Involvement of Mash1 in EGF-Mediated Regulation of Differentiation in the Vertebrate Retina

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It is believed that signaling through the epidermal growth factor (EGF) receptor plays a critical role in the development of Drosophila eyes. In the present study we have analyzed the role that EGF-mediated signaling plays in vertebrate retinal development. We have observed that during late retinal neurogenesis EGF delays rod photoreceptor differentiation and that this effect of EGF involves the modulation of expression of a homologue of Drosophila proneural genes, Mash1. EGF causes a significant decrease in Mash1 expression and an increase in the proportion of proliferating cells in vitro. The decrease in Mash1 expression is accompanied by a concomitant decrease in opsin expression, a marker for overt rod photoreceptor differentiation. Withdrawal of EGF leads to an increase in both Mash1 and opsin expression; however, the onset of expression of Mash1 precedes that of opsin. Our study identifies a proliferative intermediate precursor, characterized by Mash1 expression, that is the target of EGF-mediated suppression of rod photoreceptor differentiation. Based on the evolutionarily conserved roles of EGF- and Notch-mediated signaling in the delay of differentiation in proliferating precursors we propose that these distinct signaling mechanisms act in concert to ensure the fidelity of the strict temporal and spatial nature of cell fate determination in the retina.

INTRODUCTION

Cell interactions play a vital role in cell fate specification in the developing eyes of both invertebrates and vertebrates. In Drosophila, genetic as well as molecular perturbations have shown that the specification of retinal neurons depends on sequential and localized cell interactions (reviewed in Banerjee and Zipursky, 1990). Evidence has emerged based on a variety of experimental approaches that cell fate determination in the vertebrate retina is also largely lineage independent and that it is influenced by a changing environment. Some of the earliest results that supported this notion were from cell ablation studies carried out in Xenopus and goldfish retina (reviewed in Reh, 1990). In these vertebrates selective destruction of dopaminergic neurons resulted in replacement of ablated neurons suggesting an influence of the environment on neuronal differentiation. Subsequently, lineage studies using a retroviral marker in rodents (Price et al., 1987; Turner and Cepko, 1987; Turner et al., 1990) and tracer dye in Xenopus (Holt et al., 1988; Wetts and Fraser, 1988) showed that the dividing neuroepithelial cells in the developing retina represent a common pool of multipotential precursors that are capable of giving rise to various neurons and glia. Both the lineage and cell ablation studies suggested that, in the developing vertebrate retina, decisions taken by the multipotential progenitors to acquire a particular fate are regulated by local cell interactions.

Cell interactions in the retina can be mediated through membrane-anchored ligand and receptor interactions or through the interactions of diffusible factors and their receptors. The regulation of retinal differentiation by Notch signaling is an example of the former mechanism (Cagan and Ready, 1989; Fortini et al., 1993; Austin et al., 1995; Ahmad et al., 1995, 1997). It is believed that the interaction of the Notch receptor with its membrane-anchored ligands, Delta or Serrate, results in a cascade of events the purpose of which is to keep a precursor uncommitted until proper cues for differentiation appear in the environment (reviewed in Artavanis-Tsakonas et al., 1995). We have recently shown that the dominant ligand for the Notch-1 receptor in the vertebrate retina is Delta-1 and that Delta-1 activation of
Notch signaling participates in the specification of retinal neurons (Ahmad et al., 1997). On the other hand, in vitro coculture studies of retinal cells from different embryonic stages have shown that differentiation as well as maturation of retinal neurons is influenced by environmental cues and that these cues could be diffusible factors elaborated by the already differentiated and/or differentiating cells (Watanabe and Raff, 1990, 1992; Reh, 1992; Altschuler and Cepko, 1992). Known diffusible factors that have been shown to affect retinal neurogenesis include EGF (Anchan et al., 1991), TGFβ (Anchan et al., 1991; Lillien and Cepko, 1992), bFGF (Hicks and Courtois, 1992), aFGF (Lillien and Cepko, 1992), taurine (Altschuler et al., 1992), CNTF (Ezzedine et al., 1997), and retinoic acid (Kelley et al., 1994). These factors appear to affect neurogenesis differently; while EGF, TGFβ, and aFGF have been shown to promote cell proliferation, bFGF, taurine, and retinoic acid promote differentiation of rod photoreceptors (rods). CNTF, on the other hand, has been shown to suppress rod differentiation. There are as of yet uncharacterized diffusible factors that have been shown to influence rod differentiation. The mechanisms by which these known and unknown diffusible factors regulate rod differentiation are not well understood. The intracellular pathway may involve the regulation of transcription factors that participate in the activation of phenotype-specific genes. For example, the differentiation of rods may involve the participation of Mash1, a mammalian homologue of the Drosophila proneural gene that encode the bHLH transcription factors (Johnson et al., 1990). Evidence shows that Mash1 is essential for neurogenesis; Mash1 is expressed specifically in a subset of neuronal precursors in both the PNS and the CNS (Lo et al., 1991; Guillemot and Joyner, 1993), and a targeted disruption of the Mash1 gene results in an abnormality in the generation of autonomic and olfactory neurons (Guillemot et al., 1993). The involvement of Mash1 in retinal neurogenesis is suggested by its expression in the developing retina in the region which harbors rod precursors (Ahmad, 1995; Jasoni and Reh, 1996) and by its ability to interact specifically with the opsin promoter (Ahmad, 1995). The notion that Mash1 may participate in rod differentiation in response to epigenetic cues is supported by evidence that Mash1 and other bHLH transcription factors such as MyoD have been observed to be regulated in response to growth factors (Olson, 1992; Shah et al., 1994).

In the present study we have analyzed EGF-mediated cell interactions during retinal neurogenesis. Signaling through the EGF receptor has been shown to play a critical role in the development of Drosophila eyes (Banerjee and Zipursky, 1990; Schweitzer and Shilo, 1997). Analysis of the EGF homologue, glial growth factor (GGF), in peripheral neurogenesis in vertebrates has suggested that EGF-mediated signaling may modulate the expression of proneural homologues such as Mash1 (Shah et al., 1994). Here we show that Mash1 is likely to participate in the intracellular cascade of EGF-mediated cell interactions in retinal development. We have observed that during late retinal neurogenesis when rods are born, Mash1 expression identifies an intermediate stage of precursors before they display differentiation characteristics. This stage of precursors is likely to be the target of EGF regulation. Treatment of explants obtained from E18/E20 retina with EGF causes a significant decrease in Mash1 immunoreactivity and an increase in the proportion of proliferating cells. The decrease in Mash1 immunoreactivity is accompanied by a concomitant decrease in opsin immunoreactivity, a marker for overt rod photoreceptor differentiation. Withdrawal of EGF from the culture leads to an increase in both Mash1 and opsin immunoreactivities. Also, the temporal onset of Mash1 expression precedes that of opsin following EGF withdrawal suggesting that rod differentiation involves an intermediate step which is characterized by the onset of Mash1 expression. Therefore, it is likely that EGF suppression of rod differentiation involves the regulation of Mash1 in intermediate precursors such that the restoration of Mash1 expression upon EGF withdrawal restores opsin expression, hence reestablishing terminal differentiation of rods. Based on the evolutionarily conserved roles of EGF- and Notch-mediated signaling in the delay of differentiation in proliferating precursors we propose that these distinct signaling mechanisms act in concert to ensure the fidelity of the strict temporal and spatial nature of cell fate determination in the retina.

MATERIALS AND METHODS

Animals

Timed pregnant Sprague-Dawley rats were obtained from Sasco Laboratories. The gestation day was confirmed by the morphological examination of embryos (Christie, 1964).

Explant Culture

Retinas were harvested from stage-specific (E18 or E20) embryos in Hank’s balanced salt solution (HBSS). The retinal explants were cultured in 24-well plates in DMEM:F-12 medium containing 1× N2 supplement (Gibco), 1% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 95% humidity and 5% CO₂. Following 3–4 days of incubation with and without EGF (Gibco, 20 ng/ml) the explants were washed extensively (six times) in culture medium to remove EGF. Some of the explants were fixed for immunocytochemistry and some were incubated for 3 days without EGF before fixation. Tissues were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, and frozen in OCT embedding medium until sectioning. For dissection into single-cell suspension explants were incubated in HBSS (Ca²⁺ and Mg²⁺ free) containing 0.25% trypsin, 1 mM EDTA, and 20 µg/ml DNAase I at 37°C for 20 min. Trypsin was neutralized by washing the tissue in HBSS containing 20% FBS. Cells were dissociated by trituration (10–15 times) in the culture medium and
plated at a density of $10^4$–$10^5$ cells/glass coverslip treated with 50–100 $\mu$g/ml of poly-l-lysine. Cells were allowed to adhere to coverslips for 2–3 h at 37°C before fixation in 4% paraformaldehyde. Each explant culture was analyzed for cell viability using the trypan blue dye-exclusion method. Experiments were performed at least three times and repeated in triplicate.

To label dividing cells in vivo, pregnant rats were injected with BrDu (5 mg/200 g body wt) intraperitoneally, 4 h before euthanization. To label dividing cells in vitro, explant cultures were treated with either BrDu (10 $\mu$M) or $[^{3}H]$thymidine ($1 \mu$Ci/ml, 64 Ci/mmol, ICN) for 2–4 h before fixation for immunocytochemistry or emulsion autoradiography (Ahmad et al., 1995).

**Immunocytochemistry**

Immunocytochemistry was carried out as previously described (Ahmad et al., 1997). Briefly, whole sections or dissociated cells were incubated in PBS containing 5% normal goat serum and 0.2% Triton X-100 followed by an overnight incubation in MASH1 antibody (1:1 dilution; hybridoma supernatant; Lo et al., 1991) or Ret-P1 (1:1000 dilution; Barnstable, 1980), HPC1 (1:100 dilution; Barnstable, 1980), and $\beta$-tubulin (1:400 dilution; Sigma), at 4°C. The sections were examined for epifluorescence using a Leica DMR microscope following incubation in anti-mouse IgG conjugated to CY3 or FITC. To identify BrDu-incorporated cells in sections or dissociated cells BrDu immunocytochemistry was carried out as previously described (Soriano and del Rio, 1991). Briefly, sections or cells on the glass coverslips were incubated at 37°C for 45 min in 2 N HCl to denature DNA followed by a 10-min incubation at room temperature in 0.1 M boric acid. Sections or cells were washed in PBS, followed by incubation in anti-BrDu (1:400 dilution, Boehringer-Mannheim) for 1 h at room temperature. The sections or cells were washed in PBS, incubated with secondary antibody, and visualized as described above.

**RESULTS**

**In E18 Retina, MASH1 Immunoreactivity Is Predominantly Localized in Proliferating Precursors**

In the rodent retina differentiation of rods occurs primarily between E18 and P3 and follows the same temporal pattern in vitro as in vivo (Watanabe and Raff, 1990). Since rods are the predominant neurons (73% of all cell types) in the rodent retina (Sidman, 1961; Young, 1985) and one of the last to differentiate, the outer neuroblastic layer in the perinatal retina consists largely of rod precursors. To address this hypothesis we wanted to determine the spatial distribution of proliferating and differentiated cells in E18 retina in vivo.

Pregnant rats at day 18 gestation were injected with BrDu intraperitoneally. After a 4-h incubation embryos were removed by cesarean section, and eyes were enucleated and processed for immunocytochemical analyses as described under Materials and Methods. In sections of embryonic retina proliferating cells, as identified by the incorporation of BrDu, were predominantly localized in the outer neuroblastic layer (Fig. 1A). The density of anti-BrDu immunoreactivity suggested that the outer neuroblastic layer consists largely of proliferating cells. This is consistent with the observation that more than 60% of cells in E18 retina are proliferative (Alexiadis and Cepko, 1996). The other dominant cells that were localized in the outer neuroblastic layers were those expressing Mash1. Double-immunocytochemical analysis carried out on retinal sections showed that the majority (~75%) of Mash1-positive cells were BrDu-positive as well (Fig. 1B). However, the proportion of Mash1-positive proliferating cells is higher than that reported by Jasoni and Reh (1996) in E18 retina. This difference is likely due to the difference in techniques used to identify Mash1-positive cells; while we used immunocytochemical methods, Jasoni and Reh utilized situ hybridization to localize Mash1 transcripts. The expression of Mash1 in proliferating cells suggests that the decision for neuronal commitment is made before precursors exit mitosis. A small proportion of Mash1-positive proliferating cells (~20%) were BrDu-negative, suggesting that Mash1 expression is maintained in postmitotic precursors before overt expression of differentiation markers.

In late retinal neurogenesis rods are one of the first cell types born. In E18 retina opsin immunoreactivity, as judged by staining with the opsin-specific antibody, Ret-P1, can be detected in few cells toward the scleral surface in the outer neuroblastic layer (Fig. 1C). However, immunocytochemical analyses carried out using anti-CRALBP and anti-PKC to identify Muller and bipolar cells, respectively, the other cell types born during late neurogenesis, were negative (data not shown). Cells born during early retinal neurogenesis, i.e., amacrine and ganglion cells, can be readily detected in E18 retinal sections using immunocytochemical analyses with HPC1 (recognizes syntaxin) (Fig. 1D) and anti- $\beta$-tubulin (Fig. 1E) antibodies, respectively. Therefore, it is likely that Mash1-expressing precursors at this stage of development represent an intermediate stage of rod differentiation in late neurogenesis.

**EGF Promotes Cell Proliferation in the Outer Neuroblastic Layer in Vitro**

It has been shown previously that EGF can stimulate cell proliferation in dissociated cultures of embryonic and neonatal retinal cells (Anchan et al., 1991; Reh, 1992). We wanted to determine if EGF has a similar effect on retinal cells in explant culture. Analysis of proliferation and differentiation in explant culture is important in view of the fact that conflicting results have been obtained in explant and dissociated retinal cell cultures in the context of epigenetic cues. While several investigators have observed rod differentiation in dissociated retinal culture (Hicks and Courtois, 1992; Reh, 1992; Watanabe and Raff, 1992; Altshuler and Cepko, 1992), Sparrow et al. (1990) observed that rods tend to differentiate more robustly in explant culture than in monolayer culture. Additionally, different observations have been reported regarding the effect of bFGF on rod differ-
FIG. 1. Spatial distribution of proliferating and differentiated cells in the embryonic retina. Retinas obtained from BrDu-treated gestation day 18 embryos were fixed, cryosectioned, and processed for immunocytochemical analyses. Double-immunocytochemical staining revealed that BrDu-incorporated cells (A) and Mash1-positive cells (B), identified by anti-BrDu and anti-Mash1, are preferentially localized in the outer neuroblastic layer (Nbl). Mash1⁺ and BrDu⁺ cells are identified by arrowheads and Mash1⁺ and BrDu⁺ cells by arrows. Opsin-expressing cells, identified by Ret-P1 staining, are localized toward the scleral (arrow) surface in the Nbl layer (C). Amacrine (D) and ganglion cells (E) are identified by anti-syntanxin (HPC1) and anti-β-tubulin, respectively, in the inner retina (IR). Scale bar = 40 μm.

EGF Treatment Alters Mash1 and Opsin Immunoreactivities

To test the hypothesis that EGF delays the progression of rod precursors from the intermediate stage to terminal differentiation, we analyzed the effect of EGF treatment on Mash1 and opsin immunoreactivities in explant culture. Retinal explants obtained from E20 retina were cultured in the presence or absence of EGF for 4 days. The incubation time in culture was extended by a day so that at the end of the treatment the explant age corresponded to PN2/PN3 retina assuming that the normal rat gestation is E20±E21. At this time the proportion of opsin-positive cells increases, thereby providing an opportunity to assess the effects of EGF on rod differentiation (Watanabe and Raff, 1990). Immunocytochemical analysis carried out on sections of untreated explants at the end of 4 days in culture (DIC) revealed that Mash1 immunoreactivity was localized to the outer neuroblastic layer as observed in vivo (Figs. 3A and 3B). Treatment of the explants with EGF resulted in a significant decrease in Mash1 immunoreactivity (Figs. 3C and 3D). However, when EGF was subsequently removed and the culture was continued for 3 days, Mash1 immunoreactivity was restored (Figs. 3E and 3F). Since Mash1 is required during neurogenesis (Guillemot et al., 1993) and is a putative regulator of rod differentiation (Ahmad, 1995), we wanted to know if alteration in Mash1
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expression had any consequence on opsin expression and therefore on the differentiation of rod precursors. In untreated explants opsin immunoreactivity was detected near the scleral side of the outer neuroblastic layer (Figs. 3G and 3H). This pattern of staining is similar to that observed in PN2 retina in vivo (Barnstable, 1987). When explant cultures were treated with EGF for 4 DIC, opsin immunoreactivity, as with Mash1, decreased in comparison to that in untreated controls (Figs. 3I and 3J). As observed for Mash1, opsin immunoreactivity increased following the withdrawal of EGF, suggesting that opsin expression is also suppressed in the presence of EGF (Figs. 3K and 3L). Interestingly, Mash1 immunoreactivity was distributed more toward the vitreal surface of the outer neuroblastic layer. The absence of Mash1 immunoreactivity near the scleral side of the outer neuroblastic layer is likely due to displacement of Mash1-positive cells by rods that differentiated following EGF withdrawal. To ascertain the specificity of EGF action on rod differentiation, we analyzed its effects on the ganglion and amacrine cell markers β-tubulin and syntaxin, respectively. Both β-tubulin (Figs. 4A–4F) and syntaxin (Figs. 4G–4L) immunoreactivities were localized to the inner retina and their relative levels remained unchanged in explant cultures in the presence or absence or after the removal of EGF.

The concomitant alteration in opsin and Mash1 immunoreactivities in response to the addition and removal of EGF suggested that Mash1 expression is required for rod differentiation. This notion was further analyzed by correlating the proportion of opsin- and Mash1-positive cells in response to EGF. Retinal explants from E20 retina were cultured as described above and at the end of each treatment explants were dissociated, plated on poly-D-lysine-treated glass coverslips, and subjected to immunocytochemistry. The proportion of Mash1-labeled cells decreased twofold in the presence of EGF in comparison to untreated controls (35.45 ± 4.75% vs 17.40 ± 1.10%; P < 0.05) (Figs. 5A–5D). A similar observation was made when dissociated cells were analyzed for RetP-1 immunoreactivity (Figs. 5E–5H). The proportion of cells labeled with Ret-P1 decreased by more than half in the presence of EGF in comparison to untreated controls (48.73 ± 5.21% vs 19.18 ± 3.91%, P < 0.05). Therefore, a strong correlation was observed between the decrease in the proportion of opsin- and Mash1-positive cells in response to EGF treatment. The cells showed that the relative number of retinal cells increases upon EGF treatment compared to untreated controls (B). E18 retinal explants incubated with [3H]thymidine for the last 4 h of culture to label cells in the S-phase of the cell cycle. Analysis of [3H]thymidine incorporation showed that there are more cells in the S-phase of the cell cycle in EGF-treated explants than in untreated controls (A). Dissociation of explants into single

![Graph A](image1.png)

![Graph B](image2.png)

![Images](image3.png)

**FIG. 2.** Effect of EGF treatment on cell proliferation in the embryonic retina. Explants from E18 retina were cultured in the presence or absence of EGF (20 ng/ml) and incubated with [3H]thymidine for the last 4 h of culture to label cells in the S-phase of the cell cycle. Analysis of [3H]thymidine incorporation showed that there are more cells in the S-phase of the cell cycle in EGF-treated explants than in untreated controls (A). Dissociation of explants into single
FIG. 3. Effect of EGF-treatment on Mash1 and opsin immunoreactivities. Retinas obtained from gestation day 20 embryos were cultured as explants in the presence or absence of EGF (20 ng/ml) for 4 days followed by another 3 days of culture after an extensive wash to remove EGF. After 4 or 7 days in culture explants were fixed, cryosectioned, and subjected to immunocytochemical analyses using anti-MASH1 and Ret-P1 antibodies to detect Mash1 (A–F) and opsin (G–L) immunoreactivities, respectively. In the presence of EGF, Mash1 (C and D) and opsin (I and J) immunoreactivities decrease compared to untreated controls (A and B; G and H). Upon EGF removal both Mash1 (E and F) and opsin (K and L) immunoreactivities are relatively restored. A, C, E, G, I, and K are Nomarski images. Scale bar, 40 \( \mu \)m.

The Onset of Mash1 Expression Precedes Opsin Expression Following EGF Withdrawal

The concomitant decrease in Mash1 and opsin immunoreactivities following EGF treatment and their rebound expression after the withdrawal of EGF suggested that Mash1 participates in the regulation of opsin during rod differentiation. Based on these observations, if Mash1 were a participant in the transcriptional regulation of the opsin gene during rod differentiation then Mash1 expression would precede opsin expression. In addition, it is expected that as the suppressive effect of EGF on differentiation is relieved the proportion of dividing precursors should decline as more cells begin to differentiate. As a consequence, the proportion of proliferating Mash1-positive cells should decrease as more of them quit dividing in order to move to the next stage of differentiation.

To test these hypotheses we carried out temporal analyses of the appearance of opsin and Mash1 immunoreactivities with respect to BrDu incorporation following the withdrawal of EGF from E20 retinal explant culture (Fig. 6). At the end of 4 DIC, EGF was withdrawn and culture was continued for 0, 12, 24, and 48 h. Near the end of each time point cultures were treated with BrDu for 2 h. The explants were dissociated and plated on poly-D-lysine-treated glass coverslips followed by double immunostaining. Cells were counterstained with DAPI to visualize nuclei. At 0 h the largest proportion of cells (69.6 ± 2.9\% were those that had incorporated BrDu. The proportions of Mash1-positive and RetP1-positive cells were 25.7 ± 2.3 and 16.3 ± 2.6\%, respectively (see also Figs. 7A–7C). At this stage a majority of Mash1-positive cells were BrDu positive. Twelve hours following EGF withdrawal the proportion of cells that had incorporated BrDu decreased significantly relative to those at 0 h (46.6 ± 5.2\% vs 69.6 ± 2\%, \( P < 0.05 \)). A small but significant increase in the proportion of Mash1-positive cells was observed relative to those at 0 h (25.7 ± 2.3\% vs 28.6 ± 3\%, \( P < 0.05 \)). No significant change was observed in the relative numbers of opsin-positive cells (16.3 ± 2.6\% vs 16.5 ± 2.5\%, \( P > 0.05 \)). At 24 h, relative to those at 12 h, there was a significant increase in the proportion of cells expressing Mash1 (44 ± 3.8\% vs 28.6 ± 3\%, \( P < 0.05 \)) and...
FIG. 4. Effect of EGF treatment on β-tubulin and syntaxin immunoreactivities. Retinas obtained from gestation day 20 embryos were cultured as explants, treated, and processed as described in the legend to Fig. 4. Immunocytochemical analyses using HPC1 and anti-β-tubulin antibodies detected β-tubulin (a RGC marker) (A–F) and syntaxin (an amacrine cell marker) (G–L) immunoreactivities, respectively. In the absence (A, B, G, H), presence (C, D; I, J), and withdrawal (E, F; K, L) of EGF, syntaxin and β-tubulin immunoreactivities remain unchanged. A, C, E, G, I, and K are Nomarski images. Scale bar = 40 μm.

opsin (30.2 ± 3.5% vs 16.5 ± 2.5%, P < 0.05), whereas the proportion of BrDu-positive cells declined (36.2 ± 5.3% vs 46.6 ± 5.2%, P < 0.05). At 48 h, relative to those at 24 h, the proportion of Mash1-positive cells increased (47.9 ± 4.7% vs 44 ± 3.8%, P < 0.05), whereas the proportion of BrDu-positive cells decreased further (30.5 ± 3.5% vs 36.2 ± 5.3%, P < 0.05) (see also Figs. 7D–7F). In contrast to those at 0 h a majority of Mash1-positive cells at 48 h were BrDu-negative. At this time the proportion of opsin-positive cells increased significantly (63.5 ± 2.8%) compared to all previous time points, suggesting that there is a delay before opsin expression could be established after EGF withdrawal. During this lag time the precursors begin the process of differentiation as the inhibitory effect of EGF is lifted and Mash1 expression is restored.

DISCUSSION

It is believed that cell interactions play a critical role in the differentiation of retinal neurons. Several growth factors, both known and unknown, have been found to participate in the regulation of the fate of retinal progenitors. Among the known growth factors, EGF is a likely candidate that participates in the regulation of the progenitors that give rise to rod photoreceptors. Both EGF and EGF kinase receptor are expressed in the developing retina at the time of rod differentiation (Anchan et al., 1991; Lillien, 1995). The functional role of EGF in late retinal neurogenesis was provided by two different approaches; Anchan et al. (1991) and later Reh (1992) showed that EGF promotes the proliferation of retinal progenitors and suppresses rod differentiation in dissociated retinal cultures. Recently, Lillien (1995) showed that overexpression of the EGF receptor under experimental conditions antagonizes rod differentiation. In addition, signaling through the EGF receptor has been shown to play an important role in retinal development in Drosophila (Schweitzer and Shilo, 1997).

While the experiments mentioned above demonstrated the involvement of EGF in the differentiation of progenitors into rods, the underlying mechanisms of EGF action during retinal development remained elusive. However, insight into the mechanisms by which EGF may modulate retinal progenitor fate is provided by the role GGF plays in cell fate choice of neural crest stem cells (NCSC) (Shah et al., 1994). GGF is a Schwann cell mitogen of neuronal origin and belongs to the EGF/TGFα superfamily (Lemke and Brockes, 1984; Marchionni et al., 1993; Plowman et al., 1993). Shah et al. (1994) observed that NCSC, when grown in standard medium, differentiate into both neurons and glia. However, in the absence of GGF, neurogenesis is completely abolished while gliogenesis remains unaffected. It is believed
FIG. 5. Effect of EGF treatment on precursors and differentiating cells. Retinas obtained from gestation day 20 embryos were cultured as explants in the presence or absence of EGF (20 ng/ml) for 4 days followed by dissociation of cells, fixation, and immunocytochemical analyses using anti-Mash1, RetP-1, and HPC1 antibodies. The proportion of Mash1$^+$ (precursors) and opsin$^+$ (differentiating rods) cells decreases in the presence of EGF (C, D, G, H) in comparison to untreated controls (A, B, E, F). The proportion of syntaxin$^+$ cells remains unchanged in treated (K, L) and untreated (I, J) explant cultures. A, E, I, C, G, and K are Nomarski images. Scale bar, 20 μm. Cells dissociated from E20 retinal explants in culture in the presence or absence of EGF were analyzed as described above and counted. The proportion of Mash1$^+$ and opsin$^+$ cells decreases concomitantly in the presence of EGF, whereas the proportion of syntaxin$^+$ cells remains unchanged in comparison to untreated controls (M).
that the effect of GGF is instructive; it is also believed that GGF suppresses Mash1 expression thereby suppressing the induction of the neuronal pathway of differentiation. A similar mechanism can be invoked by which EGF delays rod differentiation by suppressing the expression of Mash1 in rod precursors. This is suggested by several lines of evidence. First, Mash1 is expressed in the outer neuroblastic layer which harbors rod precursors. Second, there is a direct correlation between the change in the proportion of opsins and Mash1-labeled cells in response to EGF-mediated suppression of differentiation. Third, there is a reciprocal change in the proportion of cells that have incorporated BrDu and those that express Mash1 when the suppressive effect of EGF is removed; the restoration of Mash1 expression in precursors after EGF withdrawal is followed by an increase in the proportion of rods (opsin-positive cells).

The direct correlation between the increase in the proportion of Mash1-positive and opsin-positive cells suggests that Mash1-positive cells represent rod precursors. Our study identifies at least two different stages of rod precursors in which Mash1 is expressed; one is mitotic (BrDu⁻, Mash1⁺) and the other is postmitotic (BrDu⁺, Mash1⁻). The expression of Mash1 in dividing precursors suggests that the decision to commit to a neuronal fate takes place before the cell exits mitosis. A similar observation that cell fate can be modulated in the S-phase of the cell cycle has been shown using heterochronic transplantation of cerebral cortical cells (McConnel, 1988; McConnel and Kaznowski, 1991). It is likely that at this decision-making stage EGF antagonizes differentiation. This notion is supported by the observation that the proportion of cells that were Mash1 positive and BrDu-negative at the beginning of EGF treatment and those that were opsin-positive at the end of treatment was similar (≈20%). The Mash1-positive and BrDu-negative cells most likely represent a population that was refractory to EGF and continued to differentiate as rods, whereas proliferative Mash1-positive cells represent a population in which Mash1 expression can be suppressed in response to EGF, thereby delaying neuronal differentiation (Fig. 8). Therefore, Mash1 expression represents an intermediate stage of neurogenesis, the regulation of which can either facilitate or delay neuronal differentiation.

Intermediate neuronal precursors expressing Mash1 have also been observed during autonomic neurogenesis (Sommmer et al., 1995). These precursors express Mash1 and the neuronal marker, neurofilament, before the expression of overt neuronal markers such as SCG 110, peripherin, and neuron-specific enolase. However, in Mash1 knock-out mice, the same precursors express neurofilament but fail to express peripherin or SCG10 and have a nonneuronal morphology. The sequential expression of Mash1, neurofilament, and autonomic neural markers suggests that the differentiation of autonomic neurons requires several intermediate steps and that Mash1 promotes the differentiation of committed neuronal precursors. Multiple stages of differ-

**FIG. 6.** Temporal changes in the proportions of proliferating, Mash1-positive, and opsin-positive cells following EGF withdrawal. E20 retinal explants were cultured for 4 days in the presence of EGF (20 ng/ml) followed by incubation in culture medium without EGF for 0, 12, 24, and 48 h. The explants were exposed to BrDu (10 μM) for 2 h prior to dissociation. The dissociated cells were analyzed for BrDu and Mash1 and opsin immunoreactivities and counted. The relative proportion of Mash1⁻ and opsin⁻ cells increases with time, whereas that of BrDu⁺ cells decreases. The relative increase in the proportion of Mash1⁺ cells precedes that of opsin⁻ cells.

**FIG. 7.** Temporal changes in the proportion BrDu⁺/Mash1⁻ and BrDu⁻/Mash1⁺ following EGF removal. The proportion of intermediate precursors (BrDu⁺/Mash1⁻ and BrDu⁻/Mash1⁺) was analyzed by double immunocytochemistry using anti-BrDu (B, E) and Mash1 (C, F) antibodies on dissociated cells obtained from explant cultures at 0 and 48 h after the withdrawal of EGF. The proportion of BrDu⁺/Mash1⁻ cells (arrows) decreases, whereas that of BrDu⁻/Mash1⁺ (arrowheads) increases with time. A and D are Nomarski images. Scale bar, 25 μm.
FIG. 8. Schematic representation of EGF-mediated signaling during retinal neurogenesis. Differentiation of retinal neurons requires several sequential steps in which Mash1-expressing cells represent intermediate precursors. The progenitors and Mash1-expressing proliferating precursors are the target of EGF, probably elaborated by postmitotic Mash1-expressing precursors to maintain a pool of progenitors and precursors uncommitted for differentiation at a later stage. The expression of Mash1 in the intermediate precursors causes an upregulation of Delta1, whose interaction with Notch1 keeps the progenitors uncommitted. Therefore, signaling through EGF and Notch receptors probably acts in concert for stereotypical cell fate determination in the retina.

Differentiation and the role of Mash1 are also observed in olfactory epithelium neurogenesis. One of these steps is Mash1-dependent since Mash1 is expressed in precursors in the intermediate stage of neurogenesis (Gordon et al., 1995) and a severe reduction in the number of olfactory receptor neurons is observed in Mash1 knock-out mice (Guillemot et al., 1993). It is likely that differentiation of rods also involves several sequential steps and that one of the intermediate steps is regulated by Mash1. Therefore, EGF suppression of Mash1, likely during the proliferating phase, compromises rod differentiation.

In Mash1 knock-out mice, no retinal abnormalities have been reported (Guillemot et al., 1993). However, when explant culture was carried out on embryonic retina (E17.5) obtained from Mash1 knock-out mice, differentiation of late-born retinal neurons, i.e., rods and bipolar cells, was delayed (Tomita et al., 1996). There are two implications based on these observations. First, it is entirely possible that a decrease in Mash1 expression in response to EGF treatment may delay differentiation of bipolar cells in the explant culture. This is due to the fact that Mash1 defines an intermediate stage of neurogenesis and that specific cell types (rods or bipolar cells) are likely to be sorted out in response to temporarily arrayed environmental cues available to these intermediate precursors at a particular time. Our culture conditions, i.e., the time of harvesting of retina (E18/E20) and incubation time (4 days), were conducive to studying the differentiation of rods and not bipolar cells. Second, the fact that differentiation of neurons is delayed in vitro and the fact that no retinal abnormalities were observed in vivo in Mash1 knock-out mice suggest that Mash1 function is compensated. Such functional compensation by bHLH transcription factors is known to occur during development (reviewed in Weintraub, 1993; Rawls and Olson, 1997). In the developing retina several proneural bHLH factors are expressed in addition to Mash1. These include NeuroD (Ahmad, 1996; Acharya et al., 1997), neurogenins and ATH3 (Sommer et al., 1996; Takebayashi et al., 1997; Ahmad, unpublished observation). The expression of NeuroD during rod differentiation and its ability to bind the E-box element in the proximal promoter of the opsin gene (Ahmad et al., unpublished observation) make it a likely candidate to functionally compensate for Mash1 deficiency in the developing retina. This hypothesis is currently being tested by reducing Mash1 and NeuroD expression in explant cultures using antisense oligonucleotides.

The involvement of signaling through the EGF receptor in retinal development is evolutionarily conserved. In flies, it plays a significant role in the induction of photoreceptors. The Drosophila compound eye is made up of reiterated units, ommatidia, in which the photoreceptors are born in a stereotypical temporal and spatial order (Tomlinson and Ready, 1987). The fact that these photoreceptors are not clonally related and are born in a strict sequence suggests that, as in vertebrates, cell interactions play a key role in the development of fly eyes (Lawrence and Green, 1979; Tomlinson and Ready, 1987). Genetic and molecular perturbation analyses have shown that one of the mechanisms by which these cell interactions are mediated is by signaling through the Drosophila EGF receptor (DER) (Banerjee and Zpursky, 1990; Schwitzer and Shilo, 1997). The gain of function mutation of DER resulted in a defect in ommatidial spacing with a remarkable decrease in the number of ommatidia (Baker and Rubin, 1989). Recently Freeman...
Ahmad, Dooley, and Afit (1996), using the dominant negative form of DER, observed that DER is used reiteratively for the differentiation of all cell types in developing ommatidia including cone and pigment cells.

The pleitropic function of DER, as indicated by the embryonic lethality of its null mutation (Baker and Rubin, 1989; Banerjee and Zippursky, 1990), its involvement in ommatidial spacing, and its reiterative usage in sorting out retinal cell fate, is remarkably similar to Notch function (Artavanis-Tsakonas et al., 1995). As with the EGF receptor, Notch function is evolutionarily conserved. In the vertebrate retina the Notch1 receptor and its ligand Delta1 are expressed during successive stages of neurogenesis and are utilized in sorting out retinal cell fate (Ahmad et al., 1995, 1997; Austin et al., 1995; Dorsky et al., 1997). Notch signaling is initiated when the Notch receptor interacts with one of its ligands, Delta1, which is expressed by intermediate precursors, most likely in response to proneural homologues such as Mash1 (Myat et al., 1996; Ahmad et al., 1997). As a consequence of signaling, progenitors and early precursors expressing the Notch receptor remain uncommitted due to inhibition of proneural homologues. Consequently the ligand Delta1 is repressed in these cells. Downregulation of Delta1 in uncommitted neighboring cells relieves the intermediate precursors from signaling through Notch thereby facilitating differentiation by amplifying initially subtle differences in signaling (reviewed in Tanabe and Jessel, 1996).

The sensitivity of precursors to signaling through Notch and EGF receptors and the similarity of their functions suggest that during retinal development these two distinctive signaling mechanisms complement each other in keeping cells uncommitted (Fig. 8). While Notch signaling can keep a cell uncommitted by repressing proneural homologues, signaling through EGF receptor is likely to occur by regulating the decision of a cell to quit mitosis or to reenter the cell cycle. To quit or not to quit mitosis is an important decision for eye development in Drosophila. Cells that quit mitosis participate in the formation of clusters (developing ommatidia) at the morphogenetic furrow, whereas those that reenter the cell cycle join clusters at a later stage as late-born neurons (Banerjee and Zipursky, 1990). Therefore, in gain of function of DER (Baker and Rubin, 1989), more cells in the eye imaginal disc remain in the cell cycle and fewer ommatidia are formed. Similarly, in the vertebrate retina, signaling through the EGF receptor may regulate the progression of progenitors (BrDu, Mash1) and precursors (BrDu, Mash1) toward differentiation. While the present study identifies an EGF-sensitive intermediate precursor, evidence has emerged in support of the presence of EGF-responsive progenitors with stem cell properties in the developing retina (Ahmad and Dooley, 1997). Therefore, it is likely that the delay in rod differentiation observed in this study is due to progenitors and precursors choosing to reenter the cell cycle in the presence of exogenous EGF at the expense of rod differentiation.

In vivo, EGF signaling is likely to be induced by differentiating cells in order to maintain a population of progenitors that can differentiate at a later stage. This notion is supported by the observation that the early-born photoreceptors in Drosophila ommatidia may be the source of Spitz, a DER-activating ligand during photoreceptor differentiation (Freeman, 1996; Schwetzer and Shilo, 1997). It is also likely that in cells that have reentered the cell cycle, any premature trigger of the process of differentiation is prevented by suppressing the homologues of proneural genes in response to Notch signaling. This notion is supported by the fact that EGF-responsive retinal progenitors express Notch1 (Ahmad and Dooley, 1997). Evidence for genetic interaction between Notch and DER loci during Drosophila eye development has been recently reported using second-site mutagenesis screening to isolate enhancers and suppressors of eye phenotypes in response to activated Notch (Verheyen et al., 1996). It is likely that such an interaction is conserved in vertebrates and that signaling through Notch and EGF receptors acts in concert to ensure the fidelity of the strict temporal and spatial nature of cell fate determination in the retina.

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