Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system

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The developing central nervous system is partitioned into compartments by boundary cells, which have different properties than compartment cells, such as forming neuron-free zones, proliferating more slowly and acting as organizing centers. We now report that in mice the bHLH factor Hes1 is persistently expressed at high levels by boundary cells but at variable levels by non-boundary cells. Expression levels of Hes1 display an inverse correlation to those of the proneural bHLH factor Mash1, suggesting that downregulation of Hes1 leads to upregulation of Mash1 in non-boundary regions, whereas persistent and high Hes1 expression constitutively represses Mash1 in boundary regions. In agreement with this notion, in the absence of Hes1 and its related genes Hes3 and Hes5, proneural bHLH genes are ectopically expressed in boundaries, resulting in ectopic neurogenesis and disruption of the organizing centers. Conversely, persistent Hes1 expression in neural progenitors prepared from compartment regions blocks neurogenesis and reduces cell proliferation rates. These results indicate that the mode of Hes1 expression is different between boundary and non-boundary cells, and that persistent and high levels of Hes1 expression constitutively repress proneural bHLH gene expression and reduce cell proliferation rates, thereby forming boundaries that act as the organizing centers.

KEY WORDS: bHLH, Floor plate, Isthmus, Rhombomere boundary, Roof plate, Zona limitans intrathalamica, Mouse

INTRODUCTION

The developing central nervous system (CNS) is partitioned into many compartments, which form units of distinct populations of neurons (Lumsden and Krumlauf, 1996; Kiecker and Lumsden, 2005; Irvine and Rauskolb, 2001; Simeone, 2002). These compartments are demarcated by boundary cells, which regulate neuronal specification in the neighboring compartments. For example, the zona limitans intrathalamica (ZLI), a boundary between the prethalamus and the thalamus, regulates specification of prethalamic and thalamic neurons by secreting Sonic hedgehog (Shh) (Kiecker and Lumsden, 2004). The isthmus, a boundary between the midbrain and the hindbrain, regulates their development by secreting Wnt1 and Fgf8 (Joyner et al., 2000; Mason et al., 2000; Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). The roof plate and the floor plate, located at the dorsal and ventral regions of the neural tube, respectively, also have similar features. They separate the right and left sides of the neural tube and regulate the dorsoventral patterning of the nervous system by expressing Wnt and BMP family members dorsally and Shh ventrally (Lee and Jessell, 1999; Placzek and Briscoe, 2005). Thus, these structures not only separate the CNS into many units but also regulate neuronal specification of each unit by acting as organizing centers. Genes regulating boundary formation have been intensively analyzed: the isthmus is formed between the ZLI and the Shh expression domains (Broccoli et al., 1999; Millet et al., 1999) whereas the ZLI is formed between Six3 and Irx3 expression domains, depending on Shh (Kobayashi et al., 2002; Braun et al., 2003; Zeltsner, 2005).

Boundaries are formed by specialized neuroepithelial or radial glial cells, which have properties that are different from those in compartments. These boundary cells are embedded in a specialized extracellular matrix and proliferate more slowly than compartment cells. Furthermore, neurogenesis is delayed or does not occur in boundaries (Lumsden and Keynes, 1989; Guthrie et al., 1991; Kahane and Kalcheim, 1998; Trokovic et al., 2005). Such boundary cell-specific properties are well documented, but little is known about how these properties are regulated. Recent studies revealed that, in zebrafish, activation of Notch signaling regulates rhombomere boundary formation by segregating boundary cells from rhombomeres and inhibiting neurogenesis (Cheng et al., 2004). Although roles of Notch signaling in boundary formation remains to be determined in mouse, we previously found that the repressor-type basic helix-loop-helix (bHLH) gene Hes1, one of the Notch effectors (Jarriault et al., 1995; Kageyama and Nakanishi, 1997; Ohtsuka et al., 1999), is involved in formation of the isthmus (Hirata et al., 2001). In the absence of Hes1 and its related gene Hes3, the isthmic cells are differentiated into neurons and lose their organizer activities prematurely, indicating that Hes1 and Hes3 contribute to maintenance of a neuron-free zone and the organizing center at the isthmus (Hirata et al., 2001). Similarly, the Hes-related genes her5 and him (her11) in zebrafish and XHR1 in Xenopus regulate formation of a neuron-free zone at the isthmus (Geling et al., 2004; Ninkovic et al., 2005; Takada et al., 2005). However, Hes1 is also expressed in compartments, where neurogenesis occurs actively, and it remains to be determined why Hes1-expressing cells in compartments undergo neurogenesis whereas those in boundaries do not. In addition, it is not known whether Hes genes play a role in formation of boundaries other than in the isthmus.

We have found that the mode of Hes1 expression is different in boundary and non-boundary regions in mice. In non-boundary regions, Hes1 is expressed at variable levels: some cells express Hes1 at high or intermediate levels whereas others express no Hes1. Downregulation of Hes1 expression leads to neurogenesis by
derepression of proneural bHLH factors such as Mash1 (Ascl1 – Mouse Genome Informatics). By contrast, boundary cells consistently express Hes1 at high levels and are negative for proneural bHLH gene expression. In the absence of Hes genes, however, proneural bHLH genes are ectopically expressed in boundaries, resulting in ectopic neurogenesis and impairment of the organizer activities. Conversely, persistent Hes1 expression in compartmental neural progenitors leads to blockade of neurogenesis and reduction of proliferation rates, properties that are reminiscent of boundary cells. Thus, persistent and high levels of Hes1 expression regulate boundary formation in the developing CNS.

MATERIALS AND METHODS

Mutant mice

Hes1, Hes3 and Hes5 mutant mice were reported previously (Hirata et al., 2001; Ohtsuka et al., 1999). All animals used for this study were maintained and handled according to the protocols approved by Kyoto University.

In situ hybridization

In situ hybridization with digoxigenin-labeled antisense RNA probes was performed as described previously (Tomita et al., 2000).

BrdU labeling

Pregnant mice were injected intraperitoneally with 120 µg BrdU/g body weight. After 30 minutes, sections of embryos were prepared for immunohistochemistry, as described previously (Ohsawa et al., 2005).

Scanning electron microscopic analysis (SEM)

SEM analysis was done, as described previously (Hatakeyama et al., 2004).

Antibodies

Anti-Hes1 antibody was generated, as previously described (Hatakeyama et al., 2006). Other antibodies used are as follows: anti-BrdU (Becton Dickinson), anti-Shh (Developmental Studies Hybridoma Bank), anti-Ki67 (PharMingen), anti-Mash1 (PharMingen), anti-phosphorylated histone H3 (Sigma), anti-GFP (Molecular Probes), anti-nestin (PharMingen), anti-β-tubulin III (TUJ1) (Babco), anti-GFAP (Sigma), and anti-cyclin D1 (Santa Cruz). Biotinylated donkey anti-guinea pig IgG (Jackson), fluorescein avidin D (Vector), Fluorolink Cy3-conjugated goat anti-mouse IgG (Amersham Pharmacia), Fluorolink Cy2-conjugated goat anti-rabbit IgG (Amersham Pharmacia) and FITC-conjugated goat anti-mouse IgG (Vector) were used as secondary antibodies.

Immunohistochemistry and immunocytochemistry

For immunohistochemistry, embryos were fixed in cold 4% paraformaldehyde (PFA) in PBS overnight, rinsed with cold PBS three times and equilibrated in cold 25% sucrose in PBS overnight. Embryos were embedded in OCT compound, frozen at –80°C and sectioned at 15 µm thickness. Sections were washed three times in PBS and preincubated in blocking buffer (1.5% normal goat serum and 0.1% Triton X-100 in PBS) for 1 hour. Sections for Hes1 immunostaining were washed three times in PBS, autoclaved in 0.01 M citrate buffer pH 6.0 (105°C, 10 minutes), and washed three times again in PBS before preincubation.

Sections were next incubated in blocking buffer with primary antibodies at 4°C overnight or for 2 days (for Hes1). After being washed in PBS three times, sections were incubated with the secondary antibody in blocking buffer at room temperature for 1 hour and then washed in PBS three times. For Hes1, the tertiary antibody reaction was performed. Samples were then treated with propidium iodide (PI) or DAPI and were mounted with Fluoromount G (SouthernBiotech).

For immunocytochemistry, cells on chamber slides were washed in ice-cold PBS three times, and fixed in 4% PFA at room temperature for 15 minutes. Samples were then washed in PBS three times and preincubated with blocking buffer at room temperature for 20 minutes. Antibody reactions were done as described above. TUNEL was performed using a standard detection kit (Roche).

Measurement of relative Hes1 protein levels

To measure a relative Hes1 protein level on images, each nucleus was circled according to the nuclear staining, and signal intensity of Hes1 immunostaining and nuclear staining in each circle was measured using the program Image-Pro Plus (Ver. 5.1, 1, 32; MediCybernetics, Silver Spring, MD, USA). The background of a non-Hes1-expressing region was subtracted from each Hes1 signal. This Hes1-specific signal value was divided by the nuclear signal value to normalize the staining efficiency and categorized as (+) for a value of <= 0.3, (+) for >0.3, (+++) for >0.8 and (++) for >1.3. During M phase, some cells express Hes1 protein in the cytoplasm, but they were excluded from the analysis because cytoplasmic Hes1 does not function in transcriptional repression. As a control experiment, we successfully detected the relative difference in Hes1 protein levels between non-Hes1-expressing cells (+) and transfected Hes1-expressing cells (+ to ++).

Neural progenitor cell (NPC) culture

NPC and retrovirus preparation was done, as described previously (Hirata et al., 2000; Ohtsuka et al., 2001; Hatakeyama and Kageyama, 2002). 10 µl of the viral solution was added to each well of poly-L-lysine-coated 8-well plates. After 4 hours, the virus-containing medium was discarded, and the cells were cultured in fresh NPC medium. We usually obtained 20-30 colonies in each well of the eight-well plates.

RESULTS

Persistent and high levels of Hes1 expression and low proliferation rates in boundaries

Hes1 mRNA is expressed at high levels by the neuroepithelial cells of boundaries (Fig. 1A,E,M,P) (Hirata et al., 2001; Hatakeyama et al., 2004; Ohtsuka et al., 2006). We previously found that Hes1 mRNA is increased when Hes1 protein levels are low because Hes1 protein represses its own transcription by negative feedback (Takebayashi et al., 1994; Hirata et al., 2002). To determine the levels of Hes1 protein expression in boundaries, we performed immunohistochemistry. Similar to the mRNA expression, Hes1 protein is also expressed at high levels in the ZLI, the isthmus, the interhormbbomeric boundaries, the roof plate and the floor plate (Figs 1-3), suggesting that the negative feedback does not work in boundary cells.

In the isthmus, Hes1 protein is expressed at a high level by neuroepithelial cells (Fig. 1B,C), which also express Wnt1 (Fig. 1D,E, arrows). These Hes1-expressing cells do not display efficient BrdU uptake (Fig. 1F,G, arrows) (see also Trokovic et al., 2005), compared to the adjacent compartment cells. In the ZLI, Hes1 protein is highly expressed (Fig. 1H,L, asterisks), and the cells that express Hes1 at high levels also express Shh (Fig. 1I-K, brackets). Hes1-expressing cells in the ZLI do not display efficient BrdU uptake (Fig. 1L-O, asterisks), compared to the adjacent compartment cells. Thus, in the isthmus and the ZLI, Hes1-positive cells express Wnt1/Shh and do not actively proliferate. Hes1 protein is also highly expressed in the interhormbbomeric boundaries (Fig. 1Q-S). These Hes1-expressing cells are mostly negative for phosphorylated histone H3 (pH3), an M phase-specific marker (Fig. 1T-V, arrows), whereas many of the adjacent rhombomeric cells are positive for pH3 (Fig. 1U,V), indicating that Hes1-expressing cells do not actively proliferate in the interhormbbomeric boundaries.

In the spinal cord, Hes1 is expressed in the roof plate and the floor plate as well as in the regions in between along the DV axis (Fig. 2A). In the roof plate and the floor plate, all cells express Hes1 protein at a high level (Fig. 2H,N). The roof plate and the floor plate cells are mostly negative for Ki67, a marker for proliferating cells (Fig. 2A-C, arrowheads), and for BrdU uptake (Fig. 2D-G) whereas many of non-boundary cells are positive for Ki67 (Fig. 2A,C, bracket) and BrdU uptake (Fig. 2D,E). In addition, pH3 is expressed...
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Whereas Hes1, a transcriptional repressor for proneural bHLH gene expression, is expressed in both boundaries and non-boundaries, proneural bHLH genes are expressed only in non-boundary regions. In the spinal cord, the proneural bHLH factor Mash1 is expressed in the same regions as Hes1 at E10.5 (Fig. 4A-D). To reveal the relationship between Hes1 and Mash1 expression, we performed double immunohistochemistry. In non-boundary regions, cells expressing Hes1 at a high level do not express Mash1 (Fig. 4E-G, green cells in H) whereas those expressing Hes1 at an intermediate level also express Mash1 at an intermediate level (Fig. 4E-G, striped cells in H). In contrast, those expressing no Hes1 protein express Mash1 at a high level (Fig. 4E-G, a red cell in H). These results indicate that there is an inverse correlation between Hes1 and Mash1 expression in non-boundary cells. Since Hes1 can repress Mash1 expression by binding to the Mash1 promoter (Chen et al., 1997; Castella et al., 1999), it is probable that decreases in Hes1 protein levels lead to de-repression of Mash1 in non-boundary cells.

In boundaries, other proneural bHLH genes such as neurogenin 2 (Ngn2) and Math1 (Atoh1 – Mouse Genome Informatics) are not expressed either, although they are highly expressed in non-boundary regions (see Fig. 6). These results raise the possibility that persistent and high levels of Hes1 expression constitutively repress proneural bHLH gene expression in boundaries, thereby making neuron-free zones.

Fig. 1. High levels of Hes1 expression in boundaries. (A) Whole-mount in situ hybridization at E10.5. Hes1 mRNA is expressed at high levels in the ZLI (left arrow) and the isthmus (right arrow). Left and right lines indicate sections shown in (H-O) and (B,C), respectively. (B) Immunohistochemistry for Hes1. Hes1 protein is expressed at high levels in the isthmus (boxed) and the floor plate (asterisk). (C) A higher magnification of a boxed region in (B). Inset in (C) shows Hes1 protein expression in the isthmus and E9.5. (D-G) Serial parasagittal sections of the isthmic region at E11.5 (boxed in (A)). Wnt1-expressing cells (D, arrow) also express Hes1 (E, arrow) at high levels in the isthmus.

These cells do not efficiently take up BrdU (F,G, arrows), compared with the surrounding region. (H-K) Hes1 protein is expressed at high levels in the ZLI (H, asterisk). Cells expressing Hes1 (I, bracket) also express Shh (J,K, brackets) in the ZLI. Inset in (J) shows a lower magnification of sti in situ hybridization for Shh. (L-O) Hes1 protein (L) and Hes1 mRNA (M) are highly expressed in the ZLI (asterisks). Cells in the ZLI display lower BrdU uptake (N,O, asterisks). (P) Flat-mount in situ hybridization of Hes1 at E9.5. The midbrain-hindbrain region was cut along the dorsal midline. Hes1 mRNA is expressed in the interrhombomeric boundaries. Arrowheads indicate the isthmus. Top is rostral. (Q-V) Frontal section of the hindbrain at E9.5. Cells at interrhombomeric boundaries express Hes1 protein at high levels. They are mostly negative for phosphorylated histone 3 (pH3) (U,V, arrows), indicating that interrhombomeric boundary cells do not actively proliferate. The boxed region in Q is enlarged in the insets in Q-S. PI, propidium iodide. Scale bars: 500 μm in O; 200 μm in V, 100 μm in C,G; 50 μm in K.

by non-boundary cells but not by the roof plate and the floor plate cells (data not shown). Quantification of the Hes1 protein levels relative to the nuclear staining with propidium iodide (PI) revealed that almost all roof plate and floor plate cells express Hes1 protein at high levels (Fig. 2H-J,N-Q). High levels of Hes1 protein expression in these cells were confirmed in all sections of different embryos at E10.5 (data not shown). These results indicate that boundary cells express Hes1 protein at high levels, and that these Hes1-expressing cells do not efficiently proliferate, compared to non-boundary cells. We also examined other stages. As at E10.5, the roof plate and the floor plate cells express Hes1 protein at high levels at E9.5 (Fig. 3A-H), E11.5 (Fig. 3I-P) and E13.5 (Fig. 3Q-X), indicating that boundary cells persistently express Hes1 protein at high levels.

In non-boundary regions, Hes1 protein is also expressed in the nuclei of neuroepithelial and radial glial cells. In Hes1-positive cells non-boundary regions of the spinal cord, some cells express Hes1 protein at a high level whereas others express it at an intermediate level (Fig. 2K-M). Cells that do not express Hes1 protein are also present in the same regions (Fig. 2K-M). Similarly, Hes1 protein levels are variable in non-boundary regions just dorsal to the floor plate (Fig. 2N-P, bracket) and in other compartments (data not shown, see Fig. 1C, in compartments near the isthmus). Quantification analysis showed that Hes1 protein levels are variable in non-boundary cells (Fig. 2Q). These results indicate that the mode of Hes1 expression is different in boundary and non-boundary cells.

Inverse correlation between the Hes1 and Mash1 expression levels

As at E10.5, the roof plate and the floor plate cells express Hes1 protein at high levels at E9.5 (Fig. 3A-H), E11.5 (Fig. 3I-P) and E13.5 (Fig. 3Q-X), indicating that boundary cells persistently express Hes1 protein at high levels. In non-boundary regions, Hes1 protein is also expressed in the nuclei of neuroepithelial and radial glial cells. In Hes1-positive cells non-boundary regions of the spinal cord, some cells express Hes1 protein at a high level whereas others express it at an intermediate level (Fig. 2K-M). Cells that do not express Hes1 protein are also present in the same regions (Fig. 2K-M). Similarly, Hes1 protein levels are variable in non-boundary regions just dorsal to the floor plate (Fig. 2N-P, bracket) and in other compartments (data not shown, see Fig. 1C, in compartments near the isthmus). Quantification analysis showed that Hes1 protein levels are variable in non-boundary cells (Fig. 2Q). These results indicate that the mode of Hes1 expression is different in boundary and non-boundary cells.
Fig. 2. Hes1 expression in the spinal cord. (A-C) Double immunostaining of Hes1 and Ki67 at E10.5. Hes1-expressing cells in non-boundary regions are mostly positive for Ki67 (C, bracket). In contrast, Hes1-expressing cells in the roof plate and the floor plate are negative for Ki67 (C, arrowheads). (D-G) BrdU uptake in the spinal cord at E11.5. BrdU-positive cells are present in non-boundary regions but very rare in the roof plate (E, upper box, and F) or the floor plate (E, lower box, and G). Boxed regions in E are enlarged in F and G. (H-J) Hes1 protein expression in the roof plate at E10.5. Inset in H shows Wnt1 expression in the roof plate. (K-M) Hes1 protein expression in non-boundary regions at E10.5. (N-P) Hes1 protein expression in the ventral spinal cord including the floor plate at E10.5. Inset in N shows Shh expression in the floor plate. In non-boundary regions just dorsal to the floor plate, Hes1 protein is expressed at variable levels (P, bracket). (Q) Hes1 protein expression (top). (Bottom) Relative intensity of Hes1 signal was measured (see Materials and methods); error bars represent s.e.m. (a total of 108 roof plate cells, 109 floor plate cells and 1,106 non-boundary cells in six different sections were examined). Scale bars: 100 μm (C-E), 50 μm (F, G and P).

Defects in structures and organizer activities of boundaries in Hes-null mice

To investigate the requirement of persistent Hes1 expression for boundary formation, we next performed loss-of-function analyses. The ZLI normally expresses Hes1 only but, because Hes5 is ectopically upregulated in the ZLI of Hes1-null mice (Hatakeyama et al., 2004), we examined Hes1;Hes5 double-null mice. Although these double-null mice exhibit severe structural defects of the nervous system (Hatakeyama et al., 2004), the forebrain and midbrain are relatively intact at E10.5. However, the ZLI is lacking in the double-null mice (Fig. 5C,D, asterisks), whereas it is clearly observed in the wild type (Fig. 5A,B, arrows). Furthermore, Shh is expressed in the ZLI of the wild-type around E10.5 (Fig. 5I, arrowhead) but it is not detectable in Hes1;Hes5 double-null mice (Fig. 5J, asterisk). In the wild type, the adjacent compartments express Lfng but the ZLI does not (Fig. 5K, arrowhead), and misexpression of Lfng has been shown to repress ZLI formation (Zeltser et al., 2001). Similarly, in the wild type the adjacent compartments express Pax6, although the ZLI does not do so (Fig. 5M, arrowhead). In contrast, in Hes1;Hes5 double-null mice, the Lfng- and Pax6-negative domain is missing (Fig. 5L,N, asterisks). In addition, the bHLH-PAS genes Sim1 and Sim2 are expressed in the ZLI of the wild type (Ema et al., 1996), whereas this expression domain is lacking in Hes1;Hes5 double-null mice (data not shown). These results indicate that the ZLI is not formed in the absence of Hes1 and Hes5.

Hes1 and Hes3 are expressed in the isthmus, and in the absence of Hes1 and Hes3, the isthmus is not properly maintained and prematurely loses Fgf8 expression (Hirata et al., 2001). Similarly, in the Hes1;Hes5 double-null mice, the isthmus is only partially formed (Fig. 5C,D, arrowheads), suggesting that Hes3 may partially compensate for this defect. We further examined the expression of Hes3 in Hes1;Hes5 double-null, Hes1;Hes5 double-null and Hes1;Hes3;Hes5 triple-null mice. Hes3;Hes5 double-null mice were found to be normal and used as a control. In the isthmus of Hes1;Hes5 double-null mice, Wnt1, Fgf8 and En1 are expressed normally (Fig. 5O,R,U, arrowheads). In the isthmus of Hes1;Hes5 double-null mice, however, expression of Wnt1, Fgf8 and En1 is reduced (Fig. 5P,S,V, arrowheads). In the isthmus of Hes1;Hes3;Hes5 triple-null mice, the expression of Wnt1, Fgf8 and En1 is more severely reduced, although they are not completely lost (Fig. 5Q,T,W, asterisks). We observed these defects at both E8.5 (data not shown) and E9.5 (Fig. 5). These results indicate that in the absence of Hes1, Hes3 and Hes5, the isthmus is not properly formed and loses much of the isthmus-specific gene expression. This defect occurs even earlier than in Hes1;Hes3 double-null mice, which display loss of Wnt1 and Fgf8 expression around E10.5 (Hirata et al., 2001). In the wild-type and Hes3;Hes5 double-null embryos the expression domain of Otx2 is rostral whereas that of Gbx2 is caudal to the isthmus (Fig. 5X,Aa), but these domains are not clearly separated in
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**Ectopic neurogenesis in boundaries of Hes-null mice**

We next examined the expression of the proneural bHLH genes *Mash1*, *Ngn2* and *Math1* in boundaries of Hes-mutant mice. *Mash1* and *Ngn2* are widely expressed in compartments but not in the ZLI or the isthmus of the wild-type and *Hes1;Hes5* double-null embryos (Fig. 6A, arrowheads, and data not shown). In *Hes1;Hes5* double-null embryos, however, the *Mash1*-negative domains in the ZLI and the isthmus become narrower or lost in the ventral region (Fig. 6B, asterisk) while the *Ngn2* expression domain extends rostrally, resulting in the loss of the *Ngn2*-negative domain in the ZLI (Fig. 6E, asterisk). Furthermore, in *Hes1;Hes3;Hes5* triple-null embryos, the *Mash1*- and *Ngn2*-negative domain in the ZLI is missing, resulting in continuous expression of *Mash1* and *Ngn2* in the ventral part of the forebrain (Fig. 6C, F, asterisks). The *Mash1*- and *Ngn2*-negative domain in the isthmus is also missing in *Hes1;Hes3;Hes5* triple-null embryos (Fig. 6C, F, arrowheads). *Math1* is expressed in the dorsal part of the neural tube caudal to the isthmus in wild-type (Akazawa et al., 1995; Vernay et al., 2005) and *Hes3;Hes5* double-null (Fig. 6G) embryos. However, in *Hes1;Hes5* double-null mice, *Math1* expression is extended rostrally into the midbrain without interruption by the isthmus (Fig. 6H, arrow), and in *Hes1;Hes3;Hes5* triple-null mice this ectopic expression is further upregulated (Fig. 6I, arrow). Similarly, in the hindbrain of the wild-type and *Hes3;Hes5* double-null embryos, *Ngn2* and *Math1* are not expressed at the interrhombomeric boundaries (Fig. 6M, arrowheads, and data not shown) whereas these proneural bHLH gene-negative regions at the interrhombomeric boundaries are somewhat obscure in *Hes1;Hes5* double-null mice (Fig. 6N, arrowheads) and mostly lacking in *Hes1;Hes3;Hes5* triple-null mice (Fig. 6O, bracket). Thus, in Hes-null mutants, the regions negative for proneural bHLH gene expression are missing in the ZLI, isthmus and interrhombomeric boundaries.

In the wild-type hindbrain, the interrhombomeric boundaries are formed at E9.5 and E10.5 (Fig. 5G, bracket) but are somewhat ambiguous at E10.5 (Fig. 5H, bracket) in *Hes1;Hes5* double-null hindbrain. Thus, the interrhombomeric boundaries are not properly maintained in the absence of Hes genes.

In *Hes1;Hes3;Hes5* triple-mutant mice, Wnt1 expression in the roof plate is still maintained in the midbrain-hindbrain regions (Fig. 5Q) but is mostly missing in the spinal cord (Fig. 6U, asterisk). Similarly, *Shh* expression in the floor plate is downregulated in *Hes1;Hes5* double-null mice (Fig. 6Z, arrowheads) and is more severely reduced or lost in *Hes1;Hes3;Hes5* triple-mutant mice (Fig. 6Aa, Aa, asterisks). Thus, in the absence of Hes genes, the organizer activities in these structures are not properly maintained.

**Development**

**Fig. 3. Persistent Hes1 expression in the roof plate and the floor plate.** (A-X) In situ hybridization for *Wnt1* and *Shh*. *Hes1* immunostaining and PI staining were performed with embryos at E9.5, E11.5 and E13.5. Cells in the roof plate and the floor plate persistently express *Hes1* protein at high levels during this period. Scale bar: 50 μm.

**Fig. 4. Inverse correlation between Hes1 and Mash1 expression levels in the spinal cord.** (A-D) Double immunostaining for *Hes1* and *Mash1* at E10.5. Boxed region in C is enlarged in D. (E-G) A higher magnification of a boxed region in D. Cells expressing *Hes1* at a high level (green) do not express *Mash1* whereas a cell expressing *Mash1* at a high level (red) does not express *Hes1*. Cells expressing *Hes1* at an intermediate level express *Mash1* at an intermediate level. (H) The results in E-G are shown schematically. Green and red cells are *Hes1* positive only and *Mash1* positive only, respectively, whereas striped cells are *Hes1;Mash1* double-positive. Scale bars: 100 μm (C), 50 μm (D).
**Fig. 5. Boundary defects in the developing nervous system.**

(A-H) SEM analysis of wild-type (A,B,E,F) and Hes1;Hes5 double-null (C,D,G,H) embryos. Parts of A and C are enlarged in B and D, respectively. At E10.5, the ZLI (arrows) and the isthmus (arrowheads) are clearly formed in the wild type (A,B). By contrast, in Hes1;Hes5 double-null embryo (C,D), the ZLI is lacking (asterisks) and the isthmus is only partially formed (arrowheads). The interrhombomeric boundaries are clearly formed in the wild type at E9.5 (E, arrowheads) and E10.5 (F, arrowheads). They are not significantly affected at E9.5 (G, bracket) but become ambiguous at E10.5 (H, bracket) in Hes1;Hes5 double-null embryos. (I,J) In situ hybridization for Shh at E10.5. Shh is expressed in the wild-type ZLI (I, arrowhead) but not in Hes1;Hes5 double-null ZLI (J, asterisk). (K-N) In situ hybridization for Lfng at E9.5 (K,L) and Pax6 at E10.5 (M,N). In the wild-type ZLI (K,M), Lfng and Pax6 are not expressed (arrowheads). In contrast, in Hes1;Hes5 double-null embryos (L,N), the Lfng- and Pax6-negative regions are lacking (asterisks). (O-Cc) In situ hybridization for Wnt1 (O-Q), Fgf8 (R-T), En1 (U-W), Otx2 (X-Z), and Gbx2 (Aa-Cc) at E9.5 and E8.5 (insets of X-Cc). Wnt1, Fgf8 and En1 are normally expressed in the isthmus of Hes1;Hes5 double-null embryos (O,R,U, arrowheads). This expression is downregulated in Hes1;Hes5 double-null embryos (P,S,V, arrowheads) and more severely impaired in Hes1;Hes3;Hes5 triple-null embryos (Q,T,W, asterisks). In Hes3;Hes5 double-null embryos, Otx2 is expressed rostrally whereas Gbx2 is expressed caudally to the isthmus (X,Aa, arrowheads). The Otx2 and Gbx2 expression domains are less clearly separated in Hes1;Hes5 double-null embryos at E9.5 (Y,Bb, asterisks), and their borders become even more ambiguous in Hes1;Hes3;Hes5 triple-null embryos at E8.5 and E9.5 (Z,Cc, asterisks).

6X, asterisk). These results indicate that in the absence of Hes genes, proneural bHLH genes are ectopically expressed in the roof plate and the floor plate.

We next examined expression of delta-like 1 (Dll1), one of the earliest markers for post-mitotic neurons. In the wild-type and Hes3;Hes5 double-null embryos, Dll1 is not expressed in boundaries (Fig. 6J, arrowheads, and data not shown). In contrast, in Hes1;Hes5 double- and Hes1;Hes3;Hes5 triple-null embryos, such Dll1-negative domains are missing (Fig. 6K,L, asterisks). Dll1 expression is also enhanced in all compartments of Hes1;Hes5 double- and Hes1;Hes3;Hes5 triple-null embryos (Fig. 6K,L). In addition, we found that neurons (TuJ1-positive cells) are ectopically differentiated in the roof plate and the floor plate of Hes1;Hes3;Hes5 triple-null embryos (data not shown). Because cell death is not increased in Hes-null mice at these stages (data not shown), it is probable that the cells that should normally form boundaries ectopically express proneural bHLH genes and are differentiated into neurons in the absence of Hes genes. These results support the conclusion that persistent and high levels of Hes1 expression are essential in vivo for the formation of neuron-free zones at boundaries.

**Persistent Hes1 expression reduces cell proliferation and maintains undifferentiated cells**

We next examined the effects of persistent Hes1 expression on neural progenitors, which were prepared from telencephalic compartments of E11.5 mouse embryos. Neural progenitors were infected with the retrovirus CLIG, which directs EGFP expression (Hojo et al., 2000), and CLIG-Hes1, which directs persistent Hes1 expression in addition to EGFP expression (Fig. 7A). Because retrovirus is infectious only to dividing cells, we were able to monitor the fate of the virus-infected neural progenitors. Each neural progenitor infected with CLIG or CLIG-Hes1 proliferated and formed a clone consisting of EGFP-positive progeny. Cells infected with CLIG proliferated efficiently and
formed large clones (Fig. 7B-D,H). The average size of each clone was about 135±12 cells at day 5. In contrast, cells infected with CLIG-Hes1 proliferated less efficiently and formed smaller clones (Fig. 7E-H). The average size of each clone was about 22±2 cells at day 5. We also performed TUNEL assays to determine whether cell death is responsible for the reduction in clonal sizes. Although there was some tendency to increased cell death when infected with CLIG-Hes1 compared to CLIG, the effect was not sufficient for the reduction of the growth rate of cells infected with CLIG-Hes1 (Fig. 7I), suggesting that persistent Hes1 expression reduces the cell proliferation rate without significant cell death. In agreement with this notion, ratios of cells positive for cyclin D1, a G1-specific marker (Momota and Holland, 2005), were increased when CLIG-Hes1 was infected, although the Ki67-positive ratios were not significantly changed (Fig. 7J). Thus, it is likely that persistent Hes1 expression prolongs the G1 phase, thereby reducing cell proliferation.

Cell type composition was also examined. Clones infected with CLIG consisted of progenitors (nestin-positive cells), neurons (TuJ1-positive cells; Fig. 7K-N) and astrocytes (GFAP-positive cells; Fig. 7S-V). In contrast, cells infected with CLIG-Hes1 were mostly negative for TuJ1 (Fig. 7O-R, closed arrowheads) and GFAP (Fig. 7W-Z, arrowheads) even after 5 days in culture. These results indicate that forced Hes1 expression endows compartment cells in culture with properties that are reminiscent of boundary cells: reduction of cell proliferation and blockade of cell differentiation.

**DISCUSSION**

**Persistent and high levels of Hes1 expression are required for formation of neuron-free zones and organizing centers in boundaries**

Here, we found that Hes genes regulate boundary formation in the developing nervous system. Although Hes1 is expressed in both boundary and non-boundary regions, the mode of Hes1 expression is different between the two structures. Wnt1-expressing cells and Shh-
expressing cells persistently express Hes1 at high levels in the isthmus and ZLI, respectively. Similarly, all cells in the roof plate and the floor plate persistently express Hes1 protein at high levels. In contrast, levels of Hes1 protein expression are variable in non-boundary regions. Expression levels of the proneural bHLH factor Mash1 display an inverse correlation to levels of Hes1, suggesting that downregulation of Hes1 leads to upregulation of Mash1 in non-boundary regions while persistent and high levels of Hes1 expression constitutively repress Mash1 and generate neuron-free zones in boundaries. Consistent with this notion, inactivation of Hes genes ectopically upregulates Hes1 expression (Kageyama and Nakanishi, 1997), it is probable that sustained activation of Notch signaling maintains persistent and high levels of Hes1 in boundaries.

Although Hes1 is expressed in most boundaries, there are no significant boundary defects in Hes1-null embryos (Hirata et al., 2001). Hes3 is highly expressed and compensates for Hes1 deficiency in the isthmus. Hes5 is not expressed in boundaries of wild-type embryos, but it is ectopically expressed in boundaries of Hes1-null embryos (Hatakeyama et al., 2004). Thus, all three Hes genes are able to regulate boundary formation, in addition to non-boundary development, although it remains to be determined whether or not the mode of Hes3 and Hes5 expression is different between boundary and non-boundary regions, like Hes1.

Fig. 7. Reduction of cell proliferation and neurogenesis by persistent Hes1 expression. (A) The schematic structures of recombinant retroviruses. (B-G) Immunostaining for GFP. E11.5 telencephalic neural progenitors infected with CLIG or CLIG-Hes1 were examined at different time points. (H) Clonal sizes (with s.e.m.) of the virus-infected cells. Clonal sizes of CLIG-Hes1-infected cells are smaller than those of CLIG-infected cells. Experiments were repeated at least six times at each time point. (I) Ratios (with s.e.m.) of TUNEL-positive cells in the virus-infected cells at 72 hours after infection (at least 2,000 cells in three independent experiments were examined). Although there is a tendency for cell death to increase in CLIG-Hes1 infection compared to CLIG infection, the effect is not sufficient for the reduction of growth rates in CLIG-Hes1 infection. (J) Ratios (with s.e.m.) of proliferating cells in the virus-infected cells at 72 hours after infection (at least 500 cells in three independent experiments were examined). Ki67-positive cell ratios are similar between CLIG- and CLIG-Hes1-infected cells, whereas a ratio of cells positive for the G1 phase-specific cyclin D1 is higher in CLIG-Hes1 infection, suggesting that Hes1 overexpression prolongs G1 phase. (K-Z) Immunocytochemical staining of cells infected with CLIG (K-N,S-V) and CLIG-Hes1 (O-R,W-Z). After 5 days in culture, some CLIG-infected cells have differentiated into TuJ1-positive neurons (K-N) or GFAP-positive astrocytes (S-V) whereas virtually none of the CLIG-Hes1-infected cells (closed arrowheads) are neurons (O-R) or astrocytes (W-Z). Some neurons (TuJ1-positive cells) and astrocytes (GFAP-positive cells) are indicated by open arrowheads (R,V).
In addition to ectopic neurogenesis, the organizer activities are severely impaired in the absence of Hes genes. In Hes1;Hes3;Hes5 triple-null mice, the isthmus is not properly formed, resulting in severe downregulation of Wnt1 and Fgf8 expression. Likewise, in Hes1;Hes5 double-null mice, neither the ZLI structure nor the Shh expression occurs. Furthermore, Wnt1 and Shh expression is reduced or lost in the roof plate and the floor plate, respectively, of Hes1;Hes3;Hes5 triple-null mice. These results indicate that persistent and high levels of Hes1 expression lead to maintenance of neuroepithelial cells of boundaries that act as the organizing centers.

**Persistent versus variable Hes1 expression in the developing nervous system**

We found that both Hes1 mRNA and Hes1 protein are highly expressed in boundaries. This expression mode is unusual because in most cells, when Hes1 protein is highly expressed, Hes1 mRNA is downregulated by negative feedback (Hirata et al., 2002). There are multiple Hes1-binding sites in the Hes1 promoter, and Hes1 protein, a transcriptional repressor (Sasai et al., 1992), inhibits its own transcription by directly binding to its own promoter (Takebayashi et al., 1994; Hirata et al., 2002). It appears that this negative feedback does not work in boundary cells, unlike most other cells. Hes1 protein should be functional, because inactivation of Hes genes leads to ectopic expression of proneural bHLH genes in boundaries. Thus, it remains to be determined why Hes1 represses proneural bHLH gene expression but not its own in boundaries. It has been shown that the Hes1-binding site of the Hes1 promoter is the N box (CACNAG) (Takebayashi et al., 1994) whereas that of the Mash1 promoter is the class C site (CACCG) (Chen et al., 1997). Thus, one possible mechanism is that the N box-binding activity of Hes1 is modulated by post-translational modification or by heterodimer formation in boundaries. Another possibility is that a boundary-specific factor binds to the N box sites of the Hes1 promoter to prevent Hes1 negative feedback.

In non-boundary regions, Hes1 expression levels are variable, but it is not yet known how Hes1 expression changes during neuronal differentiation. There could be at least two ways of changing the Hes1 expression levels during transition from Hes1-positive undifferentiated cells to Hes1-negative post-mitotic neurons. One is that Hes1 expression might be gradually downregulated and finally disappear while Mash1 expression is gradually upregulated. The other possible way is that Hes1 expression oscillates during transition from undifferentiated cells to neurons, as we previously found that Hes1 expression oscillates with a periodicity of about 2 hours in many cell types (Hirata et al., 2002; Masamizu et al., 2006). Activation of the Hes1 promoter increases Hes1 protein, which in return represses its own transcription by directly binding to its own promoter (Takebayashi et al., 1994). When the transcription is repressed by this negative feedback, Hes1 protein soon disappears because it is rapidly degraded by the ubiquitin-proteasome pathway. Disappearance of Hes1 protein then allows the next round of transcription. In this way, Hes1 autonomously starts oscillatory expression by negative feedback (Hirata et al., 2002). Since this oscillation is observed in many cell types, Hes1 expression could oscillate during transition from undifferentiated dividing cells to post-mitotic neurons. Further studies will be required to determine which way is the case for changes in Hes1 expression. As discussed below, Hes1 oscillation could be required for efficient growth of neural progenitors.

**Persistent and high Hes1 expression reduces cell proliferation rate**

We found that persistent and high Hes1 expression not only blocks neurogenesis but also reduces cell proliferation rate. It has been reported that persistent Hes1 expression also inhibits both differentiation and proliferation of PC12 pheochromocytoma cells (Castella et al., 2000). In the latter cells, overexpression of Hes1 represses transcription of the cyclin-dependent kinase (CDK) inhibitor p21. Hes1 also has been shown to repress the expression of the CDK inhibitors p21 and p27 in other cells (Castella et al., 2000; Kabos et al., 2002; Murata et al., 2005). However, because p21 and p27 usually retard G1 progression, the relationship between repression of p21 and p27 expression and cell cycle arrest is not clear. It was shown that both p21 and p27 also promote the assembly of cyclin D1-CDK4 complexes and the nuclear import of cyclin D, thereby inducing cell cycle progression (LaBaer et al., 1997; Cheng et al., 1999). Thus, both p21 and p27 are positive and negative regulators of G1-phase progression, and persistent repression of p21 and p27 by Hes1 could lead to reduction in cell proliferation rates.

It has been also reported that persistent Hes1 expression downregulates proliferating cell nuclear antigen (PCNA), an essential DNA replication factor, and leads to cell cycle arrest (Castella et al., 2000; Ström et al., 2000). In addition, Hes1 has been shown to repress expression of E2F, a transcription factor required for transition from G1 to S phase (Hartman et al., 2004). Hes1 forms a heterodimer complex with the Hes-related bHLH factor Hey/Hesr/Herp and represses transcription of E2F by directly binding to its promoter. Thus, persistent Hes1 expression blocks cell proliferation by repressing several genes that are essential for cell cycle progression. However, loss of Hes1 also inhibits proliferation of neural progenitors by upregulation of CDK inhibitors and proneural bHLH genes, inducing premature neurogenesis (Kabos et al., 2002; Hatakeyama et al., 2004). Thus, Hes1 is definitely required for maintenance of neural progenitors, raising the possibility that oscillatory rather than persistent Hes1 expression is required for efficient growth of neural progenitors.

We thank Dr Laure Bally-Cuif for critical reading of the manuscript, and Drs Makio Fujikoa, Hiroshi Kiyonari and Kazuki Nakao for discussion and technical assistance. This work was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from Japan Society for the Promotion of Science. J.H.B. was supported by the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan. J.H. was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

**References**


activators of cyclin D-dependent kinases in murine fibroblasts. EMBO J. 18, 1571-1583.


