

## Roles of the Basic Helix-Loop-Helix Genes *Hes1* and *Hes5* in Expansion of Neural Stem Cells of the Developing Brain\*

Received for publication, March 19, 2001, and in revised form, May 24, 2001  
Published, JBC Papers in Press, June 8, 2001, DOI 10.1074/jbc.M102420200

Toshiyuki Ohtsuka<sup>‡§</sup>, Masami Sakamoto<sup>‡</sup>, François Guillemot<sup>¶</sup>, and Ryoichiro Kageyama<sup>‡¶</sup>

From the <sup>‡</sup>Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan and the <sup>¶</sup>Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM, Université Louis Pasteur, Collège de France, 67404 Illkirch, Centre de l'Université de Strasbourg, France

**Neural stem cells, which differentiate into neurons and glia, are present in the ventricular zone of the embryonal brain. The precise mechanism by which neural stem cells are maintained during embryogenesis remains to be determined. Here, we found that transient misexpression of the basic helix-loop-helix genes *Hes1* and *Hes5* keeps embryonal telencephalic cells undifferentiated although they have been shown to induce gliogenesis in the retina. These telencephalic cells later differentiate into neurons and astroglia when *Hes* expression is down-regulated, suggesting that *Hes1*- and *Hes5*-expressing cells are maintained as neural stem cells during embryogenesis. Conversely, in the absence of *Hes1* and *Hes5*, neural stem cells are not properly maintained, generating fewer and smaller neurospheres than the wild type. These results indicate that *Hes1* and *Hes5* play an important role in the maintenance of neural stem cells but not in gliogenesis in the embryonal telencephalon.**

During neural development, neural stem cells undergo self-renewal and neuronal and glial differentiation (1). As differentiation proceeds, neural stem cells are decreased in number but remain in the brain until adulthood (2–4). However, the precise mechanism by which neural stem cells are maintained remains to be determined.

It has been shown that the basic helix-loop-helix (bHLH)<sup>1</sup> genes *Hes1* and *Hes5* are expressed in the ventricular zone of the developing nervous system, which contains neural stem cells (5–8). Misexpression of *Hes1* and *Hes5* inhibits neuronal differentiation, whereas mice that are mutant for *Hes1* and *Hes5* display premature neurogenesis (7, 9–17), indicating that these *Hes* genes are negative regulators of neuronal differentiation. However, it is not known whether *Hes*-expressing cells remain as neural stem cells or become glia in the embryonal brain. In the retina, both *Hes1* and *Hes5* are expressed by

differentiating Müller glia, and misexpression of *Hes1/5* promotes the generation of Müller glia at the expense of neurons, whereas the inactivation of *Hes1/5* results in defects of Müller glial development (18, 19). Thus, these *Hes* genes are capable of not only inhibiting neurogenesis but also promoting retinal gliogenesis. Interestingly, the membrane protein Notch, which controls *Hes1/5* expression, has also been shown to inhibit neurogenesis while inducing gliogenesis (11, 19–24), suggesting that *Hes1* and *Hes5* regulate neurogenesis and gliogenesis as Notch effectors. Strikingly, in the peripheral nervous system (PNS), even transient activation of Notch signaling is sufficient to irreversibly inhibit neurogenesis and instruct gliogenesis (22). However, these findings contrast the previous observation that Notch signaling is involved in the maintenance of the undifferentiated state and only allows, rather than instructs, gliogenesis (25, 26). It is likely that different cells may respond in different ways to the same signal, and it remains to be determined whether in the brain, the Notch-*Hes* pathway instructs cells to become glia, as in the retina and PNS, or to remain undifferentiated.

Here, to determine the functions of *Hes1* and *Hes5* in the developing brain, we performed gain-of-function and loss-of-function studies. We found that transient misexpression of *Hes1* or *Hes5* in the embryonal brain inhibits both neurogenesis and gliogenesis, in contrast with the retina and PNS, and keeps cells undifferentiated. However, these undifferentiated cells later become neurons and glia when *Hes* expression is down-regulated, suggesting that *Hes1*<sup>+</sup> and *Hes5*<sup>+</sup> cells remain as neural stem cells. Conversely, in the absence of *Hes1* and *Hes5*, neural stem cells are not properly maintained, generating fewer and smaller neurospheres. These results indicate that *Hes1* and *Hes5* play an important role in the maintenance of neural stem cells but not in gliogenesis in the embryonal brain.

### EXPERIMENTAL PROCEDURES

*Introduction of Expression Vectors into Telencephalic Cells of Mouse Embryos by Electroporation*—Rat *Hes1* and *Hes5* cDNAs were inserted into the *EcoRI* site of pCLIG (18), which directs gene expression from the upstream long terminal repeat promoter with cytomegalovirus enhancer. This vector also directs enhanced green fluorescent protein (EGFP) expression through the internal ribosomal entry site.

For the introduction of DNA, pregnant mice were deeply anesthetized, and a ventral midline incision was made to perform *in utero* manipulation. The expression vector (5  $\mu$ g) in solution containing 0.05% trypan blue as a tracer was injected through the uterine wall with a glass micropipette into the telencephalic vesicle of each embryo *in utero*. 1  $\mu$ g of pCL-EGFP was also co-injected with the expression vectors in many cases because EGFP<sup>+</sup> cells can be more easily identified (the same results were obtained). The volume of the injected DNA solution was kept minimal (mostly about 1  $\mu$ l). After injection, electroporation was carried out as described previously (27). Electrodes CUY 650–5 ( $\phi$ : 5 mm) or CUY 650P10 ( $\phi$ : 10 mm) were placed outside of the uterus on both sides of the head of the embryo *in utero*. A square electroporator

\* This work was supported by Special Coordination Funds for Promoting Science and Technology and research grants from the Ministry of Education, Science, Sports, and Culture of Japan and the Japan Society for the Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Dept. of Biological Sciences, Stanford University, 385 Serra Mall, Stanford, CA 94305-5020.

¶ To whom correspondence should be addressed: Institute for Virus Research, Kyoto University, Shogoin-Kawahara, Sakyo-ku, Kyoto 606-8507, Japan. Tel.: 81-75-751-4011; Fax: 81-75-751-4807; E-mail: rkageyam@virus.kyoto-u.ac.jp.

<sup>1</sup> The abbreviations used are: bHLH, basic helix-loop-helix; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; GFAP, glial fibrillary acid protein; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; PNS, peripheral nervous system; kb, kilobase pairs; E, embryonic day; P, postnatal day.

CUY21 EDIT (TR Tech, Tokyo, Japan) was used to deliver five 50-ms pulses of 50 V with 950-ms intervals. Then the abdominal wall of the pregnant mouse was sutured. Embryos or neonates were harvested 2–7 days after electroporation. The brains were excised, fixed in 4% paraformaldehyde in PBS for 30 min at 4 °C, cryoprotected in 25% sucrose in PBS overnight, and embedded in OCT compound. 16  $\mu$ m-thick cryosections were cut and examined immunohistochemically.

**Neurosphere Assay**—The primary sphere formation assay was performed essentially as described previously (17, 28). The uterus of the pregnant mouse was dissected at E11.5, and the heads of the embryos were excised. With fine forceps, the epidermis, mesenchyme, and meninges were removed. The neuroepithelium of the dorsal telencephalon was transferred into the neurosphere culture medium (Dulbecco's modified Eagle's medium/F-12 (1:1) (Life Technologies, Inc.) supplemented with 100  $\mu$ g/ml transferrin (Sigma), 25  $\mu$ g/ml insulin (Sigma), 20 nM progesterone (Sigma), 30 nM sodium selenite (Sigma), 60  $\mu$ M putrescine (Sigma), 20 ng/ml epidermal growth factor (EGF; Life Technologies, Inc.), and 20 ng/ml basic fibroblast growth factor (bFGF; Life Technologies, Inc.), washed in PBS once, and digested with 0.25% trypsin-EDTA (Life Technologies, Inc.) and DNaseI (50  $\mu$ g/ml) for 15 min at 37 °C. 0.25% trypsin inhibitor and PBS were added, and the cells were spun down, resuspended in the neurosphere culture medium, and dissociated completely by pipetting. 50  $\mu$ l of the cell suspension (at a density of  $1 \times 10^6$  cells/ml) were plated in each well of 96-well ultralow attachment plates (Corning). The numbers of primary spheres were counted, and the sphere sizes were compared at days 10 and 14.

For the secondary sphere formation assay, primary spheres were collected at day 7, spun down at 600 rpm for 1 min, and digested with 0.25% trypsin-EDTA and DNaseI (50  $\mu$ g/ml) for 15 min at 37 °C. 0.25% trypsin inhibitor and PBS were added, and the cells were spun down, resuspended in the neurosphere culture medium, and dissociated completely by pipetting. 40  $\mu$ l of the cell suspension (at a density of  $5 \times 10^4$  cells/ml) were plated in each well of 96-well ultralow attachment plates. The numbers of secondary spheres and the sphere sizes were examined at day 7.

**Sphere Differentiation Assay**—Secondary spheres were collected and plated onto polyethylenimine- (Sigma) and fibronectin- (Life Technologies, Inc.) coated Lab-Tek chamber slides (Nalge Nunc) in the differentiation medium (Dulbecco's modified Eagle's medium/F-12 (1:1) supplemented with 2% fetal bovine serum, 25  $\mu$ g/ml transferrin, 15  $\mu$ g/ml insulin, 20 nM progesterone, 30 nM sodium selenite, 3.3 ng/ml EGF, 1.7 ng/ml bFGF, 10 ng/ml nerve growth factor (Life Technologies, Inc.), and 10 ng/ml cholera toxin (Sigma)). After 7 days of culture, cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and examined immunocytochemically. Neuronal and glial differentiation was defined by triple-labeled immunocytochemistry using anti-MAP2 (neuron), anti-GFAP (astrocyte), and anti-O4 (oligodendrocyte) antibodies described below.

**Immunohistochemistry and Immunocytochemistry**—Fixed cryosections were washed three times with PBS and preincubated in PBS containing 5% normal goat serum and 0.1% Triton X-100 for 30 min and then incubated in 1% normal goat serum and 0.1% Triton X-100 with the following antibodies. Primary antibodies used are as follows: rabbit anti-GFP (diluted 1:500; Medical and Biological Laboratories), rabbit anti-GFP (1:1000; Molecular Probes), mouse anti-MAP2 (1:500; Sigma), mouse anti- $\beta$ III-tubulin (1:2000; BAbCO), mouse anti-RC2 (IgM, 1:2; Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti-nestin (1:500; PharMingen), mouse anti-GFAP (1:400; Sigma), rabbit anti-GFAP (1:10; Dako), mouse anti-S100 $\beta$  (1:250; Sigma), mouse anti-O4 (IgM, 1:5; Chemicon), mouse anti-GalC (IgG3, 1:20; Chemicon), mouse anti-Ki67 (1:100; PharMingen), mouse anti-Myc (1:500; Invitrogen), and rabbit anti-Myc (1:500; Medical and Biological Laboratories). To detect these antibodies, biotinylated goat anti-mouse IgM (7.5  $\mu$ g/ml; Vector), fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200; Vector), fluorescein isothiocyanate-conjugated goat anti-mouse IgM (1:500; ICN/CAPPEL), 7-amino-4-methylcoumarin-3-acetic acid-conjugated goat anti-rabbit IgG (1:100; Jackson), Fluorolink Cy3-labeled goat anti-mouse IgG (1:400; Amersham Pharmacia Biotech), Cy3-conjugated goat anti-mouse IgM (1:400; Jackson), and Fluorolink Cy3-labeled streptavidin (1:1000; Amersham Pharmacia Biotech) were used. Fluorescently labeled preparations were imaged using a Carl Zeiss LSM510 confocal microscope or a Carl Zeiss Axiophoto2 microscope equipped with an AxioCam color CCD camera. The images were processed by Photoshop 4.0 software (Adobe).

***Hes1*<sup>-/-</sup> and *Hes5*<sup>-/-</sup> Mutant Mice**—Previously generated *Hes1*<sup>-/-</sup> and *Hes5*<sup>-/-</sup> mutant mice (15, 16) were crossed to ICR mice for several generations. For genotyping of *Hes1*<sup>-/-</sup> mutant mice, the 0.9-kb *Bam*HI-*Pvu*II fragment was used as a probe as described previously

(15). This probe detected a 12-kb wild-type band and a 2.3-kb mutant band. For genotyping of *Hes5*<sup>-/-</sup> mutant mice, a 0.9-kb *Sma*I-*Bam*HI fragment was used as a probe (16). This probe detected a 7-kb wild-type band and a 4-kb mutant band. *Hes1*<sup>-/-</sup>-*Hes5*<sup>-/-</sup> double-null embryos were obtained by crossing *Hes1*<sup>+/-</sup>-*Hes5*<sup>-/-</sup> or *Hes1*<sup>+/-</sup>-*Hes5*<sup>+/-</sup> mice.

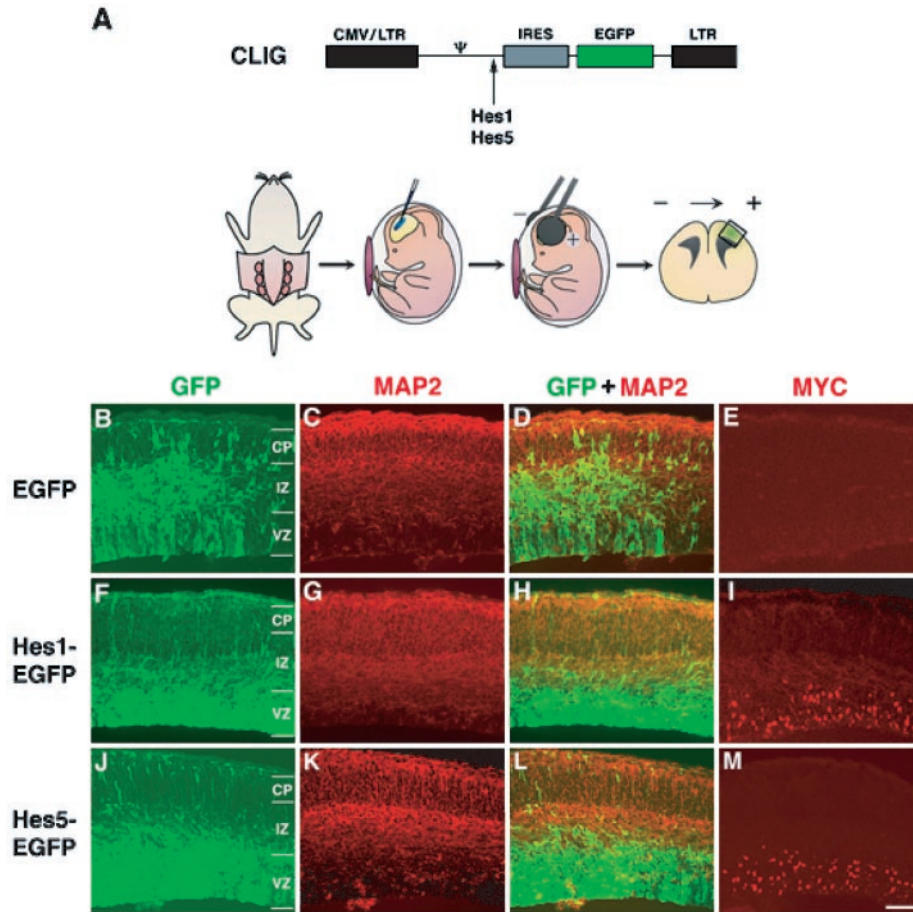
## RESULTS

**Misexpression of *Hes1* and *Hes5* Maintains Undifferentiated Cells in the Ventricular Zone of the Embryonal Telencephalon**—During neural development, *Hes1* and *Hes5* are specifically expressed in the ventricular zone (5, 6, 8). To examine their functions in the developing brain, we injected the expression vectors into the telencephalic vesicle of mouse embryos and introduced the vector DNA into telencephalic cells by electroporation (Fig. 1A). By this method, the exogenous genes can be transiently misexpressed in embryonal telencephalic cells.

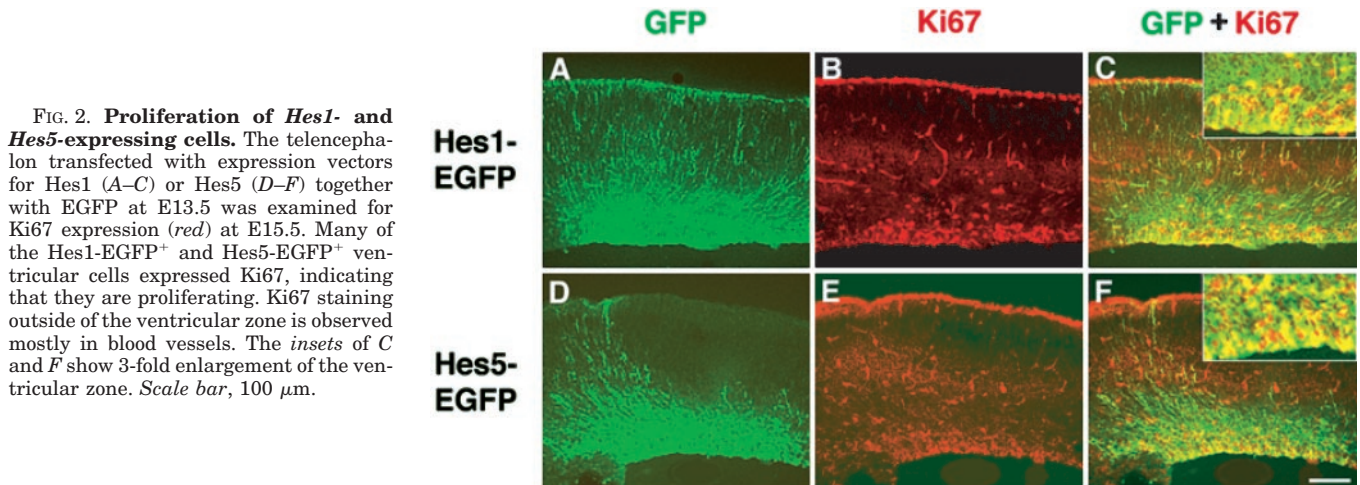
EGFP alone or together with *Hes1* or *Hes5* was misexpressed in telencephalic cells at E13.5, and after 2 days (E15.5), the fates of the transfected cells (EGFP<sup>+</sup>) were determined. When EGFP alone was misexpressed, the majority of the EGFP<sup>+</sup> cells migrated out of the ventricular zone into the outer layers during the E13.5-E15.5 period (Fig. 1B). Some of them reached the cortical plate and expressed the neuronal markers MAP2 (Fig. 1, B–D) and  $\beta$ III-tubulin (data not shown), indicating that many of the ventricular cells underwent neurogenesis during the E13.5-E15.5 period. This result is consistent with the previous observation that neurogenesis proceeds during the E13.5-E15.5 period (29). In contrast, when *Hes1* or *Hes5* was misexpressed together with EGFP, almost all transfected cells (EGFP<sup>+</sup>) remained in the ventricular zone (Fig. 1, F and J). These transfected cells were negative for MAP2 (Fig. 1, G, H, K, and L) and  $\beta$ III-tubulin (data not shown), indicating that *Hes1* and *Hes5* inhibit neurogenesis. Because these EGFP<sup>+</sup> cells were also negative for the astrocytic markers GFAP and S100 $\beta$  and the oligodendrocytic marker GalC (data not shown), *Hes1* and *Hes5* did not promote gliogenesis during the E13.5-E15.5 period. To verify the expression of *Hes1* and *Hes5*, three repeats of the Myc epitope were fused in frame to *Hes1* and *Hes5* at the amino terminus, and the Myc expression was examined. We found that the majority of the cells in the EGFP<sup>+</sup> region displayed a high level of nuclear staining of Myc (Fig. 1, I and M). We also observed inhibition of neurogenesis when *Hes1* and *Hes5* without Myc tags were misexpressed (data not shown), thus excluding the possibility that the Myc epitope affected the *Hes* functions.

To further determine whether the *Hes1*-EGFP<sup>+</sup> and *Hes5*-EGFP<sup>+</sup> ventricular cells are in a mitotic phase, we performed immunohistochemistry for Ki67, a nuclear antigen expressed by proliferating cells. Many of the *Hes1*-EGFP<sup>+</sup> (Fig. 2, A–C) and *Hes5*-EGFP<sup>+</sup> (Fig. 2, D–F) ventricular cells were positive for Ki67. These results demonstrated that *Hes1* and *Hes5* are capable of maintaining undifferentiated and proliferating cells in the ventricular zone.

**Misexpression of *Hes1* or *Hes5* Induces Radial Glia**—Interestingly, many of the *Hes1*-EGFP<sup>+</sup> and *Hes5*-EGFP<sup>+</sup> ventricular cells extended vertical processes through the intermediate zone and cortical plate toward the pial surface (Figs. 1 (F and J) and 2 (A and D)). This morphology is typical of radial glial cells, which have cell bodies in the ventricular zone and extend long processes from the ventricular zone to the pial surface (30). To determine whether these transfected cells are radial glia, we examined radial glial markers RC2 and nestin. The cell bodies and processes of almost all *Hes1*-EGFP<sup>+</sup> and *Hes5*-EGFP<sup>+</sup> cells were positive for RC2 (Fig. 3, A–C and G–I) and nestin (data not shown). In addition, the end-feet of *Hes1*-EGFP<sup>+</sup> and *Hes5*-EGFP<sup>+</sup> processes were positive for RC2 (Fig. 3, D–F and J–L). These results indicate that misexpression of *Hes1* and *Hes5* promotes the generation of radial glia during



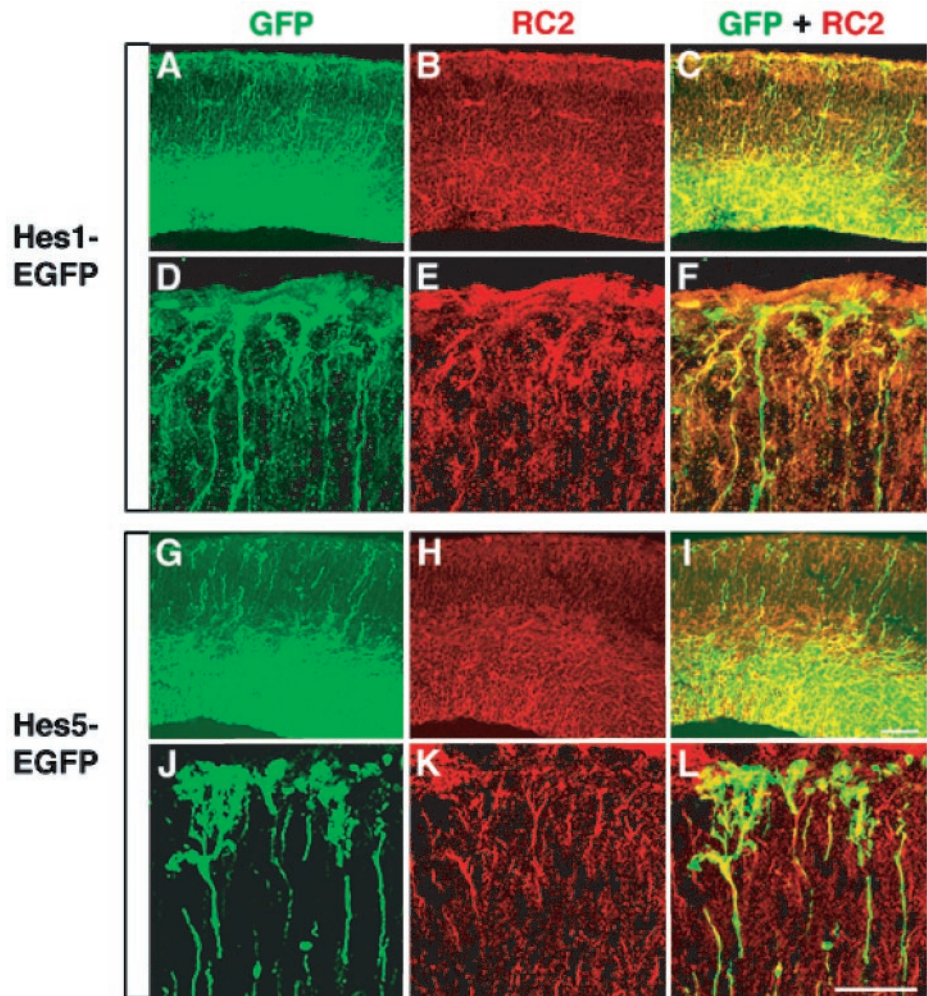
**FIG. 1. Maintenance of ventricular cells and inhibition of neuronal differentiation by *Hes1* and *Hes5*.** A, schematic structure of the expression vector CLIG and strategy for vector introduction to embryonal telencephalon (*top*). Arrow, *Hes1* and *Hes5* cDNAs were inserted into the upstream of the internal ribosomal entry site (*IRES*). *CMV*, cytomegalovirus. *LTR*, long terminal repeat. Vector DNA was injected into the telencephalic vesicle of mouse embryos *in utero* (*bottom*). DNA was then transiently introduced into telencephalic cells by electroporation. After several days, the fates of the transfected cells were determined. Panels B–M, EGFP alone (B–E) or together with *Hes1* (F–I) or *Hes5* (J–M) fused with the Myc tag was misexpressed by transfecting expression vectors by electroporation into the telencephalic cells of E13.5 embryos. After 2 days, the sections of the telencephalon were examined by immunohistochemistry with anti-EGFP (green), anti-MAP2 (red), and anti-Myc antibody (red). Fluorescently labeled preparations were imaged using a Carl Zeiss LSM510 confocal microscope. When only EGFP was misexpressed (B–D), many of the EGFP<sup>+</sup> cells migrated out of the ventricular zone (VZ) and differentiated into neurons (MAP2<sup>+</sup>, D). Adjacent section of B–D (E). When *Hes1* or *Hes5* was misexpressed (F–M), virtually all EGFP<sup>+</sup> cells remained in the ventricular zone and were negative for MAP2 (H and L). Adjacent sections of F–H and J–L (I and M). Many cells expressed *Hes1*/*Hes5* in the EGFP<sup>+</sup> region. Interestingly, some of *Hes1*<sup>+</sup> or *Hes5*<sup>+</sup> cells extended vertical processes to the pial surface (F and J), suggesting that they may be radial glia. CP, cortical plate; IZ, intermediate zone. Scale bar, 100  $\mu$ m.



**FIG. 2. Proliferation of *Hes1*- and *Hes5*-expressing cells.** The telencephalon transfected with expression vectors for *Hes1* (A–C) or *Hes5* (D–F) together with EGFP at E13.5 was examined for Ki67 expression (red) at E15.5. Many of the *Hes1*-EGFP<sup>+</sup> and *Hes5*-EGFP<sup>+</sup> ventricular cells expressed Ki67, indicating that they are proliferating. Ki67 staining outside of the ventricular zone is observed mostly in blood vessels. The insets of C and F show 3-fold enlargement of the ventricular zone. Scale bar, 100  $\mu$ m.

the E13.5–E15.5 period. It was believed previously that radial glial cells are the specialized glia that guide the migration of neurons (30). However, recent studies revealed that radial glial

cells are identical to neural stem cells (31, 32). Thus, these results strongly suggest that *Hes1* and *Hes5* may play an important role in the maintenance of neural stem cells but do not



**FIG. 3. Generation of radial glia by *Hes1* and *Hes5*.** *Hes1* (A–F) and *Hes5* (G–L) together with EGFP were misexpressed in the telencephalic cells at E13.5, and 2 days later, the fates of the transfected cells were examined by immunohistochemistry with anti-RC2 antibody (red). Cell bodies as well as many of the vertical processes of the transfected cells (EGFP<sup>+</sup>) are positive for RC2, a specific marker for radial glia. The end-feet of the processes located at the pial surface are also positive for RC2 (D–F and J–L). Scale bars, 100  $\mu$ m (A–C and G–I) and 50  $\mu$ m (D–F and J–L).

induce gliogenesis in the embryonal brain, unlike in the retina and PNS. This effect is very similar to that of the active form of Notch1, which also predominantly induces radial glia when misexpressed at an earlier stage of development (23), consistent with the notion that Notch1 and *Hes1/5* function in the same pathway (11, 20, 33).

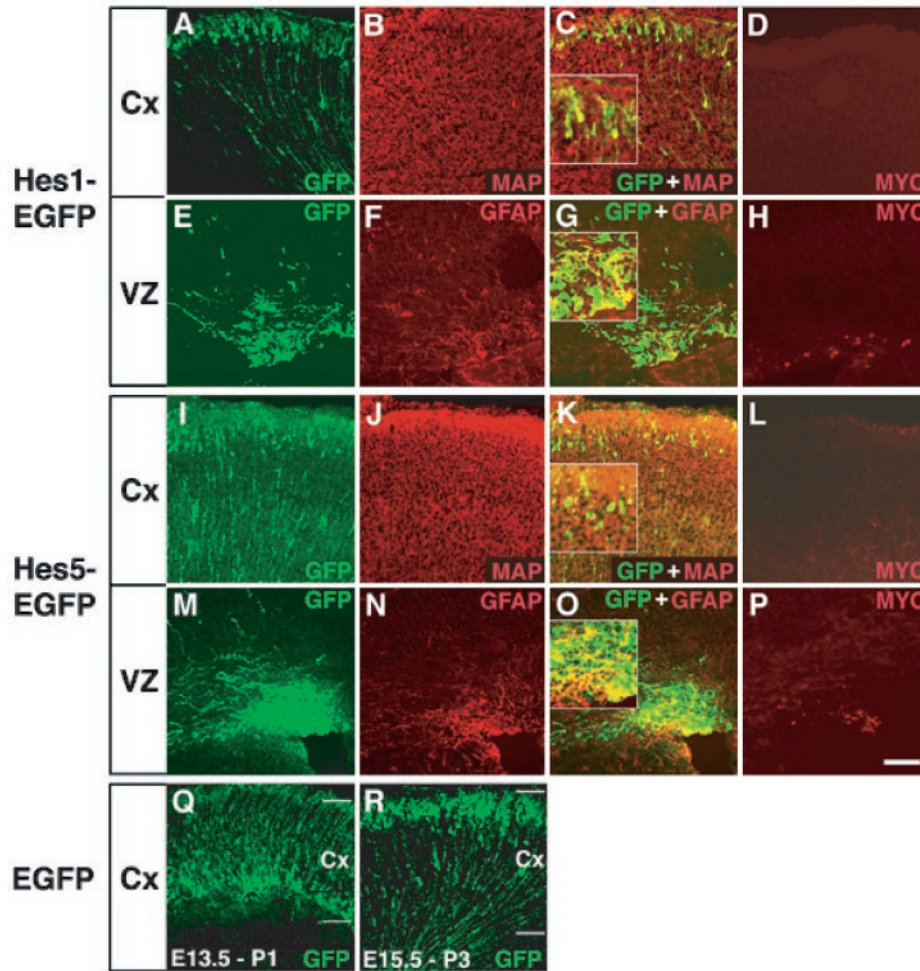
**Cells That Are Inhibited from Differentiation by *Hes1* or *Hes5* Later Differentiate into Neurons and Glia**—If the *Hes1*-EGFP<sup>+</sup> and *Hes5*-EGFP<sup>+</sup> ventricular cells are neural stem cells, they should differentiate into neurons and glia when *Hes1* and *Hes5* expression disappears. To determine the later fates of the ventricular cells that are inhibited from differentiation by *Hes1* and *Hes5*, the telencephalons transfected at E13.5 were examined postnatally. Because *Hes1* and *Hes5* proteins are unstable compared with EGFP,<sup>2</sup> *Hes* expression disappeared in many of the transfected cells during embryogenesis (Fig. 4, D, H, L, and P), whereas EGFP expression remained postnatally in many cells (Fig. 4, A, E, I, and M), which enabled us to determine the postnatal fates of the transfected cells (EGFP<sup>+</sup>).

When EGFP alone was misexpressed at E13.5, the majority of the EGFP<sup>+</sup> cells differentiated into neurons present in the deep layers of the cortex at postnatal day 1 (P1) (Fig. 4Q). When *Hes1* or *Hes5* together with EGFP was misexpressed at E13.5, many of the transfected cells now migrated out of the ventricular zone and differentiated into neurons, which were located in the superficial layers of the cortex at P1 (Fig. 4, A–C and I–K). Because these EGFP<sup>+</sup> cells in the cortex lost *Hes1* and

*Hes5* expression (Fig. 4, D and L), it is likely that the cells that lost expression of *Hes1* and *Hes5* underwent neuronal differentiation. Because these neurons were present in more superficial layers than the neurons of EGFP misexpression alone (Fig. 4, A and I versus Q), the former neurons were born later than the latter. When EGFP alone was misexpressed at E15.5, neurons were generated in the superficial layers (Fig. 4R), suggesting that *Hes1* and *Hes5* delayed neuronal differentiation for at least 2 days. We also found that many of the EGFP<sup>+</sup> cells remained near the ventricle and that those cells differentiated into GFAP<sup>+</sup> astrocytes at P1 (Fig. 4, E–G and M–O). These results demonstrated that the cells that are inhibited from differentiation by *Hes1* or *Hes5* later differentiate into neurons and glia, consistent with the above notion that *Hes1* and *Hes5* maintain neural stem cells in the embryonal brain. Immunostaining indicated that some of the cells near the ventricle still expressed *Hes1* and *Hes5* (Fig. 4, H and P), and it is possible that these *Hes1*<sup>+</sup> or *Hes5*<sup>+</sup> cells could be astrocyte-like neural stem cells.

**The Sphere-forming Activity Is Reduced in the Absence of *Hes1* and *Hes5***—The misexpression study indicated that *Hes1* and *Hes5* are capable of maintaining neural stem cells in the embryonal brain. To further determine whether *Hes* genes are required for the maintenance of neural stem cells, we performed a neurosphere assay. Neural stem cells plated in low attachment dishes expand in the presence of EGF and bFGF and form ball-like structures called neurospheres (34). Dissociated telencephalic cells were prepared from wild-type, *Hes1*<sup>-/-</sup>, *Hes5*<sup>-/-</sup>, and *Hes1*<sup>-/-</sup>*Hes5*<sup>-/-</sup> mouse embryos of

<sup>2</sup> T. Ohtsuka and R. Kageyama, unpublished data.



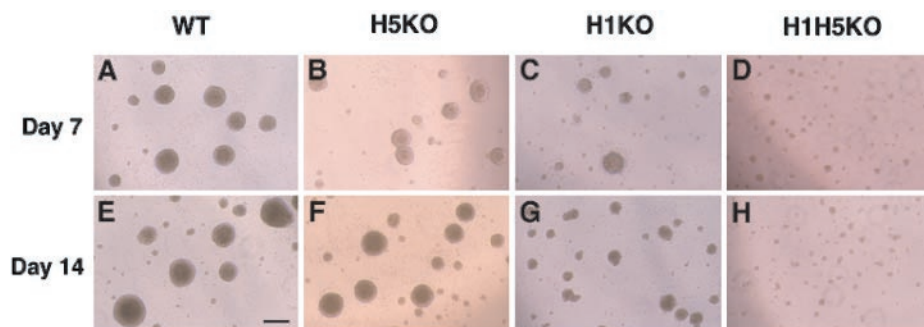
**FIG. 4. Neuronal and astrocytic differentiation of the transfected cells in the postnatal telencephalon.** Panels A–P, the telencephalon transfected with the expression vectors for *Hes1* (A–H) and *Hes5* (I–P) together with EGFP at E13.5 was examined by immunohistochemistry at P1. A–C, EGFP<sup>+</sup> cells present in the superficial layers of the cortex (Cx) were positive for MAP2 (red), suggesting that some of the cells transfected with the *Hes1*-EGFP expression vector at E13.5 differentiated into neurons by P1. D, In an adjacent section of A–C, immunohistochemistry for Myc was performed. The EGFP<sup>+</sup> cells present in the cortex lost *Hes1* expression. E–G, many of the EGFP<sup>+</sup> cells present near the ventricle were positive for GFAP (red), suggesting that they differentiated into astrocytes by P1. H, an adjacent section of E–G, where immunohistochemistry for Myc was performed. Only some of the EGFP<sup>+</sup> ventricular cells expressed *Hes1* at P1. I–K, EGFP<sup>+</sup> cells present in the superficial layers of the cortex were positive for MAP2 (red), suggesting that some of the cells transfected with the *Hes5*-EGFP expression vector at E13.5 differentiated into neurons by P1. L, in an adjacent section of I–K, the EGFP<sup>+</sup> cells present in the cortex lost *Hes5* expression. M–O, many of the EGFP<sup>+</sup> cells present near the ventricle were positive for GFAP (red), suggesting that they differentiated into astrocytes by P1. P, an adjacent section of M–O. Only some of the EGFP<sup>+</sup> ventricular cells expressed *Hes5* at P1. Q, EGFP vector alone was introduced at E13.5, and the fates of the transfected cells were examined at P1. The majority of the EGFP<sup>+</sup> cells differentiated into neurons present in the deep layers of the cortex. R, the EGFP vector alone was introduced at E15.5, and the fates of the transfected cells were examined at P3. The majority of the EGFP<sup>+</sup> cells differentiated into neurons present in the superficial layers of the cortex. The insets of C, G, K, and O show 2-fold enlargement. Scale bar, 100  $\mu$ m.

E11.5 and cultured in the presence of EGF and bFGF. After 7 days, many neurospheres were formed in cultures prepared from wild-type, *Hes1*<sup>-/-</sup>, and *Hes5*<sup>-/-</sup> embryos (Fig. 5, A–C). After 14 days, these neurospheres became larger, although *Hes1*<sup>-/-</sup> spheres were smaller than the wild-type and *Hes5*<sup>-/-</sup> spheres (Fig. 6, E–G). In contrast, *Hes1*<sup>-/-</sup>-*Hes5*<sup>-/-</sup> neurospheres were significantly smaller in size and did not seem to expand during the culture period (Fig. 5, D and H).

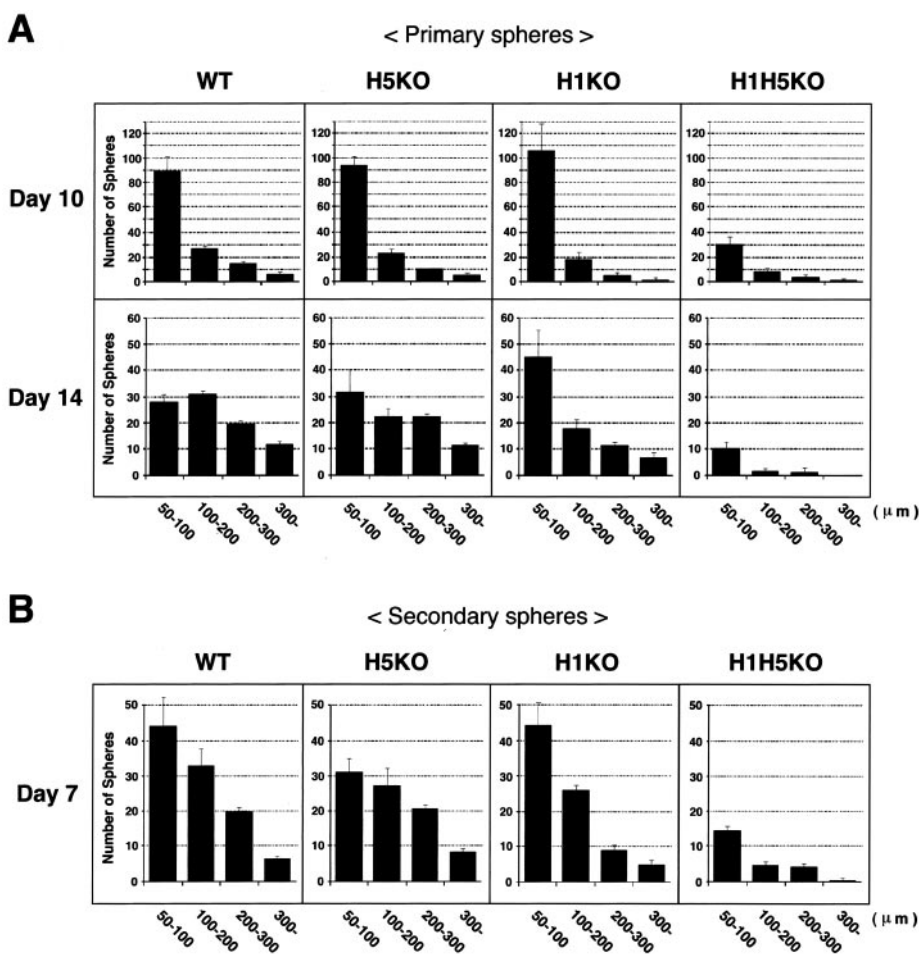
To quantify the number and size of neurospheres, an equal number ( $5 \times 10^4$ ) of cells prepared from the E11.5 telencephalon of wild-type, *Hes1*<sup>-/-</sup>, *Hes5*<sup>-/-</sup>, and *Hes1*<sup>-/-</sup>-*Hes5*<sup>-/-</sup> embryos were plated and cultured in low attachment dishes. Because it was sometimes difficult to distinguish between small neurospheres and dying cell clusters, only spheres larger than 50  $\mu$ m in diameter were counted. At day 10, the majority of neurospheres was still less than 100  $\mu$ m in diameter in all cultures (Fig. 6A). However, whereas the total number of spheres larger than 50  $\mu$ m was over 130 in the wild-type, *Hes1*<sup>-/-</sup>, and *Hes5*<sup>-/-</sup> cultures, it was approximately only 40 in

*Hes1*<sup>-/-</sup>-*Hes5*<sup>-/-</sup> cultures at day 10 (Fig. 6A), indicating that the number of sphere-forming cells was significantly reduced in the absence of *Hes1* and *Hes5*. At day 14, larger spheres were increased in the wild-type, *Hes1*<sup>-/-</sup>, and *Hes5*<sup>-/-</sup> cultures, although the average size of *Hes1*<sup>-/-</sup> spheres was smaller (average size: wild type, 174.4  $\mu$ m; *Hes1*<sup>-/-</sup>, 139.2  $\mu$ m; and in *Hes5*<sup>-/-</sup>, 173.0  $\mu$ m) (Fig. 6A). In contrast, larger spheres were not increased in *Hes1*<sup>-/-</sup>-*Hes5*<sup>-/-</sup> cultures at day 14 (average size: 91.9  $\mu$ m), suggesting that the expansion of *Hes1*<sup>-/-</sup>-*Hes5*<sup>-/-</sup> spheres stopped (Fig. 6A). Furthermore, whereas there were on average of 11.6 neurospheres that were larger than 300  $\mu$ m in diameter in the wild-type and *Hes5*<sup>-/-</sup> cultures and 6.3 neurospheres in *Hes1*<sup>-/-</sup> cultures, there were no such neurospheres in *Hes1*<sup>-/-</sup>-*Hes5*<sup>-/-</sup> cultures at day 14 (Fig. 6A). These results indicate that *Hes1* and *Hes5* are required for the normal expansion of neurospheres.

To examine the self-renewal activity of sphere-forming cells, the above neurospheres were dissociated, and an equal number ( $2 \times 10^3$ ) of cells were replated to form secondary spheres. At



**FIG. 5. Defective neurosphere formation in the absence of *Hes1* and *Hes5*.** Cells were isolated from wild-type (WT) (A and E), *Hes5*<sup>-/-</sup> (B and F), *Hes1*<sup>-/-</sup> (C and G), and *Hes1*<sup>-/-</sup>*Hes5*<sup>-/-</sup> telencephalon (D and H) of E11.5 embryos and cultured for 7 days (A–D) or 14 days (E–H) in low attachment dishes. There were many large spheres in the wild-type and *Hes5*<sup>-/-</sup> cultures (A, B, E, and F). *Hes1*<sup>-/-</sup> spheres were smaller, but still many of them were larger than 100  $\mu\text{m}$  in diameter (C and G). In contrast, *Hes1*<sup>-/-</sup>*Hes5*<sup>-/-</sup> spheres were significantly smaller (D and H). Scale bar, 200  $\mu\text{m}$ .



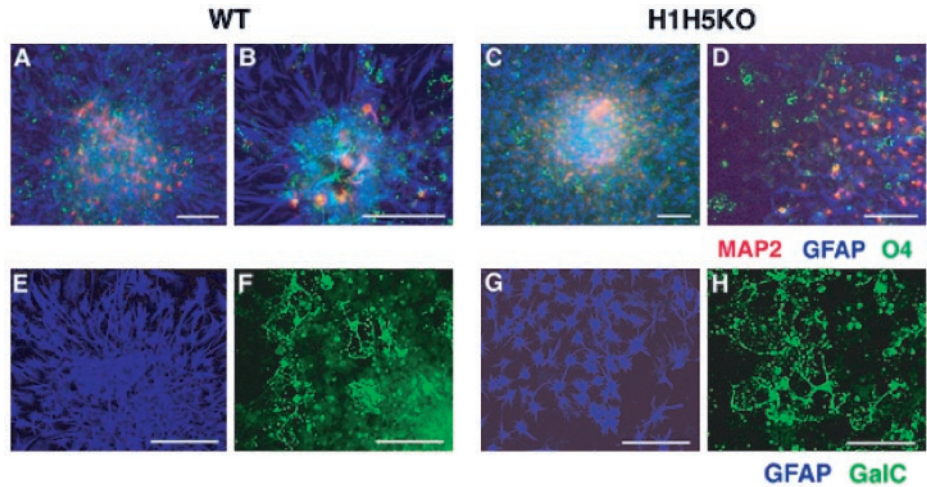
**FIG. 6. Quantification of neurosphere formation activities.** A, the size and number of primary spheres.  $5 \times 10^4$  cells prepared from E11.5 telencephalon were plated and cultured in low attachment dishes. Only spheres larger than 50  $\mu\text{m}$  in diameter were counted. The average of the following number ( $n$ ) of independent experiments is shown with S.E. Wild type (WT),  $n = 7$ ; *Hes5*<sup>-/-</sup>,  $n = 3$ ; *Hes1*<sup>-/-</sup>,  $n = 4$ ; *Hes1*<sup>-/-</sup>*Hes5*<sup>-/-</sup>,  $n = 3$ . B, the size and number of secondary spheres.  $2 \times 10^3$  cells prepared from primary spheres were plated and cultured in low attachment dishes. Only spheres larger than 50  $\mu\text{m}$  in diameter were counted. The average of the following number ( $n$ ) of independent experiments is shown with S.E. WT,  $n = 3$ ; *Hes5*<sup>-/-</sup>,  $n = 5$ ; *Hes1*<sup>-/-</sup>,  $n = 5$ ; *Hes1*<sup>-/-</sup>*Hes5*<sup>-/-</sup>,  $n = 5$ .

day 7, there were ~90–100 spheres larger than 50  $\mu\text{m}$  in the wild-type, *Hes1*<sup>-/-</sup>, and *Hes5*<sup>-/-</sup> cultures, whereas there were only 20 in *Hes1*<sup>-/-</sup>*Hes5*<sup>-/-</sup> cultures (Fig. 6B). These results indicate that the number of cells with the self-renewal activity was significantly reduced in the absence of *Hes1* and *Hes5*. In addition, from  $2 \times 10^3$  replated cells, 5–8 spheres became larger than 300  $\mu\text{m}$  at day 7 in the wild-type, *Hes1*<sup>-/-</sup>, and *Hes5*<sup>-/-</sup> cultures, whereas virtually no such spheres were formed in *Hes1*<sup>-/-</sup>*Hes5*<sup>-/-</sup> cultures (Fig. 6B). Thus, the growth rate of sphere-forming cells was also significantly reduced in the absence of *Hes1* and *Hes5*.

*Hes1*<sup>-/-</sup>*Hes5*<sup>-/-</sup> Double-mutant Sphere-forming Cells Are Multipotential—To examine the differentiation activity of sphere-forming cells, secondary spheres were plated down in

polyethylenimine- and fibronectin-coated dishes and cultured in a differentiation medium. Almost all spheres (>90%) from the wild-type and *Hes1*<sup>-/-</sup>*Hes5*<sup>-/-</sup> cultures generated mixtures of neurons, astrocytes, and oligodendrocytes (Fig. 7, A–D). Because it is likely that each sphere arose from a single cell, such sphere-forming cells may represent multipotential neural stem cells. These results indicate that, although the number and growth rate of neural stem cells were severely reduced in the absence of *Hes1* and *Hes5*, their multipotentiality of differentiation was not lost. In addition, cells of secondary spheres from both the wild-type and *Hes1*<sup>-/-</sup>*Hes5*<sup>-/-</sup> cultures very efficiently differentiated into astrocytes and oligodendrocytes (Fig. 7, E–H). Thus, *Hes1* and *Hes5* mutation did not affect the generation of astrocytes and oligodendrocytes, con-

**FIG. 7. Multipotential differentiation activity of neurosphere-forming cells.** Secondary spheres of the wild type (WT) (A, B, E, and F) and *Hes1*<sup>-/-</sup>-*Hes5*<sup>-/-</sup> (C, D, G, and H) were plated in polyethylenimine- and fibronectin-coated dishes and cultured in the differentiation medium. The cell types were examined by immunocytochemistry with anti-MAP2 (neuron), anti-GFAP (astrocyte), anti-O4 (oligodendrocyte), and anti-GalC antibodies (oligodendrocyte). Both wild-type and *Hes1*<sup>-/-</sup>-*Hes5*<sup>-/-</sup> secondary spheres differentiated into mixtures of neurons, astrocytes, and oligodendrocytes, indicating that these sphere-forming cells are multipotential. Scale bar, 100  $\mu$ m.



sistent with the above observation that neither *Hes1* nor *Hes5* directly induced gliogenesis in the embryonal brain.

#### DISCUSSION

*Hes1* and *Hes5* Regulate Maintenance of Neural Stem Cells in the Embryonal Telencephalon—We found that misexpression of *Hes1* and *Hes5* in the developing telencephalon keeps undifferentiated cells in the ventricular zone and that many of the *Hes1*<sup>+</sup> and *Hes5*<sup>+</sup> ventricular cells have a morphology of radial glial cells, which are now identified as neural stem cells. Furthermore, these ventricular cells, which are inhibited from differentiation by *Hes1* and *Hes5*, later differentiate into neurons and glia when *Hes* gene expression is down-regulated. Thus, the main function of *Hes1* and *Hes5* in the embryonal telencephalon is the maintenance of neural stem cells but not the promotion of gliogenesis, unlike in the retina and PNS. Consistent with this notion, neurosphere assays demonstrated that, in the absence of *Hes1* and *Hes5*, neural stem cells prepared from embryonal telencephalon do not efficiently expand, generating fewer and smaller neurospheres. Virtually no *Hes1*<sup>-/-</sup>-*Hes5*<sup>-/-</sup> neural stem cells form spheres larger than 300  $\mu$ m in diameter, whereas wild-type stem cells efficiently form such large spheres. It is likely that most of *Hes1*<sup>-/-</sup>-*Hes5*<sup>-/-</sup> neural stem cells stop cell division and differentiate into neurons and glia. These results are consistent with the previous observation that mutations for *Hes1* and/or *Hes5* lead to premature neuronal differentiation (7, 11, 15, 17).

It remains to be determined how *Hes1* and *Hes5* maintain the undifferentiated state of neural stem cells. It has been shown that *Hes1* is able to down-regulate the expression of p21, a cyclin-dependent kinase inhibitor, which is required for exit from the cell cycle (35). *Hes1* represses p21 expression not only by directly binding to its promoter but also by inhibiting Mash1-dependent activation (35). It is likely that this repression of p21 expression leads to cell proliferation and the maintenance of the undifferentiated state.

Despite the severe abnormalities, *Hes1*<sup>-/-</sup>-*Hes5*<sup>-/-</sup> neural stem cells still proliferate and form small neurospheres. This observation indicates that there may be another *Hes1/5*-related gene involved in the maintenance of neural stem cells. It has been shown that a splicing variant of *Hes3*, named *Hes3b*, is expressed at a high level in the midbrain-hindbrain and at lower levels in other regions (13, 36). *Hes3b* encodes a complete bHLH domain and inhibits neuronal and glial differentiation similarly to *Hes1* but in contrast to the other splicing variant *Hes3a* that has an incomplete basic region and does not inhibit neuronal differentiation (6, 13). Therefore, it is likely that *Hes3b* may also contribute to the maintenance of neural stem cells in the embryonal brain.

We previously found that the number of sphere-forming cells was reduced in the absence of *Hes1* (17). However, in the present study, although the growth rate of *Hes1*<sup>-/-</sup> neurospheres was reduced, their number was not significantly affected, suggesting that *Hes1*<sup>-/-</sup> phenotypes became weaker. This change may be attributable to different genetic backgrounds of *Hes1*-mutant mice; the previous genetic background is the mixture of ICR and 129 strains, whereas the current one is substantially ICR.

*Hes1* and *Hes5* Do Not Induce Astrocytes or Oligodendrocytes in the Embryonal Telencephalon—In the embryonal telencephalon, *Hes1* and *Hes5* do not induce the development of astrocytes or oligodendrocytes but maintain undifferentiated cells in the ventricular zone. Consistent with this misexpression study, the efficiency of gliogenesis is not affected by *Hes1* and *Hes5* mutation; *Hes1*<sup>-/-</sup>-*Hes5*<sup>-/-</sup> cells differentiate very efficiently into astrocytes and oligodendrocytes. Therefore, it is unlikely that the Notch-*Hes1/5* pathway determines the fate of astrocytes and oligodendrocytes in the developing telencephalon. In contrast, in the PNS, neural crest stem cells differentiate into neurons in the presence of BMP2 but, upon Notch activation, they adopt the glial fate, indicating that Notch directs the fate switch from neurons to glia (22). Strikingly, even transient activation of Notch signaling is sufficient for gliogenesis from neural crest stem cells. In a sharp contrast to neural crest stem cells, many of the *Hes1*<sup>+</sup> and *Hes5*<sup>+</sup> ventricular cells of the telencephalon later differentiate into neurons, indicating that the Notch-*Hes* pathway does not irreversibly induce gliogenesis in the brain. Alternatively, Notch signaling may play an important role in the generation of late-born neurons in the cortex by transiently preventing neurogenesis. Thus, different cells respond in different ways to the Notch-*Hes* pathway, but the molecular basis for these diverse responses remains to be determined.

*bHLH* Genes in Neuron/Glia Fate Determination—In the retina, Notch signaling has been found to promote Müller glial development at the expense of the neuronal fate (18, 19). Interestingly, induction of Müller glial development is also observed in double-mutant mice for the neuronal bHLH genes *Mash1* and *Math3* (37, 38). In these mutant retina, the cells that normally differentiate into bipolar neurons adopt the Müller glial fate (37, 38). In other regions of the nervous system of *Mash1*-*Math3* double-mutant mice, the cells that normally differentiate into neurons adopt the astrocytic fate, resulting in a loss of neurons and premature generation of astrocytes (37). Similarly, the double mutation of *Mash1* and *Neurogenin2*, another neuronal bHLH gene, leads to a decrease of neurons and premature generation of astrocytes (39). These results indicate that neuronal

bHLH genes such as *Mash1*, *Math3*, and *Neurogenin* not only determine the neuronal fate but also actively inhibit gliogenesis. Consistent with this notion, overexpression of *Neurogenin1* actively inhibits astrocyte differentiation (40). These results raised the possibility that down-regulation of neuronal bHLH genes is one of the mechanisms that initiates gliogenesis. Because *Hes1* and *Hes5* functionally antagonize neuronal bHLH factors (6, 9, 41), it is likely that misexpression of *Hes1* and *Hes5* may inactivate *Mash1* and *Math3*, thereby inducing gliogenesis in the retina. However, misexpression of *Hes1* and *Hes5* did not induce astrocyte development in the embryonal brain. We found that some of the *Hes1*<sup>+</sup> and *Hes5*<sup>+</sup> cells later differentiate into astrocytes at P1. However, this cell type is unlikely to be directly induced by *Hes1* and *Hes5* but most likely to be a secondary fate from neural stem cells because these astrocytes appear only around the perinatal period. Thus, the Notch-Hes pathway may only secondarily promote astrocyte development in the brain by maintaining neural stem cells until astrocytic differentiation occurs. Recent studies suggest that misexpression of *Id1*, a helix-loop-helix factor that dominant-negatively regulates neuronal bHLH factors, generates astrocytes (42). However, these astrocytes also appear only postnatally, and therefore, the astrocytic fate choice by *Id1* could be indirect.

Currently, we do not have a clear answer as to why activation of the Notch-Hes pathway induces gliogenesis in the retina but not in the brain, although inactivation of *Mash1*, *Math3*, and *Neurogenin2* induces gliogenesis in both the retina and brain. It is possible that the Notch-Hes1/5 pathway may suppress astrocytic genes in addition to *Mash1*, *Math3*, and *Neurogenin* in the brain as observed for oligodendrocyte development in the optic nerve, which is inhibited by the Notch-Hes5 pathway (43). It has been shown that Notch signaling is modulated by many factors, such as *Numb* and *Mastermind* (21). Thus, the Notch-Hes1/5 pathway could be modulated by such factors so that neurogenesis but not gliogenesis is blocked. Further characterization of Notch modulators is necessary to understand the diverse functions of Notch signaling.

**Acknowledgments**—We thank Drs. Takaki Miyata, Yuki Nakamura, and Tetsuichiro Saito for technical advice. Monoclonal antibody to RC2 was obtained from the Developmental Studies Hybridoma Bank.

#### REFERENCES

- Qian, X., Shen, Q., Goderie, S. K., He, W., Capela, A., Davis, A. A., and Temple, S. (2000) *Neuron* **28**, 69–80
- Johansson, C. B., Momma, S., Clarke, D. L., Risling, M., Lendahl, U., and Frisén, J. (1999) *Cell* **96**, 25–34
- Chiasson, B. J., Tropepe, V., Morshead, C. M., and van der Kooy, D. (1999) *J. Neurosci.* **19**, 4462–4471
- Doetsch, F., Caillé, I., Lim, D. A., Garcia-Verdugo, J. M., and Alvarez-Buylla, A. (1999) *Cell* **97**, 703–716
- Akazawa, C., Sasai, Y., Nakanishi, S., and Kageyama, R. (1992) *J. Biol. Chem.* **267**, 21879–21885
- Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R., and Nakanishi, S. (1992) *Genes Dev.* **6**, 2620–2634
- Tomita, K., Ishibashi, M., Nakahara, K., Ang, S.-L., Nakanishi, S., Guillemot, F., and Kageyama, R. (1996) *Neuron* **16**, 723–734
- Allen, T., and Lobe, C. G. (1999) *Cell. Mol. Biol.* **45**, 687–708
- Ishibashi, M., Moriyoshi, K., Sasai, Y., Shiota, K., Nakanishi, S., and Kageyama, R. (1994) *EMBO J.* **13**, 1799–1805
- Ström, A., Castella, P., Rockwood, J., Wagner, J., and Caudy, M. (1997) *Genes Dev.* **11**, 3168–3181
- Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F., and Kageyama, R. (1999) *EMBO J.* **18**, 2196–2207
- Castella, P., Wagner, J. A., and Caudy, M. (1999) *J. Neurosci. Res.* **56**, 229–240
- Hirata, H., Ohtsuka, T., Bessho, Y., and Kageyama, R. (2000) *J. Biol. Chem.* **275**, 19083–19089
- Zheng, J. L., Shou, J., Guillemot, F., Kageyama, R., and Gao, W.-Q. (2000) *Development* **127**, 4551–4560
- Ishibashi, M., Ang, S.-L., Shiota, K., Nakanishi, S., Kageyama, R., and Guillemot, F. (1995) *Genes Dev.* **9**, 3136–3148
- Cau, E., Gradwohl, G., Casarosa, S., Kageyama, R., and Guillemot, F. (2000) *Development* **127**, 2323–2332
- Nakamura, Y., Sakakibara, S., Miyata, T., Ogawa, M., Shimazaki, T., Weiss, S., Kageyama, R., and Okano, H. (2000) *J. Neurosci.* **20**, 283–293
- Hojó, M., Ohtsuka, T., Hashimoto, N., Gradwohl, G., Guillemot, F., and Kageyama, R. (2000) *Development* **127**, 2515–2522
- Furukawa, T., Mukherjee, S., Bao, Z.-Z., Morrow, E. M., and Cepko, C. L. (2000) *Neuron* **26**, 383–394
- Kageyama, R., and Nakanishi, S. (1997) *Curr. Opin. Genet. Dev.* **7**, 659–665
- Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999) *Science* **284**, 770–776
- Morrison, S. J., Perez, S. E., Qiao, Z., Verdi, J. M., Hicks, C., Weinmaster, G., and Anderson, D. J. (2000) *Cell* **101**, 499–510
- Gaiano, N., Nye, J. S., and Fishell, G. (2000) *Neuron* **26**, 395–404
- Tanigaki, K., Nogaki, F., Takahashi, J., Tashiro, K., Kurooka, H., and Honjo, T. (2001) *Neuron* **29**, 45–55
- Nye, J. S., Kopan, R., and Axel, R. (1994) *Development* **120**, 2421–2430
- Dorsky, R. L., Rapaport, D. H., and Harris, W. A. (1995) *Neuron* **14**, 487–496
- Funahashi, J., Okafuji, T., Ohuchi, H., Noji, S., Tanaka, H., and Nakamura, H. (1999) *Dev. Growth Differ.* **41**, 59–72
- Reynolds, B. A., and Weiss, S. (1996) *Dev. Biol.* **175**, 1–13
- Jacobson, M. (1991) in *Developmental Neurobiology*, Plenum Press, New York
- Rakic, P. (1971) *Brain Res.* **33**, 471–476
- Malatesta, P., Hartfuss, E., and Götz, M. (2000) *Development* **127**, 5253–5263
- Noctor, S., Flint, A. C., Weissman, T. A., Dammerman, R. S., and Kriegstein, A. R. (2001) *Nature* **409**, 714–720
- de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J., and Conlon, R. A. (1997) *Development* **124**, 1139–1148
- Reynolds, B. A., and Weiss, S. (1992) *Science* **255**, 1707–1710
- Castella, P., Sawai, S., Nakao, K., Wagner, J. A., and Caudy, M. (2000) *Mol. Cell. Biol.* **20**, 6170–6183
- Lobe, C. G. (1997) *Mech. Dev.* **62**, 227–237
- Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemot, F., and Kageyama, R. (2000) *EMBO J.* **19**, 5460–5472
- Hatakeyama, J., Tomita, K., Inoue, T., and Kageyama, R. (2001) *Development* **128**, 1313–1322
- Nieto, M., Schuurmans, C., Britz, O., and Guillemot, F. (2001) *Neuron* **29**, 401–413
- Sun, Y., Nadal-Vicens, M., Misono, S., Lin, M. Z., Zubiaga, A., Hua, X., Fan, G., and Greenberg, M. E. (2001) *Cell* **104**, 365–376
- Chen, H., Thiagalingam, A., Chopra, H., Borges, M. W., Feder, J. N., Nelkin, B. D., Baylin, S. B., and Ball, D. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 5355–5360
- Cai, L., Morrow, E. M., and Cepko, C. L. (2000) *Development* **127**, 3021–3030
- Wang, S., Sdrulla, A. D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., Weinmaster, G., and Barres, B. A. (1998) *Neuron* **21**, 63–75