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Soluble factors and the development of rod photoreceptors

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Abstract. Photoreceptors are the most abundant cell type in the vertebrate neural retina. Like the other retinal neurons and the Müller glia, they arise from a population of precursor cells that are multipotent and intrinsic to the retina. Approximately 10 years ago, several studies demonstrated that retinal precursor cells (RPCs) are competent to respond to environmental factors that promote cell type determination and differentiation. Since those studies, significant effort has been directed at identifying the molecular nature of these environmental signals and understanding the precise mechanisms they employ to drive RPCs towards the different retinal fates. In this review, we describe the recent progress toward understanding how environmental factors influence the development of vertebrate rod photoreceptors.

Key words. Retina; signaling factors; retinoic acid; Hedgehog; fibroblast growth factor; epidermal growth factor; ciliary neurotrophic factor.

Introduction

As in many other areas of the vertebrate central nervous system (CNS), the retina is derived from a spatially restricted domain of neuroepithelial cells [retinal precursor cells (RPCs)] that is partitioned after neurulation, but before the onset of neuronal differentiation. Once this domain is established, the retina undergoes a rapid and dramatic expansion in size by RPC proliferation. At the same time, neurogenesis initiates and the retinal cell types are born in an orderly, sequential fashion. Ganglion cells, cone photoreceptors, and horizontal cells are born first, amacrine cells and rod photoreceptors are born during the middle phase of neurogenesis, and rod photoreceptors, bipolar cells, and the Müller glia are born during the late phase of neurogenesis [1, 2]. This order is generally conserved in vertebrates, although there is substantial overlap in the birthdates of most or all cell types in species with rapid development [3–5]. It is now generally accepted that the retinal neuroepithelial cells are multipotent and not lineage restricted [4, 6–8]. Furthermore, several in vitro studies have demonstrated that developmental changes in the microenvironment influence the potential of RPCs to generate different cell types [9–12]. Since then an increasing number of candidate signaling molecules have been identified that influence the generation, differentiation, and survival of retinal cell types, especially photoreceptors. Therefore, the study of rod photoreceptor development is providing a foundation for understanding the mechanisms of neural precursor cell's response to environmental cues to promote differentiation.

Photoreceptors have a complex, unique, and highly specific phenotype, many aspects of which could arise from interaction with other cells. Potential sources of interaction could be cells in the immediate environment, such as the Müller glia and horizontal cells [13], as well as adjacent tissue such as the retinal pigmented epithelium [14]. Small soluble signaling molecules could mediate interactions between developing photoreceptors and other retinal cell types [9, 10, 12, 15]. The complexity of the rod phenotype could also arise from the interplay of a host of different extracellular signals. Alternatively, it is possible that the program of coordinated gene expression necessary to define the functional photoreceptor could be activated by a master photoreceptor differentiation factor. There are examples of both types of regu-

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lation in other developing systems. In this review, we will summarize evidence that the vertebrate photoreceptor phenotype arises through a series of cell-cell interactions, each necessary for some aspect of the differentiation process.

There are several distinct stages in the development of photoreceptors. In the first stage, a nascent photoreceptor arises from mitotic division of the RPC. Hinds and Hinds [13] described these newly generated photoreceptors as cells that had lost their apical (vitreal) process, but that retained a connection to the outer limiting membrane of the retina. These early photoreceptors have a simple morphology reminiscent of RPCs. Although they do not yet express opsins, they are likely to express the photoreceptor-specific homeodomain gene, *crx* [16, 17]. In the next stage of photoreceptor development, cone photoreceptors adopt an elongated cuboidal morphology, whereas rod photoreceptors remain RPC like [18]. Expression of the general photoreceptor genes *recoverin* and *IRBP* (interphotoreceptor retinoid binding protein) is initiated, but rod- and cone-specific genes such as those for the opsins are not yet activated. In the third stage of development, opsin expression is induced and synaptogenesis occurs. Lastly, the outer segments elongate.

In classical embryology, the requirements of inductive interactions in the development of organ rudiments are assessed by isolating tissue anlagen and assaying the extent of differentiation in vitro. A similar approach has been taken with RPCs. RPCs from embryonic chick retinas are capable of substantial photoreceptor differentiation in vitro. Adler [19] found that isolated chick RPCs cultured at low density (without intercellular contact) undergo a limited number of cell divisions and develop morphological features characteristic of cone photoreceptors [20]. From these studies, it appears that a remarkable degree of differentiation of chicken cone photoreceptors develops independently of cell-cell signaling. However, one caveat in this interpretation is that the culture medium itself may contain one of the important signaling molecules.

Table 1. The number of rod photoreceptor cells that differentiate in embryonic day 17 rat retinal cultures depends on the overall cell density and the number of days in vitro.

Total	Days in vitro				
cell number plated	1	3	6	9	12
2.5×10^2	0	0	0	0	0
2.5×10^3	0	0	0	0	0
2.5×10^4	0	0	0	16	101
2.5×10^5	0	0	130	33,497	105,969

The number of rod photoreceptor cells that differentiate in E17 rat retinal cultures was determined by fixing the coverslip cultures in 4% paraformaldehyde after the number of days listed above, and incubating them with one or two monoclonal antibodies to rhodopsin, and counting the immunoreactive cells. In those cases where the total number of immunoreactive cells was less than 500, the entire coverslip was scanned at $\times 40$, and the total number of opsin+ cells is listed in the table. For the high-density 9- and 12-day values, the number of immunoreactive cells was estimated by counting all opsin+ cells in more than six fields (at $\times 40$) on each of three coverslips and the mean value was multiplied by the ratio: area of coverslip/area of field. A minimum of three coverslips were examined for each value; the standard errors were $<10\%$ of the means.

In marked contrast to the apparent autonomy of chick cone photoreceptor differentiation, rod photoreceptors are dependent on other cells for differentiation. Isolated RPCs from embryonic rat retina grown in a low density environment do not develop characteristics of rod photoreceptors [21, 22]. Instead, a critical density of cells is necessary for the expression of rod-specific proteins such as rhodopsin [10–12, 15]. Table 1 shows the relationship between cell density and rhodopsin expression for embryonic day (E)17 rat retinal cells cultured for 6 days. This requirement of a critical cell density for the expression of rhodopsin indicates that some interactions among retinal cells are necessary for the development of at least part of the rod photoreceptor phenotype.

With the progress made in defining appropriate in vitro conditions, in identifying cell-type-specific markers, and most importantly, in formulating a working model for the development of rod photoreceptors, it is now possible to identify and characterize the functional relationships of extracellular signaling molecules during the stages of rod photoreceptor development. In the next section of this review, we analyze the existing evidence that extracellular signaling molecules influence specific aspects of rod photoreceptor development. The signaling molecules described below are divided into three sections based on their effects on rod photoreceptor development: (i) stimulatory, (ii) inhibitory, and (iii) both stimulatory and inhibitory. The latter factors have been found to act in opposition when analyzed across species, as in the case of ciliary neurotrophic factor (CNTF) and activin, or they have pleiotropic effects on photoreceptor development within a single species, but at different temporal stages, such as fibroblast growth factor (FGF2).

Stimulatory factors

Retinoic Acid

Retinoic acid (RA) regulates many aspects of development. Perhaps the most well-established action is control of homeobox gene expression and concomitant regulation of the anterior-posterior axis in vertebrate embryos [23]. In addition, RA regulates the development of diversity in spinal motoneurons; the initial group of motoneurons produces RA which then stimu-

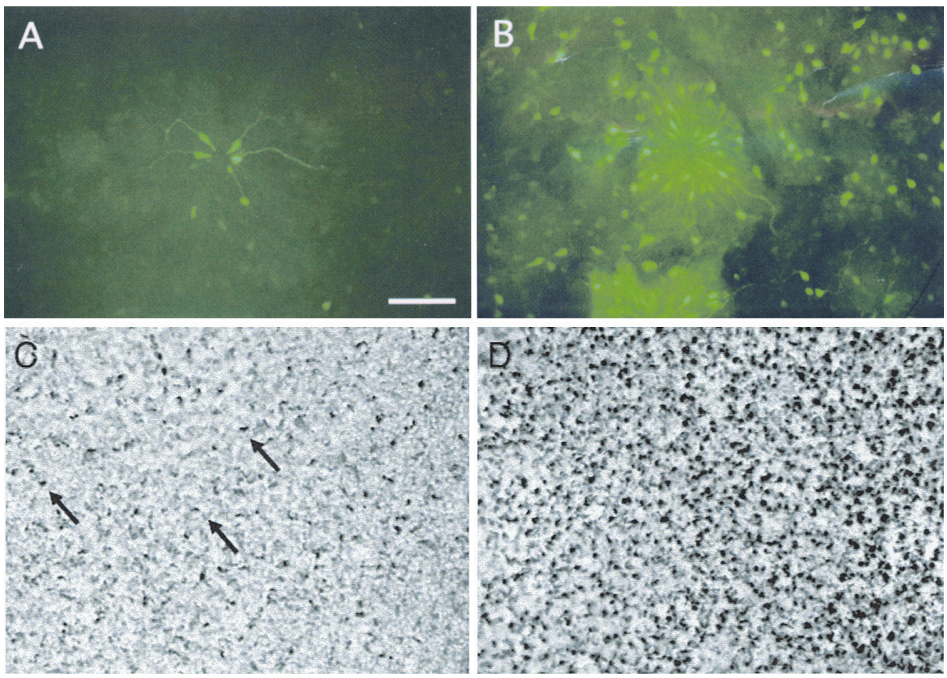

Figure 1. Retinoic acid (RA) promotes rod photoreceptor differentiation in vitro and in vivo. (*A*, *B*) Cell cultures of embryonic rat retinal cells in control or RA-treated conditions. An antibody against recoverin was used to label the photoreceptor cells, scale bar in A (same in B), 100 μm [modified from ref. 25]. (*C*, *D*) Micrographs of flat-mounted newborn rat retinas, showing rod photoreceptors labeled with an antibody against rhodopsin (4D2; arrows). (*C*) Control retina. (*D*) Retina from an animal treated with RA in utero at embryonic day 18 and 20 and allowed to survive to birth [modified from ref. 27].

lates the production of a second type of motoneuron [24]. Thus, RA acts as a signal in a sequential cell induction process.

RA is produced at high concentrations in the developing retina, and several lines of evidence implicate a role for this molecule in photoreceptor development.

1) Cell culture studies have shown that the addition of RA to dissociated embryonic mammalian retinal cells causes an increase in the number of rod photoreceptors in a dose-dependent manner. Kelley et al. [25] used two photoreceptor-specific antibody markers, anti-recoverin (which is expressed in all photoreceptors) and anti-rhodopsin (which is specific for rod photoreceptors) to assess the effects of RA. They found an increase in the number of cells expressing these markers after 2–8 days in vitro (fig. 1). The effect on photoreceptor differentiation was specific, since the other major cell type produced at this point in development, amacrine cells, did not increase with RA treatment, but were inhibited instead.

2) In vivo experiments have largely supported these cell culture findings. Zebrafish embryos treated with RA show precocious development of rod photoreceptors, while cone photoreceptor maturation is inhibited [26]. Injections of RA into pregnant rats on the 18th day of gestation cause precocious rod photoreceptor development in the newborn pups [27] (fig. 1).

3) In zebrafish, rod photoreceptor differentiation is delayed following citral-mediated inhibition of RA synthesis [26]. In RA receptor (RAR)β/RARγ double-mutant mice, rod photoreceptors fail to express rhodopsin in some regions of the retina [14].

Taken together, these studies support the hypothesis that RA is an important regulatory factor for the development of rod photoreceptors. RA is present in the retina as early as optic cup stages and work by Drager and others has clearly demonstrated the importance of RA in orchestrating the growth of the ventral retina at these early stages [28]. RA is likely synthesized by RPCs as well as by postmitotic neurons, with the highest RA concentrations in the ganglion cells, as indicated by RARE-reporter constructs and antibodies to the retinal dehydrogenase, RALDH2 [29]. As development proceeds, the highest concentrations of RA shift from the retina to the retinal pigmented epithelium (RPE) and in postnatal and adult mice, ocular RA is synthesized predominantly in the RPE [30].

From these studies, a picture of the involvement of RA in rod photoreceptor development is beginning to emerge. The multipotent retinal progenitor responds to

intraretinal RA released from the ganglion cells to shift from producing predominantly amacrine cells to generating rod photoreceptors. As development proceeds, an additional source of RA from the RPE further biases the progeny of RPCs towards the rod cell fate, such that by birth, nearly 90% of the cells being generated in the retina differentiate as rods. This model is similar to that recently proposed by Sockanathan and Jessell [24] in which RA is part of a cascade of sequential inductions that produce motoneuron diversity in the cervical spinal cord.

While this is an attractive model for the role of RA in rod photoreceptor development, there are still some unresolved questions. For example, it is not clear at what specific stage of rod photoreceptor development RA is required. To address this question, Kelley et al. [25] labeled RPCs with bromodeoxyuridine (BrDU) prior to the addition of RA to the cultures. They reasoned that if previously generated (i.e., postmitotic) rods in the cultures were driven by RA to express rhodopsin prematurely, then these cells would be labeled with the rhodopsin antibody, but not BrDU. Alternatively, if the RA acted on RPCs to direct them to the rod photoreceptor cell fate, then cells should be double-labeled with both BrDU and rhodopsin antibodies. They found that the double-labeled population of cells accounted for nearly all of the effect of RA treatment. Therefore, they proposed that RA affects the choice of cell fate by the RPC. However, results from the deletion of various RAR family members (i.e., RAR, RXR) in single or compound knockout mice are not entirely consistent with this conclusion. When single RARs were deleted, no effect on rod photoreceptor development was observed [23]. In addition, in RARβ2/RARγ2 double-null mutant mice, the rod layer is largely intact, and only in regions where the RPE is disrupted is there a failure of rhodopsin expression [14]. Therefore, the defect in rod differentiation may be secondary to an earlier defect in RPE development. While it is difficult to sort out cause and effect in these mutant mice, one thing is clear: even without the two RARs that are predominant in the retina, the number of rods generated during early retinal development seems to be normal. Instead, RARs seem to be required at a relatively late stage of rod photoreceptor differentiation (i.e., they may be involved in regulating rhodopsin expression). An alternative explanation is that there is genetic compensation in the double-null mutant mice, and that all three RARs would have to be eliminated to prevent rod photoreceptor development.

Hedgehog

One of the most exciting signaling mechanisms identified in recent years is the Hedgehog (Hh) pathway. Originally identified as a mutation affecting segment polarity during segmentation of the *Drosophila* embryo [31], Hh signaling was subsequently found to be a critical component for the patterning of many epithelial tissues in metazoans. In the developing *Drosophila* retina, Hh protein is secreted by differentiating photoreceptors in the posterior eye imaginal disk and is necessary for propagation of the morphogenetic furrow across the unpatterned and undifferentiated anterior neuroepithelium [32–34]. In the vertebrate spinal cord, Sonic Hh is secreted by the notochord and floor plate, inducing adjacent neural tube precursors to adopt ventral fates [35]. Disruption of Hh signaling in the nervous system leads to severe neural tube defects such as spina bifida and holoprosencephaly, and deregulated activation in adult animals and humans is associated with skin and brain tumors [36]. Hh proteins are secreted ligands, and several different *hh* genes have been identified in vertebrates [37]. The Hh receptor complex is expressed on adjacent cells and is composed of two multipass transmembrane proteins, Patched and Smoothened [38].

We and others recently began to assess the functions of Hh signaling during eye development [39, 40]. In a reverse transcription-polymerase chain reaction survey of rat ocular tissues, we found that Sonic Hh and Desert Hh are expressed in the neural retina, and expression of Sonic Hh begins at E16 and persists through adulthood. In the RPE, Indian Hh expression starts at E13 and persists through adulthood.

In high-density cultures of dissociated E18 retinal cells treated with recombinant Sonic Hh protein (SHH-N), we observed increased proliferation, which suggests that Hh is a retinal mitogen. The mitogenic effect of SHH-N was also observed by Jensen and Wallace [40] in reaggregated pellet cultures of E18 mouse retinal cells. In our experiments [39], the mitogenic effect was transient upon addition of SHH-N at the beginning of the culture. By 6 days in vitro (DIV), the number of progenitor cells in control cultures caught up to that in the SHH-N-treated cultures. Analysis of differentiated cell types in these cultures revealed that the number of photoreceptors increased in the SHH-N-treated cultures, whereas amacrine and ganglion cells were unchanged in number from control cultures. When we assayed for the rod photoreceptor phenotype, we found that rhodopsin-positive rods appeared earlier in the SHH-N treated cultures. By 7 DIV, the number of rod photoreceptors were approximately tenfold higher than in control cultures. By 14 DIV, the number of recoverin-positive cells were equal between SHH-N treated and control cultures, but the increase in rhodopsin-expressing photoreceptors observed at 7 DIV in the SHH-N-treated cultures remained (fig. 2).

What is Hh signaling doing to promote rod photoreceptor differentiation? Our results suggest that it may be acting at two levels. First, Hh signaling may promote the proliferation of retinal progenitors that are highly biased to give rise to rod photoreceptors. Consistent with this, an RPC response to Hh may be direct since *patched 1* mRNA is expressed by mouse RPCs [40]. Although there is no evidence for a rod-restricted progenitor cell in mammals, lineage studies of neonatal rat RPCs revealed several clones composed solely of rod photoreceptors [6]. Second, Hh signaling promotes the maturation of rod photoreceptors. In control cultures, rhodopsin-expressing cells reach a plateau at approximately 25% of the photoreceptor population whereas in SHH-N-treated cultures, almost 100% of the photoreceptor population express rhodopsin (fig. 3). These results suggest that in control cultures, a limiting factor is necessary for rod maturation, and SHH-N satisfies this requirement. At present, it is not known whether Hh signaling acts directly on developing photoreceptors or whether it is required for rod maturation in vivo.

Taurine

Taurine has also been shown to promote rod photoreceptor differentiation in rat retina. Using low-density cell cultures of P0 rat retina, Altshuler et al. [41] found that a low-molecular-weight fraction from conditioned medium could satisfy the requirement for high cell density in rod differentiation. Taurine, an amino acid previously known to be critical for rod photoreceptor survival [42], was found to be present in the medium, and when added to the culture caused an increase in the number of rhodopsin-expressing cells. The authors argued that this effect was not due to selective survival, but was due to differentiation, since taurine did not significantly affect overall cell number in the cultures. In the newborn rat retina, taurine is localized primarily in ganglion cells [43]. At 2 days of age, taurine is present in amacrine cell bodies and in the processes of photoreceptors. By day 6, taurine is present in photoreceptor cell inner and outer segments, and in horizontal cells and their lateral processes. Localization of taurine in ganglion cells is transient, but persists in photoreceptors, bipolar cells and some amacrine cells throughout life. Thus, taurine is present in the retina at the time when many of the photoreceptors are generated.

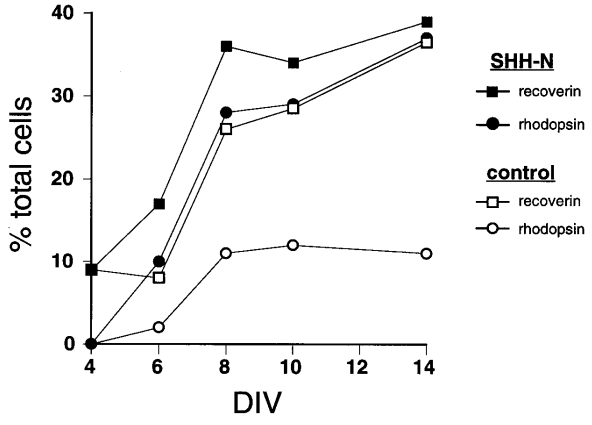

Figure 2. SHH-N promotes rhodopsin expression in cultured rat retinal cells. E18 rat retinal cells were cultured for 4, 6, 8, 10, and 14 DIV in control medium (open squares and circles) or in medium supplemented with SHH-N protein (filled squares and circles), and the percentages of total cells in the cultures that expressed recoverin (squares) and rhodopsin (circles) were determined. In control cultures, the percentage of cells that expressed recoverin increased with time in culture up to approximately 40%; however, the percentages of cells that expressed rhodopsin reached a plateau at 10%, even after 14 DIV. In contrast, the percentages of recoverin-positive and rhodopsin-positive cells increased in parallel in the SHH-N treated cultures. Both antibodies labeled approximately 40% of the retinal cells after 14 DIV [modified from ref. 39].

Laminin $\beta 2$

Another molecule that may be involved in rod differentiation is laminin $\beta 2$, also known as S-laminin. This molecule is concentrated in the matrix of the neuromuscular junction and in the subretinal space during early stages of retinal histogenesis [44]. Laminin $\beta 2$ is synthesized by the progenitor cells [45]. Hunter et al. [46] found that blocking antibodies inhibit the number of rhodopsin immunoreactive rod photoreceptors in high-density rat retinal cultures. Moreover, plating retinal progenitor cells on laminin-$\beta 2$-rich matrices causes an increase in the differentiation of rods in the cultures.

Inhibitory factors

Epidermal growth factor and transforming growth factor-α

Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) were the first factors reported to inhibit rod photoreceptor differentiation [11, 47]. The activation of the EGF receptor (EGFR) by these factors causes RPCs to proliferate in vitro. Anchan et al. [48] first reported that these factors were mitogens from retinal RPCs, and they were subsequently shown to be mitogenic for progenitor cells in many areas of the CNS, including the subventricular zone [49]. Although they are mitogens, the suppressive effect of TGF-α and EGF may not be prevention of RPC differentiation since retinal neurons other than rod photoreceptors are generated in the presence of saturating concentrations of either factor [47, 48, 50]. In addition, other mitogens for RPCs, like TGF-$\beta 3$ and SHH-N, do not inhibit photoreceptor differentiation [39, 40, 50]. Thus, mitogenic stimulation is not an obligatory block to photoreceptor development.

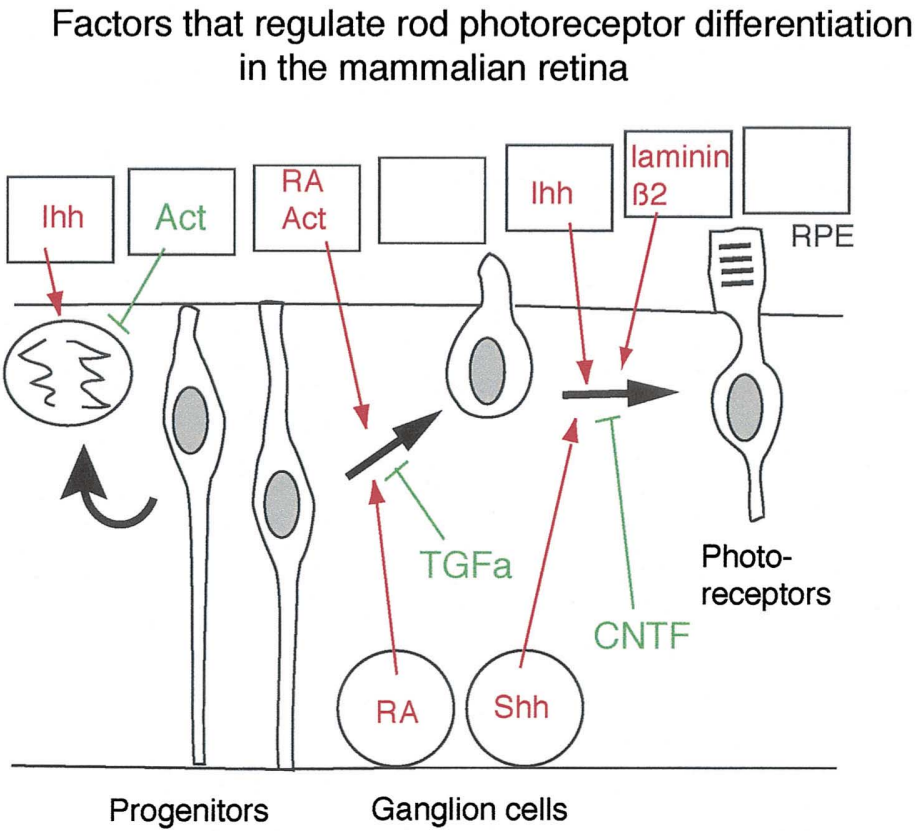

Figure 3. Factors that regulate rod photoreceptor differentiation in the mammalian retina. The stages of rod photoreceptor differentiation are shown along with the developmental stages at which particular factors are thought to act. Many different molecules have been tested in various cell cultures assays and in vivo systems for effects on rod photoreceptor differentiation; the ones that have been shown to have positive (rod-promoting) effects are shown in the figure in red, while those that suppress rod differentiation in mammalian retina are shown in green. Where known, the cells that produce each factor are identified (RA, retinoic acid; Act, activin; TGFa, transforming growth factor-alpha; CNTF, ciliary neurotrophic factor; Ihh, Indian Hh; Shh, Sonic Hh).

It appears then that activation of the EGFR by this class of ligands selectively suppresses the differentiation of rod photoreceptors. Removal of EGF or TGF-α from the culture medium allows at least some of the RPCs to differentiate into rod photoreceptors [11]. However, when RPCs are forced to express higher than normal levels of EGFR by retroviral infection in vitro, the RPCs generate Müller glial cells with a greater frequency than in controls [51]. Thus, although the activation of the EGFR maintains the RPCs in an undifferentiated state, they are still capable of generating rod photoreceptors.

While manipulations of EGFR signaling both in vitro and in vivo show that it can control the number of rod photoreceptors in the retina, it is not yet clear whether this is an endogenous function. Mice lacking the EGFR do not have reported defects in retinal development [52, 53], and this could be due to compensatory mechanisms. There are three other members of the EGF receptor family of tyrosine kinases; erbB2, erbB3, and erbB4. Although studies of the erbB4 ligand, neuroregulin, failed to show any effect on rod photoreceptor development [54, 55], animals deficient in erbB4 or erbB2 have some retinal defects. It is also possible that the EGFR is normally important in controlling the cell fate decision between rod photoreceptors and Müller glia, but in its absence a different receptor tyrosine kinase such as the FGF receptor (FGFR) can replace it. As described below, some studies have found that FGFR signaling can inhibit or delay rod photoreceptor development similar to that observed with TGF-α and EGF.

Pleiotropic stimulatory and inhibitory factors

Ciliary neurotrophic factor

The cytokine CNTF exhibits strong effects on photoreceptor development in chick and rat in vivo and in

vitro. CNTF was originally identified and purified as survival-promoting activity for chick ciliary ganglion neurons [56]. Several studies have shown that CNTF acts as a pleiotropic growth factor on neurons and glia in the developing and mature nervous system in multiple ways [57, 58]. In the vertebrate retina, CNTF regulates the differentiation of rod photoreceptors during a transient period of development and, surprisingly, the effects are opposite in chick and rat retina using rhodopsin immunoreactivity as a marker. CNTF expression has been noted in the embryonic retina and in the Müller glia of postnatal retina. Thus, the factor is present at the appropiate time during retinal development in vivo [59–61].

In chick, in vitro studies revealed that CNTF has a positive effect on rod photoreceptor differentiation [62, 63] promoting the maturation of early, postmitotic photoreceptors into rod photoreceptors that express rhodopsin. In accord with the in vitro effects of CNTF, postmitotic, rhodopsin-negative photoreceptor cells in the outer nuclear layer (ONL) of the intact retina and as in dissociated cultures express CNTF receptor (CNTFR) [64, 65]. This expression is transient and down-regulated prior to onset of rhodopsin expression. In vitro, CNTF treatment does not cause an increase in the number of cells double-labeled with ^{3}H-thymidine and rhodopsin antibody [62]. In addition, the total cell number in these cultures and the number of immature photoreceptor cells that express the CNTFR does not change after CNTF treatment [62, 64], arguing against a survival effect. Thus, CNTF could act by promoting immature photoreceptor cells to express rhodopsin, similar to the action of SHH-N.

In contrast, CNTF treatment inhibits rod differentiation in the rodent retina in vitro [61, 63, 66, 67]. In agreement with this observation, more rods develop in retinal explant cultures of mice lacking a functional CNTFR, or following treatment of wild-type rat retinal cultures with neutralizing anti-CNTF antibody [66, 67]. Therefore, CNTF appears to act as a specific negative regulator of rod development in rodents. Although CNTFR is not expressed in the ONL of the newborn rat retina, it is possible that progenitor cells or newly developing rods in the inner nuclear layer express the receptor [61]. CNTF does not interfere with proliferation, cell death or survival, but causes a decrease in the number of recoverin-positive photoreceptors [66], suggesting that CNTF exerts inhibitory influences on immature photoreceptors. It is not clear what subsequently happens to these cells. From experiments with CNTF-treated rat retinal explant cultures came the proposal that many of the cells that would normally differentiate into rods instead differentiated as bipolar cells [67]. In contrast, treatment with CNTF or another closely related cytokine (leukemia inhibitory factor) does not promote the differentiation of bipolar cells in dissociated or slice cultures [66, 68]. Instead, rod development is arrested in these cultures at a postmitotic, rhodopsin-negative stage, although rhodopsin-positive rods reappear in retinal slice cultures after long cultivation, even in the continuous presence of CNTF [68; H.-D. Hofmann, personal communication]. Thus, together with other extrinsic signals, CNTF may coordinate the time course of rod photoreceptor cell differentiation by delaying the final maturation until other retinal components have reached an appropiate functional state. However, further studies are necessary to distinguish between two hypotheses: (i) arrest of photoreceptor differentiation, or (ii) conversion of immature photoreceptor cells into bipolar cells.

Activin

Activin is another factor that appears to have opposite effects on chick and rat retina. In the developing chick retina, activin suppresses the differentiation of photoreceptors [69]. While the number of morphologically identifiable photoreceptors in the cultures does not change in the presence of activin, activin inhibits the expression of visual pigments. At the same time, in the chick retina, activin promotes the development of amacrine cells. The opposite response is observed in the rodent retina. Addition of activin to rat retinal cultures causes an increase in the number of rod photoreceptors immunoreactive for rhodopsin and recoverin [70, 71]. In addition, activin has a small but significant inhibitory effect on cellular-RA-binding-protein-immunoreactive amacrine cell differentiation. Thus, activin treatment resembles that of RA in many respects, and it is possible that these two factors act via a common pathway.

Fibroblast growth factor

The first secreted factor shown to have a positive effect on photoreceptor differentiation was FGF1 (acidic FGF). Hicks and Courtois [72] showed that addition of either purified bovine FGF1 or a partially purified FGF fraction [containing both FGF1 and FGF2 (basic FGF)] stimulated rhodopsin expression levels in dissociated P0 rat retinal cells grown as monolayers. In a subsequent study, Hicks and Courtois [73] demonstrated that addition of FGF2 to these cultures caused an increase in the number of rhodopsin-expressing photoreceptors. This effect was specific to FGF2, since neither EGF or nerve growth factor elicited a similar response. Furthermore, in their assay, other cell types were largely unaffected, and the increase in rhodopsin-expressing photoreceptors did not appear to be linked to enhanced proliferation or survival. Thus, they suggested that FGF2 is a differentiation factor for imma-

ture rods (defined as postmitotic and rhodopsin negative) that is limiting in monolayer culture.

The precise role of FGF2 in photoreceptor development is not quite that simple. Zhao and Barnstable [74] reported that in explants of E16 rat retina, exogenous FGF2 did not have an effect on rhodopsin expression. Although one could argue that different culture conditions may account for these contrasting results, another explanation is that the response to FGF2 in developing photoreceptors changes over time. Consistent with this, we also observed that FGF2 did not increase the number of rhodopsin-expressing cells in dissociated monolayer cultures of E18 rat retinal cells [75]. In addition, FGF2 blocks the induction of rhodopsin expression by positive factors such as SHH-N, RA, and taurine [E. M. Levine and T. A. Reh, unpublished data]. Furthermore, after P7, FGF2 is no longer stimulatory for rhodopsin expression [73].

An interesting study by McFarlane et al. [76] investigated the consequences of interfering with FGFR signaling using dominant negative FGFR constructs injected into *Xenopus* blastomeres that contribute progeny to neural retina. They found that expression of a dominant negative FGFR, which blocks FGF-mediated signal transduction in RPCs, resulted in a 50% loss in amacrine cells and photoreceptors, and a 3.5-fold increase in Müller glia. In a parallel experiment, they observed an increase in the percentage of photoreceptor cells when RPCs expressed a dominant negative FGFR that blocks FGFR activation through non-FGF ligands (without affecting FGFR activation through FGFs). Although this study does not address the specific requirements of FGF signaling on rod photoreceptor development, it does suggest that FGFR activation by a combination of different ligands can directly influence the fates of RPCs.

Conclusions

The above review highlights the range of factors that have been implicated in the process of rod photoreceptor development (fig. 3). Most of the work described here was done utilizing a battery of in vitro culture systems, and while the precise roles of these factors in regulating rod photoreceptor development in vivo is not as well established, the in vitro approach has been both informative and productive. This becomes clear when one considers that the described factors represent only a fraction of the various compounds that have been tested, and so there is some specificity to the observed effects. Therefore, we are faced with the following questions: Why are there so many rod photoreceptor differentiation factors? Why do only some RPCs become rod photoreceptors when exposed to these factors? Do these factors act at specific points in, or throughout, the differentiation process? In the following discussion, we attempt to address these questions.

Why are there so many rod photoreceptor differentiation factors?

It is possible that the in vitro findings may reveal an artificially high number of soluble photoreceptor differentiation factors; in vivo, the concentration of these factors may be much lower, not reaching the levels applied to cultures. While all of the factors shown to have effects on rod differentiation are known to be present in the developing retina, it is difficult to know their actual levels in vivo. It may be that the choice of a particular cell fate and subsequent differentiation is regulated by a combination of low-level signals, but in vitro, an overwhelming amount of a single signal can drive the process in a particular direction, perhaps towards rod photoreceptor differentiation.

Why do only some RPCs become rod photoreceptors when exposed to these factors?

It is also important to remember that the retinal cells used in these assays are not a homogeneous population at any stage of development. Postmitotic neurons are generated in the retina soon after optic cup formation and for the rest of retinal development. The progenitor cells themselves are not homogeneous but, rather, there are at least two and perhaps three different types of RPCs, based on differential patterns of gene expression [see for example ref. 77] and response to factors [47, 48]. It is not known whether all of the factors that affect rod photoreceptor differentiation do so by acting directly on receptors present on the RPCs. Alternatively, a particular factor may act to cause one of the other retinal cell types in the culture to secrete the 'real' rod-inducing activity. Expression of receptors for EGF, FGFs, RA, and Hh proteins have all been identified on RPCs, and so these factors could act directly on these cells. However, the situation is a bit more complicated for CNTF; in the chick, receptors for CNTF are expressed by newly differentiating photoreceptors, while in mammalian retina, there appears to be only a very low level of CNTFR expression in the neuroblastic layer or the outer nuclear layer, while the inner retinal neurons express a much higher level of the receptor. Further studies examining the effects of these factors on isolated retinal cells in low-density cultures will be necessary to resolve the issue.

Do these factors act at specific points in, or throughout, the differentiation process?

The development of any complex phenotype, such as the rod photoreceptor, is likely to involve several sequential intercellular interactions. Therefore, it is likely that rod photoreceptor differentiation is a stepwise process. The contrasting signals of EGFR activation and RAR activation may regulate the choice of fate between rod photoreceptors and the alternate identities of Müller and amacrine cells. At least one study also proposed that CNTF could perform a similar function for the cell fate decision between the bipolar cell and the rod photoreceptor. A second stage of rod photoreceptor development, the activation of genes encoding phototransduction proteins, also appears to be under the control of intercellular signaling. Several factors can have effects on this stage of differentiation, including RA, Hh proteins, taurine, CNTF, and laminin $\beta 2$. Is it possible that all of these factors are normally involved in the regulation of rhodopsin expression? While this seems excessive, it should be noted that the synthesis of rhodopsin is a highly regulated process, since either too much or too little of this protein is fatal to the cell. Rhodopsin expression varies with the time of day [78], with the level of illumination and in different regions of the retina [79, 80], and these patterns may be due to extracellular regulatory factors. The upstream promoter for rhodopsin contains no fewer than seven important regulatory sites. At least one of these, Ret1, has a binding activity that is influenced by FGF2 [81]. Several transcription factors, like Crx and Nrl, have been identified that interact with the promoters of phototransduction genes [16, 17, 82, 83]. It is reasonable to propose that some of the factors that regulate rod photoreceptor differentiation will do so by regulating the expression of these key transcription factors.

The relative simplicity of the cell culture assays that were developed to study the intercellular interactions required for rod photoreceptor differentiation has given rise to a host of candidate extracellular factors that positively and/or negatively regulate the differentiation of these cells. Considerable effort is now required to define more precisely the relative role for these molecules in the development of the complex rod photoreceptor phenotype in vivo. It is likely that these developmentally important factors will also be critical in the maintenance of the rod photoreceptor throughout life. What we learn from these developmental studies will then have ramifications for the preservation of the mature retinal structure and potential for understanding and treating retinal disease.

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