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bHLH genes *cath5* and *cNSCL1* promote bFGF-stimulated RPE cells to transdifferentiate toward retinal ganglion cells

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Abstract

The molecular mechanism of retinal ganglion cell (RGC) genesis and development is not well understood. Published data suggest that the process may involve two bHLH genes, *ath5* and *NSCL1*. Gain-of-function studies show that *ath5* increases RGC production in the developing retina. We examined whether two chick genes, *cath5* and *cNSCL1*, can guide retinal pigment epithelial (RPE) cells to transdifferentiate toward RGCs. Ectopic expression of *cath5* and *cNSCL1* in cultured chick RPE cells was achieved through retroviral transduction. *cath5* alone was unable to induce de novo expression of early RGC markers, such as RA4 antigen, neurofilament (160 kDa), and a neurofilament-associated antigen. However, *cath5* induced the expression of these proteins when the RPE cells were cultured with medium supplemented with bFGF. Since bFGF alone can induce only RA4 antigen, the expression of the additional RGC markers reflects a synergism between *cath5* and bFGF in promoting RPE transdifferentiation toward RGCs. Morphologically, the $RA4^+$ cells in bFGF + *cath5* cultures appeared more neuron-like than those generated by bFGF alone. *cNSCL1* also promoted bFGF-stimulated RPE cells to transdifferentiate toward RGCs that expressed RA4 antigen, N-CAM, Islet-1, neurofilament, and neurofilament-associated antigen. We found that *cath5* induced *cNSCL1* expression, but not vice versa. Our data suggest that *cath5* or *cNSCL1* alone was insufficient to induce RPE transdifferentiation into RGCs, but could further neural differentiation initiated by bFGF. We propose that intrinsic factors act synergistically with extrinsic factors during RGC genesis and development.

Keywords: Cell fate; Development; Neuron differentiation; RPE transdifferentiation; bHLH; *cath5*; *cNSCL1*; Retinal ganglion cells

Introduction

Retinal ganglion cells (RGCs) in the vertebrate eye are responsible for relaying visual signals to the brain. Damage to RGCs and their axons (the optic nerve) may result in irreversible visual loss. Glaucoma, a group of diseases that can lead to damage to the optic nerve and result in blindness, affects about 3 million Americans and is considered a leading cause of blindness in the United States (http://www.nei.nih.gov/health/glaucoma/glaucoma_facts.htm). Understanding how RGC fate is specified and how RGCs develop may help in the design of strategies to prevent or halt this debilitating blinding disease.

The detailed molecular mechanisms underlying RGC genesis and development are not well understood. Published studies indicate that the production of RGCs, the first group of cells generated in the vertebrate retina, is subjected to intricate regulation involving both intrinsic factors and extrinsic factors, including fibroblast growth factors (FGFs; Guillemot and Cepko, 1992; McCabe et al., 1999; Zhao and Barnstable, 1996), insulin (Fischer et al., 2002), and sonic hedgehog (Neumann and Nuesslein-Volhard, 2000; Zhang and Yang, 2001).

Recent studies suggest that genes encoding the basic helix-loop-helix (bHLH) family of transcription factors and homologous to *Drosophila* proneural genes *achaete-scute* and *atonal* play critical roles in neural cell type specification. Published studies indicate that *ath5*, a vertebrate homolog of *Drosophila atonal*, plays an important role in RGC development. Targeted mutation of mouse *ath5* (*math5*) leads to the absence or dramatic reduction of RGCs (Brown et al., 2001; Wang et al., 2001) and a loss of photic entrainment (Wee et al., 2002). Injection of *Xenopus ath5* (*Xath5*) mRNA into *Xenopus* embryo at two-cell stage promotes retinal progenitor cells to differentiate toward

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RGCs (Kanekar et al., 1997). Lipofection of *math5* into the frog retina, however, results in an increase in bipolar cells (Brown et al., 1998). In chick retina, retroviral-driven expression of *cath5* and *math5* increase RGC number (Liu et al., 2001).

Chicken *NSCL1*, like mammalian *NSCL1* (Begley et al., 1992; Brown et al., 1992), is specifically expressed in the nervous system (Li et al., 1999a). In the developing retina, expression of *cNSCL1* is cell-type specific; it is first expressed transiently in differentiating ganglion cells and later in retinal glia (Li et al., 1999b). Widespread misexpression of *cNSCL1* driven by a replication-competent retrovirus results in the death of the embryos in the middle of gestation (Li et al., 1999a). The embryos exhibit gross abnormalities, such as severe retardation in limb growth, anomalous brain development, and small eyes with reduced cell proliferation activity and increased cell death (Li et al., 1999a,b).

The retina is anatomically encircled by a layer of homogenous-looking, pigmented, nonneural cells—the retinal pigment epithelium (RPE). Unlike retinal neurons, RPE cells can reenter the cell cycle when stimulated, and thus can be readily cultured. Developmentally, RPE is closely related to the retina, as both are developed from the same optic vesicle. This common lineage may dictate common features in gene expression, rendering it possible to sway the gene expression program of RPE to that of retinal neurons with appropriate factors. Indeed, experiments have shown that RPE tissue at early developmental stages [e.g., embryonic day 4 (E4) and younger in chick or E14 and younger in rat; Zhao et al., 1995] can transdifferentiate into a neural retina under the induction of bFGF (Coulombre and Coulombre, 1965; Guillemot and Cepko, 1992; Opas and Dziak, 1994; Orts-Ilorca and Genis-Galvez, 1960; Park and Hollenberg, 1989; Pittack et al., 1991, 1997). Recent studies from our laboratory demonstrate that various stimuli can guide dissociated E6 chicken RPE cells toward distinct neural pathways. For example, these cells can be coaxed by *neuroD* to differentiate toward the photoreceptor pathway (Yan and Wang, 1998, 2000a,b) and by *neurogenin2* (*ngn2*) toward multiple types of retinal neurons (Yan et al., 2001). When cultured in the presence of bFGF, the RPE cells transdifferentiate into cells that express RA4 antigen (Yan and Wang, 2000b), a marker for ganglion cells in the chick retina (Waid and McLoon, 1995). All these indicate that cultured RPE cells could be used to examine the function of factors involved in retinal cell fate determination and as a source of cells for de novo production of retinal neurons, which might benefit studies of cell-based therapies aiming at preserving or restoring vision in patients with retinal neuron degenerations such as retinitis pigmentosa and glaucoma.

In this study, we examined whether *cath5* and *cNSCL1* would induce cultured RPE cells to transdifferentiate toward RGCs. We report that neither *cNSCL1* nor *cath5* alone was able to induce detectable level of RPE cell transdifferentiation toward RGCs, but they both enhanced molecular and morphological differentiation of bFGF-initiated transdifferentiation.

Materials and methods

RPE cell culture and retroviral infection

RPE from the central and central-peripheral region of the E6 chicken eyes was dissected free from other ocular tissues as previously described (Yan and Wang, 1998). Pooled RPE tissues were incubated with typsin–EDTA, and the dissociated cells were cultured with Medium 199 + 10% fetal calf serum either with, or without, bFGF supplement at a final concentration of 10 ng/ml. At about 50% confluence, 5–15

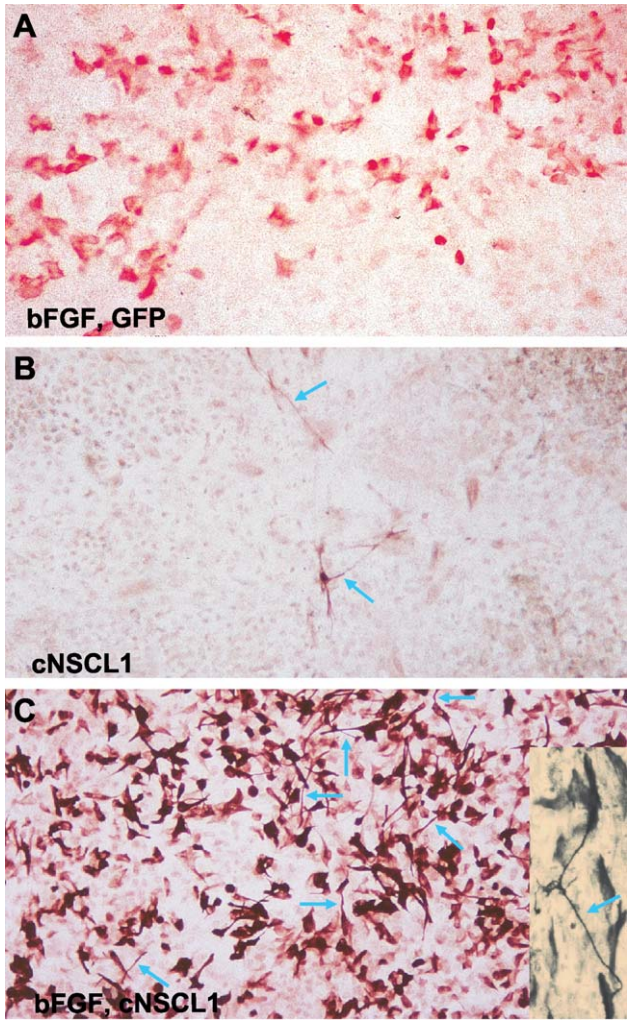

Fig. 1. Immunostaining of RPE cell cultures with monoclonal antibody RA4. (A) Infected with RCAS-GFP and cultured in the presence of bFGF. (B) Infected with RCAS-cNSCL1. (C) Infected with RCAS-cNSCL1 and cultured in the presence of bFGF. Inset: A higher magnification image showing a neural-like $RA4^+$ cell. Arrows point to cells with thin processes.

μl of concentrated retrovirus expressing *cNSCL1*, *cath5*, or GFP as a control, was added to the culture in 35-mm dish. Around 8 days after the administration of the virus, cells in the culture were harvested for RT-PCR or were fixed with ice-cold 4% paraformaldehyde in PBS for 30 min and subjected to immunocytochemistry.

Generation of recombinant retroviruses

The coding sequence of *cath5* was PCR amplified from first-strand cDNA of E6 chick retina with primers ATCCATGGAAACCTGTCAATCCAGTC and ATGTCGACTAATTAGCTATTTGAAAG. The PCR product was cloned into pGEMT. After sequence verification, the DNA fragment was subcloned first into shuttle vector Cla12Nco and then into RCAS, which was derived from Rous Sarcoma Virus and constructed by Hughes et al. (1987). Recombinant RCAS pro-viral DNA with *cath5* in the correct orientation was transfected into chick embryonic fibroblast cells. Viral particles were harvested and concentrated from cell culture medium as previously described (Yan and Wang, 1998). Generation of RCAS-cNSCL1 and RCAS-GFP has been previously described (Li et al., 1999a; Yan and Wang, 1998). The titers of the concentrated viruses ranged from 5×10^7 to 2×10^8 pfu/ml.

Immunocytochemistry

Monoclonal antibody RA4 (used at a 1:1000 dilution) was a gift from Dr. Steven McLoon (University of Minnesota). Monoclonal antibodies against Brn3A (1:200) and NeuN (1:100) were purchased from Chemicon. Monoclonal antibody (clone HM-2) against microtubule-associated proteins (MAP2) was purchased from Sigma and used at a 1:200 dilution. Four monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (Iowa University): anti-Islet-1 (clone 39.4D5, 1:100; developed by Dr. Thomas Jessell); 3A10 (1:100; developed by Dr. Thomas Jessell); 4H6 (1:100; developed by Dr. Willi Halfter); antineural cell adhesion molecule (N-CAM; clone 5e; 1:500; developed by Dr. Urs Rutishauser). Standard immunocytochemistry was performed with either ABC-peroxidase or alkaline phosphatase-conjugated secondary antibodies (Vector Laboratories) as described by the manufacturer. The number of positive cells was counted from more than 20 view areas with a 20× objective in each 35-mm dish, and the total number of positive cells was calculated per specified area. The means and SDs from three dishes in one experiment were calculated with a computer program (Origin 7.0). Experiments were repeated at least three times in their entirety, from RPE dissection to cell counting.

RT-PCR

RPE cells infected with RCAS expressing *cath5*, *cNSCL1*, or GFP were harvested from a 35-mm dish, and their total RNA was isolated using a High Pure RNA Tissue Kit (Roche). First-strand cDNA was synthesized using oligo-dT as the primer. After a fivefold dilution, 1 μl of cDNA was used as template in each PCR. *cath5* cDNA was amplified 30 cycles with the primers used for its initial cloning into pGEMT. *cNSCL1* cDNA was amplified 30 cycles with primers previously described (Li et al., 1999a). Ribosomal protein s17 was used as an internal control and used to normalize the amount of cDNA in each sample, with primers of GTGATCATCGAGAAG and AGCAACATAACGAGC annealed at 44°C (Wang and Adler, 1994). A series of fourfold dilutions of the GFP

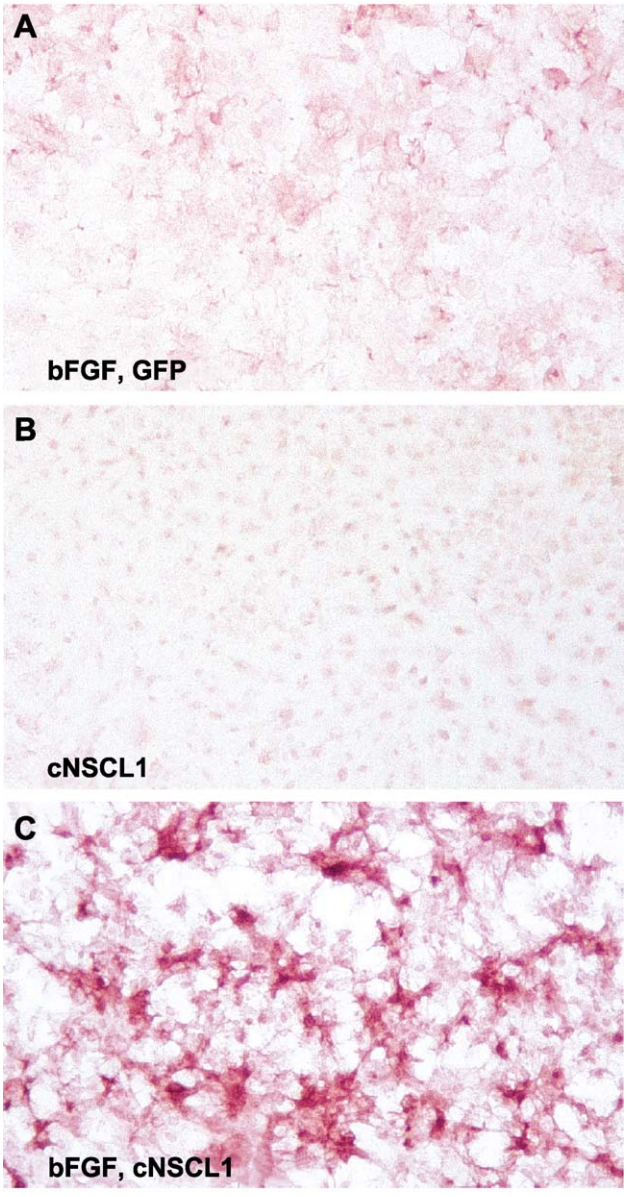

Fig. 2. Immunostaining of RPE cell cultures with antibody against N-CAM. (A) Infected with RCAS-GFP and cultured in the presence of bFGF. (B) Infected with RCAS-cNSCL1. (C) Infected with RCAS-cNSCL1 and cultured in the presence of bFGF.

cDNA sample was included to monitor that the PCR of s17 was not saturating with 20 cycles of amplification.

Results

Effect of cNSCL1 on RPE transdifferentiation

Since *cNSCL1* is transiently expressed in developing RGCs and its bHLH domain shows sequence homology with the *Drosophila* proneural gene *atonal*, we addressed the question of whether *cNSCL1* has proneural activity sufficient to induce an RGC fate. We tested this question using the RPE cell transdifferentiation assay we recently developed (Yan and Wang, 1998). Cells in cultures derived from E6 chick RPE have previously been shown to transdifferentiate toward photoreceptor cells and RGCs upon ectopic expression of *ngn2* driven by RCAS retrovirus (Yan et al., 2001), or toward photoreceptor cells selectively upon retroviral transduction of *neuroD* (Yan and Wang, 1998, 2000a,b). Therefore, we used RCAS retrovirus to ectopically express *cNSCL1* in cultured RPE cells. Since RCAS is replication-competent, it spreads through and causes a thorough infection of a dividing cell population. We found that by day 4, nearly 100% of the cells in the culture became infected with the virus based on immunostaining for a viral protein p27 (data not shown). The cell densities of cultures infected with RCAS-cNSCL1 appeared similar to those of control cultures infected with RCAS-GFP at various time points examined with an inverted microscope, suggesting that ectopic *cNSCL1* expression did not significantly affect the proliferation or death of cultured RPE cells. There was a moderate increase in pigmentation in cultures infected with RCAS-cNSCL1 (data not shown).

The possibility of de novo emergence of RGCs from the RPE cell culture infected with RCAS-cNSCL1 was examined by immunostaining with monoclonal antibodies for early RGC markers, including RA4 antigen, N-CAM, Islet-1, MAP2, and neurofilament-associated antigen recognized by monoclonal antibody 3A10. Among them, only RA4$^+$ cells were detected, but their number was small and was similar to that in the control culture infected with RCAS-GFP. Thus, *cNSCL1* appeared unable to induce early RGC markers, including RA4 antigen, under these conditions.

Synergism between cNSCL1 and bFGF on RPE transdifferentiation

Guillemot and Cepko (1992) have proposed that RGC fate is favored by bFGF and aFGF. In our RPE transdifferentiation assay, culturing RPE cells in the presence of bFGF induces RA4 antigen (Yan and Wang, 2000b), but the RA4$^+$ cells lack morphological traits typical of RGCs (Figs. 1A and 4A). In addition, bFGF treatment did not induce a significant number

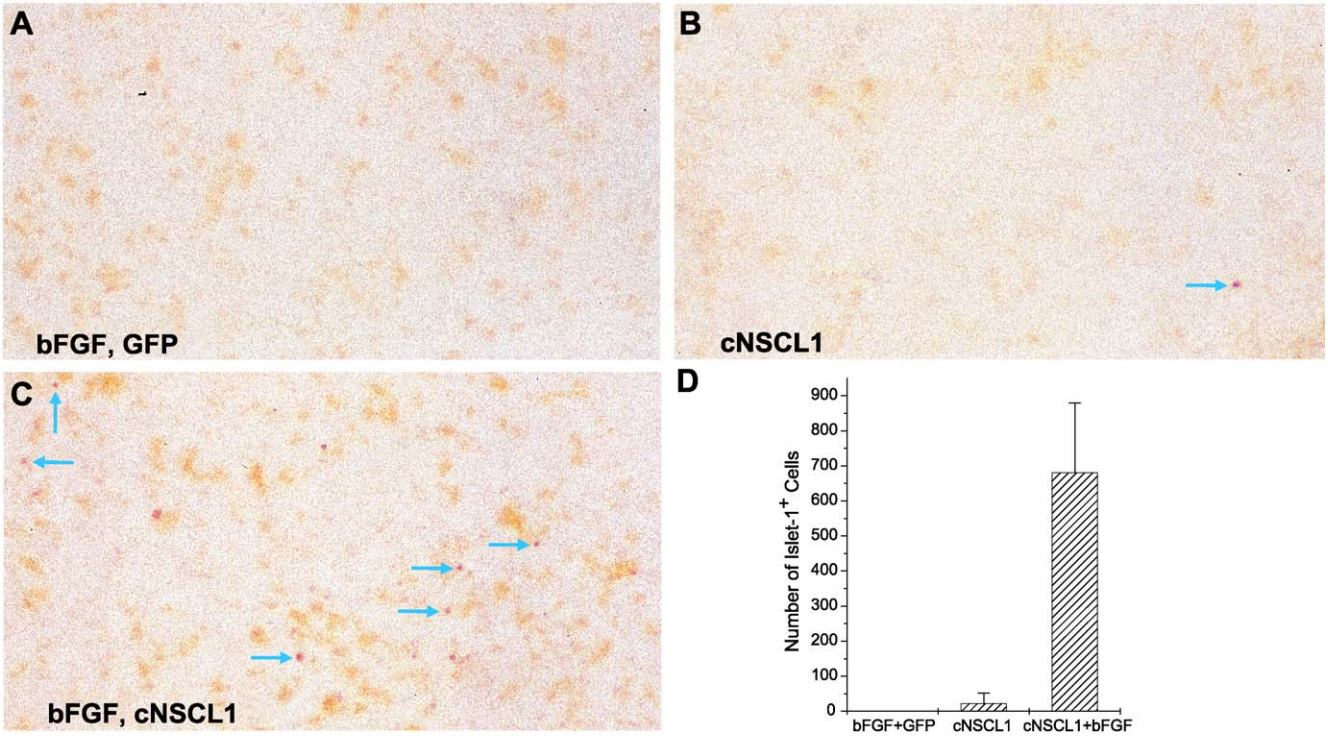

Fig. 3. Immunostaining of RPE cell cultures with anti-Islet-1 antibody. (A) Infected with RCAS-GFP and cultured in the presence of bFGF. (B) Infected with RCAS-cNSCL1. (C) Infected with RCAS-cNSCL1 and cultured in the presence of bFGF. Arrows point to immunoreactive nuclei. (D) Quantification of Islet-1$^+$ cells in RPE cultured with and without *cNSCL1* and bFGF treatments. Three dishes per treatment group were counted. Shown are the means ± SDs of calculated total number of positive cells per 35-mm dish.

Table 1
Summary of the expression of RGC markers in cultured RPE cells under various conditions

	GFP		cNSCL1		cath5	
bFGF	–	+	–	+	–	+
RA4	++	+++	++	+++	++	+++
RA4, neuron-like	–	–	–	+	–	+
NF	–	–	–	+++	–	+++
NF-Assoc. Ag	–	–	–	+++	–	+++
Islet-1	–	–	–	+	–	–
MAP2	–	–	–	+	–	++
N-CAM	–	–	–	u.c.	n.d.	n.d.

NF: neurofilament. NF-Assoc. Ag: neurofilament-associated antigen. The number of positive cells per 35-mm dish were grouped as "–" for less than 100; "+" for a few hundred; "++" for a few thousand; "+++" for tens of thousands. "u.c." means unsuitable for counting either because of the staining patterns or because the cells are too many to count. "n.d." indicates not determined.

of cells to express other early RGC markers, such as N-CAM, Islet-1, MAP2, neurofilament-associated antigen, and neurofilament (Figs. 2A, 3A, 5A, 6A, and 7; data not shown). This indicates that the extent of transdifferentiation toward RGCs is very limited.

When the bFGF-treated RPE cell culture was infected with RCAS-cNSCL1, a large number of cells expressed RA4 antigen (Fig. 1C). There were tenfold more $RA4^+$ cells in the presence of both bFGF and *cNSCL1* than *cNSCL1* alone and twofold more than bFGF alone (Fig. 7). These $RA4^+$ cells were morphologically more neuron-like with longer and thinner processes compared with those in cultures with bFGF alone (Fig. 1). Nonetheless, their morphological traits still significantly diverge from those typical of RGCs, and the majority of the cells were monopolar with a single process that is wider than what is found on a neuron.

Synergism in induction of other early RGC markers was also observed between bFGF and *cNSCL1*. While neither bFGF nor *cNSCL1* induced N-CAM expression, $N\text{-}CAM^+$ cells were abundantly detected in the RPE cultures in the presence of both (Fig. 2). Similarly, cells positive for Islet-1 were detected in bFGF-treated cultures that also ectopically expressed *cNSCL1*, but were rarely detected in cultures with bFGF treatment or *cNSCL1* expression alone (Fig. 3). Furthermore, neurofilament-associated antigen recognized by 3A10 was not induced by either bFGF or *cNSCL1* alone, but its expression was observed in a large number of cells in the presence of both (Fig. 7; Table 1). Despite the synergism, however, no significant number of cells in the bFGF + *cNSCL1* cultures expressed detectable levels of MAP2, Brn3A, or NeuN (data not shown), which are expressed in RGCs of the developing chick retina.

One characteristic of bFGF-induced RPE transdifferentiation, i.e. RA4 antigen expression, is that the positive cells appeared patchy. This is in contrast to the transdifferentiation induced by *neuroD* or *ngn2*, in which transdifferentiating cells appear scattered or in isolation. Like in cultures with bFGF alone, positive cells in bFGF + *cNSCL1* cultures were patchy, implying that their origin was largely due to bFGF.

Effect of cath5 on RPE transdifferentiation and its synergism with bFGF

Published studies indicate that vertebrate *ath5* plays an important role during retinal neurogenesis. Lack of *math5* leads to a retina with fewer or no RGCs, demonstrating that *math5* is required for RGC development. In various species, misexpression or overexpression of *ath5* in the developing retina leads to an increase in RGC production, indicating that *ath5* alone is sufficient to guide a retinal cell to differentiate into an RGC. These observations impelled us to test the possibility that *cath5* would guide cultured RPE cells to differentiate toward RGCs. Ectopic expression of *cath5* in cultured RPE cells resulted in a few $RA4^+$ cells, the

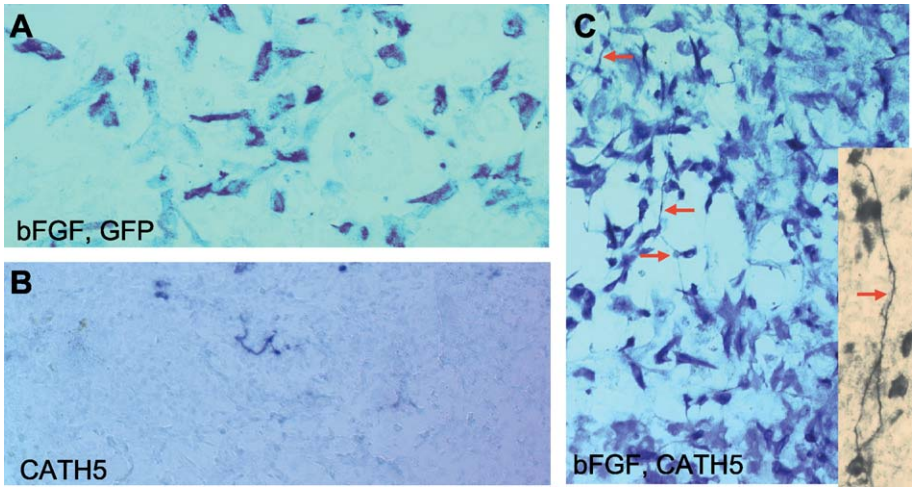

Fig. 4. Immunostaining of RPE cell cultures with monoclonal antibody RA4. (A) Infected with RCAS-GFP and cultured in the presence of bFGF. (B) Infected with RCAS-cath5. (C) Infected with RCAS-cath5 and cultured in the presence of bFGF. Inset: A higher magnification image showing a neural like $RA4^+$ cell. Arrows point to cells with long, thin processes.

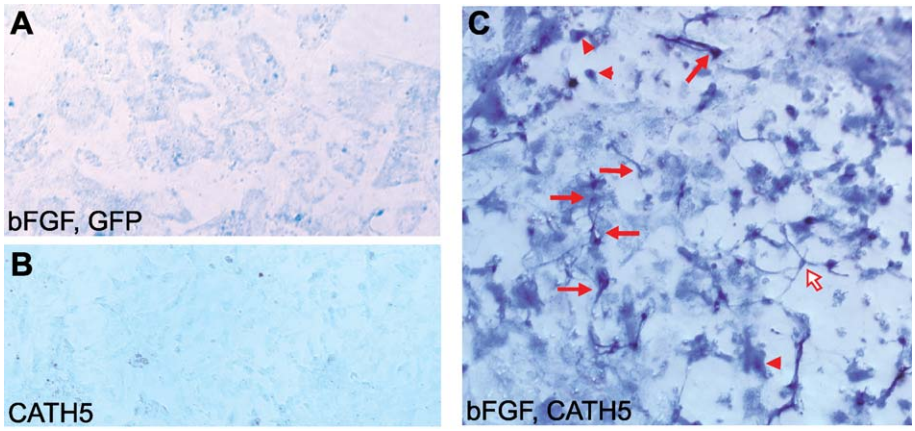

Fig. 5. Immunostaining of RPE cell cultures with monoclonal antibody 3A10. (A) Infected with RCAS-GFP and cultured with bFGF in the culture medium. (B) Infected with RCAS-cath5. (C) Infected with RCAS-cath5 and with bFGF in the culture medium. Arrowheads point to cells without obvious processes. Arrows point to cells with long, thin processes, but are monopolar. The open arrow points to a cell with multiple, long processes typical of neurons.

number of which, however, was not more than that in the control (Figs. 4 and 7). Few cells in RCAS-cath5 cultures were immunostained with 3A10 (Figs. 5 and 7), antibodies against neurofilament (Fig. 6), Islet-1, Brn3A, NeuN, or MAP2 (data not shown).

In RPE cultures treated with bFGF and ectopically expressing *cath5*, however, a large number of cells expressed RA4 antigen (Fig. 4). There were twofold more $RA4^+$ cells in the presence of both bFGF and *cath5* than bFGF alone and tenfold more than with *cath5* alone (Fig. 7; Table 1). Morphologically, these $RA4^+$ cells appeared more neuron-like compared with those in cultures with bFGF alone. Some cells exhibited long, thin processes (arrows in Fig. 4C). However, the majority of the $RA4^+$ cells lacked typical RGC morphologies.

In the presence of bFGF, *cath5* also induced in a large number of cells to express the neurofilament-associated antigen (Figs. 5 and 7). Some $3A10^+$ cells, particularly those at the edge of the culture dish, where cells were denser, exhibited typical neural morphologies with multiple long, thin processes (arrows in Fig. 5). Staining with monoclonal antibody 4H6 showed that the 160-kDa neurofilament protein was also induced (Fig. 6). We did not find a significant number of cells expressing detectable levels of Islet-1, MAP2, NeuN, or Brn3A in the presence of both bFGF and *cath5*.

To compare *cath5* and *cNSCL1* in their ability to promote RPE transdifferentiation toward RGCs, we infected the same batch of RPE cultures with RCAS-cath5 or RCAS-cNSLC1. We found that the numbers of $RA4^+$ and $3A10^+$ cells produced by either gene were similar in the presence or absence of bFGF (Fig. 7; Table 1).

Regulatory hierarchy between cath5 and cNSCL1

To study the potential regulatory hierarchy between *cath5* and *cNSCL1*, we examined their induction with RT-PCR using total RNA isolated from RPE cell cultures infected with RCAS-cath5, RCAS-cNSCL1, or RCAS-GFP. The RPE cells were cultured either in the presence or in the absence of bFGF. Ribosomal protein s17 was used as an internal control for normalizing the amount of first-strand cDNA in each sample. No *cath5* PCR products were apparent in RPE cell cultures infected with RCAS-cNSCL1, either in the presence or absence of bFGF, indicating that *cNSCL1* did not induce *cath5* expression under these conditions (Fig. 8).

cNSCL1 PCR products were detected in RPE cultures infected with RCAS-cath5 (Fig. 8). The amount of the PCR products appeared more in RPE cells cultured with medium supplemented with bFGF (band intensity IOD = 15.1) than without (IOD = 3.9). These data indicate that *cath5*-induced *cNSCL1* expression, and the induction became stronger in the presence of bFGF. No *cNSCL1* PCR products were apparent in the control cultures infected with RCAS-GFP in the presence, or absence, of bFGF. The identities of the PCR products were verified based on their calculated size and with DNA hybridization to a known sequence using the ECL Detection System (data not shown).

Discussion

In this study, we examined the possibility that *cath5* and *cNSCL1* would guide cultured RPE cells to transdifferentiate toward RGCs. The study was prompted by the sequence homology these genes have with the proneural gene atonal, their spatially and temporally restricted expression in immature RGCs, and *ath5*'s ability to increase RGCs when mis- or overexpressed in the developing retina. We found, however, that neither *cath5* nor *cNSCL1* was able to induce detectable RPE transdifferentiation toward RGCs at either the molecular or morphological level. The lack of expression of RGC markers could be due to unresponsiveness of the RPE cells to proneural induction or due to the intrinsic inability of *cath5* or *cNSCL1* to instruct an RGC fate. The

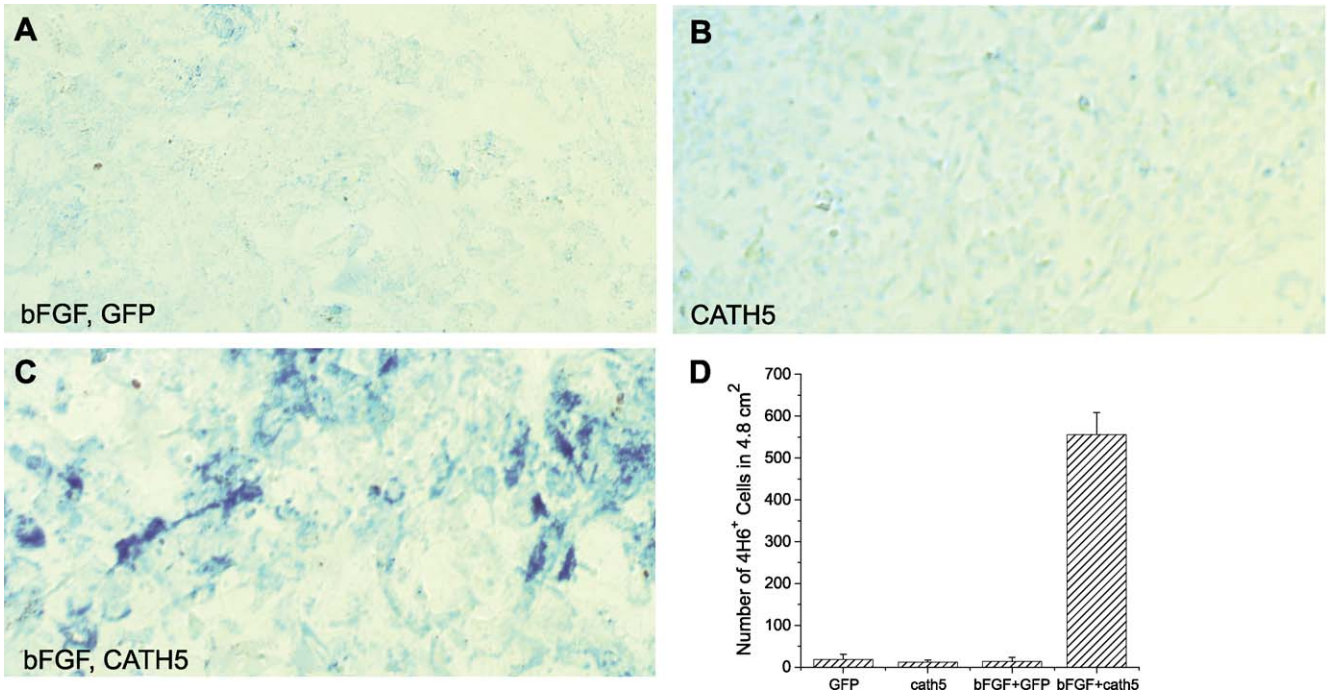

Fig. 6. Immunostaining of RPE cultures for neurofilament protein with monoclonal antibody 4H6. (A) Infected with RCAS-GFP and cultured in the presence of bFGF. (B) Infected with RCAS-cath5. (C) Infected with RCAS-GFP and cultured in the presence of bFGF. (D) Quantification of $4H6^+$ cells in RPE cultured with and without *cath5* and bFGF treatments. Three dishes per treatment group were counted. Shown are the means ± SD of calculated total number of positive cells under a 22-m m^2 coverslip.

former scenario is unlikely because previous studies with a similar strategy have demonstrated that cultured RPE cells are responsive to the induction of bHLH genes with proneural activities. For example, *ngn2* and *neuroD*, two bHLH genes also homologous to *atonal*, elicit RPE transdifferentiation into cells that morphologically and molecularly resemble RGCs or photoreceptor cells (Yan and Wang, 1998; 2000a,b; Yan et al., 2001). In light of this, the lack

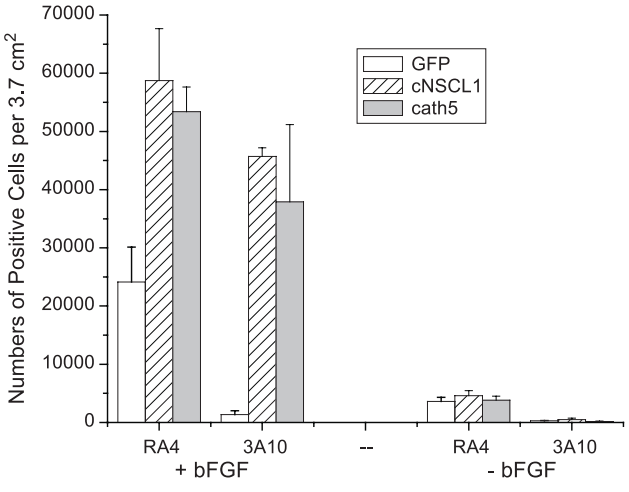

Fig. 7. A comparison of the number of $RA4^+$ cells and $3A10^+$ cells in RPE cultures in the presence or absence of bFGF and infected with either RCAS-GFP, RCAS-cath5, or RCAS-cNSCL1. Shown are means ± SD per 3.7 cm^2.

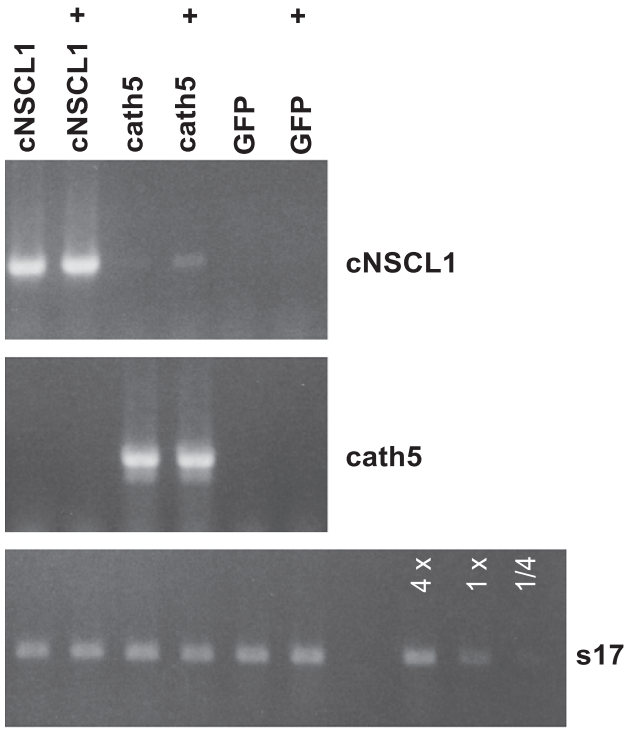

Fig. 8. An ethidium-bromide-stained agarose gel of RT-PCR products of RPE cell cultures infected with RCAS expressing the genes shown at the top. "+" indicates that bFGF was added to the cell culture medium. "4×, 1×, 1/4×" denotes different concentrations of the GFP sample used in the PCR to ensure that the amplification was not saturating.

of expression of RGC markers in RPE cell cultures ectopically expressing *cath5* or *cNSCL1* are more likely to reflect the intrinsic inability of *cath5* and *cNSCL1* to induce an RGC fate. The observation that even RA4 antigen, one of the earliest markers for chick RGCs, was not induced, presents a strong argument against *cath5* and *cNSCL1* playing a central role in the specification of an RGC fate.

While *cath5* or *cNSCL1* alone did not induce the expression of RGC markers, when expressed in RPE cells grown in the presence of bFGF they significantly increased the number of $RA4^+$ cells (compared with the number induced by bFGF alone) and induced the expression of other RGC markers. Furthermore, they promoted morphological changes in transdifferentiating cells, making them more neuron-like with long, thin processes. The enhancement in RGC differentiation suggests that *cath5* and *cNSCL1* are probably involved in RGC differentiation. This idea is consistent with a published loss-of-function study showing that in *math5*$^{-/-}$ mice, RGCs are present initially but absent at later stages (Brown et al., 2001). Nonetheless, it appears to be inconsistent with gain-of-function studies showing that *ath5* increases RGC production in the developing retina. It is also possible that sufficient FGF may be present in the developing retina and *ath5* misexpression furthers RGC differentiation and promotes commitment to the RGC fate, thus resulting in an increase in the RGC population. It should be kept in mind that transdifferentiation of RPE cells in culture into RGCs does not necessarily depict accurately what happens in vivo when neuroblasts differentiate into real RGCs.

Ectopic *cath5* expression in cultured RPE cells induced the expression of *cNSCL1*, as evidenced by the presence of *cNSCL1* PCR products. The PCR products were visible on agarose gel in cultures grown in the absence of bFGF, a condition where no detectable transdifferentiation was observed with immunostaining. This suggests that either *cNSCL1* is a direct target of *cath5* or it is in *cath5*'s close vicinity in the genetic pathway of RGC development. The amount of the PCR products increased about fourfold when the cells were grown in the presence of bFGF, illustrating a stronger *cNSCL1* induction in the presence of both *cath5* and bFGF. bFGF alone did not induce *cNSCL1*, further indicating the limited scope of bFGF-triggered gene expression. *cNSCL1*, on the other hand, did not induce *cath5* expression, illustrating a linear relation of *cath5* → *cNSCL1*.

Extrinsic factors have been known to be involved in retinal cell fate specification. Published reports indicate that RGC fate specification involves bFGF. In our RPE transdifferentiation assay, a large number of $RA4^+$ cells were detected when the cells were cultured in the presence of bFGF. This implies that bFGF might guide RPE cells to transdifferentiate toward RGCs. However, those $RA4^+$ cells lacked neural morphologies typical of RGCs, and there was little or no expression of other early RGC markers. These observations demonstrate the scope of gene expression induced by bFGF is very limited. When cells grown in the presence of bFGF ectopically expressed *cath5* or *cNSCL1*, the $RA4^+$cells became more neuron-like. Additionally, other early RGCs markers were expressed. Taking together, our data suggest that *cath5* and *cNSCL1* enhanced RGC differentiation that was initiated by bFGF. We propose that bFGF plays an instrumental role in guiding cultured RPE cells to transdifferentiate toward RGCs, but commitment to a RGC fate may require *cath5* and/or *cNSCL1*, which sustain or promote RGC differentiation to an extent that is no longer reversible. Thus, the genesis and the development of RGCs may involve interplays between intrinsic and extrinsic factors.

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