Activin A Promotes Progenitor Differentiation into Photoreceptors in Rodent Retina

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Activins are TGF β-like proteins that were first discovered for their actions on the reproductive system, but have subsequently been shown to play a role in a variety of developmental processes. Previous studies have demonstrated that activins and their receptors are present in the developing retina, as well as other regions of the embryonic nervous system. We used both in vitro and in vivo approaches to test for functions of activin during retinal development. We found that activin A treatment of embryonic day 18 rat retinal cultures causes the progenitor cells in the cultures to exit the cell cycle and differentiate into rod photoreceptors. This effect is dose-dependent and the promotion of rod photoreceptor differentiation is specific, since the other primary retinal neurons generated in these cultures, the C1+ amacrine cells, are not affected by activin A treatment. Mice with homozygous deletion of the activin A gene show a specific decrease in the number of rod photoreceptors compared to wild-type or heterozygous littermates. These data demonstrate that activin A is an important regulator of photoreceptor differentiation in the developing retina.

INTRODUCTION

The regulation of cell division and differentiation during neural development are known to involve cellular interactions and several types of signaling molecules. In the present study we have investigated the developmental role of one member of the transforming growth factor β (TGF β) superfamily of growth factors, activin A. The TGF β superfamily is made up of a large number of structurally related secreted factors including TGF β, bone morphogenetic protein (BMP), growth and differentiation factor, and activin subfamilies, as well as other related proteins. This group of factors has a variety of effects in many different systems during development, including the dorsoventral patterning of the embryo, regulation of the secretion of extracellular matrix components, induction of apoptosis, and control of cell differentiation (Q. Li et al., 1998; reviewed in Hogan, 1996; Matzuk et al., 1996).

The activins have been shown to be expressed widely in the developing nervous system. For example, activin A and activin B have been shown to be present in the developing brain and spinal cord (Feijin et al., 1994; Roberts and Barth, 1994, Andreasson and Worley, 1995). Activin A expression in the developing cortex is in the newly postmitotic cortical plate and differentiating layers and correlates with the inside-out progression of cortical development such that activin is expressed during the time of cytodifferentiation (Andreasson and Worley, 1995). Activin A has also been described to be in the developing cortex and striatum from embryonic day (E) 16 to E20, while activin B is found in the developing ventricular zone of the rat nervous system from E14 to E20). Both activin type II receptors have also been shown to be present in these regions during early nervous system development, as well as being expressed in the developing retina (Roberts and Barth, 1994). The activin type I receptors, Alk 2 and Alk 4, have also been shown to be present in early nervous system development, Alk 4 being expressed in the developing neural retina at E12.5 (Vershueren et al., 1995).

Previous reports of the functions of activin in neural development have concentrated on its role in the regulation of neurotransmitter phenotype. In vitro administration of activin A regulates the expression of somatostatin in ciliary ganglion neurons (Coulombe et al., 1993). Activin A also induces the expression of mRNA encoding several neuropeptides in sympathetic neurons (Fann and Patterson, 1994), and it has been shown to increase tyrosine hydroxylase expression in basal forebrain progenitor cells in conjunction with basic FGF (Daadi et al., 1998).
However, since activins and their receptors are expressed in the developing CNS ventricular zone, we suspected they might also play some role in the process of neurogenesis. In order to determine whether activin A has a role in regulating neuronal production, we have used a cell culture system that supports both the proliferation of these progenitor cells and the differentiation of retinal neurons (Reh and Kljavin, 1989; Anchan et al., 1991; Reh, 1992; Kelly et al., 1994). In these studies, the developing mammalian retina was chosen as a model system because the timing and antigenicity of the different cell types produced are well characterized (see Reh and Levine, 1998, for review). Ganglion cells and horizontal cells are produced first, followed in sequence by the production of cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells, and Muller glia. Previous work in our lab, and others, has shown that high-density cultures of embryonic or neonatal rat retinal cells develop at rates that approximate those found in vivo (Reh and Kljavin, 1989; Watanabe and Raff, 1990; Anchan et al., 1991; Altshuler et al., 1993; Kelly et al., 1994, 1995). Using this retinal culture system a number of factors have been shown to affect the proliferation and differentiation of retinal progenitor cells. For example, this paradigm has been used to identify EGF, TGF α, and TGF β3 as mitogens for retinal progenitor cells (Anchan et al., 1991; Lillien and Cepko, 1992; Anchan and Reh, 1995; see Cepko et al., 1996, for review). Similarly, retinoic acid, sonic hedgehog, taurine, and S-laminin have been shown to promote photoreceptor differentiation (Kelly et al., 1994; Levine et al., 1997; Altshuler et al., 1993; Hunter et al., 1992). In this study, we have used this retinal culture system and analysis of activin βA knockout mice (Matzuk et al., 1995) to investigate the influence of activin A on neural progenitor cells.

RESULTS

Activin βA Expression during Retinal Development

As noted above, activins are expressed in the developing central nervous system of the mouse and rat (Feijen et al., 1994; Roberts and Barth, 1994; Andreasson and Worley, 1995). In the eye, activin A has been shown to be expressed in the pigmented epithelium of the adult human (Jaffe et al., 1994) and the embryonic mouse (Feijen et al., 1994). We confirmed the expression of activin βA in the rat retina pigmented epithelium (RPE) by RT-PCR and in situ hybridization. RNA was extracted from embryonic and postnatal rat RPE tissue and subjected to reverse transcription prior to PCR with primers specific for the activin βA message (Fig. 1A). We found that activin βA is expressed in the developing eye as early as E15 in the rat, similar to the observations of Feijen et al. (1994) in the mouse, and continues to be expressed in the RPE in later embryonic (E18) and an early postnatal stage (P4) (Fig. 1A). For comparison, expression of activin βA was also determined for the limb bud at E14, a region that has been previously reported to express activin βA (Feijen et al., 1994). We confirmed the RT-PCR results with in situ hybridization. For these studies we used albino animals, since the pigmentation obscures the in situ signal. Figure 1B shows an example of the labeling that we observed at E18; the retinal pigmented epithelium shows a relatively higher level of signal, while a medium level of expression is observed in the inner retinal cells, and a low level of expression is present throughout the retina. A recent study of activin expression in developing chick retina found a similar level of expression in the RPE at a similar stage of development (Belecky-Adams et al., 1999). Thus, activin βA is expressed in the RPE during the period of retinal neurogenesis.

Activin A Promotes Retinal Progenitor Differentiation in Vitro

Previous work has shown that approximately 60–70% of retinal cells cultured from E18 rat are mitotically active progenitor cells on the day of plating (Reh and Kljavin 1989). Many of these cells continue to proliferate for at least 1 week in vitro, and retinal neurons of various types are generated in these cultures (see Introduction). On average these cells double their number every 2 days under control conditions (Anchan et al., 1991). Supplementation of the medium with soluble growth factors, such as TGF α or FGF can stimulate the proliferation of the progenitor cells and the production of some types of retinal neurons (Anchan et al., 1991; Lillien and Cepko, 1992; Anchan and Reh, 1995). We therefore assayed the effects of activin A in a similar manner. Cells were plated at high density and recombinant activin A added immediately after plating and maintained in the medium for the duration of the experiment.

We assayed the effects of various concentrations of activin A on retinal progenitors after 6 days of culture. We found that activin A inhibited the proliferation of the progenitor cells. From a starting population of 750,000 cells/well, the control cultures go through nearly three population doublings in 6 days in vitro to generate, on average, 4.25 million cells. In contrast, the cultures treated with 100 ng/ml activin A had only 1.15 million cells after 6 days of culture, less than one population doubling. This effect was not due to induction of cell death; examination of cells with DAPI revealed only
about 2% of the cells had pyknotic nuclear changes in either the control or the activin A-treated cultures.

To further characterize the effect of activin A on retinal progenitor cell proliferation, we used immunohistochemistry for antigens known to be expressed by progenitor cells. Labeling of cells with an antibody against nestin, an intermediate filament protein expressed by central nervous system progenitor cells, showed that the number of nestin-immunoreactive cells declined in the cultures as the concentration of activin A was increased. Figures 2A and 2C show nestin-immunolabeled cells from a control culture and an activin A-treated culture; activin A causes a decline in the percentage of nestin-labeled cells. Figure 2E quantifies the results from three experiments (see Experimental Methods for details of the quantification), showing the dose–response relationship for activin A. In the control cultures, nearly 40% of the cells are nestin immunoreactive, while only 20% of the cells in the cultures remain progenitor cells when treated with 100 ng/ml activin A. As an additional indicator of progenitor cell number, we labeled the cultures with antibodies against PCNA, a cell cycle protein expressed in all retinal progenitor cells in vivo. Figures 2B and 2D show photomicrographs illustrating a decrease in PCNA-immunolabeled cells when activin A is added to the culture. As quantified in Fig. 2F, the percentage of PCNA-labeled cells also declines with the addition of activin A to the cultures, although this effect is only statistically significant at higher activin A concentrations. While these data illustrate that activin treatment decreases the number of two progenitor cell markers in these cultures, the dose–response curves for the PCNA and nestin are not identical. This may be due to the fact that PCNA can also be expressed at a lower level in some postmitotic cells. Alternatively, TGF β-family proteins frequently have concentration-dependent actions that differ for different downstream genes.

**Activin A Causes Retinal Progenitor Cells to Differentiate as Photoreceptors in Vitro**

The initial results from the addition of activin A to the E18 rat retinal cultures indicated that activin A caused progenitor cells to exit the cell cycle and differentiate. We were interested in determining whether activin A caused the progenitor cells to differentiate into all retinal cell classes or some particular types of retinal neurons. Therefore, we labeled the cultures with several different
antibodies against proteins exclusively expressed in particular types of retinal cells. We found that the addition of activin A markedly increased the number of photoreceptors in E18 rat retinal cultures (Fig. 3). Figure 3A shows the control condition; at 6 days in vitro within a confluent field of cells there are only a few isolated cells labeled with 4d2, an antibody specific for rod opsins. Figure 3B shows a sister culture treated with activin A (100 ng/ml); there is a large increase in the number of opsin-positive cells with activin A addition to
the cultures. Figure 3C shows the dose-response relationship for this increase in photoreceptors; two separate photoreceptor-specific antibodies, 4d2 and recoverin, show a similar increase with increasing concentrations of activin A, while C1-labeled amacrine cells show no response to activin A. Scale bar, 50 µm.

FIG. 3. (A) The control condition at 6 days in vitro: within a confluent field of cells there are only a few isolated cells labeled with 4d2, an antibody specific for rod opsin. (B) A culture treated with activin A (100 ng/ml), illustrating a large increase in the number of opsin-positive cells. (C) The dose-response relationship for this increase in photoreceptors; two separate photoreceptor-specific antibodies, 4d2 and recoverin, show a similar increase with increasing concentrations of activin A, while C1-labeled amacrine cells show no response to activin A. Scale bar, 50 µm.

Cells at earlier stages of retinal development (McCaffery et al., 1993), at the developmental stages (E18–adult) used in the present studies the expression of this antigen is restricted to a subpopulation of GABAergic amacrine cells in the retina (DeLeeuw et al., 1990).

To determine whether activin caused progenitor cells to differentiate into photoreceptors directly, we performed the following experiment. We exposed retinal explants to BrdU for 2 h prior to dissociation for culture. This labeled a population of the cycling progenitor cells at the time of culture. This strategy for pulse labeling essentially “birth-dates” the cells born near the time of the pulse, since any progenitors that continue to divide will be undetectable after two to four divisions due to the dilution of the BrdU. The retinas were then dissociated and cultured as before. After 6 days in vitro, we fixed these cultures and labeled them with antibodies specific for either photoreceptors or amacrine cells. The cultures were then processed for BrdU immunohistochemistry. Any cells in S phase at the time of dissociation that subsequently differentiated as photoreceptors or amacrine cells would be double-labeled for BrdU and a cell-type-specific antigen. Figure 4 shows an illustration of single- and double-labeled amacrine cells (C1 positive, arrow; C1 positive/BrdU positive, arrowhead; Fig. 4A) and photoreceptors (recoverin positive, arrow; recoverin positive/BrdU positive, arrowhead; Fig. 4B) that we observed in the cultures. Figure 4C is a graph of the results of the cell counts from these experiments. In the control cultures, approximately 15% of the progenitor cells labeled with BrdU differentiate as photoreceptors, while nearly 30% of these cells differentiated as photoreceptors with the addition of 100 ng/ml activin A. In contrast, the number of BrdU-positive cells that are colabeled for C1 shows a slight decrease with the addition of activin A to the cultures. This indicates that activin A causes an increase in the number of progenitor cells that differentiate into photoreceptors, but not a similar increase in the formation of amacrine cells. These experiments support the hypothesis that activin A treatment is causing progenitor cells to differentiate into photoreceptors in vitro. We cannot, however, rule out that activin also increases the rate of rod photoreceptor differentiation by those cells already committed to this fate.

Activin A Is Important for Normal Photoreceptor Differentiation in Vivo

To determine if activin A is necessary for normal photoreceptor differentiation in vivo, we analyzed photoreceptor number in mice with homozygous deletion of the activin βA gene. Retinal sections from the centralmost region of both control and knockout newborn
animals were labeled with cell-type-specific antibodies. We labeled rod and cone photoreceptors with the anti-recoverin antibody (Figs. 5A and 5B) or a population of ganglion cells with the anti-Brn3.2 antibody (Xiang et al., 1993) (Figs. 5C and 5D). As shown in Table 1, we found that there was a modest but significant decrease in photoreceptors (recoverin-positive cells) in the activin βA knockout compared to the wild-type littermate controls. It may be that this decrease is not larger because the recoverin antibody also labels cone photoreceptors. Pooling the data for the litters, in the first litter (27xx) the activin βA knockouts had 35.3 ± 4.7 recoverin-positive cells in a 40× field compared to the controls with 49.6 ± 3.3 recoverin-positive cells. In the single knockout from the second litter (29xx), we found 30.4 ± 1.5 recoverin-positive cells compared with the control with 43.2 ± 5.2. This decrease was significant in each case, $P < 0.001$ (Figs. 5A and 5B). We observed that between litters there were no significant differences between the knockout and the control in the number of Brn 3.2-positive ganglion cells present (Figs. 5C and 5D) (27xx: knockout mean 120.4 ± 12 vs control mean 123.8 ± 15, $P = 0.34$. 29xx: knockout mean 64.6 ± 7.7 vs control mean 66 ± 9.3, $P = 0.40$). None of the retinas showed immunolabeling for opsin, since the protein is not expressed at high enough levels at this developmental stage (P0). These data support the in vitro data that activin A plays a role in photoreceptor differentiation in vivo.

### DISCUSSION

**Activin A Is a Mitotic Inhibitor/Differentiation Factor**

Previous reports have shown that activin and its receptors are present in the developing ventricular zone of the central nervous system. Activin and its receptors are also present in the developing retina. In this report, we have shown that activin A can promote the differentiation of retinal progenitor cells. In the activin A-treated cultures, cells went through fewer mitotic divisions, and fewer cells expressed the progenitor phenotype as evidenced by the decrease in the number of nestin- and PCNA-immunoreactive cells. This effect is not due to an induction of cell death in the progenitor cells, but rather activin A causes an increase in the number of progenitor cells that exit the cell cycle and differentiate into photoreceptor cells.

There are now several known mitogenic factors for neural progenitor cells, including FGF, EGF, TGFβ3, and sonic hedgehog (see Reh and Levine, 1998, for review). These factors all cause an increase in the proliferation of the progenitor cells in various regions of the developing CNS. In contrast, activin A is one of the few factors that causes cell cycle exit when added to dissociated cell cultures of progenitor cells. Other factors that have been reported to have a similar effect are BMPs and NT3. In other regions of the CNS, BMPs have been implicated in cell differentiation.
colleagues report an increase in the differentiation of cortical neurons from ventricular zone precursor cells in dissociated or explant cultures (W. Li et al., 1998). BMPs have also been implicated in the process of glial cell differentiation from embryonic subventricular zone cells (Gross et al., 1996). This effect on glial differentiation appears to be mediated via the SMAD1 signaling pathway, in conjunction with LIF, regulating the transcription of glial fibrillary acidic protein (Nakashima et al., 1999). In the retina, the only other factor that has been reported to cause cell cycle exit and differentiation is neurotrophin 3 (NT-3), which has also been reported to cause retinal progenitor cells to differentiate (Bovolenta et al., 1996; de la Rosa et al., 1994). Rodriguez-Tebar and colleagues found that in the embryonic chick, exogenous NT-3 added to cultured retinal cells promotes their precocious differentiation, while the addition of an antibody that blocks endogenous NT-3 causes the retinal progenitors to remain in a mitotically active state longer than they normally would (Bovolenta et al., 1996). Since these studies were carried out in the chick, it is not known whether NT-3 has a similar function in the mammalian retina. However, no retinal defects have been reported in NT-3 knockout mice (Ernfors et al., 1994; Farinas et al., 1994).

Both cell cycle proteins and cell cycle inhibitors are potential targets of activin receptor activation. In hepatoma cells, activin A inhibits cell proliferation by activating the transcription of the cdk inhibitor protein p21(waf) (Albrecht et al., 1997; Zauberman et al., 1997). In plasma-cytic cells, in addition to increasing expression of p21, activin causes a reduction in the phosphorylation of Rb

FIG. 5. Retinal sections from the central-most region of both knockout (A and C) and wild-type (B and D) newborn animals. (A and B) Sections labeled with immunohistochemistry for photoreceptors with the anti-recoverin antibody showing a decrease in photoreceptors in the knockout (−/−) compared with the control. (C and D) Sections labeled with Brn 3.2, an antibody specific for ganglion cells. Scale bar, 50 µm.
and a decline in the level of cyclin D2. It remains unknown whether p21 is regulated by activin in the nervous system; however, the related cell cycle regulator, p27, is known to be important for normal retinal development (Nakayama et al., 1995; Levine et al., unpublished observations). In addition, cyclin D1 is critical for normal retinal cell proliferation (Godbout and Andison, 1996). In the retina, activin A could be acting as a factor to regulate cell cycle progression, balancing the mitogens and thereby controlling the proportion of progenitor cells that enter S phase. Activin A could exert its inhibitory effects on retinal proliferation by upregulating the expression of cell cycle inhibitors, like p21 and p27. Alternatively, activin A could act by downregulating the expression of the cyclin genes themselves.

### Activin A Causes Rod Photoreceptor Differentiation

Activin A does not cause an increase in the differentiation of progenitor cells into all cell types in the retinal cultures, but rather it causes a specific increase in the number of rod photoreceptors. Other factors that cause specific increases in photoreceptor differentiation in these cultures include retinoic acid, sonic hedgehog, and taurine (Altshuler et al., 1993; Kelly et al., 1994; Levine et al., 1997). The effects of activin A are different from those of these other factors, but most similar to the effects seen with retinoic acid. Similar to activin A, retinoic acid promotes photoreceptor differentiation of mitotically active progenitor cells with a concurrent decrease in amacrine cell production. In comparison, neither sonic hedgehog nor taurine has effects on amacrine cell development; i.e., the promotion of the photoreceptor cell differentiation by these factors does not come at the expense of amacrine cells. Activin A also differs from these other photoreceptor differentiation factors in its effects on cell proliferation in this system; although activin A inhibits cell proliferation of the retinal progenitor cells, Shh causes an increase in cell number prior to differentiation into rod photoreceptor cells (Levine et al., 1997; Jensen and Wallace, 1997), while retinoic acid and taurine do not have any reported effects on cell proliferation in these cultures. Thus, activin A has a unique effect to promote progenitor cell exit from the cell cycle and subsequent differentiation into photoreceptors.

In the developing chick retina, activin does not promote the differentiation of photoreceptors, but rather has somewhat of the opposite effect (Belecky-Adams et al., 1999). 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. Moreover, in the chick retina, activin promotes the development of amacrine cells, while in the rodent retina, activin has a small but significant inhibitory effect on CRABP-immunoreactive amacrine cell differentiation. While we do not know why there should be this difference between the effects of activin on the chick and the rodent retinal development, there is good precedent for such differences in environmental factors in the regulation of photoreceptor differentiation between these classes. For example, CNTF and LIF promote photoreceptor differentiation in the chick retina while inhibiting rod photoreceptor differentiation in the chick retina (Kirsch et al., 1996, 1997, 1998; Ezzeddine et al., 1997). In addition, retinoic acid does not have identical effects in the two systems, since it promotes rod photoreceptor differentiation from embryonic retinal progenitor cells, but acts as a survival factor in the chick retina (Kelley et al., 1994; Stenkamp et al., 1993). These data, taken together with the present finding that activin also has opposing effects on photoreceptor development in these two classes, suggest that there may be fundamental differences in the mechanisms that control photoreceptor differentiation in birds and mammals.

Given the relatively large number of factors that promote the development of rod photoreceptor cells in retinal cultures, it will be interesting to determine whether these factors act in a single molecular pathway or alternatively act through independent parallel pathways. Several studies have noted interactions between retinoic acid and activin A in their effects on other tissues. Activin A and retinoic acid synergize in their effects on macrophage differentiation (Uchiyama and

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**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Recoverin</th>
<th>Opsin (4d2)</th>
<th>Brn 3.2</th>
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<td>64.6 ± 7.7*</td>
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<td>2992 (control)</td>
<td>43.2 ± 5.2</td>
<td>None detected</td>
<td>66 ± 9.4*</td>
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*Done in serial sections to recoverin-labeled tissue.

Note. Cell counts of immunolabeled cells were performed using cell-type-specific antibodies. Recoverin labels rod and cone photoreceptors and rod bipolar cells, Brn 3.2 labels ganglion cells. Opsin was not detected in any of the 4d2-labeled sections. Numbers represent means ± standard deviation for each animal. Animals from two separate litters counted, 27xx n = 3 sections per animal, 29xx n = 6 sections per animal. The most central region of each retina was counted. Note that sections assessed for Brn 3.2 in 2710 and 2713 were more oblique than for 2708 and 2711.
Asashima, 1992; Nusing et al., 1995), Ca²⁺ channel expression in neuroblastoma cells (Fukuhara et al., 1997), Gnot1 gene expression in the gastrulating chick embryo (Knezevic et al., 1995), and c-jun expression in P19 cells (Momoi et al., 1992). Retinoic acid induces activin receptor IIB mRNA in F9 carcinoma cells (Wan et al., 1995) or activin receptor IIA mRNA in P19 cells (Ameerun et al., 1996). However, it is not known if this is the mechanism underlying the reports of synergistic actions. In addition, retinoic acid and sonic hedgehog are known to interact in patterning chick limb buds (Helms et al., 1994; Ogura et al., 1996). Future studies will be needed to elucidate the precise mechanisms by which these pathways interact. Rod photoreceptor differentiation is a useful system to study the interactions between these pathways.

**EXPERIMENTAL METHODS**

**RNA Extraction, PCR, and in Situ Hybridization**

The pigment epithelium was dissected from the eyes of E15, E18, and P2, P4, and P7 animals and homogenized in Trizol solution, and the total RNA was extracted with 1:1 phenol:chloroform and ethanol precipitation. The total RNA was reverse transcribed with random hexamer primers for 1 h at 42°C in a 20-µl reaction containing the following: 1–3µg of retinal RNA, 50 mM KCl, 20 mM Tris–HCl, pH 8.3, 2.5 mM MgCl₂, 0.001% gelatin, 1 mM dNTPs (Pharmacia, Pleasant Hill, CA), 30 units RNasin (Promega, Madison, WI), 100 pmol random hexamers (Pharmacia), 200–400 units MoMuLV reverse transcriptase (BRL, Bethesda, MD), and 10 mM DTT. An aliquot of the cDNA (10µl) was then amplified for 30 cycles on a Perkin–Elmer thermocycler in a 50-µl reaction containing 500 ng primers, 2.5 pmol random hexamers (Pharmacia), 200–400 units MoMuLV reverse transcriptase (BRL, Bethesda, MD), and 10 mM DTT. An aliquot of the cDNA (10µl) was then amplified for 30 cycles on a Perkin–Elmer thermocycler in a 50-µl reaction containing 500 ng primers, 2.5 units Taq polymerase (BRL), and final buffer concentrations of 50 mM KCl, 10 mM Tris–HCl, pH 8.3, and 1.9–2.3 mM MgCl₂. Oligonucleotide primers were sequences specific to rat activin βA (GGCCTTTAAAGAGGCAAACC forward primer and ATGTTCACCCATTGAAAGGC reverse primer). The PCR products were analyzed by electrophoresis on a 1% agarose gel to verify that the appropriate predicted sized product was produced. In situ hybridization was carried out on embryonic retinas as described in Chow et al. (1999). The activin βA probe was generated from the PCR product cloned into pBluescript.

**Cell Culture**

Timed pregnant Sprague–Dawley rats were obtained from Simonsen Laboratories and housed at the University of Washington Department of Comparative Medicine’s facility. Pregnant females were sacrificed with CO₂ and the embryos were dissected into sterile Hanks’ buffered saline solution with Hepes buffer at 4°C. Neural retinas were dissected from the embryos and then dissociated by mild trituration after a 10- to 15-min incubation at 37°C in calcium/magnesium-free saline with trypsin (0.025%). Total cell number was determined with a hemacytometer and cells were plated onto coverslips coated sequentially with polylysine and Matrigel (1:100 dilution in HBSS; Collaborative Research) in 24-well plates at a density between 200,000 and 500,000 cells per well. All cultures were maintained at 37°C and 5% CO₂. Cultures were maintained for periods of 1 to 7 days in low-serum tissue culture medium and one-half of the medium in each well was changed every 48 h. The culture medium contained DMEM:F12 (without glutamate or aspartate), 25 mg/ml insulin, 100 mg/ml transferrin, 60 mM putrescine, 30 mM selenium, 20 nM progesterone, 100 units/ml penicillin, 100 mg/ml streptomycin, 0.05 M Hepes, and 1% fetal bovine serum (Gibco BRL). Recombinant human activin A (Austral Biological) was added on the first day of culture and replaced at each feeding.

**Determination of Cell Phenotypes**

In most experiments, after culture periods of 1 to 7 days, the cells in each well were dissociated with trypsin, counted with a hemacytometer, and then replated onto polylysine-coated coverslips for 2–6 h before fixation and subsequent immunohistochemistry. In the case of the knockout mice, eyes were fixed with 4% paraformaldehyde, embedded in OCT, and sectioned at 12 µm on a cryostat. The coverslips or slides were processed for immunohistochemistry using previously described methods (Reh and Kljavin, 1989; Kljavin and Reh, 1991). Primary antibodies used in these experiments were as follows: (1) rod-specific opsins, 4d2 monoclonal antibody from Dr. R. Molday and Dr. D. Hicks, University of British Columbia (Hicks and Barnstable, 1987); (2) recoverin, affinity-purified polyclonal rabbit antisera from Dr. J. Hurley, University of Washington (Dizhoor et al., 1991; Milam et al., 1993); (3) CRABP, C1 monoclonal antibody from Dr. J. Saari, University of Washington (De Leeuw et al., 1990); (4) nestin monoclonal antibody from the Developmental Hybridoma Bank; and (5) Brn 3.0, affinity-purified polyclonal antisera from Dr. E. Turner, University of California at San Diego. Primary antibody labeling was detected using fluoresceinated secondary antibodies. Labeled cells were viewed on a Zeiss standard compound microscope with epifluorescence illumination, and the number of labeled...
cells on each coverslip was quantified in most cases by counting all of the labeled cells in either a vertical or horizontal strip across the entire coverslip or alternatively by counting six random fields per coverslip. Sections of the knockout animal were assessed by counting fields in the central retina in several adjacent sections.

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