

The Basic Helix-Loop-Helix Gene *hesr2* Promotes Gliogenesis in Mouse Retina

Tetsu Satow,^{1,2} Soo-Kyung Bae,¹ Tomoyuki Inoue,¹ Chihiro Inoue,¹ Goichi Miyoshi,¹ Koichi Tomita,¹ Yasumasa Bessho,¹ Nobuo Hashimoto,² and Ryoichiro Kageyama¹

¹Institute for Virus Research, Kyoto University, and ²Department of Neurosurgery, Kyoto University Graduate School of Medicine, Sakyo-ku, Kyoto 606-8507, Japan

Members of a subclass of *hairy/Enhancer of split [E(spl)]* homologs, called *hesr* genes, are structurally related to another subclass of *hairy/E(spl)* homologs, *Hes* genes, which play an important role in neural development. To characterize the roles of *hesr* genes in neural development, we used the retina as a model system. *In situ* hybridization analysis indicated that all *hesr* genes are expressed in the developing retina, but only *hesr2* expression is associated spatially with gliogenesis. Each member was then misexpressed with retrovirus in the retinal explant cultures prepared from mouse embryos or neonates, which well mimic *in vivo* retinal development. Interestingly, *hesr2* but not *hesr1* or *hesr3* promoted gliogenesis while inhibiting rod genesis without affecting cell proliferation or death,

suggesting that the cells that normally differentiate into rods adopted the glial fate by misexpression of *hesr2*. The gliogenic activity of *hesr2* was more profound when it was misexpressed postnatally than prenatally. In addition, double mutation of the neuronal determination genes *Mash1* and *Math3*, which increases Müller glia at the expense of bipolar cells, upregulated *hesr2* expression. These results indicate that, among structurally related *hesr* genes, only *hesr2* promotes glial versus neuronal cell fate specification in the retina and that antagonistic regulation between *hesr2* and *Mash1–Math3* may determine the ratios of neurons and glia.

Key words: *bHLH*; Müller glia; Hes; hesr; retina; retrovirus; rod

Neurons and glial cells differentiate from common precursors. In *Drosophila*, the gene *glial cells missing (gcm)* determines the glial versus neuronal cell fate (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996), whereas in mammals *gcm* homologs are not involved in gliogenesis but regulate trophoblast cell development in the placenta (Kim et al., 1998; Anson-Cartwright et al., 2000; Schreiber et al., 2000), suggesting that the mechanism of gliogenesis has diverged during evolution. We and others have recently found that in mammals *Notch1* and its effectors, *Hes1* and *Hes5* (Kageyama and Nakanishi, 1997), promote gliogenesis at the expense of the neuronal fate (Furukawa et al., 2000; Gaiano et al., 2000; Hojo et al., 2000; Morrison et al., 2000). *Hes1* and *Hes5*, mammalian homologs of *Drosophila hairy* and *Enhancer of split [E(spl)]*, encode a transcriptional repressor with a basic helix-loop-helix (bHLH) domain and negatively regulate neuronal bHLH genes such as *Mash1* (Akazawa et al., 1992; Sasai et al., 1992; Ishibashi et al., 1995; Tomita et al., 1996a; Chen et al., 1997). It is likely that this anti-neuronal activity may contribute to promotion of gliogenesis. However, *Hes1*($-/-$)-*Hes5*($-/-$) neural precursor cells (Ohtsuka et al., 1999; Cau et al., 2000) can still differentiate into glia (our unpublished data), and therefore it is likely that there may be other bHLH genes that promote gliogenesis.

For the analysis of such factors that regulate neural develop-

ment, the retina is an ideal model system. It has only six types of neurons and one type of glia (Müller glia), which all differentiate from common precursors (Cepko, 1999). These retinal cells constitute three cellular layers: the outer nuclear layer (ONL), which contains rod and cone photoreceptors; the inner nuclear layer (INL), which contains bipolar, horizontal and amacrine interneurons, and Müller glia; and the ganglion cell layer, which contains ganglion cells. In addition to the simple structure, the majority of retinal neurons and glial cells differentiate postnatally, and therefore it is easy to examine the process of neuronal and glial fate determination.

To identify new gliogenic genes, we focused on the recently characterized bHLH genes, *hesr* family (*Hey*, *HRT*, *gridlock*, *CHF*), which belong to a related but different subclass from *Hes* genes (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999; Chin et al., 2000; Zhong et al., 2000). There are three members in *hesr* family, and each member contains three *Hes*-related domains: a bHLH domain, an orange domain, which confers the specificity of protein interaction (Dawson et al., 1995), and a domain related to the WRPW sequence, which is known to interact with corepressor (Paroush et al., 1994; Fisher et al., 1996; Grabavec and Stifani, 1996). Here, we found that *hesr* genes are expressed in the developing retina and that misexpression of *hesr2* with retrovirus promotes gliogenesis at the expense of rod genesis, whereas that of *hesr1* and *hesr3* does not. Thus, among the three structurally related members, only *hesr2* functions as a gliogenic gene and may substitute for *Hes1* and *Hes5* in the retina.

MATERIALS AND METHODS

cDNA library screening. To obtain *Hes*-related cDNAs, poly(A)⁺ RNA was prepared from mouse retina of postnatal day (P) 0, P5, and P10 and subjected to reverse transcription (RT) using oligo(dT) as a primer. The primers for

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Correspondence should be addressed to Ryoichiro Kageyama, Institute for Virus Research, Kyoto University, Shogoin-Kawahara, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: rkageyam@virus.kyoto-u.ac.jp.

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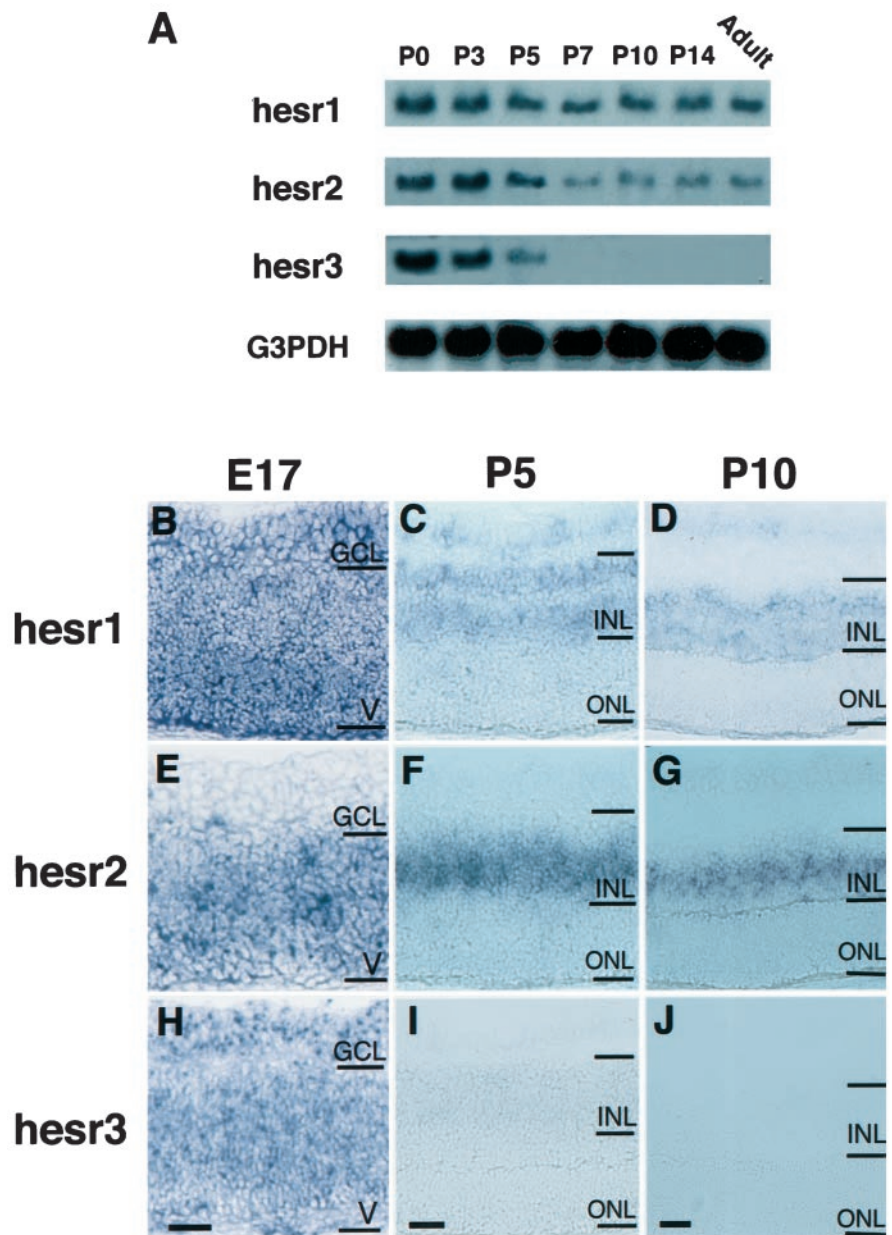


Figure 1. Expression of *hesr* genes in the developing retina. **A**, *hesr1–3* expression is examined by Northern blot analysis. All *hesr* genes are expressed at comparable levels at P0. Although the level of *hesr1* expression is relatively constant until adulthood, *hesr2* and *hesr3* expression is decreased at P5; *hesr3* expression disappears at P7. Glyceraldehyde-3 phosphate dehydrogenase (G3PDH) cDNA was used as a control. **B–J**, *hesr1–3* expression is examined by *in situ* hybridization. At E17, all *hesr* genes are expressed in the ventricular zone (V) (**B**, **E**, **H**). *hesr1* and *hesr3* are also expressed in the ganglion cell layer (GCL) (**B**, **H**). At P5, *hesr1* expression is observed mainly in the outer and inner regions of the INL, which contain horizontal and amacrine cells, respectively, as well as in the GCL (**C**). In contrast, *hesr2* is mainly expressed in the middle region of the INL, which contains bipolar and Müller glial cells (**F**). Thus, *hesr1* and *hesr2* display complementary expression patterns. *hesr3* is only weakly expressed in the INL (**I**). At P10, *hesr1* is again expressed in the outer and inner regions of the INL (**D**), whereas *hesr2* is expressed in the middle region of the INL (**G**). In contrast, *hesr3* expression is not detectable at P10 (**J**). Scale bar, 25 μ m.

hesr1 used in PCR were 5'-ATCAGTGTGCACGCACCTCC-3' and 5'-TCCAAGGGCACTGGGTACCAG-3' for upper and lower primers, respectively. The PCR product was used as a probe to screen ~200,000 clones of cDNA library produced from mouse embryos at day 9.5 (E9.5). One *hesr1* clone was obtained. To clone other Hes-related cDNAs, RT-PCR was performed again, and the fully degenerated primers corresponding to the following sequences were synthesized and used: RG(I/L/V)(M/L/V)EK and KLE(K/N)A(D/E) for the upper and lower primers, respectively. The PCR products were used as probes to screen ~1,000,000 clones of a mouse embryo (E9.5) cDNA library. Four clones for *hesr2* and three clones for *hesr3* were obtained. The protein coding region of each *hesr* was subcloned into the *EcoRI* site of the retroviral expression vector pCLIG (Hojo et al., 2000). Three copies of the Myc sequence (MEQKLISEEDLNE) were tagged in frame at the amino-terminal site of each *hesr*.

Northern blot analysis and in situ hybridization. Total RNA (15 μ g) from mouse retinas were electrophoresed on a formaldehyde/1.2% agarose gel and transferred to a nylon membrane (NEN). The full-length of each *hesr* cDNA was used as a probe, and hybridization was performed as described previously (Sasai et al., 1992). *In situ* hybridization analysis was performed as described previously (Shimizu et al., 1995; Takebayashi et al., 1997). Digoxigenin-labeled antisense RNA probes corresponding to *EcoRI-SalI* (1.8 kb), *EcoRI-HindIII* (1.6 kb), and *EcoRI-*

HindIII (1.35 kb) fragments of *hesr1*, -2, and -3, respectively, were synthesized *in vitro*. These probes were hybridized to 16 μ m cryostat sections of embryonal and postnatal retinas.

Retinal explant culture and retrovirus infection. Retroviral DNAs were transfected with LipofectAMINE (Life Technologies, Gaithersburg, MD) into ψ 2mp34, an ecotropic packaging cell line (Yoshimatsu et al., 1998). The supernatant was collected 2 d later and concentrated with Centriprep 100 (Amicon), as described previously (Ishibashi et al., 1994; Tsuda et al., 1998). The retinal explant culture and retroviral infection were performed as described previously (Tomita et al., 1996a; Hojo et al., 2000). Briefly, eyes were isolated from E17.5 mouse embryos or P1 neonates and transferred to PBS solution. The neural retina without pigment epithelium was placed on a Millicell chamber filter (Millipore; diameter 30 mm, pore size 0.4 μ m) with the ganglion cell layer upward. The chamber was transferred to a six-well culture plate. Each well contained 1 ml of culture medium (50% MEM with HEPES, 25% Hank's solution, 25% heat-inactivated horse serum, 200 μ M L-glutamine, and 5.75 mg/ml glucose). Explants were cultured at 34°C in 5% CO₂, and the medium was changed every other day. Two weeks after infection, retinas were fixed with 4% paraformaldehyde, dehydrated in 25% sucrose, and embedded in OCT compound (Miles), and cryosections (16 μ m) were made.

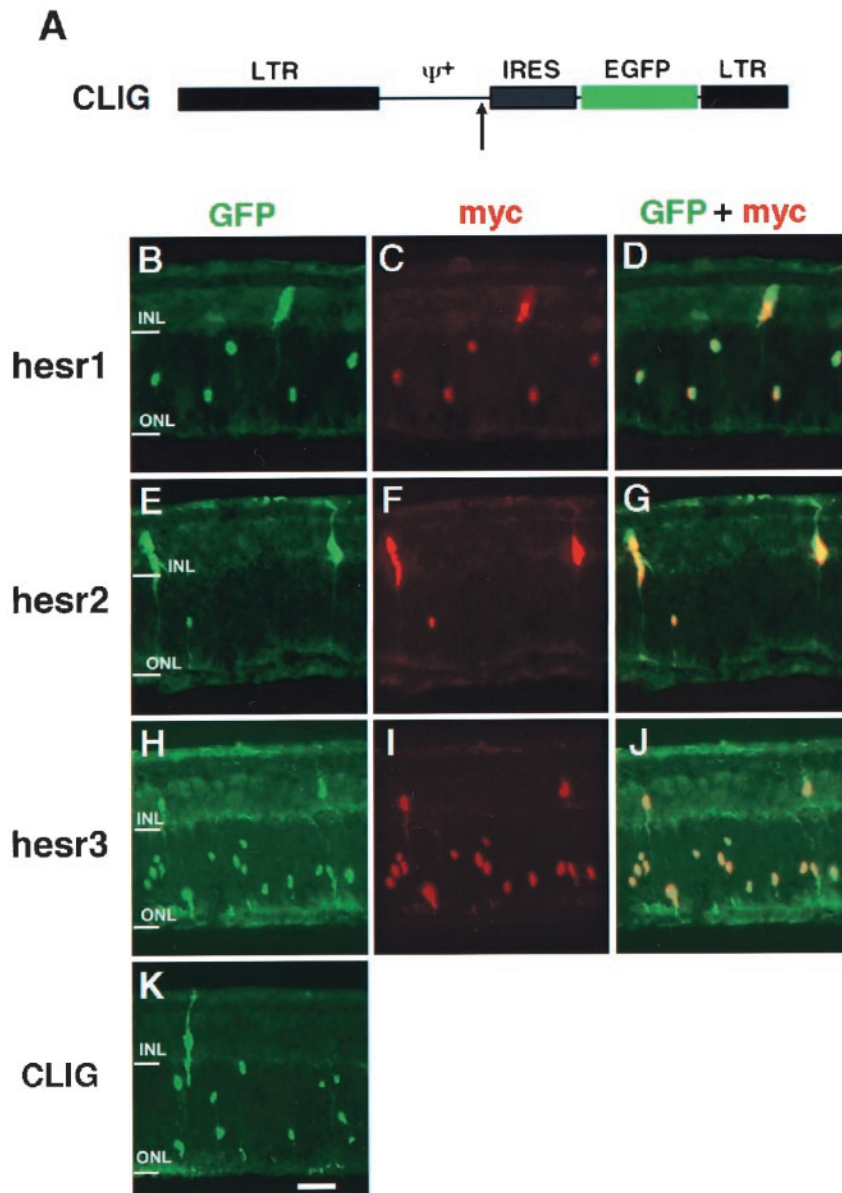


Figure 2. Misexpression of *hesr* genes in the retinal explants starting at E17.5. **A**, Schematic structure of the retroviral vector CLIG. Each *hesr* cDNA fused with three repeats of the Myc epitope is inserted in the upstream of IRES (arrow). EGFP, Enhanced green fluorescent protein; IRES, internal ribosomal entry site; LTR, long terminal repeat. **B–K**, Retinal explants were prepared from E17.5 mouse embryos and infected with CLIG-*hesr1* (**B–D**), CLIG-*hesr2* (**E–G**), CLIG-*hesr3* (**H–J**), and CLIG (**K**). After 2 weeks of culture, the explants were subjected to immunohistochemistry using anti-GFP and anti-Myc antibodies. When infected with CLIG-*hesr*, almost all virus-infected cells expressed both GFP and Myc. Rod genesis is decreased and gliogenesis is increased (**E–G**) only when CLIG-*hesr2* is applied. Scale bar, 25 μ m.

Immunostaining and terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling assay. For immunohistochemistry, sections on slides were preincubated in PBS containing 5% goat serum and 0.1% Triton X-100 for 1 hr and then incubated in 1% goat serum and 0.1% Triton X-100 with the following antibodies: rabbit anti-green fluorescent protein (GFP) (diluted 1:500; Medical and Biological Laboratories), mouse anti-Myc (1:400; Invitrogen, San Diego, CA), mouse anti-vimentin (1:1; HistoFine), and mouse anti-glutamine synthetase (GS) (1:200; Chemicon, Temecula, CA). To detect these antibodies, biotinylated anti-rabbit antibody (1:200; Vector Laboratories, Burlingame, CA), fluorescein isothiocyanate avidinD (1:1000; Vector), and Fluorolink Cy3-labeled goat anti-mouse antibody (1:400; Amersham Pharmacia Biotech) were used. Retinal cell types were determined by their morphology, their locations, and the following antibodies: anti-HPC1 (amacrine cells), anti-PKC (bipolar cells), anti-calbindin (horizontal and amacrine cells), anti-glutamine synthetase (Müller glia), anti-vimentin (Müller glia), and anti-rhodopsin (rods). For Ki-67 staining, anti-human Ki-67 antibody (1:100; PharMingen, San Diego, CA) was used. Terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling (TUNEL) assay was performed with a detection kit (Boehringer Mannheim, Indianapolis, IN). All pictures were taken by a confocal microscope (Carl Zeiss, Thornwood, NY).

Mash1–Math3 mutant mice. *Mash1*–*Math3* double-mutant mice were obtained by crossing *Mash1*(+/-)–*Math3*(-/-) male and *Mash1*(+/-)–

Math3(+/-) female mice (Tomita et al., 2000). Because double-mutant embryos survived until E15.5 but died by E17.5, embryos were harvested at E15.5.

RESULTS

Expression of *hesr* genes in the developing retina

Expression of *hesr* genes in the retina was first determined by Northern blot analysis. At P0, when there are many retinal precursors, all *hesr* genes were expressed at comparable levels (Fig. 1A). However, after P3 the expression patterns were different from each other. *hesr1* expression was maintained at a relatively constant level until adulthood (Fig. 1A). In contrast, both *hesr2* and *hesr3* expression was downregulated at P5 but the former was maintained afterward, whereas the latter became undetectable at P7 (Fig. 1A). Thus, *hesr1* and *hesr2* are expressed by both undifferentiated and differentiated cells, whereas *hesr3* is expressed transiently by undifferentiated cells.

The expression was further examined by *in situ* hybridization. At E17, all *hesr* genes were expressed in the ventricular zone, which contains common precursors for neurons and glia (Fig.

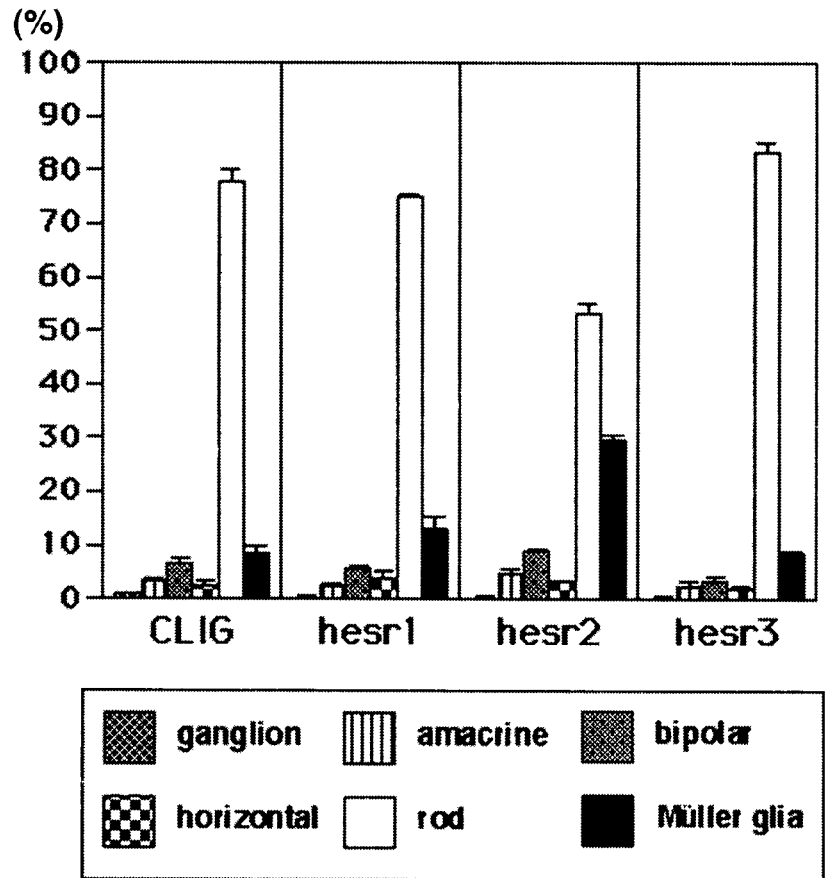


Figure 3. Ratios of the virus-infected cells in the explants starting at E17.5. Ratios of retinal cell types infected with CLIG, CLIG-*hesr1*, CLIG-*hesr2*, and CLIG-*hesr3* at E17.5 are shown. When infected with CLIG or CLIG-*hesr1*, ~80% of the virus-infected cells differentiated into rods, whereas 10% differentiated into Müller glia, indicating that *hesr1* does not affect the ratios of retinal cell types. When infected with CLIG-*hesr3*, slightly more cells differentiated into rods, although this increase is not statistically significant. In contrast, when infected with CLIG-*hesr2*, ~30% of the virus-infected cells differentiated into Müller glia, and <60% differentiated into rods. Thus, misexpression of *hesr2* displayed an approximately threefold increase of gliogenesis. Ratios with a SE are the average of at least three independent experiments.

1*B,E,H*). *hesr1* and *hesr3* were also expressed in the ganglion cell layer, which contains projection neurons (Fig. 1*B,H*). At P5, when the ventricular cells were differentiating into neurons and glial cells, which form the INL and ONL, *hesr* expression was shifted to the INL (Fig. 1*C,F,I*). *hesr1* expression was observed mainly in the outer and inner regions of the INL, which contain horizontal and amacrine cells, respectively (Fig. 1*C*). In contrast, *hesr2* was mainly expressed in the middle region of the INL, which contains bipolar and Müller glial cells (Fig. 1*F*). Thus, *hesr1* and *hesr2* displayed complementary expression patterns in the INL. At P10, when the majority of retinal cells finished differentiation, *hesr1* was again expressed mainly in the outer and inner regions of the INL (Fig. 1*D*), whereas *hesr2* was expressed in the middle region of the INL (Fig. 1*G*). In contrast, *hesr3* expression was not detectable at P10 (Fig. 1*J*). Thus, the three *hesr* genes exhibited different expression patterns in the developing retina.

Misexpression of *hesr* genes in the developing retina at E17.5

To examine the functions of *hesr* genes, each gene was misexpressed with retrovirus in the developing retina. We used a replication-incompetent retrovirus, CLIG, which directs GFP expression as a marker from the upstream long terminal repeat (LTR) promoter (Fig. 2*A*) (Hojo et al., 2000). Each *hesr* gene was inserted at the upstream of the internal ribosomal entry site (IRES) so that *hesr* and GFP genes can be expressed bicistronically (Fig. 2*A*). Retinal explants were prepared from mouse embryos at E17.5, and virus was applied on the same day. Two weeks later, by which time most retinal cells finished differentia-

tion, the fate of the virus-infected cells was examined by monitoring GFP⁺ cells.

When the control virus CLIG was applied to the retinal explants, ~80% of the virus-infected cells differentiated into rods in the ONL, whereas the others differentiated mostly into bipolar and Müller glial cells in the INL (Figs. 2*K, 3*), as described previously (Turner and Cepko, 1987). Similarly, when CLIG-*hesr1* was applied, ~80% of the virus-infected cells differentiated into rods in the ONL, and the others differentiated mostly into bipolar and Müller glial cells in the INL (Figs. 2*B, 3*). Thus, no significant effects by misexpression of *hesr1* were detectable. When CLIG-*hesr3* was applied, slightly more cells differentiated into rods (Figs. 2*H, 3*), although this increase was not statistically significant. In contrast, when CLIG-*hesr2* virus was applied, >30% of the virus-infected cells differentiated into Müller glia, whereas <60% differentiated into rods (Figs. 2*E, 3*). Thus, *hesr2* exhibited an approximately threefold increase of Müller glial cell genesis with concomitant decrease of rod genesis. These *hesr2*-induced Müller glial cells exhibited a typical morphology: a cell body in the INL and long processes to both the ganglion cell layer and the ONL (Fig. 2*E*). To verify the coexpression of *hesr* genes with GFP, three repeats of the Myc epitope were fused to the amino terminus of each *hesr*, and we confirmed double staining of GFP and Myc in almost all virus-infected cells (Fig. 2*B–J*). In separate experiments, we also misexpressed each *hesr* without the Myc tag and obtained the same results, which suggests that the Myc tag did not affect the activity of *hesr* proteins (data not shown). These data indicate that among the three structurally related members, only *hesr2* may have a gliogenic activity in the retina.

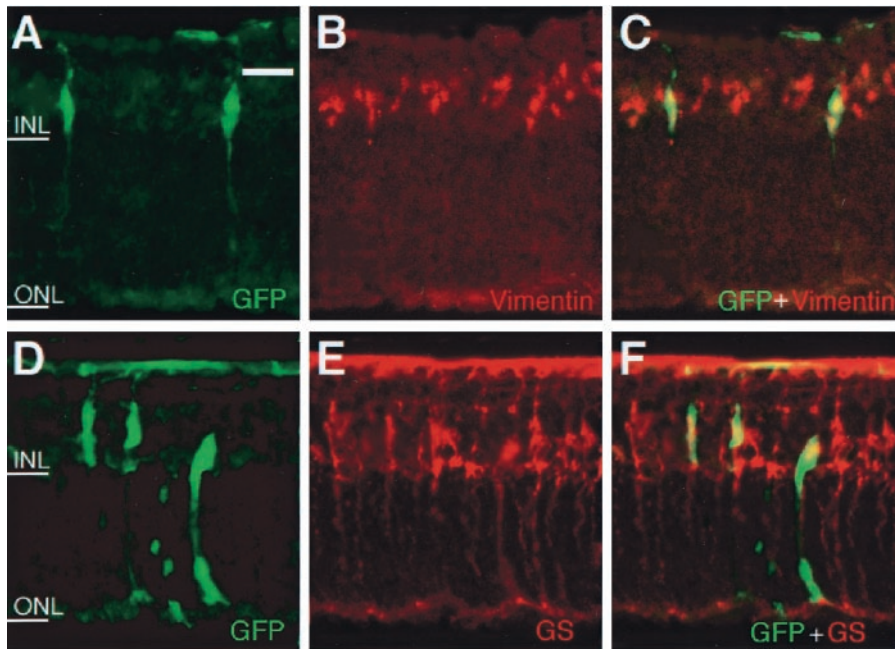


Figure 4. Misexpression of *hesr2* promotes Müller glial development. Retinal explants were prepared from E17.5 mouse embryos and infected with CLIG-*hesr2*. After 2 weeks of culture, the explants were subjected to immunohistochemistry using anti-GFP, anti-vimentin, and anti-GS antibodies. Many of the virus-infected cells expressed the Müller glial markers, vimentin and GS. Scale bar, 25 μ m.

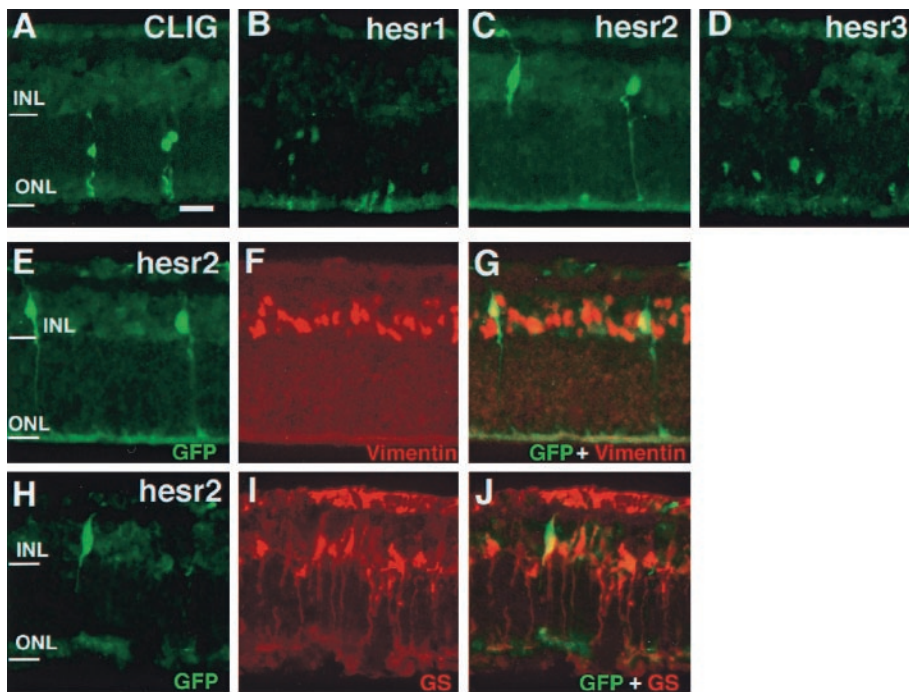


Figure 5. Misexpression of *hesr* genes in the retinal explants starting at P1. Retinal explants were prepared from P1 mouse neonates and infected with CLIG (*A*), CLIG-*hesr1* (*B*), CLIG-*hesr2* (*C*, *E–J*), and CLIG-*hesr3* (*D*). After 2 weeks of culture, the explants were subjected to immunohistochemistry using anti-GFP, anti-vimentin, and anti-GS antibodies. The majority of CLIG-, CLIG-*hesr1*-, and CLIG-*hesr3*-infected cells differentiated into rods in the ONL (*A*, *B*, *D*). In contrast, most CLIG-*hesr2*-infected cells became Müller glia (vimentin⁺, GS⁺) (*C*, *E–J*). Scale bar, 25 μ m.

We next examined whether Müller glia-like cells induced by *hesr2* expressed glia-specific markers, vimentin and GS. Many of the *hesr2*⁺ cells expressed the Müller glial markers vimentin (Fig. 4*A–C*) and GS (Fig. 4*D–F*). These results indicated that *hesr2* promotes gliogenesis but inhibits neurogenesis, as do *Hes1* and *Hes5*.

Misexpression of *hesr* genes in the developing retina at P1

Because most Müller glial cells, one of the last-born cell types, differentiate postnatally, we next examined the effects of *hesr* genes at a later stage of retinal development. Retinal explants were thus prepared at P1, and virus was applied on the same day. When the control virus CLIG was applied, the ratios of the

last-born cell types, bipolar and Müller glial cells, were slightly increased, but still, most of the virus-infected cells differentiated into rods (Figs. 5*A*, 6). Similarly, when CLIG-*hesr1* was applied, ~80% of the virus-infected cells differentiated into rods, indicating that *hesr1* did not affect the choice between the neuronal and glial fates (Figs. 5*B*, 6). When CLIG-*hesr3* was applied, ~90% of the virus-infected cells differentiated into rods and only 5% differentiated into Müller glial cells, thus suggesting that *hesr3* promotes rod genesis and inhibits gliogenesis (Figs. 5*D*, 6). In contrast, when CLIG-*hesr2* was applied, ~75% of the virus-infected cells differentiated into Müller glia, whereas only <20% became rods (Figs. 5*C*, 6). In addition, many of *hesr2*⁺ cells expressed the Müller glial

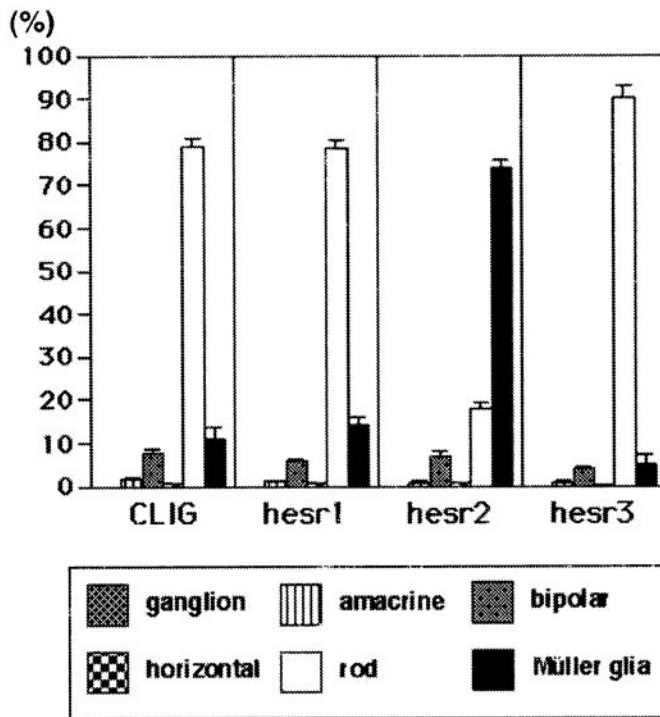


Figure 6. Ratios of the virus-infected cells in the explants starting at P1. Ratios of retinal cell types infected with CLIG, CLIG-*hesr1*, CLIG-*hesr2*, and CLIG-*hesr3* at P1. When infected with CLIG or CLIG-*hesr1*, ~80% of the virus-infected cells differentiated into rods. When infected with CLIG-*hesr3*, ~90% of the virus-infected cells differentiated into rods, indicating that *hesr3* promotes rod genesis. This change is statistically significant (*t* test; *p* < 0.0001). When infected with CLIG-*hesr2*, ~75% of the virus-infected cells differentiated into Müller glia. Thus, *hesr2* significantly promoted gliogenesis when misexpressed postnatally. Ratios with a SE are the average of at least three independent experiments.

markers, vimentin (Fig. 5E–G) and GS (Fig. 5H–J). Thus, *hesr2* exhibited a profound effect on gliogenesis when misexpressed in the postnatal retina.

Cell proliferation and death are not affected by *hesr2*

The increase of Müller glia by *hesr2* could be the result of proliferation of glial cells and apoptosis of neurons including rods, rather than conversion of precursors to the Müller glial cell fate at the expense of neurons. To distinguish between these possibilities, proliferation and death of virus-infected cells were analyzed. Cell proliferation was examined by Ki67, a nuclear antigen expressed by proliferating cells. Four or seven days after viral infection, most of the cells infected with CLIG or CLIG-*hesr2* were negative for Ki67 (Fig. 7A,B, and data not shown), indicating that *hesr2* did not promote cell proliferation. To determine the extent of cell death, the retinal explants were subjected to TUNEL assay 4 or 7 d after viral infection. Most of the virus-infected cells were negative for the TUNEL assay at both time points (Fig. 7C,D, and data not shown). These results suggested that the Müller glial cell genesis induced by *hesr2* was not the result of glial proliferation or neuronal apoptosis but most likely of conversion of precursors toward the Müller glial cell fate at the expense of neurons.

To further determine the extent of cell proliferation and survival, the clonal size of the virus-infected cells was examined. The sizes of clones infected with CLIG-*hesr* at both E17.5 (Fig. 7E) and P1 (Fig. 7F) were mostly one or two cells, and they were very close

to the size of CLIG-infected clones (Figs. 7E,F). These results indicated that *hesr2* did not affect cell proliferation or death, in agreement with the above data of Ki67 staining and TUNEL assay.

hesr2 expression in the retina double-mutant for *Mash1* and *Math3*

We have recently found that in the retina double-mutant for *Mash1* and *Math3*, the cells that normally differentiate into bipolar cells are blocked from neuronal differentiation and instead adopt the Müller glial fate (Tomita et al., 2000). To determine whether *hesr2* is involved in this increase of Müller glia, we examined *hesr2* expression in the double-mutant retina. Because all double-mutant embryos die by E17.5, retinal explants were prepared from E15.5 embryos and cultured to examine *hesr2* expression at a later stage.

At E15.5, *hesr2* expression was not affected in the double-mutant retina (Fig. 8A,D). However, at day 7 of retinal explant culture, when more Müller glial cells (vimentin⁺) were differentiating in the double-mutant than in the wild type (Fig. 8C,F), *hesr2* expression was upregulated in the INL of the double-mutant retina (Fig. 8B,E). These results suggest that upregulation of *hesr2* expression may contribute to the increase of the Müller glial cell number in the double-mutant retina. In contrast, expression of *Hes1* and *Hes5* was not altered in the double-mutant retina both at E15.5 and at day 7 of explant cultures (data not shown).

DISCUSSION

hesr2 promotes the glial versus neuronal fate choice in the retina

Previous studies revealed that two *hairy/E(spl)* homologs, *Hes1* and *Hes5*, play an important role in gliogenesis (Furukawa et al., 2000; Hojo et al., 2000). Here, we found that a related bHLH gene, *hesr2*, but not *hesr1* or *hesr3*, promotes gliogenesis and inhibits rod genesis in the retina. Analysis of Ki67 and TUNEL staining as well as of clonal sizes demonstrated that glial proliferation and neuronal apoptosis cannot account for *hesr2*-induced gliogenesis. Therefore, it is most likely that the cells that normally differentiate into rods adopt the glial fate by misexpression of *hesr2*, indicating that *hesr2* regulates the glial versus neuronal fate choice in the retina. This activity is very similar to that of *Hes1* and *Hes5*, and thus *hesr2* may substitute for the two *Hes* genes in gliogenesis.

The gliogenic activity of *hesr2* correlates very well with its expression pattern. *hesr2* is initially expressed by common precursors of neurons and glia in the ventricular zone, but during the postnatal period, *hesr2* expression is shifted to the middle region of the INL, where Müller glial cells are differentiating. Thus, the expression pattern agrees well with the function of *hesr2*, which directs precursors to adopt the glial fate. Because *hesr2* continues to be expressed until adulthood, it could also be involved in maintenance of mature glial cells in addition to glial fate determination.

Roles of *hesr2* in gliogenesis

The mechanism by which *hesr2* as well as *Hes1* and *Hes5* promote gliogenesis remains to be determined. One mechanism would be that these genes may downregulate neuronal bHLH genes such as *Mash1* and *NeuroD*, which promote neurogenesis at the expense of gliogenesis (Tomita et al., 1996b; Morrow et al., 1999). We have recently found that in mice double-mutant for the neuronal determination genes

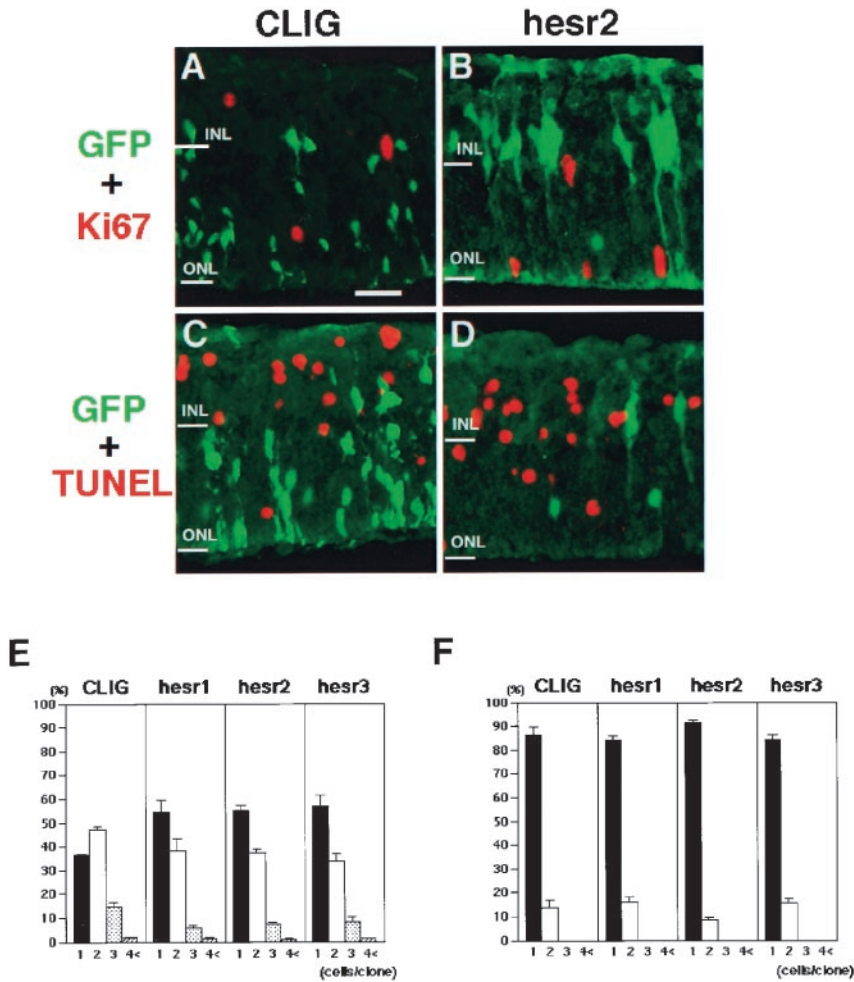


Figure 7. Proliferation and death of the virus-infected cells. *A–D*, Retinal explants infected with CLIG (*A, C*) and CLIG-*hesr2* (*B, D*) were subjected to immunohistochemistry with anti-Ki67 antibody (*A, B*) and to TUNEL assay (*C, D*) after 1 week of culture. Most of the virus-infected cells were negative for Ki67 expression and TUNEL assay. Scale bar, 25 μ m. *E, F*, Comparison of the clonal sizes of the cells infected at E17.5 (*E*) and P1 (*F*). The clonal sizes of CLIG- and CLIG-*hesr*-infected cells are very similar in all infections. These results indicate that misexpression of *hesr* did not affect cell proliferation or survival. Ratios with a SE are the average of at least three independent experiments.

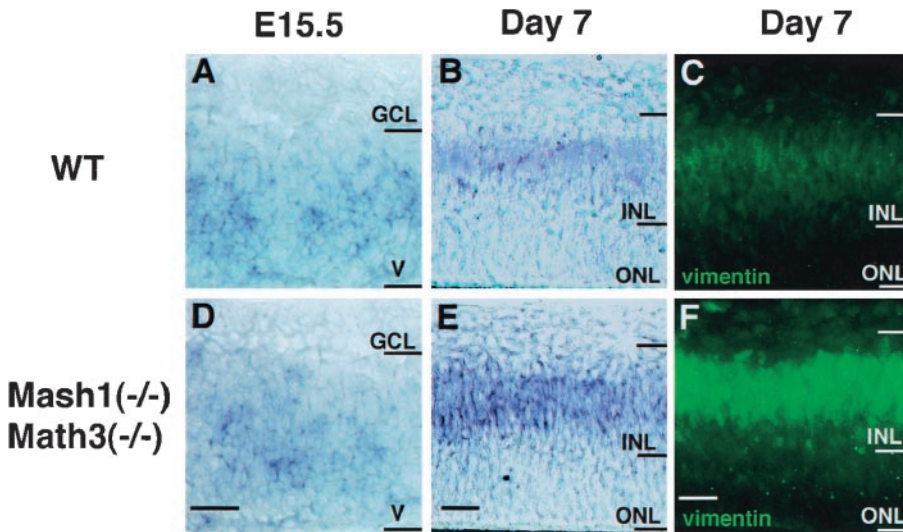


Figure 8. *hesr2* expression in the retina double-mutant for *Mash1* and *Math3*. The wild-type (*A–C*) and *Mash1-Math3* double-mutant retinas (*D–F*) were examined at E15.5 (*A, D*) and at day 7 of explant culture (*B, C, E, F*). *A, B*, *hesr2* is initially expressed in the ventricular zone (V) and later in the INL. *C*, Müller glial cells (vimentin⁺) are present in the INL. *D, E*, *hesr2* expression is not altered in the double-mutant retina at E15.5 but significantly upregulated at day 7 of explant culture, compared with the wild type. *F*, Müller glial cells (vimentin⁺) are increased in the double-mutant INL. Scale bar, 25 μ m.

Mash1 and *Math3*, the cells that normally differentiate into neurons are blocked from neuronal commitment and instead adopt the Müller glial fate (Tomita et al., 2000). Thus, downregulation of *Mash1* and *Math3* is sufficient to initiate the gliogenic program. Because *Hes1* and *Hes5* are transcriptional repressors that inhibit the activity and expression of neuronal bHLH factors (Akazawa et al., 1992; Sasai et al., 1992; Ishibashi et al., 1995; Chen et al., 1997), it is likely that *Hes1* and

Hes5 may suppress *Mash1* and *Math3*, thereby promoting gliogenesis. In addition, it was recently reported that *Id1*, which dominant-negatively regulates positive bHLH genes (Benezra et al., 1990), also promotes gliogenesis (Cai et al., 2000). Thus, *Hes1*, *Hes5*, and *Id1* may specify the glial fate by inducing the same effects as the double mutation of *Mash1* and *Math3*, although it remains to be determined whether this is the only mechanism for *Hes1/Hes5/Id1*-induced gliogenesis. It is not

known whether *hesr2* has such an inhibitory activity on *Mash1* and *Math3*, but the structural conservation suggests that it does: *hesr2* and Hes1/Hes5 share a high homology in two important domains. Hes1 and Hes5 have a conserved bHLH domain, which is important for the DNA binding and dimer formation, and the C-terminal WRPW domain, which interacts with the corepressor Groucho/TLE (Paroush et al., 1994; Fisher et al., 1996; Grabavec and Stifani, 1996). These two domains are essential for transcriptional repression activity of Hes1 and Hes5. Interestingly, *hesr2* has a conserved bHLH domain in the amino-terminal region and a WRPW-related sequence, YQPW, in the C-terminal region (Leimeister et al., 1999; Nakagawa et al., 1999; Chin et al., 2000; Zhong et al., 2000), suggesting that *hesr2* functions as a transcriptional repressor like Hes1 and Hes5. Supporting this idea, it has been shown recently that *hesr2* (CHF1) represses transcription induced by ARNT/EPAS1 (Chin et al., 2000). Interestingly, the increase of Müller glia in the *Mash1-Math3* double-mutant retina is associated with upregulation of *hesr2*, suggesting that *Mash1* and *Math3* normally inhibit gliogenesis by repressing *hesr2*. Thus, antagonistic regulation between *hesr2* and *Mash1/Math3* may determine the ratios of neurons and glia.

Is gliogenesis a default pathway after downregulation of neuronal bHLH genes? Recent studies demonstrated that oligodendrocyte development is regulated by two related bHLH genes, *Olig1* and *Olig2* (Lu et al., 2000; Zhou et al., 2000), suggesting that glial development may not be a simple default pathway but require glia-specific transcription factors. However, although *Olig1* and *Olig2* can upregulate some glial-specific gene expression, they alone are not sufficient for oligodendrocyte development (Lu et al., 2000; Zhou et al., 2000). Thus, it is possible that for glial development these glia-specific bHLH genes may depend on negatively acting genes such as *hesr2*, *Hes1*, *Hes5*, and *Id1*, which suppress neurogenesis and switch on the gliogenic program.

Interestingly, the gliogenic activity of *hesr2* seems to be stage-dependent, and when it is misexpressed at a later stage (P1), it displays a more profound effect: ~75% of *hesr2*⁺ cells differentiate into Müller glia, whereas normally ~10% become glia at this stage. Because some neuronal bHLH genes such as *Mash1* are downregulated postnatally (Tomita et al., 1996b), it is possible that *hesr2* could more effectively antagonize the neuronal bHLH genes at later stages.

Outside of the retina, *hesr* genes are also expressed in the developing nervous system. Although *hesr1* is expressed in the ventricular zone, which contains neural precursors, *hesr2* is expressed in both the ventricular zone and cortical plate (Leimeister et al., 1999), suggesting that these two *hesr* genes have distinct functions. It remains to be determined whether *hesr2* has a gliogenic activity in the cortical plate.

Other functions of *hesr* genes

hesr genes are also expressed outside of the nervous system. Although *hesr1* is expressed in the cardiac atrium, *hesr2* is in the ventricles, suggesting that the two *hesr* genes have again distinct functions in heart development (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999; Chin et al., 2000; Zhong et al., 2000). They are also expressed in the blood vessels. Interestingly, in the zebrafish mutant for *hesr2* (*gridlock*), assembly of the dorsal aortas is affected and blood flow is blocked (Zhong et al., 2000), indicating that *hesr2* plays an important role in development of aorta. However, other defects such as neural defects have

not been noted. Because *gridlock* mutation is hypomorphic rather than null, it is possible that defects are observed only in the most susceptible regions. Null mutation analysis is required to characterize the *hesr2* functions in other regions.

It is interesting that *hesr3* promotes rod differentiation in the retina, although less efficiently, suggesting that *hesr3* may have an opposite activity to *hesr2*. In this regard, the function of *hesr3* is similar to *Hes6*, which also promotes rod genesis in the retina (Bae et al., 2000). *Hes6* is structurally related to but functionally antagonizes Hes1 by direct physical interaction. When Hes1 and the positive regulator *Mash1* are coexpressed, Hes1 represses *Mash1*-induced transcription (Sasai et al., 1992). However, when *Hes6* is additionally coexpressed, *Hes6* suppresses Hes1 and thereby supports the activity of *Mash1*, which promotes neuronal specification (Bae et al., 2000). Thus, misexpression of *Hes6* promotes neuronal differentiation. It is possible that *hesr3* may also have a *Hes6*-like activity and inhibit *hesr2* by physical interaction, thereby inducing neurogenesis, although the precise mechanism remains to be determined.

In our present study, the three *hesr* genes have distinct expression patterns and functions in neural development, although they are structurally related. Further study of *hesr* genes would help determine the mechanism for the binary cell fate decision between neurons and glial cells.

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