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## Enhanced Retinal Ganglion Cell Differentiation by *ath5* and *NSCL1* Coexpression

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### Abstract

**Purpose**—The molecular mechanism underlying retinal ganglion cell (RGC) differentiation is not fully understood. In this study, the role of the basic helix-loop-helix (bHLH) genes *ath5* and *NSCL1* in RGC differentiation was examined, by testing whether their coexpression would promote RGC differentiation to a greater extent than either gene alone.

**Methods**—The replication-competent avian RCAS retrovirus was used to coexpress *ath5* and *NSCL1* through an internal ribosomal entry site. The effect of the coexpression on RGC differentiation was assayed in vivo in the developing chick retina and in vitro in RPE cell cultures derived from day 6 chick embryos.

**Results**—Coexpression of *ath5* and *NSCL1* in RPE cells cultured in the presence of bFGF promoted RPE transdifferentiation toward RGCs, and the degree of transdifferentiation was much higher than with either gene alone. Cells expressing RGC markers, including RA4, calretinin, and two neurofilament-associated proteins, displayed processes that were remarkably long and thin and often had numerous branches, characteristics of long-projecting RGCs. In the developing chick retina, retroviral expression of *NSCL1* resulted in a moderate increase in the number of RGCs, results similar to retroviral expression of *ath5*. Coexpression of *ath5* and *NSCL1* yielded increases in RGCs greater than the sum of their increases when expressed separately.

**Conclusions**—Both in vitro and in vivo data indicate that the combination of *ath5* and *NSCL1* promotes RGC differentiation to a greater degree than either gene alone, suggesting a synergism between *ath5* and *NSCL1* in advancing RGC development.

Many aspects of the molecular regulation of retinal ganglion cell (RGC) differentiation remain unclear. Published studies have shown that genes encoding transcription factors of the homeodomain family<sup>1–4</sup> and the basic helix-loop-helix (bHLH) family, including *Ath5* and *NSCL1*, play a role in regulating gene expression during RGC development in the vertebrate retina. *Ath5* is expressed in immature RGCs, and their expression becomes undetectable in mature RGCs<sup>5,6</sup> (Ma et al., manuscript submitted). Chicken *NSCL1*, like its mammalian counterpart,<sup>7,8</sup> is specifically expressed in the nervous system.<sup>9</sup> In the developing retina, *NSCL1* is first expressed in differentiating ganglion cells during early development and then in Müller glia during late development.<sup>10</sup> Retroviral expression of *NSCL1* in the developing chick retina causes premature cessation of mitotic activity, massive cell death, and microphthalmia.<sup>10</sup>

The function of *ath5* in RGC development has been the subject of extensive investigation. *Xenopus ath5* mRNA injected into *Xenopus* embryos at the two-cell stage promotes retinal progenitor cell differentiation into RGCs.<sup>11</sup> In the embryonic chick retina, retroviral

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expression of chick and mouse *ath5* increases the number of RGCs.<sup>6</sup> Targeted deletion of *ath5* in mouse results in a lack, or a dramatic reduction, of RGCs, indicating that *ath5* is indispensable for RGC development.<sup>12,13</sup> Kay et al.<sup>14</sup> have reported a lack of RGCs in the zebrafish *ath5* mutant, *lackritz*. Nonetheless, the role that *ath5* plays during vertebrate retinal development is likely to be multifaceted. Brown et al.<sup>5</sup> reported that lipofection of mouse *ath5* into the frog retina resulted in an increase in bipolar cells. Through lineage tracing, Yang et al.<sup>15</sup> found that photoreceptors and amacrine cells, in addition to RGCs, may express *ath5* at certain times in their lives. Our recent RPE transdifferentiation assays indicate that chick *ath5* (also referred to as *cath5*) can lead to the photoreceptor pathway (Ma et al., manuscript submitted).

We have previously investigated the roles of *ath5* and *NSCL1* in RGC genesis using the RPE transdifferentiation assay we developed<sup>16–20</sup> (Ma et al., manuscript submitted). The assay takes advantage of the simplicity and plasticity of RPE cells to allow us to examine the proneural activities of various genes or factors. The neural retina not only naturally expresses several proneural genes, but also contains different types of cells, creating molecularly and biochemically heterogeneous cellular contexts and intercellular environments. The non-neural RPE, in contrast, is a single-layered structure that lacks the expression of many proneural genes. Furthermore, the RPE is developmentally related to the retina. RPE tissue at early developmental stages may be triggered to transdifferentiate into a neural retina,<sup>21–24</sup> and cultured RPE cells can be induced to undergo transdifferentiation into what closely resembles retinal neurons.<sup>16–19</sup> An RPE cell transdifferentiation assay showed that *ath5* and *NSCL1* promote bFGF-initiated RPE transdifferentiation toward RGCs, but alone they are insufficient to induce this change; nonetheless, the extent of transdifferentiation is rather limited.<sup>20</sup>

In this study, we examined whether *ath5* and *NSCL1* act synergistically in advancing RGC differentiation. *Ath5* and *NSCL1* were coexpressed through an internal ribosomal entry site (IRES) and a replication-competent retrovirus. We found that retroviral coexpression of *ath5* and *NSCL1* in the developing chick retina increased the number of cells that expressed RGC markers more than the sum of the increases caused when the two genes were expressed separately. In RPE cell cultures, cells morphologically resembling RGCs were readily observed in dishes infected by retrovirus coexpressing *ath5* and *NSCL1*. However, in the absence of bFGF, *ath5* and *NSCL1* coexpression did not alleviate the insufficiency in inducing RPE transdifferentiation toward RGCs.

## Methods

### Generation of Recombinant Retrovirus Coexpressing *ath5* and *NSCL1*

The IRES (nucleotide 2175–2760 in pFB-Neo; Stratagene, La Jolla, CA) was subcloned into a modified version of the adeno-associated virus (AAV) vector (Stratagene), onto which a cassette of *ath5*-IRES-*NSCL1* was built. The translation initiation of *ath5* was under the regulation of the *Src* oncogene sequence originating from the shuttle vector Cla12Nco,<sup>25</sup> and the translation initiation of *NSCL1* was regulated by the IRES. The *ath5*-IRES-*NSCL1* cassette was then inserted into an replication-competent avian RCAS proviral vector<sup>25</sup> for coexpression of *ath5* and *NSCL1* (Fig. 1). Previously described procedures were followed for transfecting chick embryonic fibroblast cells with recombinant proviral DNA and for generating high-titer viral stocks.<sup>16</sup> RCAS retroviruses expressing other genes were generated as described (RCAS-*ath5*,<sup>20</sup> RCAS-*NSCL1*,<sup>9</sup> RCAS-GFP<sup>16</sup>). Titers of the viral stocks ranged from  $5 \times 10^7$  to  $2 \times 10^8$  pfu/mL.

### Microinjection of Retrovirus into the Subretinal Space

To access the developing chick embryos, an opening of approximately  $1 \times 1$  cm was made in the eggshell. Through the opening, approximately 1  $\mu$ L of concentrated viral suspension was placed into the neural tube and the subretinal space of day 2 embryos, using a micropipette attached to a pneumatic pump with a positive-pressure delivery system. The injected viral suspension contained fast green, allowing us to monitor visually the spreading into the subretinal space. Injection was repeated two more times during the day; this procedure routinely produces a thorough infection of the retina.<sup>10,16,26</sup> Viral infection was later detected with a specific antibody against viral protein p27.

### Dissociation of Retinal Cells

Retinas were harvested at embryonic day (E)8. At this time, essentially all the RGCs have been generated and the developmental ganglion cell death that takes place between E10 and E12 has not yet begun. The right-eye (injected) retinas from three or more embryos were pooled, and their cells were dissociated by incubation with trypsin/EDTA followed by mechanical trituration. Dissociated retinal cells were seeded onto polyornithine-treated substratum for 4 hours in a humidified 37°C incubator with 5% CO<sub>2</sub>. Cells were then fixed with ice-cold 4% paraformaldehyde for 30 minutes at room temperature and subjected to double immunostaining to identify retinal ganglion cells (Brn3A<sup>+27</sup> or Islet-1<sup>+28</sup>) that had been infected with the virus (p27<sup>+</sup>). To achieve a cell density suitable for counting, cells equivalent to on thirtieth of an E8 retina were seeded into each 35-mm dish.

### Cultures of Dissociated RPE Cells

RPE was isolated from day-6 chick embryos and cultured as dissociated cells, as previously described.<sup>16,20</sup> When used, bFGF was added to the culture medium at the inception of culture at a final concentration of 10 ng/mL. When the cells reached approximately 50% confluence, which usually took approximately 3 to 4 days, the culture was inoculated with RCAS virus expressing the various genes: *ath5*, *NSCL1*, *ath5-NSCL1*, or GFP as a negative control. Cells in the infected cultures were fixed with 4% paraformaldehyde for 30 minutes and then subjected to immunocytochemical analysis.

### Immunocytochemistry

Monoclonal antibody RA4 (1:1000 dilution) was a gift from Steven McLoon (University of Minnesota, Minneapolis, MN). Monoclonal antibody (clone HM-2; 1:200 dilution) against microtubule-associated protein (MAP)-2 was purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal antibody against Brn3A (1:200 dilution) was purchased from Chemicon (Temecula, CA). Three monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (Iowa University, Iowa City, IA): anti-Islet-1 (clone 39.4D5, 1:100; developed by Thomas Jessell, Columbia University, New York, NY), 3A10 (1:100; developed by Thomas Jessell), and 4H6 (1:100; developed by Willi Halfter, University of Pittsburgh, PA). Antibody against viral protein p27 was purchased from Spafas (Preston, CT). Standard immunocytochemistry was performed with alkaline phosphatase-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA) or fluorophore-conjugated secondary antibodies (Molecular Probes, Eugene, OR), as described by the manufacturers.

### Cell Counting and Analysis

The number of cells with typical neural morphology in each well of a 24-well plate was scored from 10 viewing areas under a 20 $\times$  objective. The counts from three wells were used to calculate the mean and SD with a computer program (Origin 7.0; Microcal, Northampton, MA). Each experiment included samples infected individually with RCAS-*ath5-NSCL1*, RCAS-*ath5*,

RCAS-NSCL1, and RCAS-GFP as a negative control. Experiments were repeated at least three times in their entirety, from RPE dissection to cell counting.

For dissociated retinal cells, the number of single-labeled and double-labeled cells was counted from 20 viewing areas in one 35-mm dish with a 20× objective. The number in the 20 view areas was summed, and the means and SDs of three dishes of each experiment were calculated (Origin 7.0; Microcal). Experiments were repeated at least three times in their entirety, from microinjection to cell counting. Data from RCAS-ath5-NSCL1 were compared with those from RCAS-ath5, RCAS-NSCL1, and RCAS-GFP.

To analyze the number of RGCs on E7 retinal sections, sections were viewed under a 40× objective; 10 viewing areas of infected regions and 10 viewing areas of adjacent, uninfected regions from eight sections per retina were used. The total number of Islet-1<sup>+</sup> cells and Brn3A<sup>+</sup> cells in the selected view areas were scored and used to calculate the percentage of change in the virally infected regions versus that in the adjacent, uninfected regions. The mean and SD of three retinas were calculated (Origin 7.0; Microcal).

## Results

### Enhanced Transdifferentiation into RGCs by *ath5* and *NSCL1* Coexpression in RPE Cell Cultures

To assay whether coexpression of *ath5* and *NSCL1* would be sufficient to trigger RPE transdifferentiation into RGCs, we examined RPE cell cultures infected with RCAS-ath5-NSCL1 for the de novo generation of neuronlike cells bearing similarities to RGCs. Previously, we showed that a single proneural bHLH gene, *neurogenin2*, can induce such transdifferentiation when ectopically expressed in cultured RPE cells through RCAS retroviral transduction.<sup>19</sup> Using the same transdifferentiation system, however, we found that coexpression of *ath5* and *NSCL1* did not induce RPE transdifferentiation toward RGCs. Cultures infected with RCAS-ath5-NSCL1 contained no more cells positive for monoclonal antibody RA4 than did control cultures infected with RCAS-GFP (data not shown). RA4 is an early marker for ganglion cells in the chick retina.<sup>29</sup> Thus, simple coexpression of *ath5* and *NSCL1* did not initiate an RGC differentiation program in cultured RPE cells.

We then examined whether coexpression of *ath5* and *NSCL1* would promote RGC differentiation when the RPE cells were cultured in the presence of bFGF. Previously, we showed that bFGF can initiate RPE transdifferentiation toward RGCs, but the extent of transdifferentiation is very limited, because essentially all RA4<sup>+</sup> cells maintain the fibroblast-like morphology typically of RPE cells in low-density cultures. When the cells are manipulated to express *ath5* or *NSCL1* through retroviral transduction, not only are RA4<sup>+</sup> cells more numerous, but they show altered morphology, such as distinguishable processes<sup>20</sup> (Figs. 2B, 2C; dashed arrows). Nonetheless, the processes on the RA4<sup>+</sup> cells in cultures treated with bFGF and with RCAS-ath5 or RCAS-NSCL1 often are devoid of branches and are shorter and wider than typical neuronal processes (Figs. 2B, 2C; dashed arrows). Only on rare occasions do RA4<sup>+</sup> cells exhibit typical RGC morphology with long, thin, and often branching processes.

When bFGF-primed RPE cells were infected with RCAS-ath5-NSCL1, some of the RA4<sup>+</sup> cells exhibited morphology remarkably resembling typical RGC neurons (Fig. 2A). These cells displayed elaborate cellular processes (Fig. 3). Some cells showed multiple processes emanating from the cell bodies; some had two opposing processes, with one of them forming articulate branches at the end, indicative of fine axons reaching different targets (Fig. 3). The various morphologies of the RA4<sup>+</sup> cells suggested that different subtypes of RGCs might be present in the bFGF-primed RPE cells ectopically coexpressing *ath5* and *NSCL1*. No cells with typical neural morphology were observed in the GFP control. A small number of such cells

were detected when RPE cells were infected with either RCAS-*ath5* or RCAS-*NSCL1* ( $3.3 \pm 1.5$  and  $36.0 \pm 4.3$ , respectively, in 10 viewing areas under a  $20\times$  objective; Fig. 2D). With coexpression of *ath5* and *NSCL1*, the number increased to  $126.7 \pm 5.9$ , which is more than three times greater than the sum of either gene alone.

In addition to the RA4 antigen, cells undergoing transdifferentiation expressed other RGC markers, including calretinin, MAP2, the 160-kDa neurofilament protein recognized by monoclonal antibody 4H6, and the neurofilament-associated antigen recognized by monoclonal antibody 3A10 (Fig. 4). Some of the immunopositive cells exhibited typical neuronal morphology. We did not find detectable levels of expression of Islet-1 or Brn3A (data not shown).

### Increase in RGCs in Retinas Infected with RCAS-*ath5*-*NSCL1*

To address whether *ath5* and *NSCL1* act synergistically in vivo to promote RGCs differentiation, we examined whether *ath5* and *NSCL1* coexpression would result in an increase in cells that expressed RGC markers in the developing retina. RCAS-*ath5*-*NSCL1* was used to achieve coexpression of *ath5* and *NSCL1* in the developing retina by microinjecting the virus into the subretinal space at E2, the optic cup stage. Retinas were harvested for analysis of the number of RGCs at E7 to E8, when ganglion cell genesis is essentially finished. Another reason for using E7-to-E8 retina was to avoid complications caused by cell death that we have previously observed to take place beginning at E9 in retinas misexpressing *NSCL1*.<sup>10</sup> To facilitate cell identification and data analysis, we used monoclonal antibodies that recognize nuclear proteins Islet-1<sup>28</sup> and Brn3A.<sup>27</sup>

In initial experiments, we performed repeated microinjection, each time with  $\sim 1 \mu\text{L}$  of virus (titer  $\sim 5 \times 10^7$  pfu/mL) placed into the subretinal space and the neural tube, aiming at a thorough infection of the retina.<sup>10,16,26</sup> Because RCAS is replication-competent and because a small amount of the injected viral suspension may overflow from the injection site onto the embryo, a widespread viral infection in the embryos, including systemic regions, often occurs several days after the injection.<sup>30</sup>

Chick embryos injected with RCAS coexpressing *ath5* and *NSCL1* developed normally. They appeared indistinguishable from those not subjected to experimental manipulation and those injected with RCAS-*ath5* or RCAS-GFP. This was unexpected, as we had observed that a similar microinjection of RCAS-*NSCL1* results in severe growth retardation and embryonic lethality with no embryos surviving longer than E13. In addition, retroviral *NSCL1* expression in the developing retina causes microphthalmia and gross distortion of retinal lamination.<sup>10</sup> None of these effects was observed with embryos coexpressing *ath5* and *NSCL1*. The size and appearance of the eye and the lamination of the retina were indistinguishable from the controls (data not shown). The phenotypic disparities between expression of *NSCL1* and coexpression of *ath5* and *NSCL1* could be due to a lower *NSCL1* expression level in the coexpression construct, since the level of protein synthesis regulated through the IRES is believed to be low,<sup>31</sup> or, alternatively, to a possible attenuation of the teratogenic effect of *NSCL1* by *ath5*.

Despite their normal appearance, retinas infected with RCAS-*ath5*-*NSCL1* contained more RGCs. To reduce ambiguity associated with comparing retinal sections of different eyes, we generated partially infected retinas using a single, instead of a repetitive, microinjection. A partially infected retina allows for a direct comparison between virally infected and uninfected regions at adjacent locations in the same retinal section. We found that E7 retinas that had been infected with RCAS-*ath5*-*NSCL1* contained more Islet-1<sup>+</sup> cells in the infected regions than the adjacent, uninfected regions (Figs. 5G, 5H). An increase in Islet-1<sup>+</sup> cells was also observed in regions infected with RCAS-*ath5* (Figs. 5C, 5D) and RCAS-*NSCL1* (Figs. 5E, 5F), whereas the number of Islet-1<sup>+</sup> cells appeared similar between infected and uninfected regions in

embryos microinjected with RCAS-GFP (Figs. 5A, 5B). Cell counting revealed that the increase due to *ath5* and *NSCL1* coexpression was greater than that of expressing *ath5* or *NSCL1* alone. On average, coexpression of *ath5* and *NSCL1* resulted in a more than 45% increase in the number of Islet-1<sup>+</sup> cells, whereas expression of either alone resulted in increases of ~20% and ~10%, respectively (Fig. 5I).

The number of Brn3A<sup>+</sup> cells also increased with expression of either gene alone and with coexpression of *ath5* and *NSCL1*. Coexpression of *ath5* and *NSCL1* in the developing retina resulted in a 30% increase in cells expressing Brn3A, whereas expression of either *ath5* or *NSCL1* alone resulted in an increase of approximately 5% (Fig. 6). The increase in Brn3A<sup>+</sup> cells with coexpression was more than the sum of the increases by the expression of either gene alone.

The number of RGCs was also scored, using dissociated E8 retinal cells to avoid histologic variations. In the control retina, 6.92% ± 0.87% of the cells infected with RCAS-GFP developed into RGCs and expressed Islet-1 (Fig. 7A). In the experimental retinas, 9.58% ± 0.28% and 10.48% ± 0.61%, respectively, of retina cells infected with RCAS-*NSCL1* or RCAS-*ath5* were Islet-1<sup>+</sup>, increases of 38% and 51%, respectively, over the control. When retina was infected with RCAS-*ath5*-*NSCL1*, 15.73% ± 0.87% of the infected cells were Islet-1<sup>+</sup>, an increase of 127% over the control (Fig. 7A). Similarly, the percentage of infected retinal cells that expressed Brn3A increased from 4.4% ± 0.51% in the GFP retinas to 7.39% ± 0.51% in the *NSCL1* (68% increase), 7.35% ± 0.41 (67% increase) in *ath5* retinas, and 10.03% ± 0.64% (128% increase) in the *ath5* + *NSCL1* retinas (Fig. 7B). All the increases were statistically significant at  $P \leq 0.01$  compared with that of GFP, and the increase in coexpression over either gene alone was also significant at  $P \leq 0.01$ . The increases in Islet-1<sup>+</sup> cells in the coexpressing retinas were higher than the sum of single expression of the two genes.

## Discussion

In the developing retina, coexpression of *ath5* and *NSCL1* resulted in an increase in the number of RGCs, whereas smaller increases were observed with expression of *ath5* alone or *NSCL1* alone. Intuitively, these increases suggest that *ath5* and *NSCL1*, particularly when coexpressed, recruit more multipotent progenitors to differentiate into RGCs and thus may have played a role in determination of RGC fate. However, it is also possible that the increase was due to enhanced RGC differentiation that had been potentiated by other factor(s). In this scenario, cells fated to be RGCs remained uncommitted in the absence of *ath5* and *NSCL1*, and their predetermined differentiation along the RGC pathway was promoted by *ath5* and *NSCL1* expression. This possibility is favored by the observation that *ath5* and *NSCL1* caused an increase in the number of RA4<sup>+</sup> cells in RPE cell culture in the presence of bFGF, which by itself induces de novo expression of RA4.<sup>18,20</sup> In the absence of bFGF, neither gene nor a combination of the two had an effect on inducing RPE transdifferentiation. Thus, coexpression of *ath5* and *NSCL1* in the developing retina may have resulted in enhanced commitment to RGCs through advancing an RGC cell differentiation program initiated by another factor, such as bFGF, in cells that otherwise could be lured into other pathways by other genes or environmental cues.

The morphologic transdifferentiation observed with *ath5* and *NSCL1* coexpression was robust, and the resemblance to RGCs is indicative of the cooperation among *ath5*, *NSCL1*, and bFGF in bringing about neural differentiation that may involve a spectrum of genes. Note, however, that not every gene that is expressed by RGCs in the developing retina was expressed at a detectable level in the transdifferentiating cells. The cells did not, for instance, stain positively with antibodies against Islet-1 or Brn3A. This could be because of several factors, singly or in combination. First, it is possible that *ath5* and *NSCL1* induce a portion of genes needed for

RGC development, and thus their coexpression inherently could elicit only a limited number of genes. Second, the RPE cells provided a different cellular context than retinal cells, and thus coexpression of *ath5* and *NSCL1* in RPE cells was unable to induce the entire repertoire of genes that are otherwise downstream of *ath5* and *NSCL1* in retinal cells. It should be pointed out, nonetheless, that previous studies have indicated that RPE cells have the capacity to transdifferentiate into cells with characteristics of RGCs. For instance, ectopic expression of *ngn2* in cultured RPE cells induces de novo production of cells morphologically and molecularly resembling RGCs,<sup>19</sup> and bFGF induces RPE tissues in young chick embryos to transdifferentiate into a neural retina encompassing retinal neurons including RGCs.<sup>21–24</sup> Third, dissociated RPE cells used in this study were more fastidious than intact RPE tissue, and thus certain neural traits would only be detected with factors of high inducing power. Notably, studies have shown that dissociated RPE cells failed to manifest the neural transdifferentiation phenomenon that was observed with RPE tissue.<sup>23</sup> Fourth, the *ATH5* and *NSCL1* protein levels in the RPE cells were insufficient to induce certain genes. This is supported by the lack of embryonic lethality of the coexpression construct. Fifth, the microenvironment under our experimental conditions was not permissive for RGC development. The observed increases in *Islet-1*<sup>+</sup> and *Brna3A*<sup>+</sup> cells in the developing retina coexpressing *ath5* and *NSCL1* argues that the developing retina provides additional factors that are lacking in culture conditions and that are required for expression of these genes and for RGC differentiation.<sup>23,29–35</sup> Together with those factors, *ath5* and *NSCL1* may regulate a spectrum of targets associated with RGC development and differentiation. In light of this, in vitro-generated cells may manifest a greater degree of differentiation on exposure to the native microenvironment than when grown in culture.

Ectopic coexpression of *ath5* and *NSCL1* in cultured RPE cells in the absence of bFGF did not induce de novo expression of RA4, an outcome similar to ectopic expression of *ath5* or *NSCL1* alone. Thus, combining *ath5* and *NSCL1* did not promote an RGC fate under the experimental conditions. Coexpression of *ath5* and *NSCL1* promoted bFGF-initiated RGC differentiation and to a higher degree than either gene alone. These findings, together with the significant increase in RGCs in the developing retina with coexpression over single expression, suggest that *ath5* and *NSCL1* probably function synergistically in advancing the RGC differentiation program.

The absence of embryonic lethality after *ath5* and *NSCL1* coexpression was unexpected because, of more than 100 embryos misexpressing *NSCL1* thus far generated in our laboratory, none have survived longer than embryonic day (E)13.<sup>30</sup> The absence of embryonic lethality could be due to a lower level of *NSCL1* protein synthesized under IRES regulation.<sup>31</sup> This seems consistent with the observation that *Islet-1*<sup>+</sup> cells were detected in RPE culture infected with RCAS-*NSCL1*,<sup>20</sup> in which *NSCL1* translation was under the *Src* oncogene control, and were not detected with the coexpression construct, in which *NSCL1* translation was under IRES control. Apparently, under IRES regulation, *NSCL1* protein was synthesized at a sufficient level to exert a biological effect as manifested by the increased RGC differentiation in vivo and in vitro. Alternatively, the absence of embryonic lethality after *ath5* and *NSCL1* coexpression could be due to *ath5*'s having a certain rescue effect and thus attenuating the teratogenic effect of *NSCL1* on embryonic development. More studies are needed to verify this.

Generating RGCs in vitro is a tantalizing prospect for both basic and clinical research. The present studies show de novo generation of cells that closely resembled RGCs morphologically from bFGF-primed cultured RPE cells coexpressing *ath5* and *NSCL1*. It remains to be further investigated whether these cells have the potential to mature into functional RGCs, the development of which is likely to require intricate interplay between intrinsic and extrinsic factors present in the developing retina.

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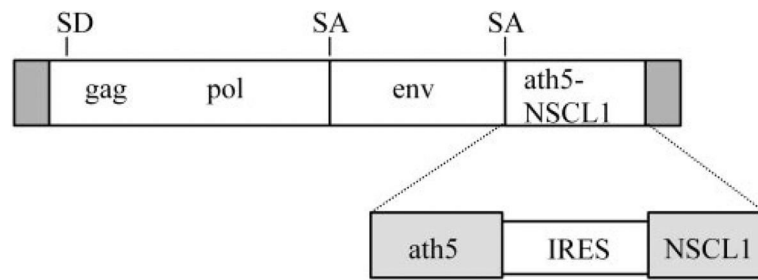
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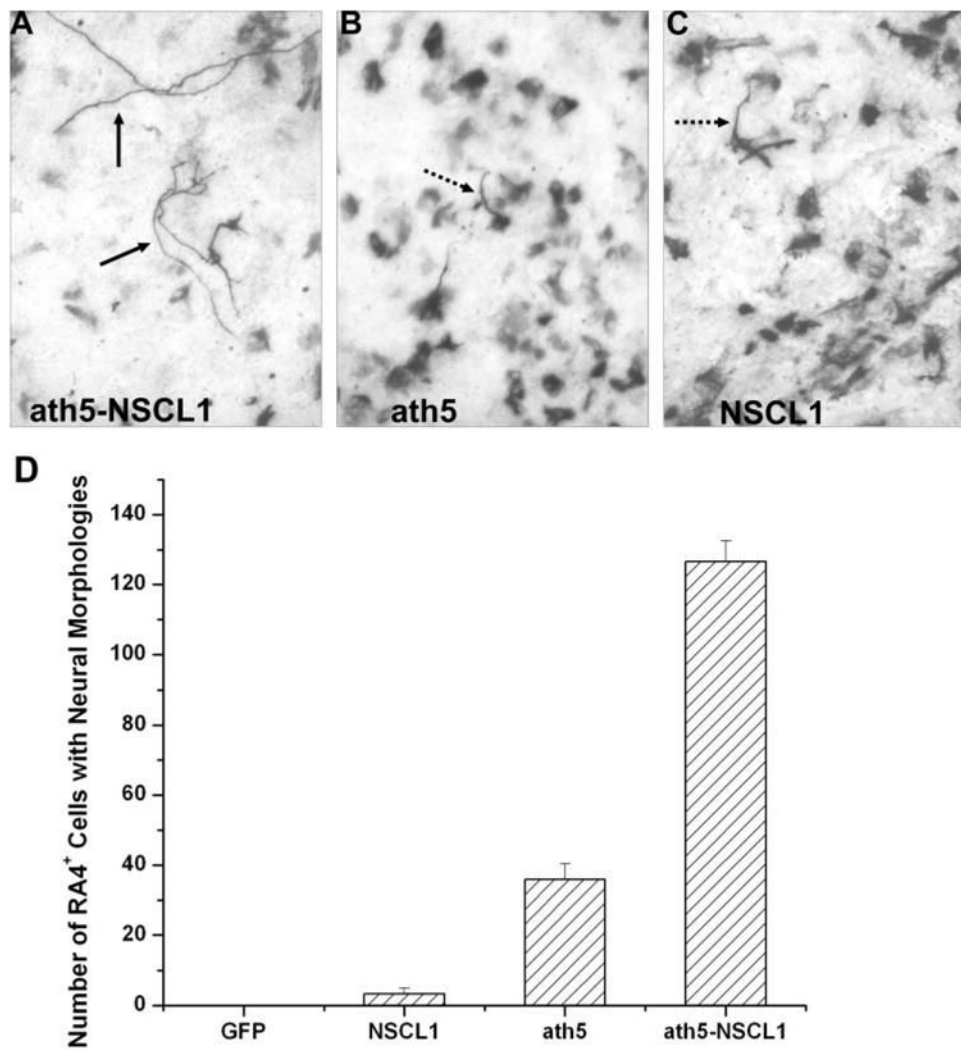
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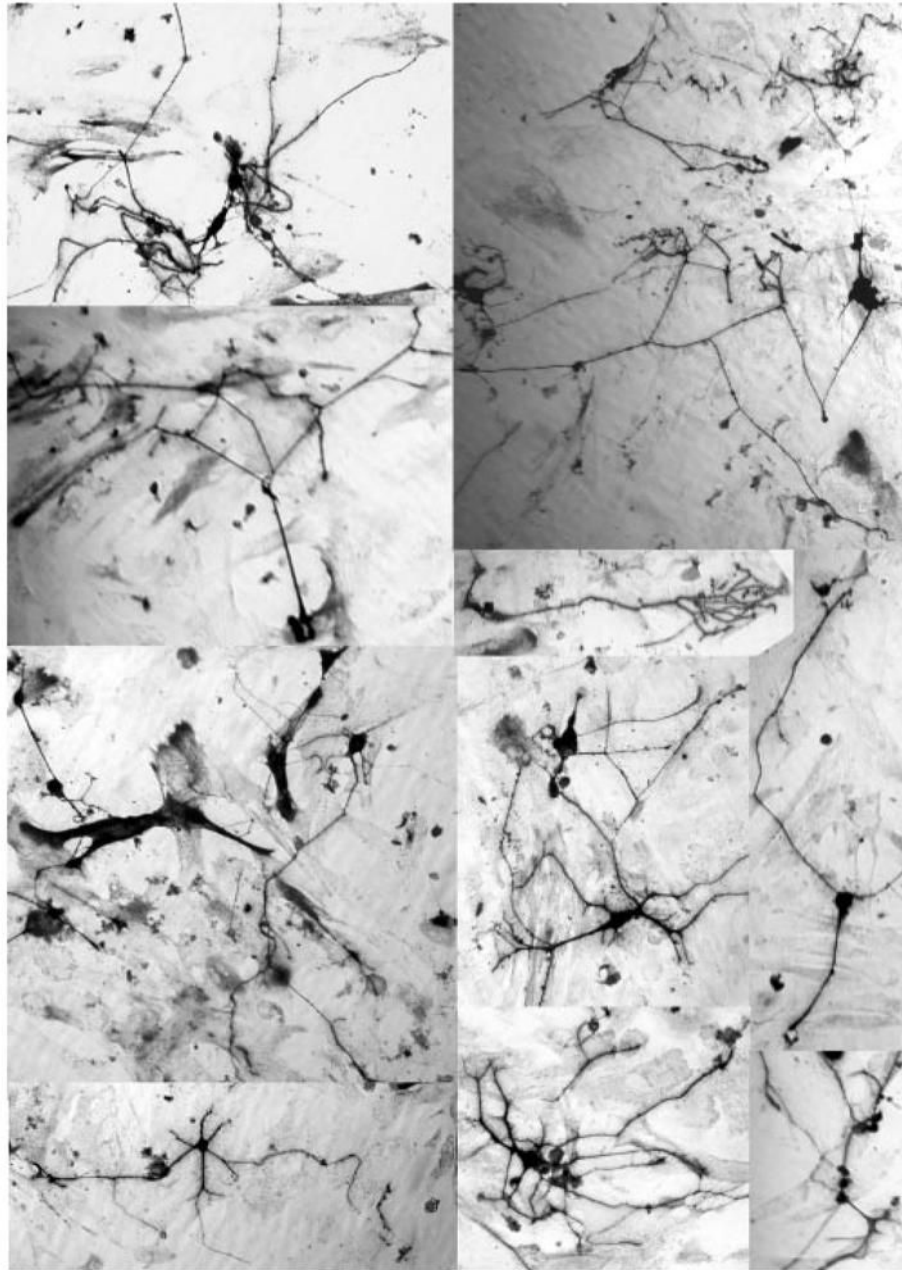
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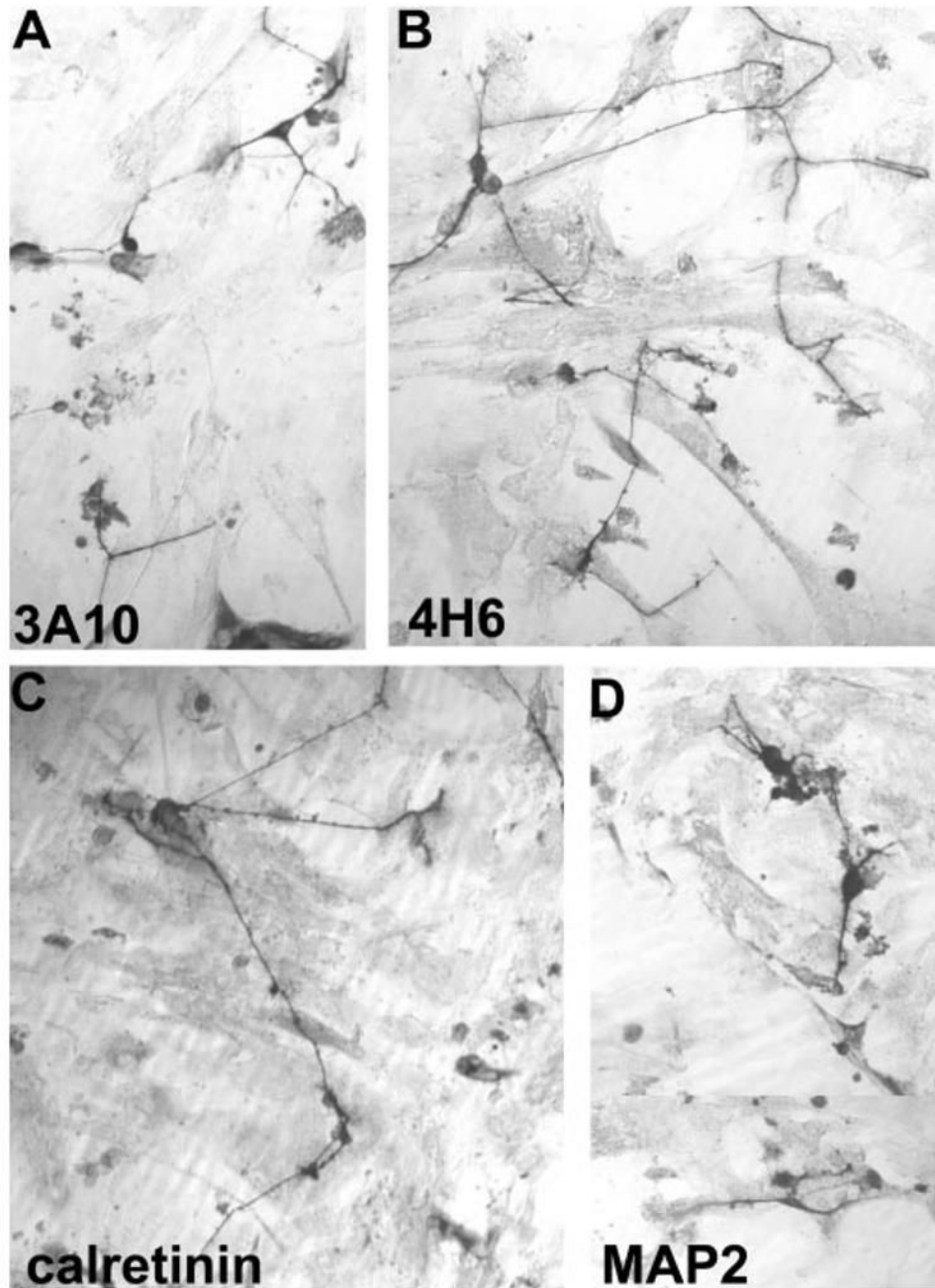
**Figure 1.** Schematic illustration of RCAS coexpressing *ath5* and *NSCL1*. SD, splicing donor; SA, splicing acceptor.



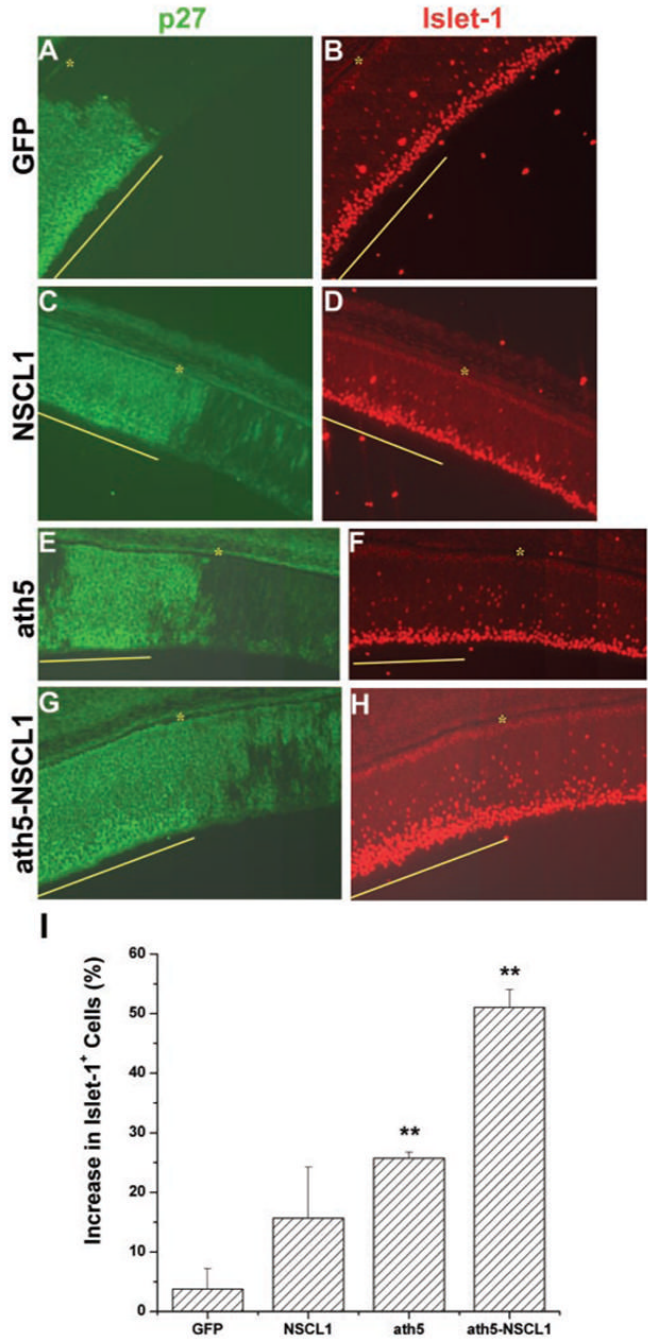
**Figure 2.** RA4<sup>+</sup> cells in bFGF-primed RPE cell cultures infected with retroviruses expressing various genes. (A–C) Morphology of RA4<sup>+</sup> cells in cultures infected with RCAS-ath5-NSCL1, RCAS-ath5, or RCAS-NSCL1. *Dashed and solid arrows*: distinguishable processes. (D) The number of RA4<sup>+</sup> cells with typical neural morphology. Shown are the mean and SD in 10 viewing areas under a 20× objective.



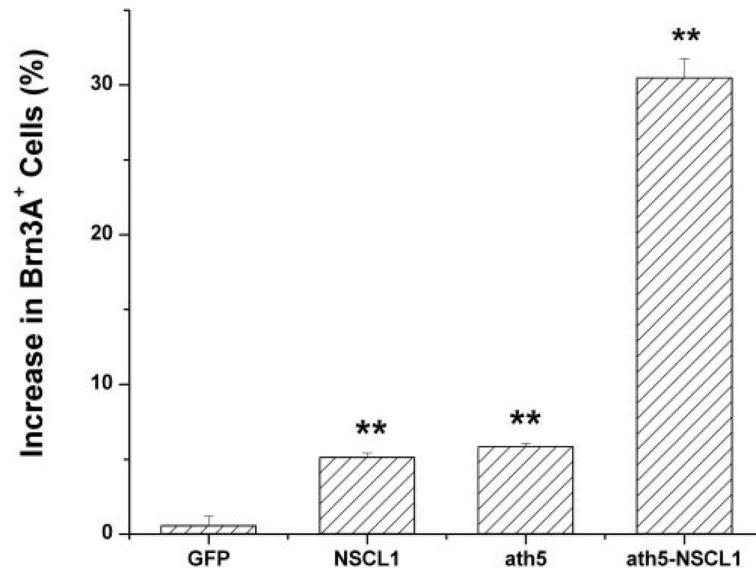
**Figure 3.** The neuronlike morphology of RA4<sup>+</sup> cells from bFGF-primed RPE cell cultures ectopically coexpressing *ath5* and *NSCL1*.



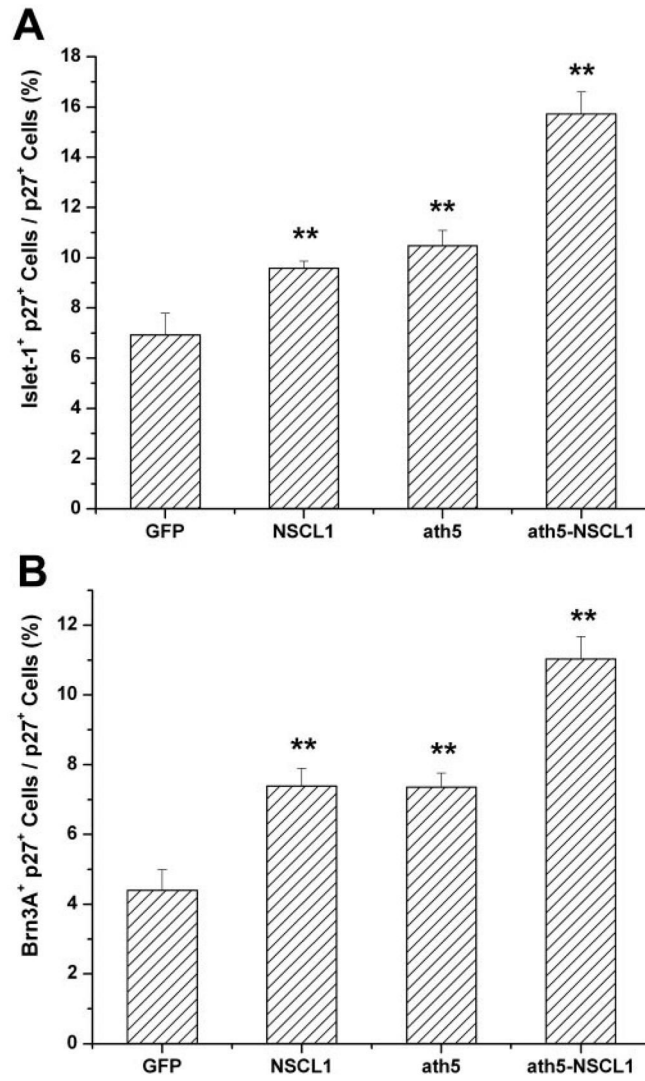
**Figure 4.** The neuronlike morphology of cells immunopositive for (A) 3A10, (B) 4H6, (C) calretinin, and (D) MAP2. RPE cultures were primed with bFGF and infected with RCAS-ath5-NSCL1.



**Figure 5.** The increase in Islet-1<sup>+</sup> cells in E7 retinas partially infected with retrovirus expressing ath5-NSCL1, ath5, or NSCL1. Each section was double stained to show the regions infected by the virus (p27 immunostaining; **A, C, E, G**) and expression of Islet-1 (**B, D, F, H**). RCAS expressing GFP (**A, B**) was used as the control. *Yellow line*: virally infected regions; (\*) RPE. **(I)** The percentage increase in the Islet-1<sup>+</sup> cells in the infected regions compared with that in adjacent, uninfected regions. \*\*Statistically significant difference at  $P \leq 0.01$  compared with the GFP sample.



**Figure 6.** The percentage of increase in Brn3A<sup>+</sup> cells in regions infected with the virus compared with that in uninfected regions in E7 retinas. \*\*Statistically significant difference at  $P \leq 0.01$  compared with the GFP sample.



**Figure 7.** The percentage of p27<sup>+</sup> cells that expressed a ganglion cell marker: Islet-1 (A) or Brn3A (B). \*\*Statistically significant difference at  $P \leq 0.01$  compared with the GFP sample.