required for the initial formation of neuroepithelial cells. However, in the absence of Hes1, Hes3 and Hes5, neuroepithelial cells are not properly maintained but have prematurely differentiated into neurons by E8.5. Thus, neuroepithelial cells are initially

Fig. 9. Ablation of radial glia by diphtheria toxin (DT) disrupts the inner and outer barriers of the developing brain. (A-L) Each vector was introduced into the telencephalic cells by electroporation at E13, and the sections were examined at E15. When pCL-GFP was introduced (A-C), both cells in the ventricular zone (nestin+) and the outer layers were found to express GFP. No dying cells (TUNEL+) were detected (C). When pNes-DT-A was introduced (E-G), many cells underwent apoptosis in the ventricular zone (TUNEL+, G). When pNes-GFP was introduced, only nestin+ ventricular cells (I,J), but not NeuN+ neurons (K), expressed GFP. The schematic structures of the vectors are shown in D,H,L. (M-Z) Each vector was introduced into the telencephalic cells by electroporation at E13, and the sections were examined at E18. When pCL-GFP was introduced (M-S), GFP was expressed in both radial glia and neurons (O) and did not affect radial glia (nestin+, P), the apical junction (ZO-1+, Q, arrowheads), the basal lamina (Laminin α1+, N, arrowheads) or neurons (NeuN+, R). Cell arrangement was not disturbed (DAPI, S). The indicated region in M is enlarged in O-S. When pNes-DT-A was introduced (T-Z), GFP+ cells were almost undetectable (T,T‘). Only a small number of GFP+ neurons is detectable, which shows the electroporated region (T,T‘). pNes-DT-A specifically killed nestin+ radial glia, which disappeared from the electroporated region (V,V‘, arrowheads). This region lost ZO-1 expression at the apical side (W, arrowheads, compare with the normal expression indicated by arrows). The electroporated region also lost laminin α1 at the basal side (X, between the two arrowheads). In this region, the cortical lamination is destroyed with rosette-like structures, and some neurons protrude into the outer region and the lumen (Y,Y‘,Z, arrowheads). The indicated region in (T) and (U) is enlarged in (T‘) and (V‘,W,Y‘,Z), respectively. Scale bars: 100 μm in A-C,E-G,I,K,N-S,T‘,V‘,W,Y‘,Z; 200 μm M,T-V,X,Y.
dependent of, but become dependent on, Hes gene activities for their maintenance before changing to radial glia. In the wild type, radial glial cells undergo an asymmetric cell division, forming one radial glial cell and one neuron from each cell division. In the absence of Hes genes, however, radial glial cells seem to be unable to undergo asymmetric cell division, forming neurons only. Thus, Hes genes are essential for maintenance of both neuroepithelial and radial glial cells. These observations also indicate that neural stem cells change their characters over time, in the following order: Hes-independent neuroepithelial cells, transitory Hes-dependent neuroepithelial cells, and Hes-dependent radial glial cells (Fig. 12A). Based on the expression patterns (Fig. 11), transitory Hes-dependent neuroepithelial cells seem to depend on Hes1 and Hes3, while Hes-dependent radial glial cells mostly depend on Hes1 and Hes5 (Fig. 12A), although dorsal radial glial cells depend on Hes3 to some extent.

Hes genes organize the brain structures by maintaining radial glial cells

Radial glial cells form intercellular junctions at the apical side

Previous morphological analyses show conflicting results about the presence of the tight junction (Aaku-Saraste et al., 1996; Bancroft and Bellairs, 1975; Decker and Friend, 1974; Duckett, 1968; Hinds and Ruffett, 1971; Möllgård et al., 1987; Revel and Brown, 1975). It was previously shown that, during neurulation, expression of the tight junction molecule occludin is lost (Aaku-Saraste et al., 1996). In the present study, however, we found that the tight junction did exist at the apical side of the neural tube on TEM analysis and that the tight junction molecules claudins were expressed by radial glial cells on immunohistochemical analysis. Thus, it is most likely that, even after neurulation, radial glial cells are anchored at the apical side by the tight junction as well as by the adherens junction. We speculate that a single strand junction identified by the freeze-fracture method (Möllgård et al., 1987) may correspond to a claudin-based tight junction. Further analysis is required to determine the features of the tight junction of the embryonal nervous system.

It was previously shown that blockade of N-cadherin activity by specific antibody or gene inactivation destroys the laminar structure of the nervous system, indicating an essential role of N-cadherin in maintenance of the nervous system morphology (Bronner-Fraser et al., 1992; Gänzler-Ordenthal and Redies, 1998; Lele et al., 2002; Luo et al., 2001; Masai et al., 2003). However, because N-cadherin is diffusely expressed by almost all neural cells including neurons, blockade of N-cadherin activity may disrupt interactions between all these cells, making it difficult to interpret the contribution of radial glial cells to the structural integrity. In the present study, we showed that, although neurons still expressed N-cadherin, loss of radial glial cells scattered many neurons out of the neural tube, indicating that diffuse N-cadherin activity is required to maintain tight cell-cell interactions.
cadherin expression is not sufficient to keep neurons inside the wall of the neural tube.

### Radial glial cells contribute to the basal lamina formation

The basal lamina is composed of networks of glycoproteins such as laminin and collagen IV (Timpl, 1996; Yurchenco and O’Rear, 1994). It was previously shown that laminin is expressed by radial glial cells (Liesi, 1985; Thomas and Dziadek, 1993). We found that, when radial glial cells disappear, both laminin-α1 mRNA in the ventricular zone and laminin-α1 protein at the basal side are lost or severely downregulated. These results suggest that radial glial cells produce, transport, and secrete laminin-α1 to the basal side. The phenotype of radial glial cell loss is very similar to that of laminin-γ1-mutant mice, which display disruption of the basal lamina and neuronal protrusion through the meninges (Halfter

---

**Fig. 11.** Premature loss of neuroepithelial and radial glial cells in Hes1;Hes3;Hes5 triple mutants. (A-D) At E8.0, like Hes1 (A), Hes3 is expressed in the nervous system (B) whereas Hes5 and Dll1 are not (C,D). (E-H) At E8.5, while Hes1 is still diffusely expressed (E), Hes3 expression domain is restricted to the medial to dorsal region (F). Hes5 and Dll1 expression is observed at this stage (G,H). (LJ) Hes3 expression is widely expressed in the developing nervous system at E8.0 (I) and E8.5 (J). The head region is indicated by arrowheads. (K-M) Hes3 expression is not significantly affected in the Hes1;Hes5 double mutant, compared with the wild type. (N-R) At E8.5, very few neurons (TuJ1⁺) are generated in both the wild type and Hes1;Hes5 double mutant (N,O) whereas in Hes1;Hes3;Hes5 triple-mutant, many neurons are prematurely differentiated (P). There are still many neuroepithelial cells (nestin⁺Ki67⁺) in the Hes1;Hes3;Hes5 double mutant (Q,R). (S) At E9.5, more neurons are prematurely differentiated in the ventral region of Hes1;Hes3;Hes5 triple-mutant spinal cord. (T-V) At E10.0, the structure of the spinal cord is severely disorganized in Hes1;Hes3;Hes5 triple mutants (T). Strikingly, virtually all cells become neurons (U), and radial glia are missing (V). Scale bars: 100 μm in A-H,N-V.

**Fig. 12.** Characters and functions of neural stem cells. (A) Embryonic neural stem cells change their characters in the following order: Hes-independent neuroepithelial cells, transitory Hes-dependent neuroepithelial cells, and Hes-dependent radial glial cells. Based on the expression patterns, transitory Hes-dependent neuroepithelial cells seem to depend on Hes1 and Hes3, while Hes-dependent radial glial cells mostly depend on Hes1 and Hes5. (B) Roles of radial glia in the inner and outer barrier formation. (a) Radial glia form the tight and adherens junctions at the apical side and contribute to the basal lamina formation at the basal side. (b) Radial glia establish the framework of the neural tube by forming the inner and outer barriers. In the absence of radial glia (Hes1⁻/⁻:Hes5⁻/⁻), neurons escape from the wall of the neural tube, thereby destroying the structural integrity.
et al., 2002). Radial glial cells thus contribute to formation of the outer barrier by expressing laminin.

Previous studies showed that the collagen IV network is produced by surrounding mesenchymal cells but not by radial glial cells (Thomas and Dziadek, 1993). In addition, in-situ hybridization analysis showed that laminin-α1 mRNA is weakly expressed by surrounding mesenchymal cells next to the spinal cord. Thus, the basal lamina is generated cooperatively at the interface by radial glia and mesenchymal cells.

**Accelerated differentiation generates neurons at the expense of the later-born cell types in the absence of Hes genes**

Neural stem cells are known to change their competency over time (Desai and McConnell, 2000). They initially give rise to neurons and later to other cell types such as ependymal cells and astrocytes. Ependymal cells are the internal lining of the neural tube and carry the adherens junction (Lagunowich et al., 1992; Mollgard et al., 1987; Redies et al., 1993), while astrocytes produce laminin (Liesi et al., 1983). Thus, in the wild type, at later stages when radial glial cells disappear, they are replaced by ependymal cells and astrocytes.

In Hes-null mice, differentiation of radial glial cells is accelerated. There could be two types of acceleration: (1) all cell types are generated rapidly; or (2) only early-born cell types are generated at the expense of the later-born cell types. In Hes-null mice, radial glial cells prematurely differentiate into neurons only without giving rise to later-born cell types. It is likely that it takes a certain period of time for neural stem cells to change their competency to generate the later-born cell types. It was previously shown that the promoter region of the astrocyte-specific gene for glial fibrillar acidic protein (GFAP) is highly methylated and silenced in neural stem cells at early stages of development (Takizawa et al., 2001). Neural stem cells at early stages are therefore refractory to inductive signals for astrocyte differentiation. However, as development proceeds, the GFAP promoter region is gradually demethylated, and neural stem cells gain the competency to become astrocytes. Thus, maintenance of radial glial cells for a certain period of time during development has two functions: increasing the cell number; and covering a full range of competency. Accelerated differentiation therefore leads to defects in both aspects, reduction of the cell number and lack of later-born cell types.

We thank Drs Sue McConnell and Toshiyuki Ohtsuka for discussion and critical reading of an early version of the manuscript, Drs Shoichiro Tsukita and Mikio Furuse for providing antibodies, discussion and technical advice and Dr Makoto Ishibashi for technical help. This work was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Japan Society for the Promotion of Science. J.H. was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

**References**


Hirata, H., Ohtsuka, T., Bessho, Y. and Kageyama, R. (2000). Generation...
of structurally and functionally distinct factors from the basic helix-loop-helix gene Hes3 by alternative first exons. J. Biol. Chem. 275, 19083-19089.


