Development/Plasticity/Repair

# A Role of *ath5* in Inducing *neuroD* and the Photoreceptor Pathway

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**Photoreceptors in the vertebrate retina are light-sensitive neurons, and their degeneration results in irreversible visual loss. Understanding how photoreceptor fate is determined is a prerequisite for developing photoreceptor replacement therapies. Previous studies identified two basic helix-loop-helix genes, *neurogenin2* (*ngn2*) and *neuroD*, participating in a genetic pathway leading to photoreceptor genesis. Here we present experimental data suggesting that *ath5*, which is known for its critical role in retinal ganglion cell development, may also lead to photoreceptor production. In the developing retina, *ath5* expression was detected in two zones of cells, and coexpression with *neuroD* was observed in the zone adjacent to young photoreceptor cells accumulating on the retinal pigment epithelial side. Retroviral-driven misexpression of *ath5* in retinal cells increased the population of photoreceptor cells, as well as ganglion cells, in a developmental stage-dependent manner that is consistent with *ath5* being involved in the development of multiple types of retinal neurons. Ectopic *ath5* expression in cultures of non-neural retinal pigment epithelial cells elicited transdifferentiation into cells that expressed photoreceptor-specific genes and displayed photoreceptor-like morphologies. Gene expression analysis showed that *ngn2* did not induce *ath5*, and *ath5* did not induce *ngn2*, but both induced *neuroD* and *RaxL*. These data suggest a pathway of "*ath5* → *neuroD* → photoreceptor genes" separate from yet convergent with the *ngn2* pathway.**

*Key words:* **gene; transcription; differentiation; regeneration; photoreceptor; retina**

## Introduction

The vertebrate retina contains five major types of neurons: photoreceptor, horizontal, bipolar, amacrine, and ganglion cells. Photoreceptor cells are light-sensitive neurons, and their degeneration can result from light damage or genetic alterations, resulting in irreversible visual loss. Coaxing self-renewable, multipotent cells to differentiate (or transdifferentiate) toward photoreceptor cells has exciting clinical implications. Such an approach, however, relies heavily on knowledge about factors sufficient to induce the photoreceptor pathway. The molecular mechanism underlying photoreceptor genesis has been subjected to intensive investigation but remains not well understood. Previous studies have implicated two basic helix-loop-helix (bHLH) genes, *neurogenin2* (*ngn2*) and *neuroD*, participating in a genetic pathway leading to photoreceptor genesis (Yan and Wang, 1998; Marquardt et al., 2001; Yan et al., 2001). In the mouse retina, regions where *Pax6* is inactivated lack *ngn2* expression and contain no photoreceptor cells (Marquardt et al., 2001). In the chick retina, *ngn2* is expressed in proliferating progenitors (Yan et al., 2001). Ectopic *ngn2* expression in non-neural retinal pigment epithelial (RPE) cell cultures induces RPE transdifferentiation into cells that molecularly and morphologically resemble retinal neurons, including photoreceptor cells and retinal ganglion cells (RGCs) (Yan et al., 2001), and induces *neuroD* and *NSCL1*, which are expressed in developing photoreceptor cells and RGCs, respectively (Yan and Wang, 1998, 2004; Li et al., 1999b). *NeuroD* is expressed in the developing retina of many species, but its function remains unresolved (Kanekar et al., 1997; Brown et al., 1998; Yan and Wang, 1998, 2000a,b, 2004; Morrow et al., 1999; Pennesi et al., 2003). A prominent role for *neuroD* in photoreceptor genesis and development has been indicated by different studies. Pennesi et al. (2003) found photoreceptor-specific defects in *neuroD* knock-out mouse retina. Studies from our laboratory show that *neuroD* is both sufficient and required for photoreceptor formation in the chick retina (Yan and Wang, 1998, 2004).

In addition to *ngn2* and *neuroD*, the developing retina expresses several other proneural bHLH genes, including *ath5* (Brown et al., 1998; Liu et al., 2001; Wang et al., 2001; Stenkamp and Frey, 2003). Published studies show that *ath5* is required for RGC development (Brown et al., 2001; Kay et al., 2001; Wang et al., 2001) and may play a role in RGC genesis (Kanekar et al., 1997; Hutcheson and Vetter, 2001; Liu et al., 2001; Xie at al., 2004). Our recent studies indicate that *ath5* enhances basic FGF (bFGF)-initiated RPE transdifferentiation toward RGCs but alone is insufficient to induce this transdifferentiation (Ma et al., 2004).

The contribution of bHLH genes to retinal development seems complex, and little is known about how they network and how, through the network, they regulate the production of di-

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verse retinal neurons, including photoreceptor cells. To evaluate the proneural activity of *ath5* and its genetic relationship with other bHLH genes, we performed gain-of-function studies using retinal cells and RPE cells. We report here experimental data suggesting that *ath5* may lead to the photoreceptor pathway by inducing *neuroD*, in addition to taking part in RGC differentiation. The *ath5*-led photoreceptor pathway may be separate from yet convergent with the *ngn2* pathway.

## Materials and Methods

*Generation of recombinant retroviruses*. Replication-competent avian (RCAS) retroviruses expressing various genes were produced as previously described [*neuroD* and GFP (Yan and Wang, 1998); *NCSL1* (Li et al., 1999a); *ngn2* (Yan et al., 2001); *ath5* (Ma et al., 2004); and *ath5* and *NSCL1* coexpression through an internal ribosomal entry site (Xie et al., 2004)]. To generate RCAS expressing *RaxL*, the coding region was first amplified by reverse transcription (RT)-PCR based on the published sequence (Chen and Cepko, 2002) and cloned into pGEMT (Promega, Madison, WI). After sequence verification, *RaxL* was subcloned into shuttle vector Cla12Nco and then inserted into RCAS (Hughes et al., 1987). The titers of the virus stocks ranged from $5 \times 10^7$ to $2 \times 10^8$ pfu/ml. Different batches of independently produced viruses were used when repeating experiments to rule out the possibility that experimental observations were attributable to retroviral recombination.

*RPE cell culture*. Chick RPE was dissected free from the neural retina at embryonic day 6 (E6) as described (Yan and Wang, 1998). Pooled RPE tissues were incubated with typsin-EDTA, and the dissociated cells were cultured with medium 199 plus 10% fetal calf serum or knock-out DMEM plus 20% serum replacement (Invitrogen, San Diego, CA). When used, bFGF was added at a final concentration of 4, 10, or 25 ng/ml. When the culture was ~50% confluent, 10–20 μl of concentrated retrovirus expressing *ath5*, *neuroD*, *ngn2*, *NSCL1*, or green fluorescent protein (GFP) as a control was added to a 35 mm dish. Cultures were maintained for an additional 4–8 d, and cells in the culture were then harvested for RT-PCR or were fixed for immunocytochemistry or *in situ* hybridization. Viral infection of the culture was verified with immunodetection of viral protein p27. For reseeding, cells from a confluent culture were trypsinized, seeded onto poly-L-ornithine-treated coverslips, and cultured for 2–3 d with medium 199 plus 10% fetal calf serum before fixation for immunocytochemistry.

*Microinjection of retrovirus into the subretinal space and retinal cell culture*. Retroviruses expressing *ath5* or GFP as a control were microinjected into the subretinal space on E2 as previously described (Yan and Wang, 1998). Retinas were dissected from the injected eyes from E4.5 to E8.5, and dissociated retinal cells were seeded onto poly-L-ornithine-treated 35 mm culture dishes at low densities ($1–5 \times 10^4$ cells/cm$^2$) to minimize the potential influence of cell–cell contact on cell fate determination. After 4 hr [0 d *in vitro* (DIV)], 2 DIV, or 4 DIV in a cell culture incubator with medium 199 supplemented with 10% fetal calf serum, the cells were fixed with ice-cold 4% paraformaldehyde.

Double staining was performed to identify cells that were infected with the virus (p27$^+$) and developed as photoreceptor cells (visinin$^+$) or RGCs (RA4$^+$). The numbers of double- and single-labeled cells (from >1000 p27$^+$ cells) were counted, and the percentages of virus-infected cells that developed as photoreceptor cells or RGCs were calculated. The means and SDs from three dishes were determined with a computer program (Origin 7.0).

*Immunocytochemistry*. Monoclonal antibody RA4 (used at 1:1000 dilution) was a gift from Dr. Steven McLoon (University of Minnesota, Minneapolis, MN) The following monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA): anti-bromodeoxyuridine (BrdU, clone G3G4, 1:100; developed by Dr. Stephen J. Kaufman), anti-islet-1 (clone 39.4D5, 1:100; developed by Dr. Thomas Jessell), and anti-visinin (clone 7G4, 1:500; developed by Dr. Constance Cepko). Standard immunocytochemistry was performed with ABC-peroxidase, alkaline phosphatase-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA), or fluorophore-conjugated secondary antibodies (Molecular Probes, Eugene, OR).

**Table 1. Primers used for RT-PCR**

| Gene | 5′ primer | 3′ primer | Product length (bp) |
|---|---|---|---|
| *ath5* | cctgtcaatccagtcatttg | gaaagtgttcagggtgatag | 435 |
| *neuroD* | ggctgcctgcagctcaac | tcactcgtggaagatggcg | 509 |
| *ngn2* | gccggtgaaggcggagag | gcgataaagtgcaggcgtag | 545 |
| *visinin* | cgatgaattcgagcgcatttac | cttgggttcgtattggatgag | 436 |
| *RaxL* | gctgctgctgaggaggaac | ggctttcatgcggagcgag | 569 |

In situ *hybridization*. Digoxigenin (Dig)-labeled antisense RNA probes for photoreceptor-specific genes were prepared as previously described [visinin (Yan and Wang, 1998); and interphotoreceptor retinoid-binding protein (IRBP), rhodopsin, and red pigment (Yan and Wang 2000a)]. Antisense RNA probes against the chick *ath5* coding sequence were synthesized using the Genius kit (Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer's instructions. Embryonic eyes were fixed with 4% paraformaldehyde as previously described (Li et al., 1999b), and *in situ* hybridization was performed with 8–10 μm frozen sections on glass slides. For double *in situ* hybridization, the procedure used for single *in situ* hybridization was followed with the following modifications. Before proteinase K digestion, tissue sections on glass slides were incubated with 2% (v/v) hydrogen peroxide for 30 min. The FITC-labeled anti-*neuroD* mRNA probe was detected with horseradish peroxidase (HRP)-conjugated anti-FITC (Roche Molecular Biochemicals) followed by incubation with fluorescein-tyramide using the Tyramide Signal Amplification (TSA) Plus kit (PerkinElmer Life Sciences, Emeryville, CA) according to the manufacturer's instructions. The HRP conjugate was then inactivated by incubating the tissue sections in 2% (v/v) hydrogen peroxide for 30 min. The anti-*ath5* mRNA signal was then visualized with HRP-conjugated anti-Dig (Roche Molecular Biochemicals). The signal was developed by rhodamine-tyramide using the TSA Plus kit.

To obtain single-cell resolution, retinal sections on glass slides were subjected to prolonged proteinase K treatment followed by a gentle smearing with a coverslip to spread some retinal cells into the vitreous area. Retinal sections and cells on the glass slide were then fixed with 4% paraformaldehyde for 10 min at room temperature and subjected to the remaining steps of *in situ* hybridization.

*Double labeling for BrdU incorporation and* ath5 *expression*. E6 embryos were treated with BrdU (50 μg in 50 μl of HBSS) dropped through an opening in the shell onto the vitelline membrane, as opposed to injecting it systemically. The embryos were incubated for 4 hr before the eyes were harvested and fixed with 4% paraformaldehyde. Previous studies showed that a 4 hr incubation with BrdU applied this way labeled cells that were confined to the ventricular zone of the developing brain (Wang and Adler, 1995), whereas longer incubation posed the risk of allowing some of the BrdU$^+$ cells to enter postmitotic stage, migrate out side of the ventricular zone, and thus become false-positive by the time of tissue fixation (our unpublished observations). Frozen sections on glass slides were first subjected to *in situ* hybridization with digoxigenin-labeled anti-*ath5* RNA probes and then to BrdU detection using a specific antibody as previously described (Li et al., 1999b).

*RT-PCR*. RPE cells infected with RCAS expressing various genes were harvested from two 35 mm dishes, and their total RNA was isolated using the acid–guanidium isothiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). First-strand cDNA was synthesized with a cDNA synthesis kit (Ambion) using oligo-dT as the primer. After dilution to 350–800 μl with Tris-EDTA, 1 μl of the cDNA was added to each 30 μl PCR. Standard PCR conditions were followed. Amplification was performed for 30 cycles using gene-specific primers (Table 1) with an annealing temperature of 56°C. Ribosomal protein s17 was amplified as an internal control to normalize the amount of cDNA in each sample (Wang and Adler, 1994) with 20 cycles of amplification using primers gtgatcatcgagaag and agcaacataacgagc annealed at 44°C.

*Detection of apoptotic cells*. The presence of apoptotic cells in the de-

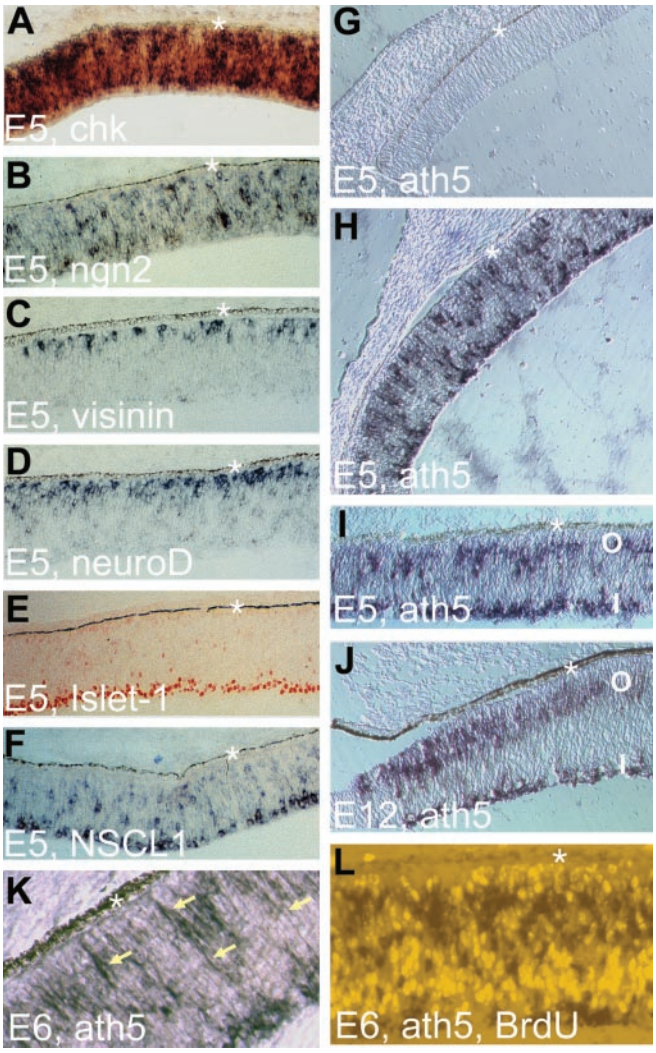


**Figure 1.** Expression of *ath5* in the developing chick retina examined with *in situ* hybridization. *A*, Expression of chromokinesin (*chk*, a marker of proliferating cells) at E5. *B*, Expression *ngn2* in cells scattered across the retina. *C*, Expression of *visinin*, a cone marker. *D*, Expression of *neuroD* in cells accumulating at the outermost portion, where young photoreceptor cells reside. *E*, Islet-1 expression in young RGCs. *F*, *NSCL1* expression in differentiating (and, in some cases, still migrating) young RGCs. *G*, Absence of *ath5* expression in the peripheral retina at E5. *H*, Expression of *ath5* in the midperipheral retina at E5. *I*, Expression of *ath5* in the central retina at E5. Note that two zones, the outer zone (O) and the inner zone (I), are clearly discernible. *J*, Expression of *ath5* in the peripheral retina at E12. *K*, *ath5* expression in the central retina at E6. Arrows, *ath5*-expressing cells with long processes. *L*, Absence of cells double-labeled for *ath5* expression (dark stain in the cytoplasm) and BrdU incorporation (bright nucleus). The asterisk denotes RPE. Magnification: *A–J*, 20×; *K*, *L*, 40×.

veloping retina infected with RCAS-ath5 and RCAS-GFP was examined with the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end-labeling (TUNEL) method using an *in situ* cell death detection kit (Roche Molecular Biochemicals) following the manufacturer's instructions.

## Results

### Coexpression of *neuroD* and *ath5* in the developing retina

To facilitate the understanding of its function and its relation with other bHLH genes, we undertook a reexamination of the spatial pattern of *ath5* expression in the developing retina. At E5, the chick retina is a pseudostratified structure composed mostly of proliferating cells distributed across the entire thickness, as shown by the expression of *chromokinesin* (Fig. 1*A*), a gene specifically expressed in proliferating cells (Wang and Adler, 1994, 1995). *ngn2* was expressed in cells scattered across the developing retina (Fig. 1*B*) (Yan et al., 2001). Young photoreceptor cells expressing *visinin* were localized on the RPE side (Fig. 1*C*), as were cells expressing *neuroD* (Fig. 1*D*). Differentiating RGCs (Islet-1$^+$) (Fig. 1*E*) accumulated on the vitreal side and transiently expressed bHLH gene *NSCL1* (Fig. 1*F*) (Li et al., 1999b). At this time, *ath5* mRNA was absent in the peripheral region (Fig. 1*G*), but was detected in the midperipheral region (Fig. 1*H*) and the central region (Fig. 1*I*) of the retina.

In E5 chick retina, there was a center-to-periphery developmental gradient in the spatial pattern of *ath5* expression. At the periphery, where development lags behind the center, cells expressing *ath5* were more or less randomly distributed (e.g., the midperiphery) (Fig. 1*H*). At the center, they became congregated at two zones (the midperiphery and the central region) (Fig. 1*I*). The inner zone of *ath5* expression coincided with the anatomical location of differentiating RGCs, and the outer zone was adjacent to young photoreceptor cells concentrating at the outer portion of the retina (Fig. 1*I*). In an E12 retina, the central and midperipheral regions no longer showed detectable levels of *ath5* expression, but the two expression zones were clearly visible at the very periphery (Fig. 1*J*).

Cells in the outer zone of *ath5* expression have long processes (Fig. 1*K*, arrows), typical of neuroblasts migrating between the apical and basal sides of the retina. This prompted us to examine whether *ath5*-expressing cells would incorporate BrdU. No double-labeled cells were found (Fig. 1*L*), suggesting that *ath5* was likely expressed in postmitotic cells.

To test the possibility that *ath5* might partake in photoreceptor genesis and induce *neuroD*, in addition to its well-known role in RGC development, we examined the embryonic retina to determine whether *neuroD* and *ath5* were coexpressed. Double *in situ* hybridization identified cells that coexpressed *neuroD* and *ath5* in the developing chick retina (Fig. 2). Double-labeled cells were always localized to the outer zone of *ath5* expression (Fig. 2*A–C*), which supports the notion that some of these cells would differentiate as photoreceptor cells. To eliminate ambiguities associated with tissue sections, we produced sections in which some individual retinal cells were dispersed across the vitreous. This allowed us to unequivocally identify individual doubled-labeled cells (Fig. 2*D–F*). Coexpression of *ath5* and *neuroD* was also observed in embryonic mouse retina (data not shown).

### Increase in photoreceptor population on *ath5* misexpression in retinal cells

The coexpression of *ath5* and *neuroD* was consistent with the possibility that *ath5* might be involved in photoreceptor production because *neuroD* is expressed in young photoreceptor cells and their precursors (Yan and Wang, 1998, 2004). However, studies published to date have given little indication of *ath5* playing a role in photoreceptor genesis. To test the possibility directly, we asked whether *ath5* could promote the photoreceptor pathway during retinal neurogenesis by examining whether retroviral-driven *ath5* misexpression in retinal precursor cells would result in an increase in the photoreceptor population. To promote viral infection of retinal cells, retrovirus RCAS-ath5 was microinjected into the subretinal space at E2, the optic cup stage, and the retinas were harvested for analysis at later stages of embryonic development, allowing the replication-competent retrovirus to spread among the proliferating retinal progenitors. To minimize the potential influence of cell–cell contacts, which may provide positive and/or negative feedbacks and thus affect cell production in the experimental retina, retinal cells were dissoci-

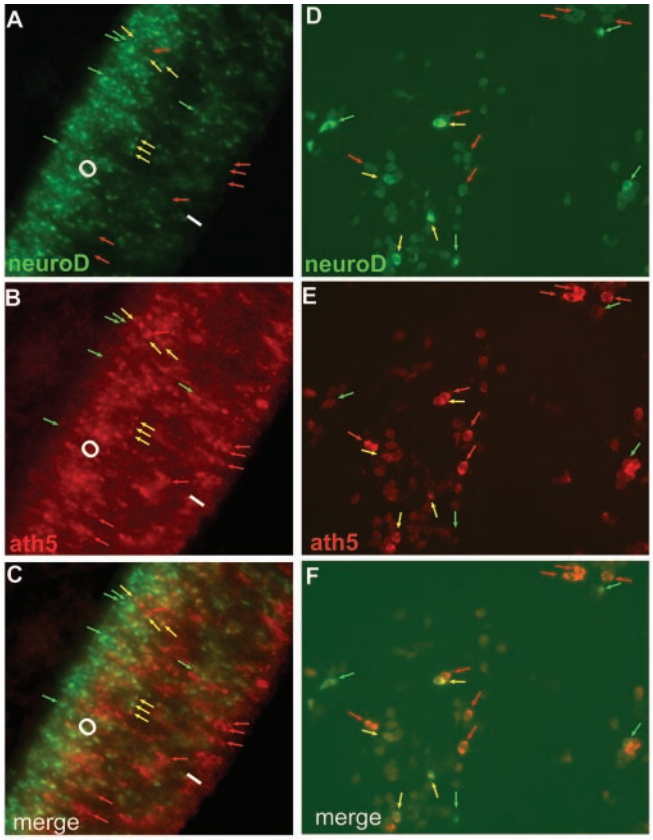


**Figure 2.** Coexpression of *neuroD* (green) and *ath5* (red) in E5 chick retina examined with double *in situ* hybridization. *A–C*, Retinal sections. *D–F*, Retinal cells dispersed into the vitreous area. Green arrows, Cells expressing *neuroD* only; red arrows, cells expressing *ath5* alone; yellow arrows, double-labeled cells. O, I, Outer and inner portions of *ath5* expression, respectively.

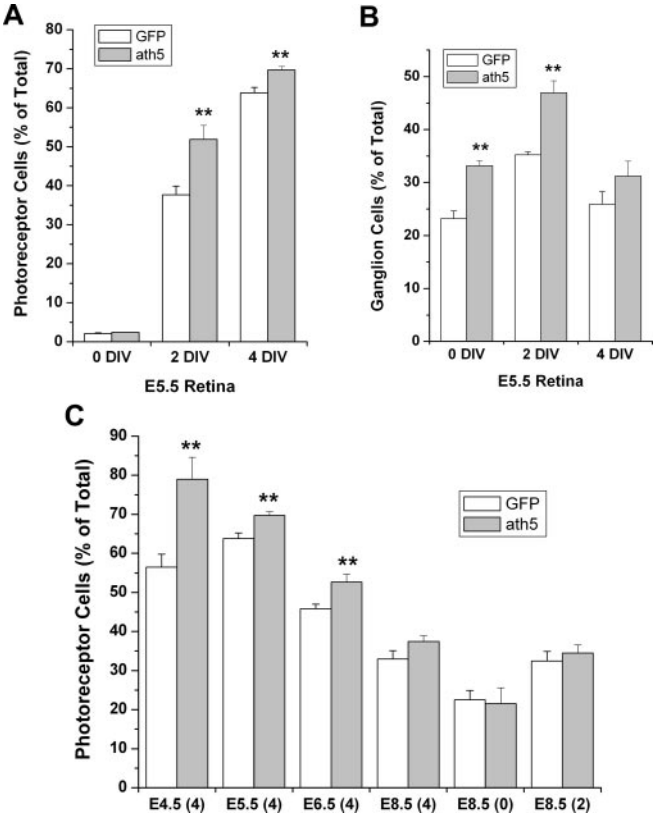


**Figure 3.** Effects of *ath5* misexpression in retinal cells on the relative populations of photoreceptors and RGCs. *A*, Photoreceptor populations in dissociated E5.5 retinal cells after 0, 2, or 4 DIV. **Significant increases at $p < 0.01$ in photoreceptor cell numbers were observed at 2 and 4 DIV. *B*, RGC populations in dissociated E5.5 retinal cells after 0, 2, or 4 DIV. Note that, unlike the photoreceptor population, the RGC population was increased in *ath5*-misexpressing samples at 0 and 2 DIV but not 4 DIV. *C*, Photoreceptor populations in dissociated retinas dissected at various developmental stages and subjected to low-density culture for the numbers of days indicated in parentheses.

ated and subjected to low-density culture for various times before fixation for analysis.

Because photoreceptor cell birth peaks at E6–E7 in the chick retina (Belecky-Adams et al., 1996), we first analyzed retina dissected at E5.5, when photoreceptor cells just begin to accumulate, so that the bulk of photoreceptor cells would be born under the low-density culture conditions. At the onset of the culture (0 DIV), the numbers of photoreceptor cells in retinas infected with RCAS-ath5 and the control RCAS-GFP were very low (~2% of total cells) and appeared statistically similar between the two groups ($p = 0.094$). After 2 DIV (equivalent to E7.5 *in vivo*), $37.7 \pm 2.2\%$ of total cells developed as photoreceptor cells in the control RCAS-GFP-infected retinas. In retinas infected with RCAS-ath5, the number became $51.8 \pm 3.6\%$, an increase of 38% (Fig. 3*A*). Subjecting E5.5 retinal cells to 4 DIV (equivalent to E9.5 *in vivo*) yielded a statistically significant ($p = 0.004$) yet smaller increase of photoreceptor cells in the retinas infected with RCAS-ath5 compared with those infected with RCAS-GFP (Fig. 3*A*). Thus, the increase in photoreceptor number appeared to decrease with longer culture time. This raised the question of whether the effect of *ath5* on photoreceptor production might be transient and developmental stage-dependent. To address this question, we analyzed the photoreceptor cell numbers from retinas dissected at different developmental stages, from E4.5 to E8.5. We observed, indeed, that increases in the photoreceptor population varied with developmental stages: the largest increase was observed with retinas dissected at E4.5, whereas no statistically significant increase was detected with retinas dissected at E8.5 regardless of 0, 2, or 4 DIV (Fig. 3*C*).

The time dependence of the photoreceptor-promoting effect of *ath5*, together with its known RGC-promoting activity, suggested the possibility that *ath5* might promote the production of different types of cells at different developmental stages. To test this possibility, we scored the RGC number at different *in vitro* time points and found that *ath5* misexpression resulted in an increase in RGCs in E5.5 retinas at both 0 and 2 DIV (Fig. 3*B*), but the increase diminished with continued culture for an additional 2 d (4 DIV) (Fig. 3*B*). Thus, the effect of *ath5* on RGCs might also be developmental stage-dependent. Additionally, RGCs, which are born earlier than photoreceptor cells, responded to *ath5* misexpression earlier than photoreceptor cells did.

TUNEL assays were used to examine whether *ath5* misexpression had a significant impact on retinal cell death. A comparison of the number of TUNEL$^+$ cells scored from 11 cross sections of E7 retina infected with RCAS-ath5 and RCAS-GFP revealed no difference (data not shown).

### Induction of RPE transdifferentiation toward photoreceptor cells by *ath5*

To assess the proneural activity of *ath5*, particularly in guiding non-neural cells to transdifferentiate into retinal neurons, we used an RPE transdifferentiation assay we have recently developed to examine whether ectopic *ath5* expression in RPE cell cultures would induce *de novo* production of cells with molecular

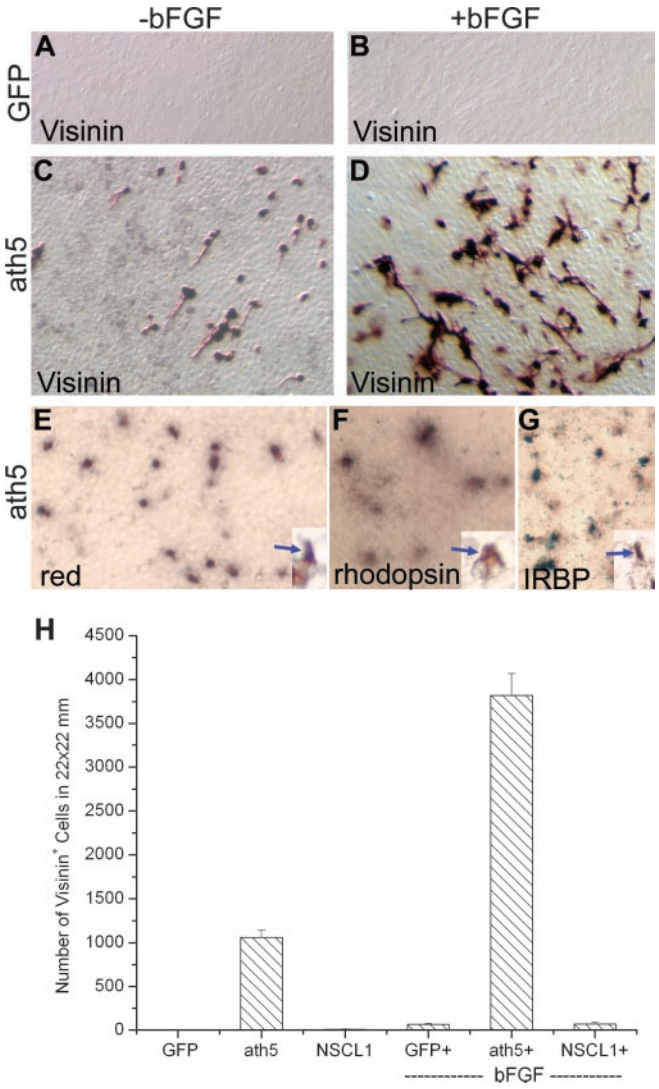


**Figure 4.** *De novo* generation of cells expressing photoreceptor-specific genes in RPE cell cultures induced by *ath5*. *A–D*, Immunostaining with a monoclonal antibody against visinin. The RPE cells were cultured in the presence (+) or absence (−) of bFGF and infected with RCAS-GFP (*A, B*) or RCAS-ath5 (*C, D*). *E–G*, *In situ* hybridization showing the expression of red pigment gene, rhodopsin, and IRBP in RPE cell cultures infected with RCAS-ath5. Insets, Positive cells at a higher magnification. *H*, Calculated number of visinin$^+$ cells. Magnification, 20×.

and morphological resemblance to RGCs or photoreceptors. Previously we have shown that cultured RPE cells are responsive to the proneural activities of *ngn2* and *neuroD*, and they may undergo transdifferentiation into cells that resemble photoreceptors and RGCs (Yan and Wang 1998, 2000a,b; Yan et al., 2001). We found previously that retroviral-driven ectopic expression of chick *ath5* promoted bFGF-initiated RPE transdifferentiation toward RGCs but alone was insufficient to induce such transdifferentiation (Ma et al., 2004). However, ectopic *ath5* expression in RPE cell culture induced *de novo* generation of cells expressing *visinin*. *Visinin* encodes a calcium-binding protein present in cones (Yamagata et al., 1990), the predominant photoreceptor type in the chick retina (Morris and Shorey, 1967). The number of visinin$^+$ cells increased severalfold when RPE cells were cultured in the presence of bFGF (Fig. 4*C,D,H*). No visinin$^+$ cells were present in the RPE cell cultures ectopically expressing GFP (Fig. 4*A,B*), *NSCL1*, or *NSCL2* (data not shown); the latter two are bHLH genes expressed in nonphotoreceptor cells in the retina (Li et al., 1999b, 2001).

Morphologically, the visinin$^+$ cells were neuron-like with a short process (Fig. 4*C,D*) in contrast to RPE cells, which have hexagonal morphologies in culture at high density or fibroblast-like morphologies at low density. This prompted speculation that *ath5* induced RPE transdifferentiation toward photoreceptor cells. To examine this possibility directly, we tested whether *ath5* induced the expression of other photoreceptor-specific genes and whether the transdifferentiating cells, when provided a more adhesive substratum, would develop morphologies typical of photoreceptor cells. Characteristic photoreceptor-specific genes include IRBP, which plays an important role in recycling and/or regeneration of retinoid (Gonzalez-Fernandez et al., 1998); the gene encoding the red pigment, a hallmark of cones that sense red light; and rhodopsin, a hallmark of rods. Whereas IRBP expression begins early during photoreceptor differentiation, genes encoding photopigments are expressed during late phases of photoreceptor development (Bruhn and Cepko, 1996); thus, their expression would indicate that *ath5* triggered the expression of genes associated with photoreceptor differentiation. Additionally, the inclusion of both cone and rod pigment genes would indicate whether *ath5* might lead to the cone pathway, the rod pathway, or both. We found that expression of IRBP, red pigment, and rhodopsin was induced in RPE cultures infected with RCAS-ath5 (Fig. 4*E–G*) and not in the control cultures infected with RCAS-GFP (data not shown) (Yan et al., 2001).

To evaluate whether those transdifferentiating cells could develop morphologies typical of young photoreceptor cells derived from the retina, we reseeded cells of RPE cultures infected with RCAS-ath5 onto coverslips coated with poly-L-ornithine (Adler, 1982) and cultured them in the same way as for dissociated retinal cells. On this substratum, visinin$^+$ cells developed highly structured morphologies resembling young photoreceptor cells derived from the developing retina (Fig. 5*D*) (Adler et al., 1984). They displayed an elongated cell body, an axon on the basal side (Fig. 5*A*, arrows), elaborate axonal arboration (Fig 5*A*, arrowheads), and an inner segment-like structure (Fig. 5*A*, open arrows). The morphologies of the visinin$^+$ cells generated by ectopic expression of *ath5* (Fig. 5*A*) were similar to those generated by expression of *neuroD* (Fig. 5*B*) and *ngn2* (Fig. 5*C*); thus, the amplitude of photoreceptor gene induction by *ath5* might be similar to that by *neuroD* and by *ngn2*.

### A genetic pathway of *ath5* → *neuroD* → *RaxL* and other photoreceptor genes

Because *neuroD* alone is sufficient to induce RPE transdifferentiation toward photoreceptor cells, and because some *ath5*-expressing cells in the developing retina coexpressed *neuroD*, we then asked whether *neuroD* was an intermediate in *ath5*-induced RPE transdifferentiation toward photoreceptor cells. RT-PCR demonstrated *neuroD* expression in RPE cell cultures infected with RCAS viruses expressing *ath5*, *neuroD* (a positive control), and *ngn2* (a positive control for *neuroD* induction) (Fig. 6). The induction of *neuroD* expression by *ath5* appeared to be stronger with bFGF than without, whereas the opposite seemed to be the case for *neuroD* induction by *ngn2*. Notably, the levels of *neuroD* induction by *ath5* and *ngn2* and the effect by bFGF were closely paralleled by the levels of *visinin* expression (Fig. 6). This was expected because *neuroD* and *visinin* are considered photoreceptor cell-specific. The RT-PCR products were verified by Southern DNA hybridization (data not shown). Neither GFP (a negative control) nor *NSCL1* (a negative control bHLH gene) induced any detectable level of expression of *neuroD* or *visinin* (Fig. 6).

Next we examined whether *RaxL* was a genetic target of *ath5*,

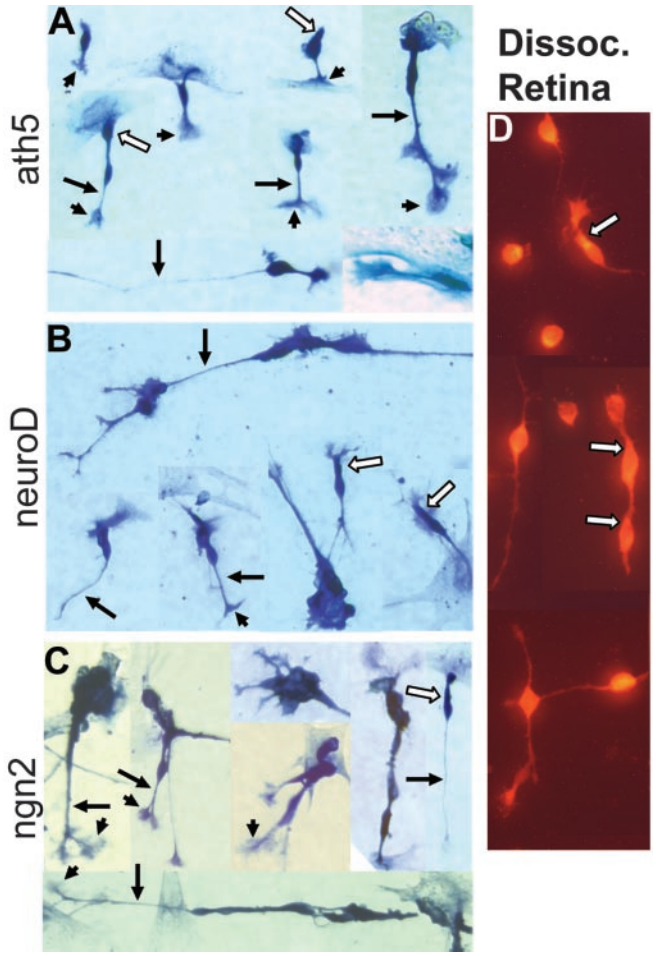


**Figure 5.** Development of photoreceptor-like morphologies after reseeding cells from RPE cell cultures infected with RCAS expressing different proneural bHLH genes onto poly-L-ornithine coated coverslips. *A–C*, Visinin $^+$ cells from RPE cell culture infected with RCAS-ath5 (*A*), RCAS-neuroD (*B*), or RCAS-ngn2 (*C*). *D*, Visinin $^+$ cells in cultures of dissociated retinal cells (Dissoc. Retina). Arrows, Axons; arrowheads, axonal arboration; open arrows, inner segment-like structure. Magnification, 40×.

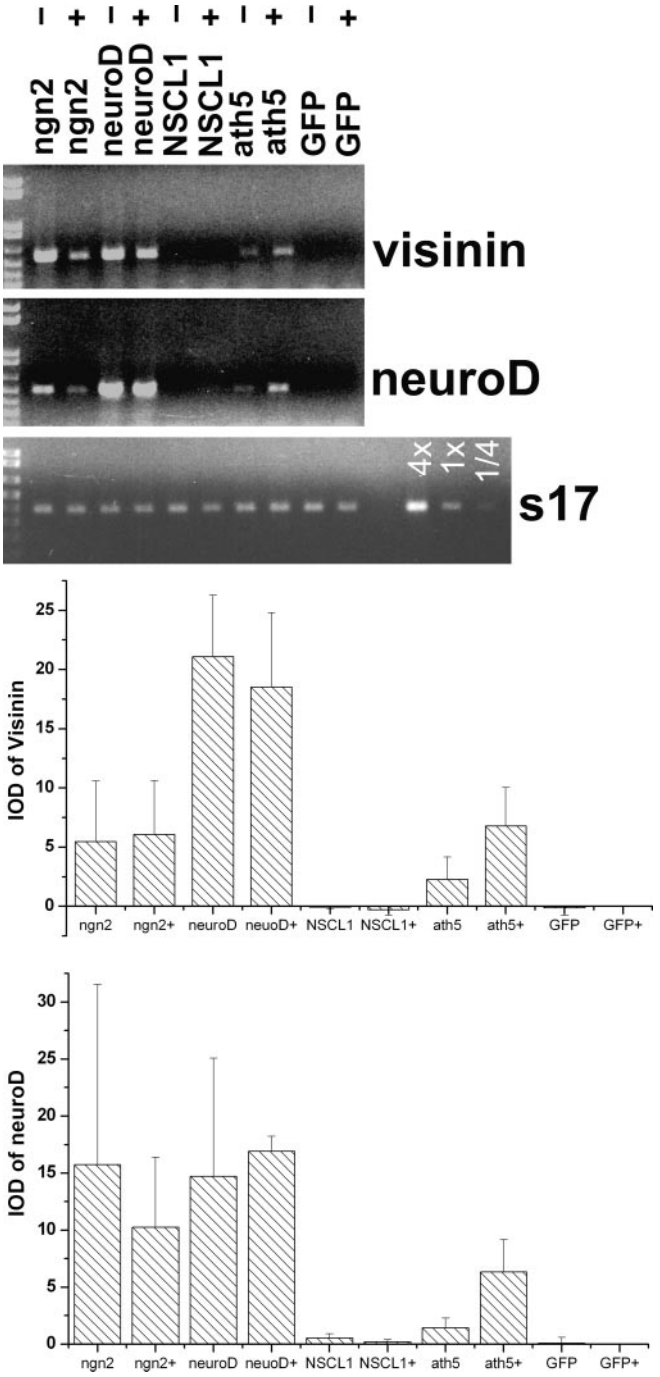


**Figure 6.** Induction of *visinin* and *neuroD* by *ath5* in RPE cell cultures. Shown are ethidium bromide-stained agarose gels of RT-PCR products and the means and SDs of the band intensities [integrated optical density (IOD)]. The statistical data were obtained by scanning ethidium bromide-stained agarose gels of three independent amplification reactions. Labels at the top indicate genes harbored in RCAS retroviruses that were used to infect RPE cell cultures. A GFP sample at various concentrations (4×, 1×, 1/4×) was included in RT-PCR for s17. +, –, RPE cells were cultured in the presence and absence, respectively, of bFGF.

*neuroD*, and *ngn2*. *RaxL* is a homeobox gene first expressed in retinal progenitor cells and later in photoreceptor cells and is required for initiating photoreceptor differentiation (Chen and Cepko, 2002). RT-PCR showed that *RaxL* was induced in RPE cells ectopically expressing *ngn2*, *neuroD*, *ath5*, and a construct coexpressing *ath5* and *NSCL1*. Only background levels of *RaxL* expression were present in the samples with *NSCL1* alone or GFP (Fig. 7).

We also examined whether *RaxL* could induce *neuroD* and whether *RaxL* alone was sufficient to guide cultured RPE cells to transdifferentiate toward photoreceptor cells. We found that RCAS-driven ectopic expression of *RaxL* in RPE cells did not induce the expression of *visinin*, assayed by immunostaining (data not shown) or by RT-PCR (Fig. 8) or the expression of MAP2 (data not shown), an early neural marker that is induced by *neuroD* (Yan and Wang, 1998). No induction of *neuroD* was detected by RT-PCR (Fig. 8), indicating a linear relationship of *neuroD* → *RaxL*. Addition of bFGF into the culture medium did not alter the inability of RaxL to induce the expression of *visinin* and *neuroD* (data not shown).

### Lack of induction of *ath5* by *ngn2* and vice versa

Three lines of evidence prompted us to ask whether *ath5* is a downstream target of *ngn2*. First, the induction of *neuroD* by *ath5* in RPE transdifferentiation suggested a possibility that *ath5* might lie between *ngn2* and *neuroD* in the pathway of *ngn2* → *neuroD* → photoreceptor genes. Second, *ngn2* might induce *ath5* while leading to the RGCs pathway because *ngn2* elicits *de novo* generation of cells that closely resemble RGCs (Yan et al., 2001), the development of which requires *ath5* (Brown et al., 2001; Kay et al., 2001; Wang et al., 2001). Third, *ngn2* is expressed in pro-

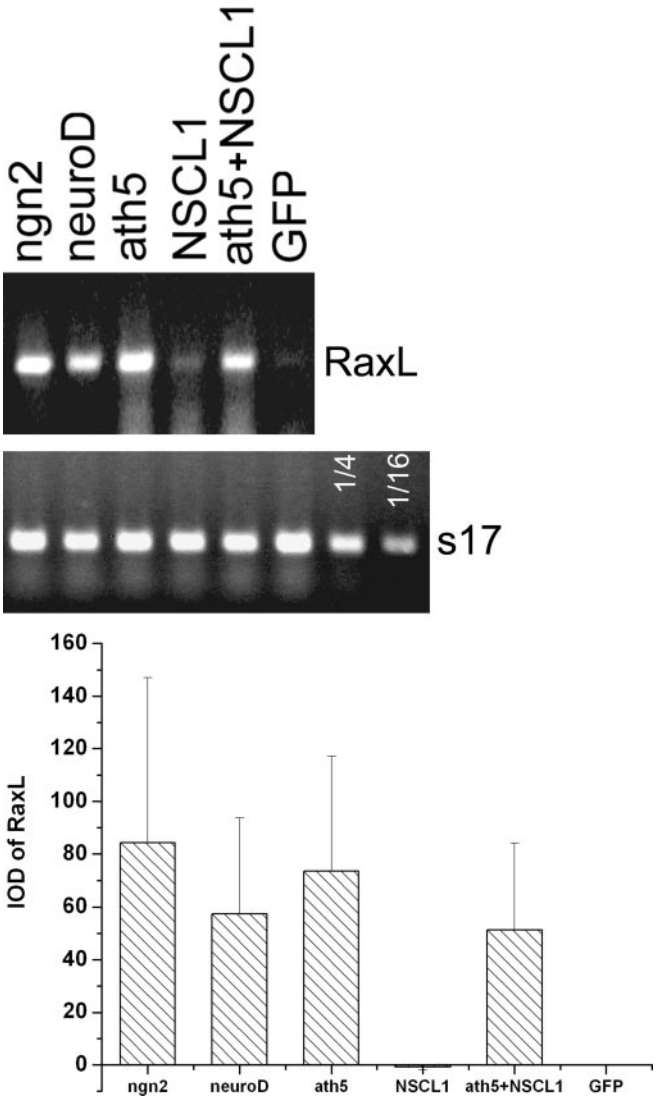


**Figure 7.** Induction of *RaxL* by *ath5*, *neuroD*, and *ngn2* in cultured RPE cells. Top, Ethidium bromide-stained agarose gels of RT-PCR products. Labels at the top indicate genes harbored in RCAS retroviruses that were used to infect RPE cell cultures. A GFP sample at 1/4 and 1/16 concentration was included in RT-PCR for s17. Bottom, Means and SDs of the band intensities [integrated optical density (IOD)]. The statistical data were obtained by scanning ethidium bromide-stained agarose gels of three independent amplification reactions.

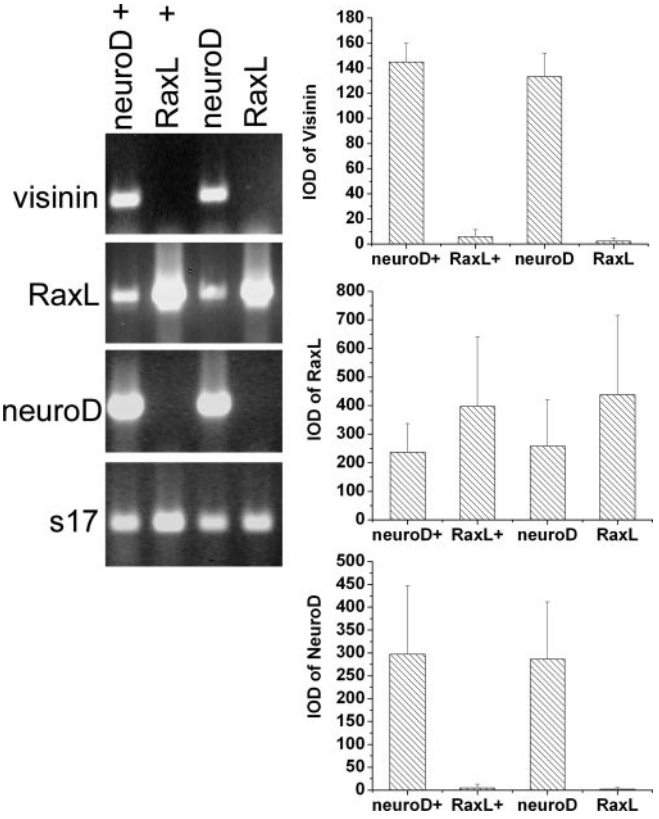


**Figure 8.** Lack of induction of *visinin* and *neuroD* by *RaxL*. Shown are ethidium bromide-stained agarose gels of RT-PCR products and the Means and SDs of the band intensities [integrated optical density (IOD)]. The statistical data were obtained by scanning ethidium bromide-stained agarose gels of three independent amplification reactions. Labels at the top indicate genes harbored in RCAS retroviruses that were used to infect RPE cell cultures. +, RPE cells cultured in the presence of bFGF.

genitor cells still in the cell cycle (Yan et al., 2001), whereas *ath5* is expressed in postmitotic cells. We found, however, no detectable induction of *ath5* by *ngn2* in the presence or absence of bFGF (Fig. 9). No significant levels of *ath5* were induced by *neuroD*, *NSCL1*, *ath3*, or *NSCL2* (Fig. 9) (data not shown). Furthermore, no induction of *ngn2* was observed by *ath5* in either the presence or absence of bFGF. Thus, *ngn2* was unable to induce *ath5*, and *ath5* was unable to induce *ngn2* under the experimental conditions.

## Discussion

In this study, we took advantage of the simplicity and plasticity offered by cultured non-neural RPE cells to assay the proneural activities of *ath5* and to examine the relationship of *ath5* with other transcription factors involved in photoreceptor genesis and differentiation. These questions could not be as easily addressed in the developing retina or cultured retinal cells because the neural retina naturally expresses several proneural bHLH genes and also contains several types of neural cells that create a cellular context that is molecularly and biochemically heterogeneous. The RPE, on the other hand, is a single-layered structure with non-neural cells and lacks the expression of the proneural bHLH genes under investigation. In addition, the RPE is developmentally related to the retina, sharing the same origin of the optic cup. This common origin may bring about common molecular properties. In fact, our previous studies showed that cultured RPE cells are responsive to the proneural activities of *ngn2* and *neuroD* and may undergo neural transdifferentiation. Furthermore, the types of cells produced by RPE transdifferentiation agree with the retinal expression patterns of the proneural genes (Yan and Wang, 1998, 2000b; Yan et al., 2001; Ma et al., 2004). In light of all this, RPE transdifferentiation toward retinal neurons might, at least to a certain extent, recapitulate the molecular events of retinal neurogenesis and offer an effective assay for proneural activities. However, because RPE cells are not identical to retinal neuroblasts, our RPE transdifferentiation assay was complemented with and supported by experiments using the developing retina and cultured retinal cells. Another important implication of our RPE transdifferentiation studies is that cultured RPE cells might be a potential source of cells to generate retinal neurons *de novo* for studies with therapeutic goals. Keep in mind, however, that further studies are needed to show whether RPE cells can be induced by proneural genes to produce functional retinal neurons.

Ectopic expression of *ath5* in RPE cell cultures resulted in the *de novo* generation of cells that expressed photoreceptor-specific

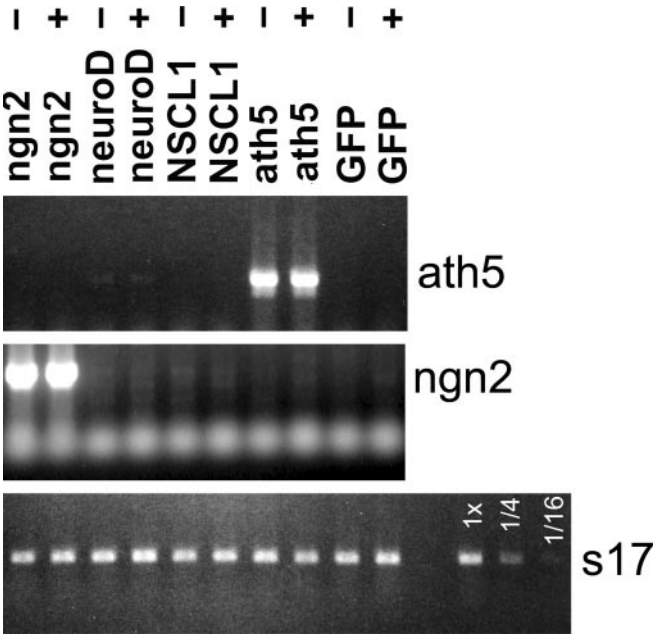


**Figure 9.** Lack of induction of *ath5* by *ngn2* and vice versa. Shown are ethidium bromide-stained agarose gels of RT-PCR products. RPE cell cultures were infected with RCAS expressing the various genes as listed at the top. +, –,RPE cells were cultured in the presence and absence of bFGF.

genes and morphologically resembled young photoreceptor cells. The expression of the red pigment gene and rhodopsin and the presence of elaborate cellular processes suggest that *ath5* was able to trigger a spectrum of genes associated with photoreceptor development and differentiation of both cones and rods. The repertoire of genes downstream of *ath5* included *neuroD*, which alone is sufficient to guide a retinal cell or a cultured RPE cell to adopt a photoreceptor fate and to induce an array of photoreceptor genes of both cones and rods (Yan and Wang, 1998, 2000a,b), including homeobox gene *RaxL* (this study). Thus, it becomes apparent that a pathway of *ath5* → *neuroD* → *RaxL* and other photoreceptor genes might underlie the *de novo* generation of photoreceptor-like cells in the RPE cell cultures ectopically expressing *ath5*.

The pathway of *ath5* → *neuroD* → photoreceptor genes might also operate during retinal neurogenesis. First, in the developing retina, more cells than what was considered to be differentiating RGCs (the inner zone) expressed *ath5*. The two-zone pattern of *ath5* expression was also observed in mouse retina expressing an ath5-LacZ knock-in (Brown et al., 2001). In the chick retina, *ath5* was previously presumed to be expressed in progenitor cells in the ventricular zone and in differentiating RGCs (Liu et al., 2001), whereas our data showing a lack of cells double-labeled with BrdU and *ath5* expression suggest that *ath5* is expressed in postmitotic cells. It is possible that cells in the outer zone of *ath5* expression could adopt a photoreceptor fate on *neuroD* expression. Second, in the developing retina, *ath5* and *neuroD* were coexpressed in some cells, particularly in those *neuroD*-expressing cells presumably still in their migratory path. Third and most importantly, *ath5* misexpression in retinal cells resulted in an increased photoreceptor cell population, in addition to an RGC population. Remarkably, the photoreceptor cell-promoting activity of *ath5* was developmental stage-dependent and lagged behind the ganglion cell-promoting activity. This temporal context-dependent effect is consistent with the notion that *ath5* may be involved in the production of multiple types of retinal cells. Notably, a recently published study by Yang et al. (2003) indicates the involvement of mouse *ath5* in the production of various types of retinal cells, including photoreceptor cells, RGCs, and amacrine cells. All these findings suggest that vertebrate retinal neurogenesis might involve the *ath5* → *neuroD* pathway for photoreceptor genesis.

The pathway appeared to be a different one from the pathway of *ngn2* → *neuroD* → photoreceptors because no *ath5* expression was detected in RPE cultures infected with RCAS-ngn2, and no *ngn2* expression was detected in RPE cultures infected with RCAS-ath5. Alternatively, *ath5* could be an intermediate between *ngn2* and *neuroD*, but its induction by *ngn2* in the RPE cell cultures was transient and escaped our detection. This scenario, although possible, seems unlikely when one considers that the expression of *NSCL1*, which is also transiently expressed in young RGCs (Li et al., 1999b), was readily detected in RPE cell cultures infected with RCAS-ngn2 by both *in situ* hybridization (Yan et al., 2001) and RT-PCR (our unpublished data). Moreover, expression of *ath5* and *neuroD* in mouse retinas lacking *ngn2* appeared comparable with that in normal retinas (our unpublished data). Thus, it is likely that there might be two pathways leading to photoreceptor genesis; one is represented by *ngn2*, and the other involves *ath5*, and they merge at *neuroD* expression. The existence of separate pathways could explain the lack of a photoreceptor deficiency in *ath5*$^{-/-}$ mouse retina (Brown et al., 2001; Wang et al., 2001). The lack of *ath5* induction by *ngn2* might also exclude *ath5* as a downstream genetic target of *ngn2* in the RGC pathway. This renders the factor that induces *ath5* intriguing. Studies by Masai et al. (2000) and Stenkamp and Frey (2003) indicate that sonic hedgehog (*shh*) may induce *ath5* expression in the developing zebrafish retina.

The life history of a retinal cell can be divided into four developmental phases: proliferation, cessation of mitosis, fate determination, and differentiation (supplemental Fig. 1, available at www.jneurosci.org/cgi/content/full/24/32/7150/DC1). Certain bHLH genes, such as *ngn2*, function in proliferating retinoblasts and may lead to certain specific types of retinal neurons. Some bHLH genes may be involved in differentiation, such as *ath5* and *NSCL1*, which promote RGC differentiation. The developmental transition from cell proliferation to cell differentiation is a critical window for retinal cell fate determination (Adler, 2000) and may involve a number of bHLH genes, including *ath5* and *neuroD*. However, not all of the genes expressed during the transition specify a particular cell type. *ath5* would be one such example: although expressed in postmitotic cells, it may render progenitors competent to differentiate into RGCs (Yang et al., 2003; Mu and Klein, 2004), participate in RGC differentiation (Ma et al., 2004), and induce *neuroD* and the photoreceptor pathway (this study). Together with published studies, our data indicate that the vertebrate retina might use complex networking of bHLH genes, and there might exist two separate pathways converging at *neuroD* expression and leading to photoreceptor genesis.

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