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Mammalian *hairy* and *Enhancer of Split* Homolog 1 Regulates Differentiation of Retinal Neurons and Is Essential for Eye Morphogenesis

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Summary

Mammalian *hairy* and *Enhancer of split* homolog 1 (*HES1*), a basic helix-loop-helix factor gene, is expressed in retinal progenitor cells, and its expression decreases as differentiation proceeds. Retinal progenitor cells infected with *HES1*-transducing retrovirus did not differentiate into mature retinal cells, suggesting that persistent expression of *HES1* blocks retinal development. In contrast, in the retina of *HES1*-null mutant mice, differentiation was accelerated, and rod and horizontal cells appeared prematurely and formed abnormal rosette-like structures. Lens and cornea development was also severely disturbed. Furthermore, in the mutant retina, bipolar cells extensively died and finally disappeared. These studies provide evidence that *HES1* regulates differentiation of retinal neurons and is essential for eye morphogenesis.

Introduction

During the development of mammalian neural retina, retinal progenitor cells give rise to a variety of morphologically and functionally distinct cell types (Sidman, 1961; reviewed by Altshuler et al., 1991; Raymond, 1991; Reh, 1991; Harris, 1991). Differentiating retinal cells migrate out of the ventricular zone and form laminar structures. Mature retina exhibits a well-organized structure and contains three cellular layers: the outer nuclear layer (rod and cone photoreceptor cells), the inner nuclear layer (bipolar, horizontal, and amacrine cells), and the ganglion cell layer.

Retinal progenitor cells differentiate into different types of cells as retinal development proceeds (Sidman, 1961; Carter-Dawson and LaVail, 1979; Young, 1985; Turner and Cepko, 1987; Turner et al., 1990). Ganglion, horizontal, cone, and amacrine cells differentiate at early stages and rod, bipolar, and Müller glial cells differentiate at later stages. In the case of rodents, retinal progenitor cells keep dividing for up to 1 week after birth, and the majority of rod, bipolar, and Müller glial cells differentiate postnatally. This postnatal development, in addition to its well-organized structure, makes the retina an ideal system to examine the mechanisms underlying the complex developmental processes of mammalian neurogenesis. However, although this system has been actively studied, the molecular mechanisms of retinal development are not yet well understood.

HES1, a member of a family of mammalian basic helix-loop-helix (bHLH) factor genes homologous to Drosophila *hairy* and *Enhancer of split [E(spl)]* (Akazawa et al., 1992; Sasai et al., 1992; Feder et al., 1993; Takebayashi et al., 1994), is expressed in the ventricular zone of the developing nervous system (Sasai et al., 1992). *HES1* expression decreases as neurogenesis proceeds, and persistent expression of *HES1* blocks neuronal and glial differentiation in the cerebral cortex (Ishibashi et al., 1994). In addition, *HES1*-null mutation leads to premature neuronal differentiation and severe neural tube defects in the cranial region (Ishibashi et al., 1995). Thus, *HES1* acts as a negative regulator of neurogenesis, like Drosophila *hairy* and *E(spl)* (Moscoso del Prado and Garcia-Bellido, 1984; Jan and Jan, 1993; Campos-Ortega and Jan, 1991; Campuzano and Modolell, 1992; Brown et al., 1995) and plays an important role in cranial neurulation.

Recent analysis suggests that *HES1* expression is regulated by the Notch signaling pathway. The activated form of Notch translocates to the nucleus, forms a complex with recombination signal-binding protein–Jκ (RBP-Jκ), a mammalian homolog of Suppressor of Hairless (Furukawa et al., 1992), and activates *HES1* transcription by interacting with its promoter element in transient transfection experiments (Jarriault et al., 1995). Interestingly, Notch is expressed in the developing retina (Weinmaster et al., 1991; Franco del Amo et al., 1992), and an activated form of Notch inhibits retinal development in Xenopus (Dorsky et al., 1995). These results raise the possibility that Notch-induced suppression of retinal development involves *HES1* induction.

In the experiments described here, we examined the expression and roles of *HES1* in the development of neural retina. We found that *HES1* expression occurs in retinal progenitor cells and is down-regulated as development proceeds. Retinal progenitor cells infected with *HES1*-transducing retrovirus did not differentiate into mature retinal cells, indicating that persistent expression of *HES1* prevents differentiation of retinal neurons. Conversely, *HES1*-deficient retinal progenitor cells differentiated prematurely into rod and horizontal cells, resulting in severely disorganized retinal structures. The results of these gain-of-function and loss-of-function analyses suggest that *HES1* prevents premature neuronal differentiation and regulates retinal development.

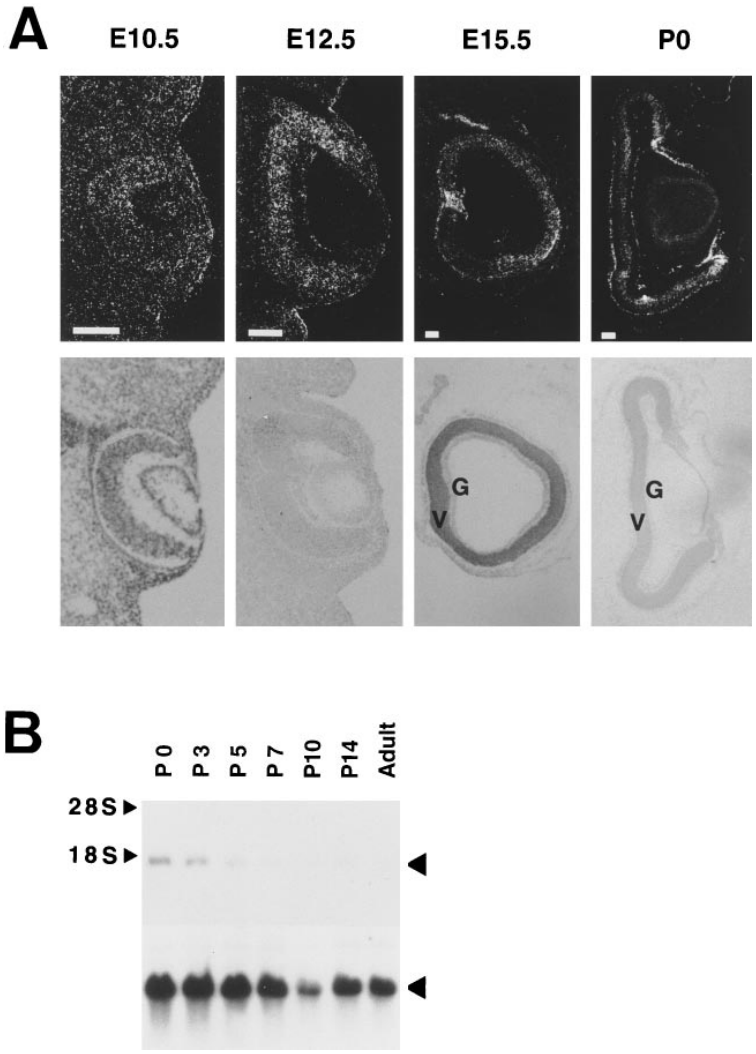

Figure 1. *HES1* Expression in the Developing Mouse Retina

(A) In situ hybridization of *HES1* (top). E10.5, E12.5, E15.5, and P0 eyes were examined. During this period, *HES1* was expressed in the ventricular zone (V) but not in the ganglion cell layer (G). Cresyl violet staining of near sections is shown in the bottom panels. Scale bar, 100 μm.

(B) Northern blot analysis of neural retina. 15 μg of total RNA prepared from neural retina at various stages was used, as indicated above each lane. *HES1* (top arrowhead) and elongation factor 1α transcripts (bottom arrowhead) are indicated on the right.

Results

Expression of *HES1* in the Developing Retina

We first examined mouse *HES1* expression in the developing retina by in situ hybridization. During the embryonic period, *HES1* was expressed at high levels in the ventricular zone of the neural retina in which retinal progenitor cells are dividing, but not in the ganglion cell layer in which differentiated neurons are present (Figure 1A). At postnatal day 0 (P0), *HES1* expression still occurred at a high level in the ventricular zone (Figure 1A). Northern blot analysis showed that *HES1* expression continued at high levels until P0, but gradually decreased around P3–P7 reaching very low levels at P10 (Figure 1B), when the last retinal progenitor cells undergo their final division and differentiate into various types of mature retinal cells. Thus, *HES1* expression occurs at high levels in neural precursor cells and decreases as differentiation proceeds. We also observed the same *HES1* expression patterns in rat retina (data not shown). In the following experiments, we examined mouse retinas for loss-of-function studies, and both rat and mouse retinas for gain-of-function studies and obtained the same results in the two species.

Retinal Organ Culture System

To investigate the roles of *HES1* in retinal development, we attempted a gain-of-function analysis by infecting retinal progenitor cells with a *HES1*-transducing retrovirus. We also attempted a loss-of-function analysis by examining the retina of *HES1*-null mice. However, because all *HES1*-null mice died either during gestation or within one day after birth (Ishibashi et al., 1995), we were not able to examine the postnatal retinal development of *HES1*-null mice. Since previous studies have demonstrated that retina in organ culture develops in a normal manner that closely mimics retinal development in vivo (Caffé et al., 1989; Sparrow et al., 1990), the problem of premature death of *HES1*-null mice was overcome by studying newborn or embryonic retinal preparations maintained in organ culture. An additional benefit of this culture system in comparison to in vivo retina is the better accessibility to retroviral infection for a gain-of-function analysis.

Table 1. Antibody Markers Used in the Analysis of Retinal Development

Retinal Cell Type	Antibody	Localization
Rod Cell	Rhodopsin	Cell bodies, outer segments
Horizontal cell	Neurofilament[a]	Processes
Bipolar cell	Protein kinase C	Processes, cell bodies
	mGluR6	Processes, cell bodies[b]
Amacrine cell	HPC-1	Processes, cell bodies
Ganglion cell	Thy-1	Processes, cell bodies

[a] Anti-neurofilament antibody reacts with both horizontal and ganglion cells in vivo, but in organ culture, it only reacts with horizontal cells because axons of ganglion cells are severed in organ culture.
[b] Staining of mGluR6 in cell bodies is only observed before terminal differentiation (Nomura et al., 1994).
References of the markers used: rhodopsin (Hicks and Barnstable, 1987); neurofilament (Dräger, 1983; Shaw and Weber, 1983); protein kinase C (Negishi et al., 1988; Greferath et al., 1990; Zhang and Yeh, 1991); metabotropic glutamate receptor 6 (mGluR6) (Nomura et al., 1994); HPC-1 (Barnstable et al., 1985); Thy-1 (Barnstable and Dräger, 1984).

Differentiation and cell death of cultured and in vivo retinas were compared by immunohistochemical (Table 1) and TdT-mediated dUTP-biotin nick end labeling (TUNEL) methods. At P5, the ventricular zone of both in vivo and cultured retinas started separating into two layers, the outer and inner nuclear layers (Figures 2A and 2B). Cells in the prospective outer nuclear layer started rhodopsin expression in both retinas at this stage (Figures 2A and 2B). TUNEL analysis indicated that cell death also similarly occurred mainly in the prospective inner nuclear layer of both retinas (Figures 2A and 2B). At P10, both in vivo and cultured retinas consisted of three well-developed cellular layers, and the cells in the inner nuclear layer expressed protein kinase C (PKC) (Figures 2C and 2D), indicating that most bipolar cells had differentiated by P10. These results thus demonstrate that retinal development in our organ culture system closely resembles the development of retina in vivo.

HES1-Transducing Retroviral Infection Blocks Retinal Development

To perform a gain-of-function analysis, two replication-defective retroviruses, SG virus and SG-HES1 virus (Ishibashi et al., 1994), were used. SG virus, a control retrovirus, directs *lacZ–neo* fusion gene expression from an

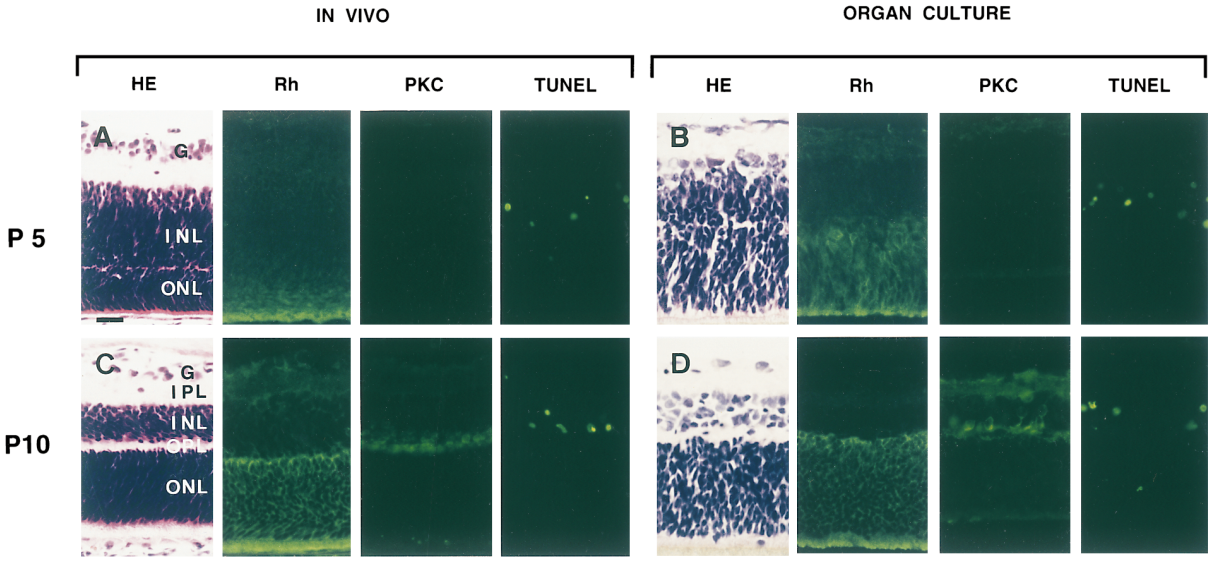

Figure 2. Comparison of the Development of Retina In Vivo and in Organ Culture

P0 retina was used for organ culture. Sections were prepared from P5 (A) and P10 (C) rat retina and retina in organ culture at days 5 (B) and 10 (D). Sections were examined by HE staining (left panel), an immunohistochemical method [middle two, anti-rhodopsin [Rh] and anti-protein kinase C [PKC]) and TUNEL (right).

(A and B) At P5, the ventricular zone started separating into two layers in both in vivo and cultured retinas. Rhodopsin expression started in the prospective outer nuclear layer but no PKC expression was detected in either retina. TUNEL analysis indicated that moderate cell death occurred mainly in the prospective inner nuclear layer.

(C and D) At P10, three cellular layers had developed in both in vivo and cultured retinas. PKC expression had begun in the inner nuclear layer of both in vivo and cultured retinas, suggesting that most bipolar cells had differentiated by P10. TUNEL analysis indicated that moderate cell death occurred in the inner nuclear layer of both retinas. Thus, retinal development in organ culture closely mimics retinal development in vivo. G, ganglion cells; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar, 25 μm.

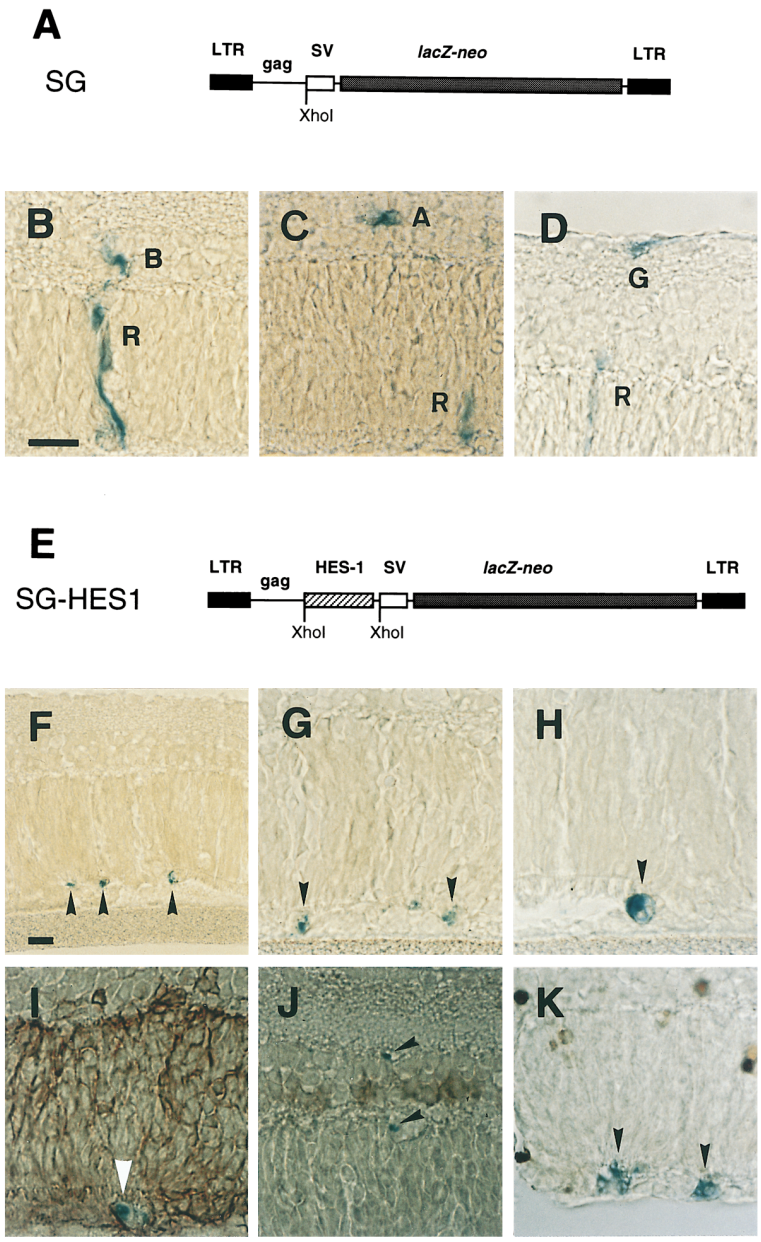

Figure 3. Infection with SG and SG-HES1 Retroviruses

SG (B–D) and SG-HES1 viruses (F–K) were used to infect retinal organ cultures at day 0, and the fates of infected cells were examined by X-Gal staining at day 14.

(A) Schematic structure of SG retrovirus. The *lacZ-neo* fusion gene is expressed from the internal SV40 promoter (Ishibashi et al., 1994).

(B) A cluster of labeled rod (R) and bipolar cell (B).

(C) Labeled amacrine cell (A) and rod.

(D) Labeled ganglion cell (G) and rod.

(E) Schematic structure of SG–HES1 virus. *HES1* expression is directed from the upstream long terminal repeat (LTR) and *lacZ-neo* fusion gene is expressed from the internal SV40 promoter.

(F) Most of SG-HES1 virus-infected cells remained at the outer edge of the neural retina (arrowheads).

(G) The labeled cells were round in appearance and did not exhibit retinal neuron-like morphology (arrowheads).

(H) This cluster consisted of three labeled cells, indicating that these virus-infected cells were dividing (arrowhead).

(I) Cells in the outer nuclear layer were positive for rhodopsin expression (brown). However, the SG-HES1 virus-infected cells were negative for rhodopsin expression (arrowhead).

(J) mGluR6 expression was observed in the inner nuclear layer (brown). Some labeled cells migrated into inner layers. However, they were also round in appearance and negative for mGluR6 expression (arrowheads).

(K) Dying cells were labeled by TUNEL (brown). Two clusters of SG-HES1 virus-infected cells were not labeled by TUNEL (arrowheads), indicating that they were not dying but proliferating. Scale bars, 25 μm (B–D, G–K). Bar, 25 μm (F).

internal SV40 promoter (Figure 3A), while SG-HES1 virus additionally directs *HES1* expression from the upstream long terminal repeat (Figure 3E). Thus, both retroviruses confer *lacZ* expression to allow the visualization of infected cells by X-Gal staining. These retroviruses were used to infect retinal organ cultures prepared from embryonic day 17.5 (E17.5)–P0, and the fates of more than 1000 cells infected with the retroviruses were examined by X-Gal staining.

In the retina, which had been infected with SG virus at day 0 of culture, X-Gal stained cells were distributed mostly in the outer and inner nuclear layers at day 14 (Figures 3B and 3C). These labeled cells appeared alone or as discrete clusters of two to three cells. They were arranged in a radial array within a cluster, and 80%–90% of the labeled cells were located in the outer nuclear layer and exhibited rod-like morphology (Figures 3B–3D). Another 10%–20% of the labeled cells were present in the inner nuclear layer and showed bipolar cell-like morphology (Figure 3B). The remaining 4%–5% represented other types of cells, including amacrine (1%–2%) and ganglion cells (2%–3%) (Figures 3C and 3D). These cell types were also confirmed by immunohistochemistry (data not shown). These results indicated that retinal progenitor cells infected with SG virus differentiated normally into mature retinal cells.

In contrast, in SG-HES1 virus-infected retinas, almost all labeled cells were round in appearance, and ~90% of the labeled cells remained at the outer edge of neural retina at day 14 (Figures 3F–3I, arrowheads). Some labeled cells (~10%) migrated into the outer and inner nuclear layers, but none of them exhibited rod, bipolar,

or any other mature retinal cell-like morphology (Figure 3J, arrowheads). Furthermore, these labeled cells were negative for rod, bipolar (Figures 3I and 3J), and other retinal neuronal markers (data not shown), indicating that retinal progenitor cells forced to express *HES1* failed to differentiate into mature retinal neurons. These SG-HES1 virus-infected cells were not dying because they were not labeled by TUNEL (Figure 3K). In addition, ~20% of them expressed proliferating cell nuclear antigen (Miyachi et al., 1978) (data not shown), suggesting that at least some of these SG-HES1 virus-infected cells were in a mitotic phase. Agreeing with this observation, some of them formed a cluster consisting of several labeled cells (Figures 3H and 3K, arrowheads).

Eye Morphogenesis of *HES1*-Null Mice

To carry out a loss-of-function analysis, we next examined eye morphogenesis of *HES1*-null mice (Ishibashi et al., 1995). As shown in Figure 4, at E10.5 and later, it was noted that eyes of *HES1*-null mice were always smaller in size than wild-type eyes. A total of 318 samples (100 *HES1* [+/+], 190 *HES1* [+/−], and 28 *HES1* [−/−] retinas) were examined, and all *HES1*-null eyes were smaller than normal and exhibited abnormal structures. At E10.5, optic cups and lens vesicles were present in wild-type eyes (Figure 4A). In contrast, in *HES1* (−/−) embryos, optic cups were formed, but they were small and deformed (Figure 4B), probably because retinal progenitor cells did not grow enough to constitute a normal-sized retina. The ventral side of the optic cups remained widely open (Figure 4B, arrowheads), periocular mesenchyme seemed to be entering the optic cup through the ventral opening, and lens development was severely disturbed (Figure 4B). In some extreme cases (2 out of 28 samples), the neural retina was almost completely lacking in *HES1*-null eyes (Figure 4D). In situ hybridization analysis showed that the neuronal marker *SCG-10* (Stein et al., 1988) was already expressed in *HES1*-null retina as early as E9.5 (Figure 4J, right panel, arrow) but not in wild-type retina (Figure 4I, right panel, arrowhead). These results suggest that the genesis of early differentiating neurons, such as ganglion cells, is accelerated without *HES1*, raising the possibility that this accelerated differentiation may affect growth of progenitor cells in *HES1*-null retina.

At E15.5 and E17.5, wild-type neural retina consisted of two layers, the ganglion cell layer and the ventricular zone, and lens fibers were well developed (Figures 4E and 4G). *HES1* (−/−) retina also consisted of two layers, like wild-type retina (Figures 4F and 4H). However, there were several abnormal rosette-like structures in the ventricular zone (Figures 4F and 4H, arrowheads). Furthermore, lens formation was severely disturbed in *HES1*-null eyes (Figures 4F and 4H). It was also noted that cornea developed poorly and was thicker than normal in *HES1*-null eyes (Figures 4F and 4H). These results indicate that development of both the neural retina and the adjacent tissues was severely affected in *HES1*-null mice.

Disorganized Development of *HES1*-Null Retina

To examine whether *HES1*-null retinal progenitor cells can differentiate into mature retinal cell types and form laminar structures, we next conducted retinal organ cultures prepared from *HES1*-null embryos that survived until near full term. Retina was prepared from E17.5 embryos. At day 0 (E17.5), retina of *HES1* (+/+), *HES1* (+/−), and *HES1* (−/−) consisted of the ganglion cell layer and the ventricular zone (Figures 5A, 5F, and 5K). Cells in the ganglion cell layer of both wild-type and *HES1*-null retinas expressed Thy-1 (data not shown), suggesting that ganglion cells differentiated normally without *HES1*. However, *HES1* (−/−) retina contained rosette-like structures in the ventricular zone (Figure 5K, see also Figures 4F and 4H). Rosette-like structures in *HES1* (−/−) retina already expressed a low level of rhodopsin at day 0 (Figure 5L). However, no rhodopsin expression was detected in wild-type retina (Figure 5B) in which rhodopsin-positive cells first appeared at day 5 of culture. Thus, rod cell differentiation prematurely occurred without *HES1*. At day 0, rhodopsin-positive cells were found only in the rosette-like structures and represented ~15% of the final number of rod cells that were observed at day 7.

At day 3, rosette-like structures in *HES1* (−/−) retina were increasingly prevalent. Cells in the rosette-like structures expressed higher levels of rhodopsin (Figure 5N) and represented ~50% of the final number of rods. Furthermore, whereas no neurofilament-positive cells were present in normal retina (Figure 5E), cells surrounding the rosette-like structures already expressed a low level of neurofilament at day 3 (Figure 5O), suggesting that horizontal cells also differentiated prematurely in *HES1* (−/−) retina. Surprisingly, in the presumptive outer nuclear layer of *HES1* (+/−) retina, rhodopsin-positive cells appeared prematurely at day 3 (Figure 5I), although the histology of *HES1* (+/−) retina was very similar to that of *HES1* (+/+) retina (compare Figures 5C and 5H). Thus, a lower level of *HES1* expression also accelerates differentiation, but does not disrupt the development of normal laminar structures.

Rhodopsin expression started uniformly in the outer nuclear layer of wild-type retina at day 5 and was clearly observed at day 7 (data not shown). In *HES1* (−/−) retina, the number of rosette-like structures, which expressed rhodopsin, further increased at days 5 and 7 (data not shown). Thus, in *HES1* (−/−) retina, some rod cells (~15%) already expressed rhodopsin at E17.5, but other rod cells started rhodopsin expression much later. These results indicate that rod cell differentiation did not occur uniformly in *HES1*-null retina, contrasting with the wild-type retina in which rhodopsin expression started more uniformly throughout the outer nuclear layer around days 5–7. Thus, *HES1* may be important not only for preventing premature differentiation but also for coordinating the timing of differentiation.

At day 10 of culture, wild-type retina exhibited three well-developed cellular layers (Figure 6A); rod cells in the outer nuclear layer (Rh^+, Figure 6B), horizontal cells (NF^+, Figure 6C), bipolar cells (PKC^+, Figure 6D), and amacrine cells ($HPC\text{-}1^+$, Figure 6E) in the inner nuclear layer, and ganglion cells in the ganglion cell layer (Figure 6A). In contrast, the laminar structure in *HES1*-null retina was completely disrupted, with many rosette-like structures present (Figure 6G). These rosette-like structures contained rod cells (Rh^+, Figure 6H) and were surrounded by horizontal cells (NF^+, Figure 6I). The top

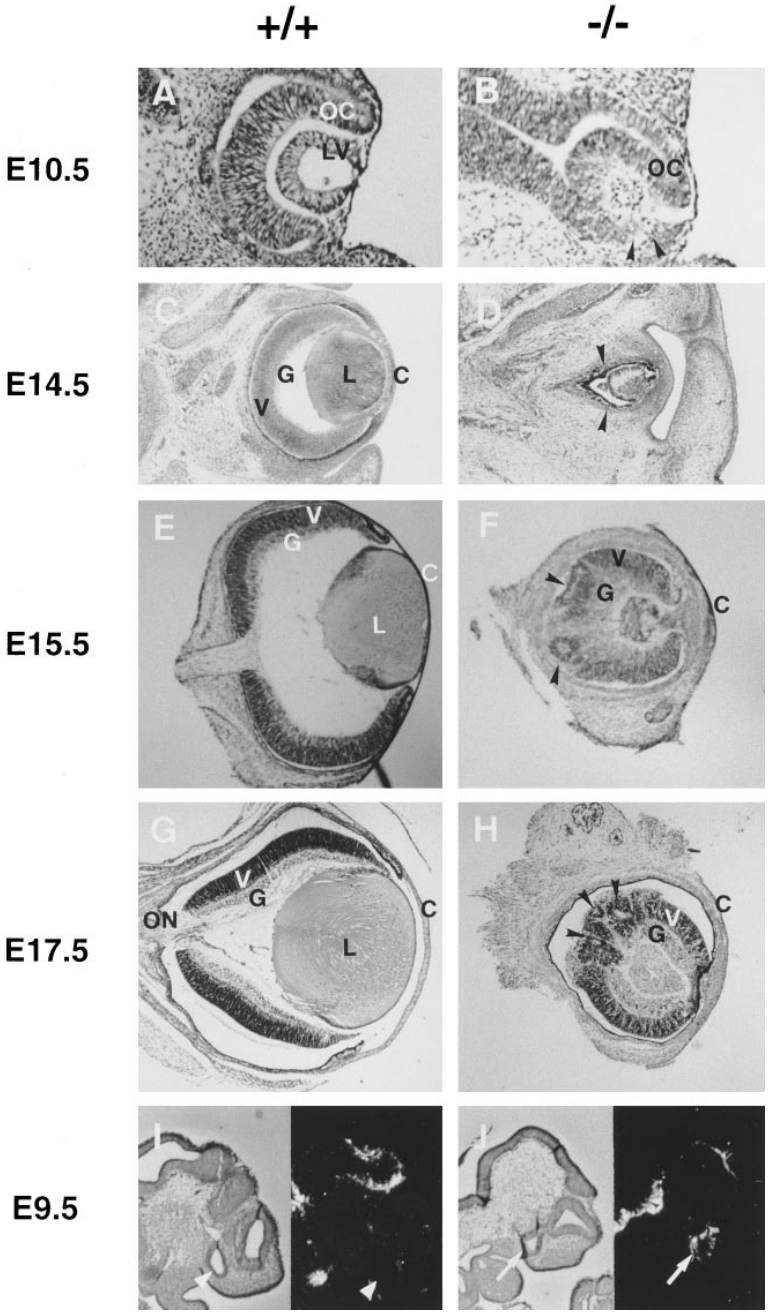

Figure 4. Eye Morphology of Wild-Type and *HES1*-Null Mice

Sections were examined by HE staining (A–H), toluidine blue staining (left panel of I and J), and in situ hybridization (right panel of I and J).

(A) Frontal section of a wild-type E10.5 eye. The optic cup and lens vesicle were formed.

(B) Frontal section of *HES1*-null E10.5 eye. The optic cup was small and deformed, and its ventral side was still widely open (arrowheads). Periocular mesenchyme seemed to be entering the optic cup through the ventral opening.

(C) E14.5 normal eye. Neural retina consisted of the ganglion cell layer and the ventricular zone. The lens contained well-developed lens fibers.

(D) *HES1*-null E14.5 eye. In this extreme case, neural retina was almost completely lacking and only a remnant of pigment epithelium was visible (arrowheads).

(E) E15.5 normal eye.

(F) *HES1*-null E15.5 eye. The size of the eye was still quite small. The *HES1*-null retina consisted of two layers, like wild-type retina. In the ventricular zone, there were abnormal rosette-like structures (arrowheads). Lens and cornea development was severely disturbed.

(G) E17.5 normal eye.

(H) *HES1*-null E17.5 eye. Neural retina consisted of the ganglion cell layer and the ventricular zone. The ganglion cell layer contained Thy-1-positive ganglion cells similar to a normal retina (data not shown), suggesting that ganglion cells differentiated normally without *HES1*. There were several rosette-like structures in the ventricular zone (arrowheads). Lens development was severely disturbed, and the cornea was thicker than normal.

(I) Parasagittal section of the head region of a wild-type E9.5 embryo. The neuronal marker *SCG10* was not yet expressed in the optic vesicle (right, arrowhead).

(J) Parasagittal section of the head region of a *HES1*-null E9.5 embryo. *SCG10* was already expressed in the optic vesicle (right, arrow).

C, cornea; G, ganglion cell layer; L, lens; LV, lens vesicle; OC, optic cup; ON, optic nerve; V, ventricular zone.

thin layer contained ganglion cells (Figure 6G), and the remaining region mostly consisted of amacrine cells (HPC-1$^+$, Figure 6K), which were increased $\sim$50% in number compared with the wild type. However, only a very few bipolar cells were found in *HES1* (−/−) retina (PKC$^+$, Figure 6J). TUNEL analysis indicated that significant cell death occurred along the outer edge of the rosette-like structures in which bipolar cells were located (Figure 6L), suggesting that the decrease in the number of bipolar cells was the result of extensive cell death. This cell death seemed to occur after day 7 because no significant difference was observed between wild-type and *HES1*-null retinas at day 7 on TUNEL analysis (data not shown). Thus, although *HES1* (−/−) retinal progenitor cells can differentiate into all the major retinal cell types that maintain their normal spatial relationships, ratios of retinal cell types were significantly changed because of increased amacrine cells and extensive bipolar cell death.

At day 14 of culture, *HES1* (−/−) retina exhibited almost the same histology as that of day 10 *HES1* (−/−) retina (Figure 7E). The rosette-like structures contained rod cells and were surrounded by horizontal and amacrine cells. However, no PKC- or mGluR6-positive cells were found at this stage in *HES1* (−/−) retina (Figures 7F and 7G), indicating that no bipolar cells were present. In addition, extensive cell death was observed along the outer edge of rosette-like structures in which bipolar

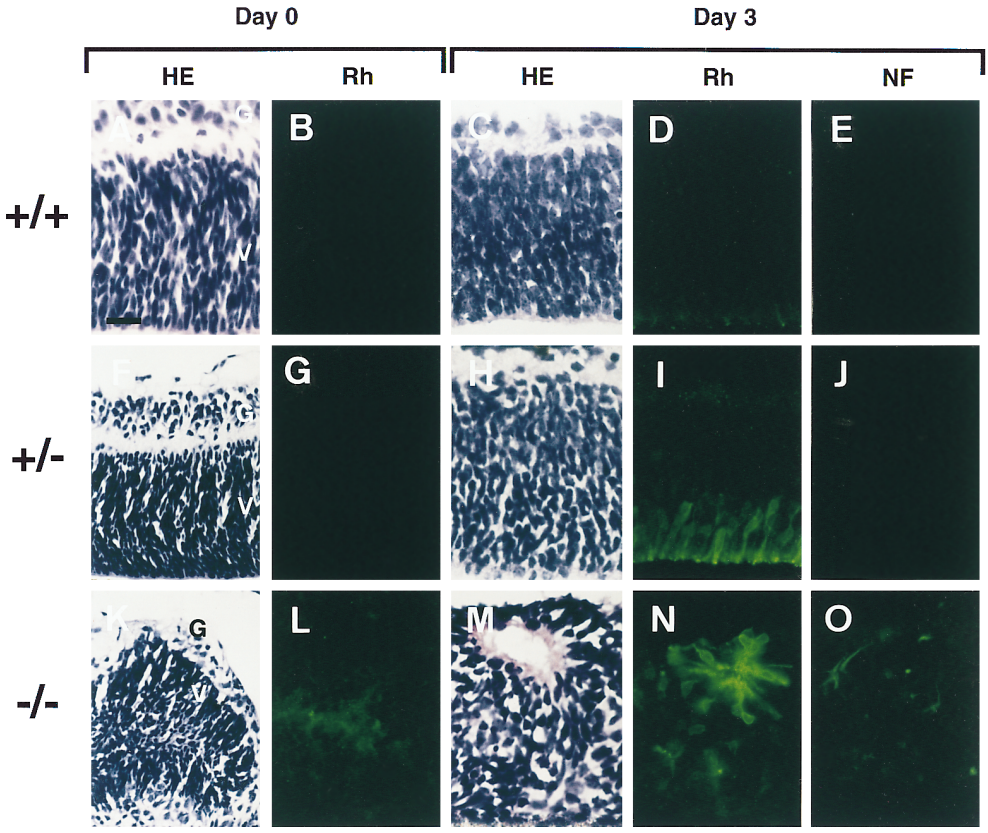

Figure 5. Retinal Organ Cultures at Days 0 and 3

Retina was prepared from E17.5 embryos of genotypes *HES1* (+/+) (A–E), *HES1* (+/−) (F–J), and *HES1* (−/−) (K–O). Sections were made at day 0 (A, B, F, G, K, and L) or day 3 (C–E, H–J, M–O) and examined by HE staining (A, C, F, H, K, and M) and an immunohistochemical method (B, D, G, I, L and N, anti-rhodopsin [Rh]; E, J, and O, anti-neurofilament [NF]).

(A and B) At day 0, *HES1* (+/+) retina consisted of two layers, the ganglion cell layer and the ventricular zone, and was negative for rhodopsin expression.

(C–E) At day 3, *HES1* (+/+) retina still consisted of two layers and was negative for rhodopsin and NF expressions.

(F and G) At day 0, *HES1* (+/−) retina consisted of two layers, like *HES1* (+/+) retina, and was negative for rhodopsin expression.

(H–J) At day 3, histology of *HES1* (+/−) retina was very similar to that of *HES1* (+/+) retina. However, rhodopsin-positive cells had already appeared in *HES1* (+/−) retina, suggesting that rod differentiation occurred prematurely. No neurofilament expression was detected at this stage.

(K and L) At day 0, *HES1* (−/−) retina also consisted of the ganglion cell layer and the ventricular zone. However, there was an abnormal rosette-like structure in the ventricular zone. Cells in the rosette-like structure already expressed rhodopsin at day 0, suggesting that rod differentiation had occurred prematurely by day 0.

(M–O) At day 3, cells in the rosette-like structure expressed rhodopsin at higher levels. In addition, neurofilament-positive horizontal cells had already appeared next to the rosette-like structure at day 3, suggesting that horizontal cell differentiation was also accelerated without *HES1*. We examined at least four independent samples for each experiment and obtained the same results. Abbreviations are identical to those of Figure 4. Bar, 25 μm.

cells were present at day 10 (Figure 7H). Thus, the few bipolar cells that were present at day 10 (Figure 6J) all seemed to have died by day 14, suggesting that *HES1* may be essential for the survival of bipolar cells.

Discussion

The bHLH factor gene *HES1* is expressed in retinal progenitor cells, and its expression is down-regulated when these cells differentiate. In this analysis, we have performed gain-of-function and loss-of-function studies of *HES1*. Persistent expression of *HES1* blocks retinal cell differentiation, whereas *HES1* deficiency accelerates neuronal differentiation and disrupts the laminar structures of the retina.

HES1 Negatively Regulates Retinal Cell Differentiation

Retinal progenitor cells infected with a *HES1*-transducing retrovirus remain small in size and do not express mature retinal markers or exhibit mature retinal cell-like morphology. Even when all normal progenitor cells had differentiated, many of the cells infected with the *HES1*-transducing retrovirus still remained at the outer edge of the neural retina, in which retinal progenitor cells were previously located. Some cells infected with the *HES1*-transducing retrovirus migrated into the inner layers, but even these cells did not express mature retinal markers. Thus, persistent expression of *HES1* inhibits differentiation of retinal cells. Similar effects were reported when the activated form of Notch was expressed in Xenopus

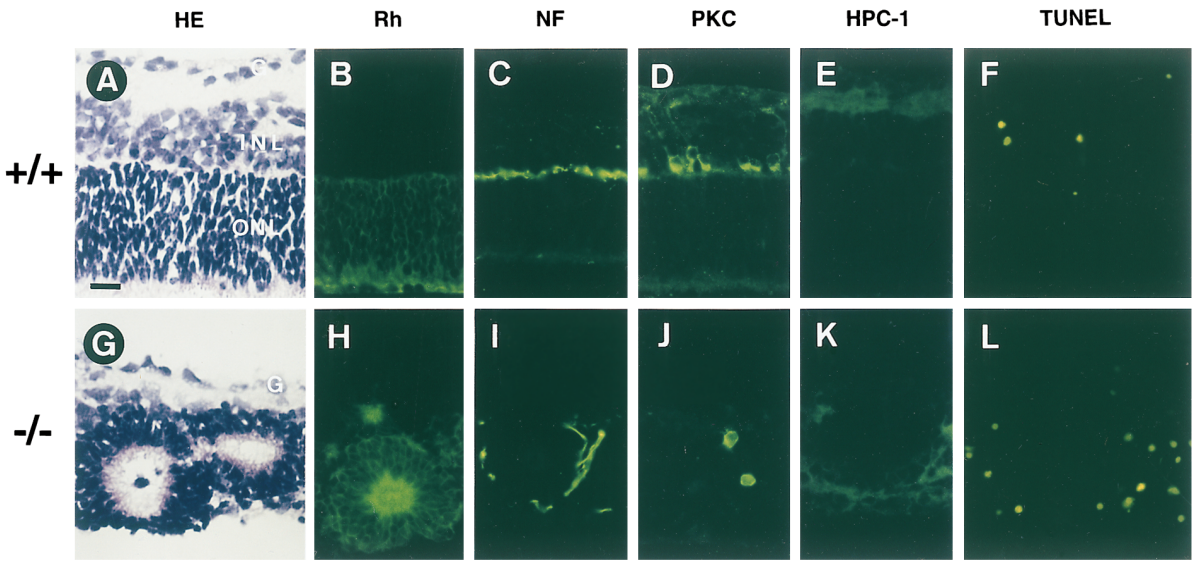

Figure 6. Retinal Organ Culture at Day 10

Retina was prepared from E17.5 embryos of *HES1* (+/+) (A–F) and *HES1* (−/−) (G–L) and cultured for 10 days. Sections were examined by HE staining (A and G), an immunohistochemical method (B and H, anti-Rh; C and I, anti-NF; D and J, anti-PKC; E and K, anti-HPC-1) and TUNEL (F and L).

(A) At day 10, *HES1* (+/+) retina exhibited three well-developed cellular layers.

(B) Rod cells (Rh^+) were present in the ONL.

(C) Horizontal cells (NF^+) were located in the outer region of the INL.

(D) Bipolar cells (PKC^+) appeared in the INL.

(E) Amacrine cells ($HPC\text{-}1^+$) were located in the inner region of the INL.

(F) Cell death ($TUNEL^+$) occurred weakly in the INL.

(G) At day *10*, *HES1* (−/−) retina contained rosette-like structures. The laminar formation was severely disrupted, but the ganglion cell layer remained (G).

(H) The rosette-like structures were comprised of rod cells (Rh^+).

(I) Horizontal cells (NF^+) surrounded the rosette-like structure.

(J) Only a few bipolar cells (PKC^+) appeared, and they were next to the rosette-like structures.

(K) Amacrine cells ($HPC\text{-}1^+$) were present outside of the rosette-like structures.

(L) Extensive cell death ($TUNEL^+$) occurred along the outer edge of rosette-like structures in which bipolar cells were present, suggesting that bipolar cells were dying. We examined at least four independent samples for each experiment and obtained the same results. Scale bar, 25 μm.

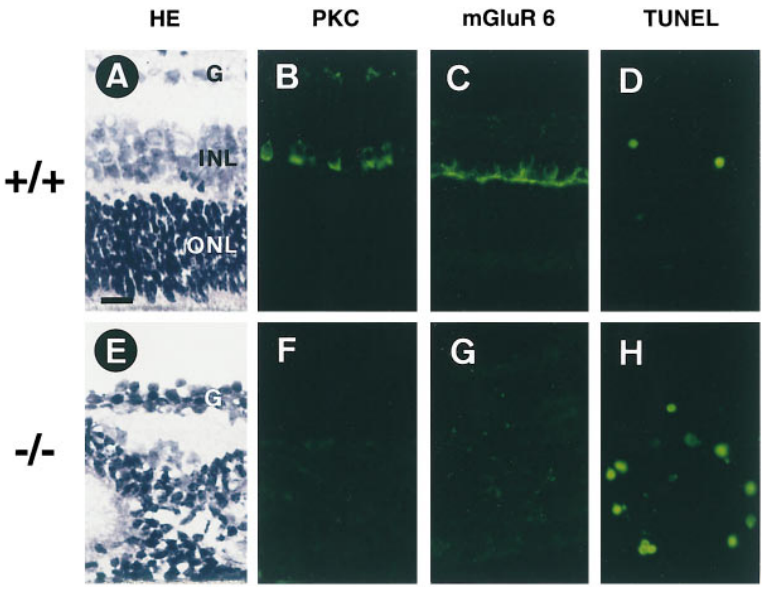

Figure 7. Retinal Organ Culture at Day 14

Retina was prepared from E17.5 embryos of *HES1* (+/+) (A–D) and *HES1* (−/−) (E–H) and cultured for 14 days. Sections were examined by HE staining (A and E), an immunohistochemical method (B and F, anti-PKC; C and G, anti-mGluR6) and TUNEL (D and H).

(A) At day 14, *HES1* (+/+) retina exhibited three well-developed cellular layers.

(B and C) Bipolar cells (PKC^+, $mGluR6^+$) were located in the INL.

(D) Cell death ($TUNEL^+$) weakly occurred in the INL.

(E) At day 14, *HES1* (−/−) retina contained rosette-like structures.

(F and G) No PKC- or mGluR6-expressing cells were present in *HES1* (−/−) retina at day 14, suggesting that bipolar cells did not survive until day 14.

(H) Extensive cell death ($TUNEL^+$) occurred along the outer edge of rosette-like structures, suggesting that the bipolar cells that were found at day 10 had all died by day 14. We examined at least four independent samples for each experiment and obtained the same results. Scale bar, 25 μm.

retina (Dorsky et al., 1995), agreeing well with the notion that the membrane protein Notch and the nuclear factor HES1 function in the same regulatory pathway (Jarriault et al., 1995; Bailey and Posakony, 1995).

HES1 Prevents Premature Neurogenesis and Controls the Timing of Differentiation of Retinal Cell Types

In *HES1*-null retina, differentiation occurred prematurely, indicating that retinal development is negatively regulated by *HES1*. This is in good agreement with the conclusions from the gain-of-function study. Interestingly, neuronal differentiation is also accelerated without *hairy* (*HES1* homolog) and *extramacrochaetae* during Drosophila eye development (Brown et al., 1995), suggesting that functions as well as structures have been well conserved between mammals and Drosophila during evolution.

It has been shown that both intrinsic properties and humoral factors regulate cell type-specific and stage-specific differentiation in the retina (Adler and Hatlee, 1989; Watanabe and Raff, 1990, 1992; Altshuler and Cepko, 1992; Repka and Adler, 1992; Altshuler et al., 1993). Embryonal retinal cells are rather unresponsive to rod-differentiation signals, and thus, their late timing of differentiation into rods is an intrinsic property of retinal progenitor cells, independent of the surrounding environment (Watanabe and Raff, 1990). Our findings of premature rod differentiation without *HES1* suggest that *HES1* may contribute to the intrinsic mechanism preventing earlier response of progenitor cells to the differentiation signals. It was noted that differentiation was not uniformly accelerated in *HES1*-null retina. For example, some rod cells already expressed rhodopsin at E17.5 while others started rhodopsin expression much later (around days 5–7 of culture), thus contrasting with the normal rod cells that started rhodopsin expression more uniformly around days 5–7. It has been shown that rod-differentiation signals appear during late embryogenesis and increase progressively until postnatal stages (Watanabe and Raff, 1992; Altshuler and Cepko, 1992). Thus, the progressive increase in rod cell number during late embryogenesis and postnatal stages in *HES1*-null retina could be a consequence of a premature competence of *HES1*-null progenitor cells to respond to rod-differentiation signals. Therefore, a function of *HES1* could be to coordinate differentiation by blocking progenitor cell competence until differentiation signals reach some level.

HES1 Regulates Eye Morphogenesis

In *HES1*-null embryos, eye cups were always small and deformed. It thus seems that development of retinal progenitor cells is affected at an early stage. This could be due to accelerated differentiation, which would deplete the population of dividing cells. Consistent with this idea, expressions of the neuronal marker *SCG-10* (Figure 4) and neurofilament 160 kDa (data not shown) were observed in the optic vesicles of *HES1* (−/−) embryos as early as E9.5. At that time, no such expressions were detected in wild-type optic vesicles (Figure 4), suggesting that neuronal differentiation occurred prematurely without *HES1*. Based upon these observations, we speculate that *HES1* regulates eye morphogenesis by preventing premature differentiation in the early retinal primordium. In addition, our data show that *HES1* is also essential for lens and cornea development. However, the defects of lens and cornea development in *HES1*-null embryos could be secondary phenotypes due to a failure of proper interaction between the optic vesicles and the surface ectoderm.

At later stages of retinal development, multiple rosette-like structures appeared and laminar formation was severely affected in *HES1*-null retina. It is possible that this altered structure leads to accelerated differentiation in *HES1* (−/−) retina. However, differentiation was already accelerated at E9.5, at which time the histology of the neural retina was apparently normal (Figure 4J). In addition, differentiation was also accelerated without any histological abnormalities in *HES1* (+/−) retina (Figures 5H and 5I). Thus, it is most likely that accelerated differentiation is the primary phenotype of *HES1* mutation and that the abnormal retinal structures are the secondary event, due to the premature and nonuniform differentiation of retinal cells.

Another important feature of *HES1*-null retina is that bipolar cell development was severely impaired while amacrine cells increased. A few bipolar cells were differentiated without *HES1*. However, apparently extensive cell death occurred and these bipolar cells did not survive. It is unclear why only bipolar cells did not survive in *HES1*-null retina. It is possible that cell–cell interaction may be important for bipolar cell maintenance and that bipolar cells cannot survive amidst the disorganization of the *HES1*-null retina. In contrast, amacrine cells increased without *HES1*. This increase was probably the result of increased amacrine cell genesis rather than diminished cell death because no significant difference in cell death was observed between wild-type and *HES1*-null retinas until P7 on TUNEL analysis. Whether or not amacrine cell genesis occurred at the expense of bipolar cell genesis is an interesting question and remains to be determined.

Positive and Negative HLH Factors Regulate Retinal Development

Accumulating evidence suggests that multiple positive and negative HLH factors play an important role in mammalian neurogenesis. Mammalian bHLH factor genes such as *Mash1*, *MATH1*, *MATH2* (*NEX1*), and *NeuroD* encode positive regulators expressed in the developing nervous system (Johnson et al., 1990; Bartholomä and Nave, 1994; Akazawa et al., 1995; Shimizu et al., 1995; Lee et al., 1995), while *HES1* is a negative regulator of neural differentiation (Sasai et al., 1992; Ishibashi et al., 1994). HES1 antagonizes the transcriptional activities of Mash1 and MATH1 (Sasai et al., 1992; Akazawa et al., 1995), and this negative regulation may account for *HES1*-induced suppression of neurogenesis. Thus, the balance between these positive and negative regulators may be critical for mammalian neurogenesis. Regarding *HES1* roles in retinal development, we speculate that

HES1 antagonizes the activity of bHLH factors that positively regulate retinal development. It is likely that the activity of such positive bHLH factors becomes dominant in *HES1*-null retina and accelerates retinal differentiation. However, it is not yet known which bHLH factors positively regulate retinal development. *Mash1* is expressed in the developing retina (Guillemot and Joyner, 1993) and thus may be involved in retinal development. In *HES1*-null embryos, *Mash1* expression is up-regulated in retina (Ishibashi et al., 1995) and it is likely that this up-regulation may contribute to premature differentiation of retinal cells. However, targeted disruption of *Mash1* does not cause any apparent abnormalities in retina at birth (Guillemot et al., 1993). Therefore, other bHLH factors may positively regulate retinal development probably in collaboration with Mash1. Further characterization of the bHLH factors expressed in retina will help to clarify the molecular mechanisms of retinal development.

Experimental Procedures

In Situ Hybridization and Northern Blot Analyses

In situ hybridization analysis of *HES1* and *SCG10* was done as previously described (Sasai et al., 1992; Guillemot et al., 1993). For *HES1*, ^{35}S-labeled cRNA corresponding to the EcoRI–SmaI fragment (723 bp) of pHES1A was used as a probe.

For Northern blot analysis, 15 μg of total RNA was electrophoresed on a formaldehyde/1% agarose gel and transferred to a nylon membrane filter. The filter was hybridized with the ^{32}P-labeled full-length rat HES1 cDNA.

Animals

SD rats and ICR mice were purchased from SLC (Shizuoka, Japan).

HES1-null mice were generated by targeted disruption of the *HES1* locus (Ishibashi et al., 1995), and their genetic background was 129/Sv × ICR. In *HES1*-null mice, the first three exons of *HES1* gene, which encode the bHLH domain (Takebayashi et al., 1994), were replaced with a neomycin-resistance expression cassette. Approximately 70% of *HES1*-null embryos exhibited open brain, but the rest were apparently normal (Ishibashi et al., 1995). However, all died during gestation or within 1 day after birth (Ishibashi et al., 1995).

Retinal Organ Culture

Eyes were isolated from rats and mice at E17.5–P0 and transferred to a petri dish containing PBS. The explant of neural retina without retinal pigment epithelium was placed onto a Millicell chamber filter (Millipore: diameter 30 mm, pore size 0.4 μm) with the ganglion cell layer upward. The chamber was transferred to a 6-well culture plate. Each well contained 0.8 ml of 50% MEM with HEPES, 25% Hank's solution, 25% heat-inactivated horse serum, 200 μM L-glutamine, and 6.75 mg/ml glucose (Stoppini et al., 1991). Organ cultures were incubated at 34°C in 5% CO_2 (Caffé et al., 1989).

Retroviral Infection

Stocks of SG and SG-HES1 retroviruses were prepared, as previously described (Ishibashi et al., 1994). The titer of each stock was 5×10^5–10×10^5 cfu/ml.

On the first day of culture, the retinal explant was infected with 50 μl of virus solution with 0.2 mg/ml polybrene. The retinal explant was fixed 14 days later with 0.5% glutaraldehyde in PBS at 4°C for 30 min, incubated in 25% sucrose in PBS at 4°C overnight, and then stained with 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 35 mM potassium ferricyanide, 35 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.02% Nonidet P-40, and 0.01% deoxycholate in PBS. Frozen sections were made at 10–16 μm thickness.

Histological Analysis

Eyeballs and retinal explants were fixed with 4% paraformaldehyde in PBS at 4°C for 30 min and incubated in 25% sucrose in PBS at 4°C overnight. Frozen sections were cut at 10–16 μm thickness and subjected to hematoxylin-eosin (HE), cresyl violet, or toluidine blue staining and immunohistochemistry. For immunohistochemistry, sections were preincubated in PBS containing 5% normal goat serum, 1% bovine serum albumin, 0.1% Triton X-100, and 0.02% sodium azide for 30 min and then incubated with the following antibodies: monoclonal antibody (MAb) against 160 kDa neurofilament (Amersham), MAb against PKC (Amersham), MAb against Thy-1.2 (Pharmingen), MAb against HPC-1 (Sigma), and rabbit polyclonal antibody against rhodopsin (LSL) and mGluR6 (Nomura et al., 1994). As a secondary antibody, biotinylated goat antibody against mouse, rat, or rabbit IgG (Vector) was used. The antibody complex was visualized by avidin-labeled fluorescein or ABC kit (Vector). The ratio of each cell type was measured by counting the cell numbers in sections or measuring the areas occupied by each cell type.

TUNEL

Frozen sections were fixed in 4% paraformaldehyde and subjected to TUNEL analysis, as previously described (Chang et al., 1993). The biotinylated dUTP incorporated by TdT was visualized by avidin-labeled fluorescein or ABC kit (Vector).

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