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Pro-photoreceptor activity of chick *neurogenin1*

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Abstract

Purpose—A better understanding of photoreceptor fate specification may lead to efficient production of photoreceptors for cell replacement studies. This study investigates the role of proneural bHLH gene *neurogenin1* (*ngn1*) in photoreceptor genesis using the chick retina.

Methods—In situ hybridization was used to delineate the spatial and temporal pattern of *ngn1* expression. RCAS retrovirus was used to drive overexpression of *ngn1* in retinal cells, and siRNA was used to reduce *ngn1* expression in loss-of-function experiments.

Results—Chick *ngn1* was transiently expressed during early phases of retinal neurogenesis, from embryonic day 3 (E3) to E6, with cells expressing *ngn1* confined to the apical side of the retinal neuroepithelium. The time window and the anatomical location of *ngn1* expression coincided with photoreceptor genesis and differed from that of other transiently expressed proneural bHLH genes, such as *ash1*, *ath3*, *ath5*, and *ngn2*. Most *ngn1*-expressing cells lacked BrdU incorporation and lacked phosphorylated histone H3. In low density cell culture, *ngn1* overexpression increased *neuroD* expression, expanded the photoreceptor population, but reduced the ganglion population. Treatment of dissociated retinal cells with siRNA against *ngn1* mRNA specifically reduced the photoreceptor population. Overexpression of *ngn1* in the retina reduced the expression of *ash1*, *ath5*, *chx10*, and *ngn2*.

Conclusions—The data suggest that *ngn1* participates in a complex transcriptional network and may play a role in guiding a progenitor cell to the photoreceptor pathway.

Keywords

gene expression; photoreceptor; retinal development; transcription factors; proneural gene

Introduction

Photoreceptors are specialized sensory neurons in the retina. Because they are terminally differentiated and do not re-enter the cell cycle for regeneration, photoreceptors lost due to various causes cannot be replenished, leading to irreversible blindness. [1,2] One of the potential therapies is cell-replacement with developing photoreceptors. [3] This promising approach faces a major roadblock – the need for a supply of photoreceptors. [4] As a result, attention has been directed at inducing photoreceptor genesis through programming or reprogramming the differentiation of cells that can be propagated in large amounts. Key to this approach is

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knowledge of the decision-making factor(s) that can guide a progenitor cell to the path of differentiating into a photoreceptor.

The photoreceptors and their "siblings" (i.e., the other five major types of retinal cells) arise from a pool of multipotent progenitors during vertebrate retinal development. [5] What dictates photoreceptor fate over other options during retinal neurogenesis has been a central question in the developmental biology of the retina. Photoreceptor development and maturation requires a number of regulatory genes, such as homeodomain genes *crx* [6–8] and *raxL*; [9] the neural retina leucine-zipper gene *NRL*; [10,11] thyroid hormone receptor *TRβ2*; [12] and basic helix loop-helix (bHLH) gene *neuroD*. [13–15] Whether there is a single key factor dictating photoreceptor fate remains elusive.

Proneural bHLH genes have been shown to play important roles in specifying neural fates/diversities in both the central and the peripheral nervous systems (CNS and PNS). The developing vertebrate retina expresses several such genes, such as *achaete-scute* homologue 1 (*ash1*), *atonal* homologue 3 (*ath3*), *ath5*, *neuroD*, *neurogenin1* (*ngn1*), *ngn2*, *ngn3*, *NSCL1*, and *NSCL2*. Proneural bHLH genes known to be expressed in retinal progenitor cells include *ash1*, *ath3*, *ngn1*, *ngn2*, and *ngn3*. [16–21] Analysis of retinal explants derived from *ash1*-null mice indicated that *ash1* participates in the production of late-born neurons, including rod photoreceptors and bipolar cells. [22] In the chick retina, *ash1* was proposed to promote amacrine cells, [23] and this was later confirmed experimentally. [24] Studies have indicated *ath3* in the production of bipolar and amacrine cells. [25–27] *Ngn2* is expressed in the proliferating zone, [25,28] including cells still in the cell cycle. [29,30] In the mouse retina, regions lacking *ngn2* expression contain no photoreceptor cells, indicating that *ngn2* has a role in photoreceptor genesis. [28] Analyses of retinas from double and triple knockoutsindicate that *ngn2* may also play an important role in horizontal cell genesis. [31] A fate mapping study showed that cells expressing *ngn2* develop into all major cell types in the mouse retina. [30] Chick *ngn3* is transiently expressed during early retinal neurogenesis, and its overexpression increases the population of ganglion cells such that they expand into the territory normally occupied by amacrine cells. Overexpression of *ngn3* induces *ngn1* expression, while suppressing the expression of *ngn2*, *ash1*, and *ath3*. [21] The role of *ngn1* in retinal cell fate specification is not well established, even though in the brain *ngn1* is known to play a determinative role in generating neural diversity. [32,33] Perron et al. [16] reported a specific, albeit moderate, increase in the photoreceptor population upon overexpression of a related gene, *ngnr1*, in Xenopus retina. Thummel et al. [20] found that zebrafish *ngn1* is expressed in developing retina and during photoreceptor regeneration after light-induced photoreceptor degeneration. These studies suggest that *ngn1* may be involved in photoreceptor generation.

We have investigated the expression of *ngn1* in the developing chick retina and the role of *ngn1* in retinal cell generation. We report that the expression of chick *ngn1* was transient and restricted to early neurogenesis, with a spatial and temporal window of expression coinciding with the generation of photoreceptor precursor cells. In retinal cell culture, *ngn1* overexpression increased the photoreceptor population at the expense of ganglion cells, while siRNA against *ngn1* reduced the photoreceptor population. Overexpression of *ngn1* in the developing retina reduced the expression of other regulatory genes. These results suggest that *ngn1* participates in regulatory networks governing retinal neurogenesis and has a role in leading progenitor cells to take the photoreceptor pathway.

Materials and Methods

Chick embryos

Fertilized, pathogen-free White Leghorn chicken eggs were purchased from Spafas and incubated in a Petersime incubator. All use of animals adhered to the ARVO Statement for the

Use ofAnimals in Ophthalmic and Vision Research and the procedures and policies set by the Institutional Animal Use and Care Committee at the University of Alabama at Birmingham.

Generation of RCAS-ngn1 retrovirus

Based on published information, [34] we amplified the coding region of chick *ngn1* with RT-PCR. After cloning and its sequence verification, the DNA was subcloned into shuttle vector Cla12Nco and then inserted into proviral vector RCAS. [35] Virus particles were produced by transfecting chick embryonic fibroblast cells with the recombinant proviral DNA. Concentrated viral stocks (~1×10^8 pfu/ml) were prepared as described. [36]

Microinjection of retrovirus into chick embryos

Concentrated RCAS-ngn1 virus, or control RCAS-GFP virus, [36] was microinjected into the neural tube and the subretinal space (between the two layers of the optic cup) of day 2.5 chick embryos (E2.5, stage 15–17), as previouslydescribed. [36] Infected eyes were enucleated at various developmental stages and fixed with ice-cold 4% paraformaldehyde, cryoprotected with OCT:sucrose (2:1), frozen with liquid nitrogen, and kept at −80°C. Infection by RCAS viruses (RCAS-ngn1 and RCAS-GFP) was visualized by immunostaining with an antibody against viral protein p27.

Low density retinal cell culture

Retinas (n=3–16) were dissected from E4.5 – E8.5 chick embryos infected with RCAS-ngn1 or RCAS-GFP as control. Retinal cells were dissociated with trypsin-EDTA and seeded into the wells of 24-well plates treated with polyornithine at a density that covered <1/5 of the surface area. After 4 days in culture with Medium 199 supplemented with 10% fetal calf serum, cells were fixed with ice-cold 4% paraformaldehyde, the subjected to immunostaining or in situ hybridization. For experiments with E4.5 and E8.5 retinas, double-labeling for viral (p27) and retinal markers was carried out. The number of the positive cells and the number of total cells were scored from 9 view areas from each culture well, each with 40–300 cells, under a 20x objective. The percentage of positive cells was calculated for one well, and the means and SDs from 3 wells were calculated using the computer program Origin 7.0 (OriginLab Corp.).

Low density retinal cell culture with siRNA

Retinal cells from E4.5 chick embryos were seeded at low density, as described in the previous subsection. Poly-ornithine-treated glass coverslips were coated with siRNA immediately before cell seeding. Two *Silencer*® Select pre-designed siRNAs against *ngn1*, uucgauuuuggugaguuugGT and uaagggugugcagcaaagcCT, were selected and synthesized by Ambion (Applied Biosystems). *Silencer*® Select Negative Control #1 siRNA from Ambion was used as a control. SiRNA and the transfection agent (siPORT™ NeoFX™ transfection agent, Ambion) were mixed following the manufacturer's instructions, and added to the cell culture at a final concentration of 300 nM. The culture was maintained for 2 or 4 days before fixation for immunocytochemistry, in situ hybridization, or TUNEL analysis. Scoring cell numbers and statistical analysis were as described in the previous subsection.

In situ hybridization

Digoxigenin (Dig) labeled antisense RNA probe against *ngn1* was prepared from linearized plasmids harboring the coding sequence using the Genius kit (Roche Molecular Biochemicals) following the manufacturer'sinstructions. Dig-labeled antisense RNA probes against chick *ash1*, [21] *ath3*, [37] *ath5*, [38] *chx10*, [37] *neuroD*, [36] *ngn2*, [29] and *ngn3*21 were prepared as described. Retinal cryosections of 10 μm thick were used for in situ hybridization following procedures described previously. [37]

Immunocytochemistry

The following monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (University ofIowa): anti-bromodeoxyuridine (BrdU, clone G3G4,1:100; developed by Dr. Stephen J. Kaufman), anti-islet-1 (clone39.4D5, 1:100; developed by Dr. Thomas Jessell), anti-AP2α(3B5, 1:50; developed by Dr. Trevor Williams), and anti-visinin (clone 7G4, 1:500; developed by Dr. Constance Cepko). Antibodies obtained from a commercial source included: monoclonal antibody against Brn3a (1:200; Chemicon); polyclonal antibody against red opsin (1:200; Chemicon); polyclonal antibody against calretinin (1:500; Chemicon), anti-phosphorylated histone H3 (1:200; Upstate Biotechnology), and polyclonal antibody against an RCAS viral protein, p27 (1:500; Spafas). Monoclonal antibody RA4 (1:1000dilution) was a gift from Dr. Steven McLoon (University of Minnesota). A monoclonal antibody against chick NeuroD was produced in our laboratory. Standard immunocytochemistry was performed with secondary antibodies conjugated with peroxidase, alkaline phosphatase (Vector Laboratories), or fluorophore (Molecular Probes).

TUNEL assay

TUNEL assay was used to detect the presence of apoptotic cells in E4.5 retinal cell culture, using the In Situ Cell Death Detection Kit (RocheMolecular Biochemicals) following the manufacturer's instruction.

Pulse-labeling chick embryos with BrdU

BrdU (50 μg in 50 μlof HBSS) was dropped through an opening in the shell onto the vitelline membrane of E5 and E7.5 chick embryos. The embryos were incubated for 3 hours before the eyes were harvested and fixedwith 4% paraformaldehyde. For double labeling, cryosections were first subjected to in situ hybridization with Dig-labeled anti-*ngn1* RNA probesand then to BrdU detection using a specific antibody as previouslydescribed. [37]

Results

Transient *ngn1* expression in early retinal development

To gain a perspective on how *ngn1* might function in the retina, its expression was examined and compared with the expression of some better-studied proneural bHLH genes. The earliest retinal expression of *ngn1* detected with Dig-labeled antisense RNA probes was embryonic day 2.5 (E2.5), when some positive cells were observed at the central region (Fig. 1A). At this time, *ath5* expression was visible in only a few cells (Fig. 1B), while no *ngn2* expression was evident (Fig. 1C). Expression of *ngn1* (Fig. 1D), ath5 (Fig. 1E), *ngn2* (Fig. 1F), and *ash1* (Fig. 1G) was visible in the E3.5 retina. Starting from E4.5, differences emerged in the spatial expression patterns of the genes. Cells expressing *ngn1* localized to the outermost zone in the pseudostratified retinal neuroepithelium at E4.5 (Fig. 1H) and E5.5 (Fig. 1J), an anatomical location of newly born photoreceptor precursors. This spatial pattern is similar to *neuroD* expression (Fig. 1L). Chick *neuroD* is expressed in young photoreceptor cells or their precursors. [14,36] However, *neuroD* expression is not evident in retinas younger than E5. [36] Thus, *ngn1* expression preceded that of *neuroD*. Cells expressing *ath5* mostly localized to the vitreal side (Fig. 1I), and cells expressing *ngn2* were distributed across the neuroepithelium (Fig. 1K). Expression of *ngn1* began to decrease in the E6 central retina (data not shown) and by E7.5 the expression of *ngn1* was no longer detectable in the central region (Fig. 1M). This temporal pattern coincided with photoreceptor genesis, which in the chick retina mostly takes place between E5 and E7. [39] The E7.5 retina continued the expression of *ath5* (Fig. 1N), *ngn2* (Fig. 1O), *ash1* (Fig. 1P), and *neuroD* (Fig. 1Q), each with its distinctive spatial pattern maintained, except *ath5*, which had an additional zone of expression in the outer portion of the retinal neuroepithelium. Overall, while partially overlapping with *ngn3* expression (Ma et al.,

2009), *ngn1* expression differed spatially and temporally from that of *ath5*, *ngn2*, *neuroD*, and *ash1*.

In addition to coinciding with the prospective location of photoreceptor cells, the localization of *ngn1*-expressing cells to the outermost region of the retinal neuroepithelium also coincided with the location of M-phase cells. To determine whether *ngn1* expression defined M-phase cells, double-labeling for phosphorylated histone H3 (phospoH3) and for *ngn3* mRNA was carried out. Double-labeled cells were detected (Fig. 2A–C), but accounted for ~10% of the phospoH3$^+$ cells and ~5% of the *ngn1* mRNA$^+$ cells. A typical view area under a 40 x objective contained 3 double-labeled cells from 26 phospoH3$^+$ cells and 60 *ngn1* mRNA$^+$ cells. To determine whether *ngn1*-expressing cells were cycling or postmitotic, double-labeling for *ngn1* expression and BrdU incorporation was carried out after pulsing the embryo with BrdU. Only a small number of double-labeled cells were detected in the retina (Fig. 2D) and the brain (Fig. 2E). In a typical view area with a 40 x objective, 4 were double-labeled, representing 7% of the 61 *ngn1* mRNA$^+$ cells and 3% of the 156 BrdU$^+$ cells. Thus, the majority of *ngn1*-expressing cells lacked phospoH3 or BrdU incorporation.

Effect of *ngn1* overexpression on retinal neurogenesis

The replication-competent avian leukosis virus RCAS [35] was used to drive the overexpression of *ngn1* in chick embryos. This virus infects an increasing number of cells over time in a population of proliferating cells, because viruses released from infected cells can infect other cells. [40,41] RCAS infection and transduction do not affect retinal development; we have observed no abnormalities either at the gross level or at the microscopic level from hundreds of embryos infected with RCAS or RCAS-GFP. [21,24,42,43] Analyses of cell populations in regions infected with RCAS-GFP against the adjacent, uninfected regions revealed no differences in the numbers of AP2$^+$ cells, Pax6$^+$ cells, chx10$^+$ cells, visinin$^+$ cells, BrdU$^+$ cells, and ngn2$^+$ cells. [24] RCAS-ngn1, or RCAS-GFP, was microinjected into the lumen of the neural tube and the subretinal space at E2.5 (~64 hours of incubation) through ~E3 (70 hours of incubation), [24] because during this time the neural tube and the subretinal space remain connected. Expression of the transgene was expected to begin at ~E3.5 and to persist thereafter until the retina was harvested for analysis, based on the fact that transgene expression becomes apparent 21–24 hours after infection. [40,41] Previous studies showed that RCAS-driven overexpression of bHLH genes may alter retinal neurogenesis in a gene-specific manner. [21, 24,44–46] Retinal sections from embryos infected with RCAS-ngn1 were examined histologically, followed by immunohistochemistry with antibodies that recognize specific cell types. Retinas from embryos (n>50) microinjected with RCAS-ngn1 displayed no obvious alterations in histology or in retinal neurogenesis. For unequivocal evidence, we compared immunostaining of regions infected by RCAS-ngn1 with adjacent, uninfected regions on the same retinal section. This is to minimize ambiguities arising from comparing retinas that may vary due to differences in developmental stages and/or the angles at which the retinas were sectioned. Molecular analyses with specific antibodies showed no changes in the populations of photoreceptor (visinin$^+$, Fig. 3A, B), amacrine (AP2α$^+$, Fig. 3C, D), or ganglion cells (Brn3A$^+$, Fig. 3E, F).

Effects on regulatory gene expression by *ngn1* overexpression

To determine whether *ngn1* overexpression might have altered gene expression without concomitant alterations in cell production, we examined the retinas infected with RCAS-ngn1 for the expression of a number of regulatory genes involved in different retinal cell populations. For the immunohistochemical analysis, uninfected regions in the same retinal sections were used as internal controls to avoid ambiguities from comparing different retinal sections that potentially differed in developmental stage or angle of sectioning. In retinas infected with RCAS-ngn1, expression of *neuroD* (Fig. 3G, H) remained unchanged, despite the spatial

similarity of their expression. Expression of *ath3*, which plays an important role in the development of progenitor cells and bipolar cells, [27] also remained unaltered with *ngn1* overexpression (Fig. 3I, J; Fig. 5G). On the other hand, expression of *chx10*, a homeodomain gene with important roles in the development of progenitor cells and bipolar cells, [47] was essentially abolished in the region infected with RCAS-ngn1, whereas in the adjacent, uninfected region a high level of *chx10* expression was observed (Fig. 3K, L; Fig. 5G). *Ngn1* overexpression also reduced the expression of *ath5* (Fig. 3M, N; Fig. 5G), which is required for ganglion cell development (for review see Mu and Klein [48]) and may also participate in the production of other retinal cells. [49]

Ash1 plays a determinative role in generating neural diversity in other regions of the CNS. [32,33] In the retina, *ash1* expression is detected during both early and late phases of neurogenesis in a subpopulation of retinal progenitor cells, and it may participate in the generation of amacrine cells and/or rod photoreceptors and bipolar cells, which are the last born neurons in the retina. In E7.5 retina, *ash1* expression is detected in cells within the proliferating zone, and the expression was unaffected by RCAS-GFP infection (Fig. 4A, B). In E7.5 retinas infected with RCAS-ngn1, expression of *ash1* was abolished at the periphery (Fig. 4C–F), where neurogenesis was active, as reflected by the presence of a large number of *ash1*-expressing cells. Expression of *ash1* was also decreased in the central retina (Fig. 4G, H), where neurogenesis was subsiding. The lack of *ash1* expression was specific to the infected regions, while the adjacent, uninfected region maintained *ash1* expression. Suppression of *ash1* expression was also observed in the brain (Fig. 4I, J).

Ngn2 is another regulatory gene involved in cell fate specification during neurogenesis in other regions of the CNS. [32,33] In the retina, *ngn2* expression spans early and late phases of cell neurogenesis, and it is believed to participate in the development of progenitor cells that eventually differentiate into all major types of retinal neurons. [28–31] Infection with RCAS-GFP does not alter *ngn2* expression. [21] In RCAS-ngn1 infected retinas, *ngn2* was down-regulated (Fig. 5A–D, G). The down-regulation was observed at places where neurogenesis was active, as reflected by the presence of a large number of *ngn2*-expressing cells (Fig. 5A, B). The down-regulation was also observed at places where neurogenesis was tapering off, as reflected by the presence of a relatively smaller number of *ngn2*-expressing cells (Fig. 5C, D). Notably, no suppression of *ngn2* expression was apparent in the brain (Fig. 5E, F). This resistance to *ngn1's* suppression could be due to either *ngn1* alone being insufficient to suppress *ngn2* expression or other genes/factors promoting *ngn2* in these particular cells, or both.

Ngn3, the third member of the *ngn* subfamily, promotes early retinal neurogenesis in the chick. [21] Infection by RCAS-ngn3 results in ectopic expression of *ngn1*, suggesting an inductive relation of *ngn3→ngn1*. [21] To examine whether *ngn1* could reciprocally induce *ngn3*, we examined E7.5 retinas infected with RCAS-ngn1 for *ngn3* expression, considering that at this developmental stage the level of endogenous *ngn3* expression is undetectable in normal retina. [21] No induction of *ngn3* was found (data not shown).

To examine the effect of *ngn1* overexpression on cell proliferation, BrdU incorporation analysis was carried out. To minimize ambiguities arising from comparing retinas having different cell proliferation activities due to difference in developmental stages, the number of $BrdU^+$ cells in regions infected by RCAS-ngn1 was compared to that in adjacent, uninfected regions of the same retinal section. Infection with RCAS-GFP did not change the number of $BrdU^+$ cells (Fig. 6A, B, E). However, in regions infected with RACS-ngn1, fewer $BrdU^+$ cells were present compared with the internal control (adjacent, uninfected region; Fig. 6C, D). Scoring the number of $BrdU^+$ cells in regions of the similar sizes showed a 40% reduction in the infected region (Fig. 6E).

Effects on retinal populations from manipulating *ngn1* expression in vitro

One reason for the lack of changes in retinal cell populations from *ngn1* overexpression could be cell contact-mediated feedback mechanisms circumventing the effect of the experimental manipulation on cell production. *Ngn1* has been shown to induce and then be a target of Notch/Delta-mediated lateral inhibition, [50–52] which plays an important role in regulating retinogenesis. [53–56] To minimize cell contact-mediated feedback modulation, low-density retinal cell culture [38,53,57,58] was used to examine whether *ngn1* could steer retinal progenitors to a particular path. Retinal cells were isolated at E4.5, when expression of *ngn1* was high (Fig. 1H), and cultured at low density for 4 days (4 DIV). We found that *ngn1* induced an 84% increase in photoreceptor population (visinin$^+$/p27$^+$), from 32% of the total P27$^+$ (infected) cells in the control (infected with RCAS-GFP) to 59% in the experimental retina (Fig. 7A; $p<0.01$). This strong photoreceptor-promoting effect was still observed at E8.5 (Fig. 7B), when the retina normally no longer expressed *ngn1*. When the number of total cells, instead of infected cells, was used in the analysis, a milder effect was observed (Fig. 7C, D), likely due to a dilution effect by uninfected cells. Consistent with the expansion of the photoreceptor population, the number of cells expressing *neuroD* was also increased (Fig. 7C, D). No significant change in AP2α$^+$ or calretinin$^+$ amacrine cells was observed (Fig. 7A, C). The number of cells expressing *chx10* was reduced (Fig. 7C). The ganglion cell population (Islet-1$^+$, RA4$^+$, or Brn3A$^+$) was also reduced. With retinal cells of E4.5, when a major portion of the cells born will take on a ganglion fate, the ganglion population was decreased by 42%, from 48% of the infected cells in the control to 28% in the experimental retinas ($p<0.01$; Fig. 7A). A milder, yet significant ($p<0.01$), reduction in ganglion population was observed with E4.8 cells (Fig. 7C). With E5.5 retinal cells, no significant reduction in the ganglion population was observed (Fig. 7D).

Low density retinal cell culture was also used in siRNA knockdown experiments. Dissociated cells from E4.5 retinas were cultured in the presence, or absence, of siRNA against *ngn1*. In initial experiments, two siRNAs against *ngn1* were used separately. We found that either of the two reduced the number of visinin$^+$ cells significantly ($P<0.01$), albeit mildly (Fig. 8A), while neither siRNA affected the number of Islet-1$^+$ cells or Pax6$^+$ cells (ganglion and amacrine). In subsequent experiments, the siRNAs were used in combination. To verify that the siRNA reduced *ngn1* mRNA, we used in situ hybridization to identify cells with detectable levels of *ngn1* mRNA in E4.5 retinal cell cultures 48 hours after siRNA treatment. In cultures receiving a control siRNA, ~10% of the cells were *ngn1* mRNA$^+$. The number was reduced by 25 fold, to 0.4% of total cells in cultures receiving siRNA against *ngn1* ($p<0.01$; Fig. 8B). At the same time, the number of NeuroD$^+$ cells was reduced by >50%, from 52.9±7.6% of the total cells in the culture treated with control siRNA to 22.6±3.4% in the experimental culture ($p<0.01$; Fig. 8B). The number of visinin$^+$ cells was significantly ($p<0.05$), yet mildly, reduced. There were no significant changes in the numbers of cells that were positive for RA4 (ganglion) or Pax6, BrdU incorporation, or expression of *ngn2* or *ash1* (Fig. 8B). There was a small, yet statistically significant ($p<0.5$), reduction in the number of TUNEL$^+$ cells, from 0.9% of the total cells in the control culture to 0.8% in the experimental culture. The results suggest that the reductions in the number of NeuroD$^+$ cells and the number of visinin$^+$ cells from treatment with siRNA against *ngn1* were unlikely due to reduced cell proliferation or increased cell death.

Discussion

In the chick retina, *ngn1* was expressed during the early phases of retinal neurogenesis, and the expression became undetectable during the later phases, when neurogenesis is still active. Expression of *ngn1* likely occurred in both proliferating and postmitotic cells, because only some of the *ngn1*-expressing cells incorporated BrdU or expressed phospoH3. Thus, it is

plausible that *ngn1* is expressed as a cell is undergoing the transition from proliferation to differentiation.

Spatially, cells expressing *ngn1* were confined to the outermost portion of the retinal neuroepithelium, the prospective location of photoreceptor cells. Transient expression has been considered a signature of genes specifying neural types. [33] Thus, the temporal and spatial patterns of *ngn1* expression are consistent with a role in photoreceptor fate specification. In support of this, overexpression of *ngn1* in low-density cell culture resulted in an expansion of the photoreceptor population and an increase in number of cells expressing *neuroD*, which participates in photoreceptor production likely through promoting differentiation and survival. [13–15,36] Conversely, the photoreceptor population was reduced when retinal cells were cultured in the presence of siRNA against *ngn1*. Not totally unexpected, in vivo overexpression of *ngn1* did not produce noticeable alterations in retinal cell populations. This could result from confounding factors in the retina, including bHLH factors cross-regulating one another through networks and/or cascades. [21,31] Indeed, we have observed that overexpression of *ngn1* diminished the expression of *ash1*, *ath5*, and *ngn2*, all of which participate in the genesis of different types of retinal cells, including photoreceptors. [22,28,30,38,49] Another potential reason for the lack of alteration in retinal cell populations from *ngn1* overexpression is the presence of a cell contact-mediated feedback mechanism theorized to participate in the global regulation over retinogenesis that results in a balanced production of all cell types. This could explain why with low density cell culture, we detected *ngn1's* photoreceptor-promoting activity. *Ngn1* promoting photoreceptor production is consistent with the limited information that is available in the literature. In Xenopus retina, the *ngn1*-related gene, *ngnr1*, specifically increases the photoreceptor population. [16] In zebrafish retina, *ngn1* is expressed both in development and during photoreceptor regeneration after light-induced photoreceptor degeneration. [20] Little is known about the function of *ngn1* in mouse retinal development. In the brain, *ngn1* plays a determinative role in generating neural diversity. [32,33]

During retinal neurogenesis, a number of proneural bHLH genes are expressed. Among them, *ngn1* showed a spatial and temporal pattern of expression similar only to that of *ngn3*. Both were switched off early, when retinal neurogenesis in the chick is still active and the expression other bHLH regulatory genes (*ath5*, *ngn2*, and *ash1*) remains high. Temporally, expression of *ngn3* precedes the expression of *ngn1*. [21] Spatially, expression of *ngn3*, but not *ngn1*, was also detected on the vitreal side, the prospective location of ganglion cells. Overexpression of *ngn3* results in an expansion of the ganglion population into the territory otherwise occupied by amacrine cells. [21] Overexpression of *ngn1*, however, reduced the ganglion cell population. Overexpression of *ngn3* leads to ectopic induction of *ngn1*. Yet, no induction of *ngn3* was observed with *ngn1* overexpression. These findings suggest *ngn1* as a downstream genetic target of *ngn3* and a linear inductive relationship of *ngn3→ngn1*.

How and why *ngn1* suppressed the expression of *ash1*, *ath5*, and *ngn2* is unclear. One simple speculation is rivalry. It is plausible that *ngn1* is expressed during the phase of photoreceptor genesis not only to promote photoreceptor production but also to suppress the expression of *ash1*, *ath5*, and *ngn2* and thus attenuate the production of other cell types. Previous studies with similar experimental approaches showed that in the chick retina, overexpression of *ath5* promotes ganglion cell production, [46] overexpression of *ash1* increases the amacrine cell population, [24] and overexpression of *ngn2* has no effects on retinal cell populations. [24] Thus, *ngn1* differed from these bHLH genes not only in its expression pattern but also in its effect on retinal neurogenesis upon overexpression.

The chick *ngn1* displayed a distinctive pattern of expression coinciding with photoreceptor genesis, exhibited a photoreceptor-promoting activity, and repressed other regulatory genes

whose expression persist through late phases of retinal neurogenesis. These properties support *ngn1* to play a role in guiding a progenitor cell to the path of developing into a photoreceptor.

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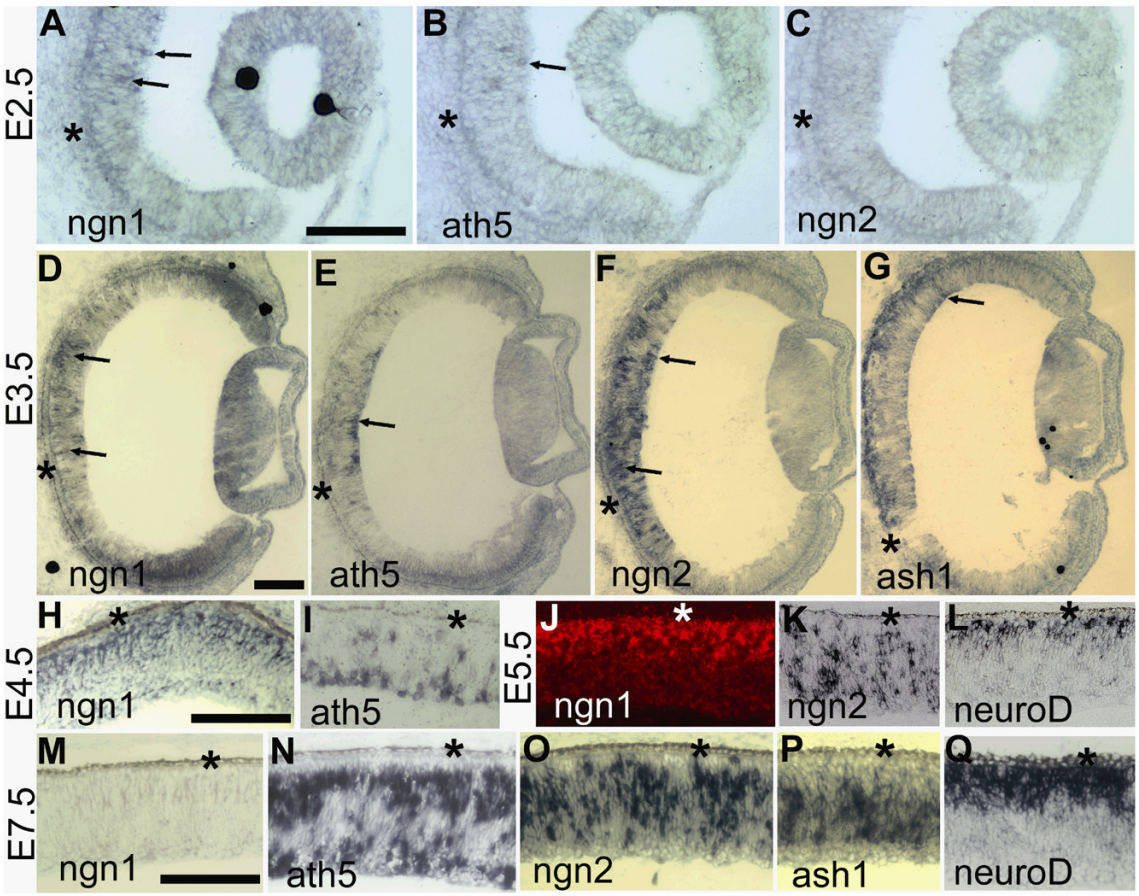

Fig. 1.

The expression pattern of *ngn1* in the developing chick retina in comparison with other proneural bHLH genes. A–C: In situ hybridization of serial sections to detect the expression of *ngn1* (A), *ath5* (B), and *ngn2* (C) in E2.5 eye. D–G: In situ hybridization of serial sections to detect the expression of *ngn1* (D), *ath5* (E), *ngn2* (F), and *ash1* (G) in E3.5 eye. H–I: Spatial expression patterns of *ngn1* (H) and *ath5* (I) in E4.5 retinas. J–L: Spatial expression patterns of *ngn1* (J), *ngn2* (K), and *neuroD* (L) in E5.5 retinas. M–Q: Spatial expression patterns of *ngn1* (M), *ath5* (N), *ngn2* (O), *ash1* (P), and *neuroD* (Q) in E7.5 retinas. In situ hybridization signals were developed with nitroblue tetrazolium, except in J, where rhodamine-tyramide was used. Arrows in A–G point to retinal cells expressing the respective gene. The RPE layer is indicated by an asterisk. Scale bars: 100 μm.

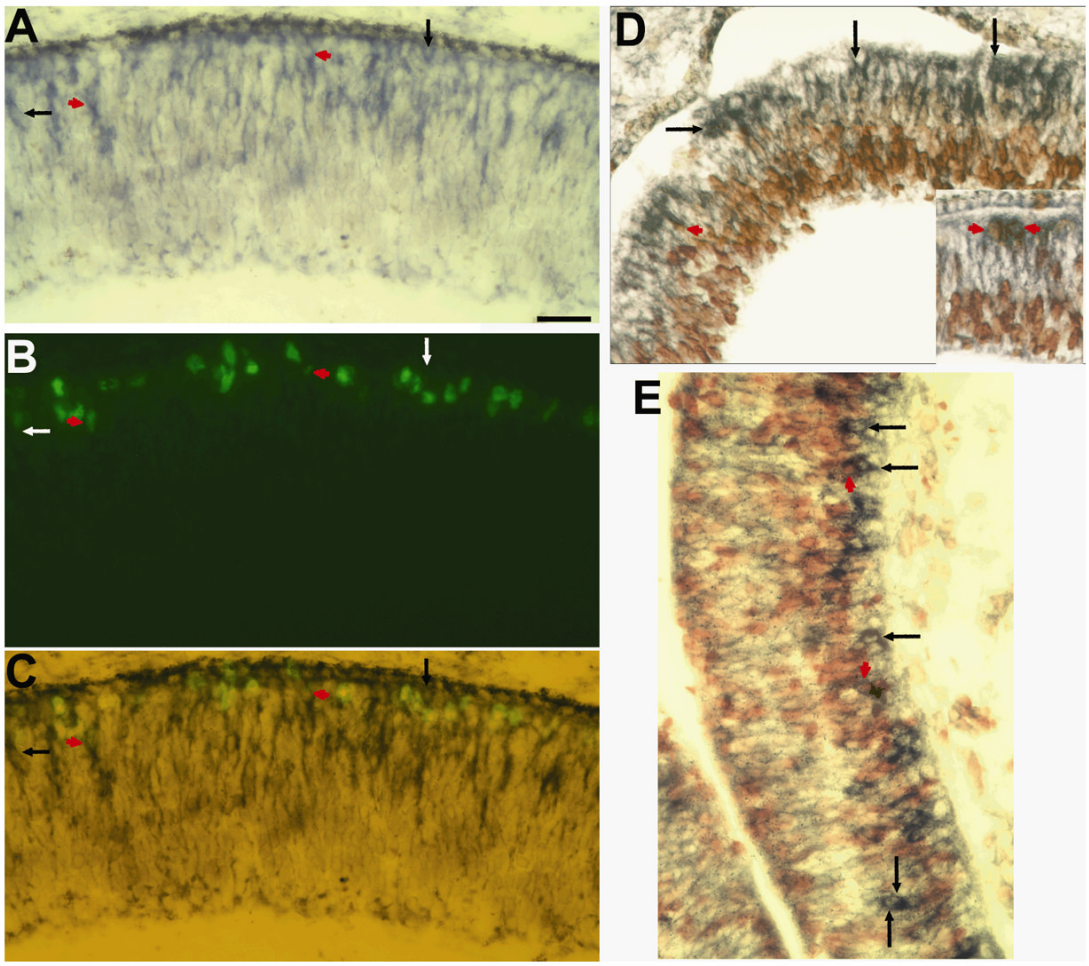

Fig. 2.

Double-labeling for *ngn1* expression and cell proliferation. A–C: Double-labeling for *ngn1* mRNA (A, blue) and phosphorylated histone H3 (B, green) in E6 retina. Panel C shows a simultaneous view of both. D,E: Double-labeling for *ngn1* mRNA (blue) and BrdU incorporation (red) in E5 retina (D) and brain (E). Inset in D provides a clearer image of double-labeled cells. Arrows point to cells positive for *ngn1* mRNA but negative for BrdU or phospoH3. Short arrows (red) point to double-labeled cells. Scale bar: 50 μm.

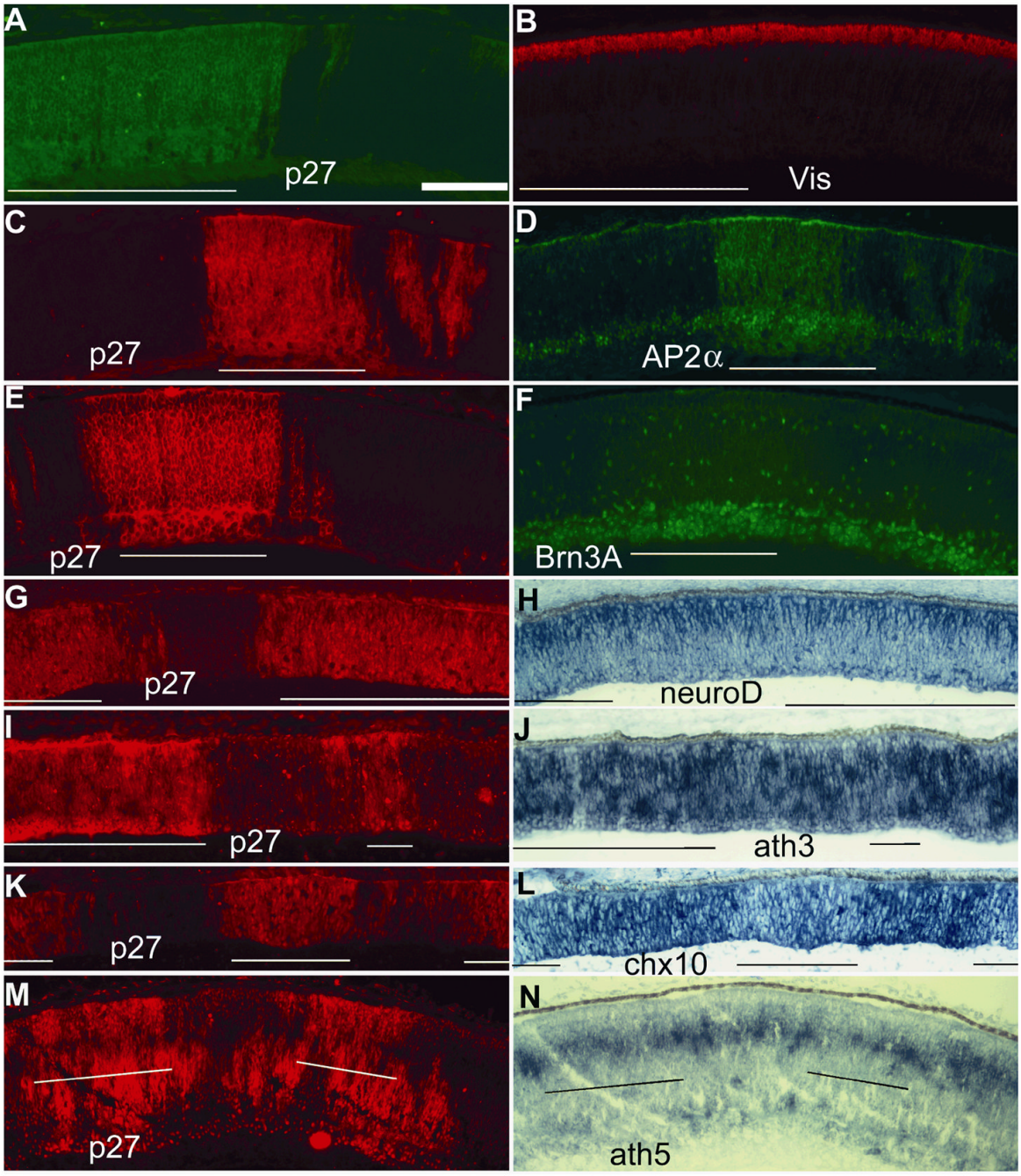

Fig. 3.

Double-staining of E7.5 retinas partially infected by RCAS-ngn1 for alterations in retinal neurogenesis. A, B: Double-labeling for RCAS viral protein p27 (A) and photoreceptor protein visinin (Vis, B). C, D: Double-labeling for viral protein p27 (C) and amacrine protein AP2α (D). E, F: Double-labeling for viral protein p27 (E) and ganglion protein Brn3A (F). G, H: Double-labeling for viral protein p27 (G) and *neuroD* mRNA (H). I, J: Double-labeling for viral protein p27 (I) and *ath3* mRNA (J). K, L: Double-labeling for viral protein p27 (K) and *chx10* mRNA (L). M, N: Double-labeling for viral protein p27 (M) and *ath5* mRNA (N). Infected regions are underlined. Scale bar: 100 μm. Of note, in some of the double-fluorescent images (C–F), the anti-p27 signals were so strong that they were also visible under the filter

used to view the anti-AP2α (D) or anti-Brn3A (F) signals, resulting in optical spillover. The genuine anti-AP2α (D) or anti-Brn3A (F) signals can be distinguished by their nucleus localization from the optical spillover of anti-P27 signals in the cytoplasm.

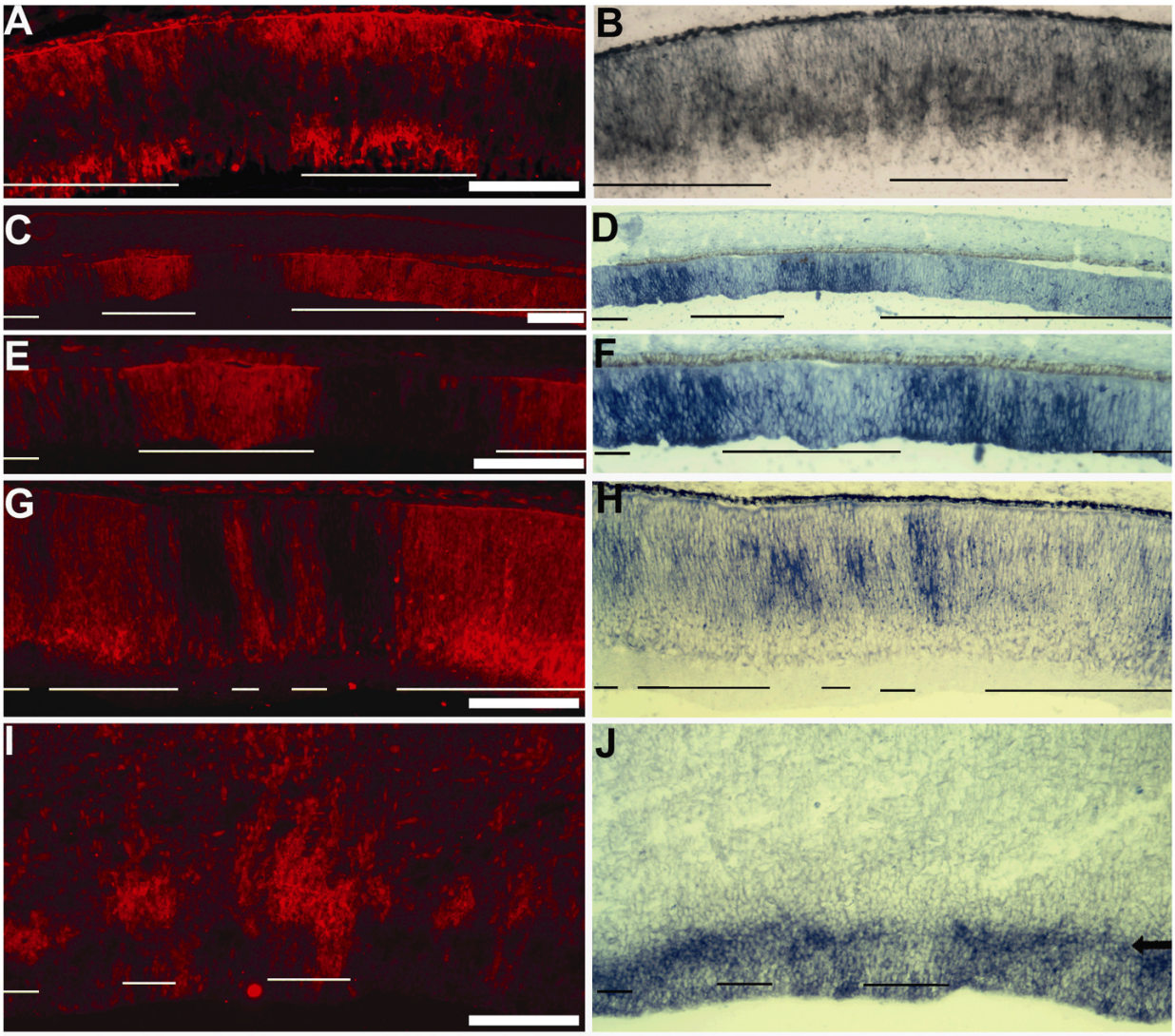

Fig. 4.

Diminished *ash1* expression in E7.5 retinas infected by RCAS-ngn1. A, B: Control RCAS-GFP retina with double-labeling for RCAS viral protein p27 (A) and for *ash1* mRNA (B). C–H: RCAS-ngn1 partially-infected retinas showing double-labeling for RCAS viral protein p27 (C, E, G) and for *ash1* mRNA (D, F, H) in peripheral retina (C–F) and central retina (G, H). C and D are lower magnifications of E and F. I, J: Double-labeling for viral protein p27 (I) and *ash1* expression (J) of E7.5 brain partially infected with RCAS-ngn1. Arrow in J points to the zone with diminished *ash1* expression. In all panels, infected regions are approximately underlined. Scale bars: 100 μm.

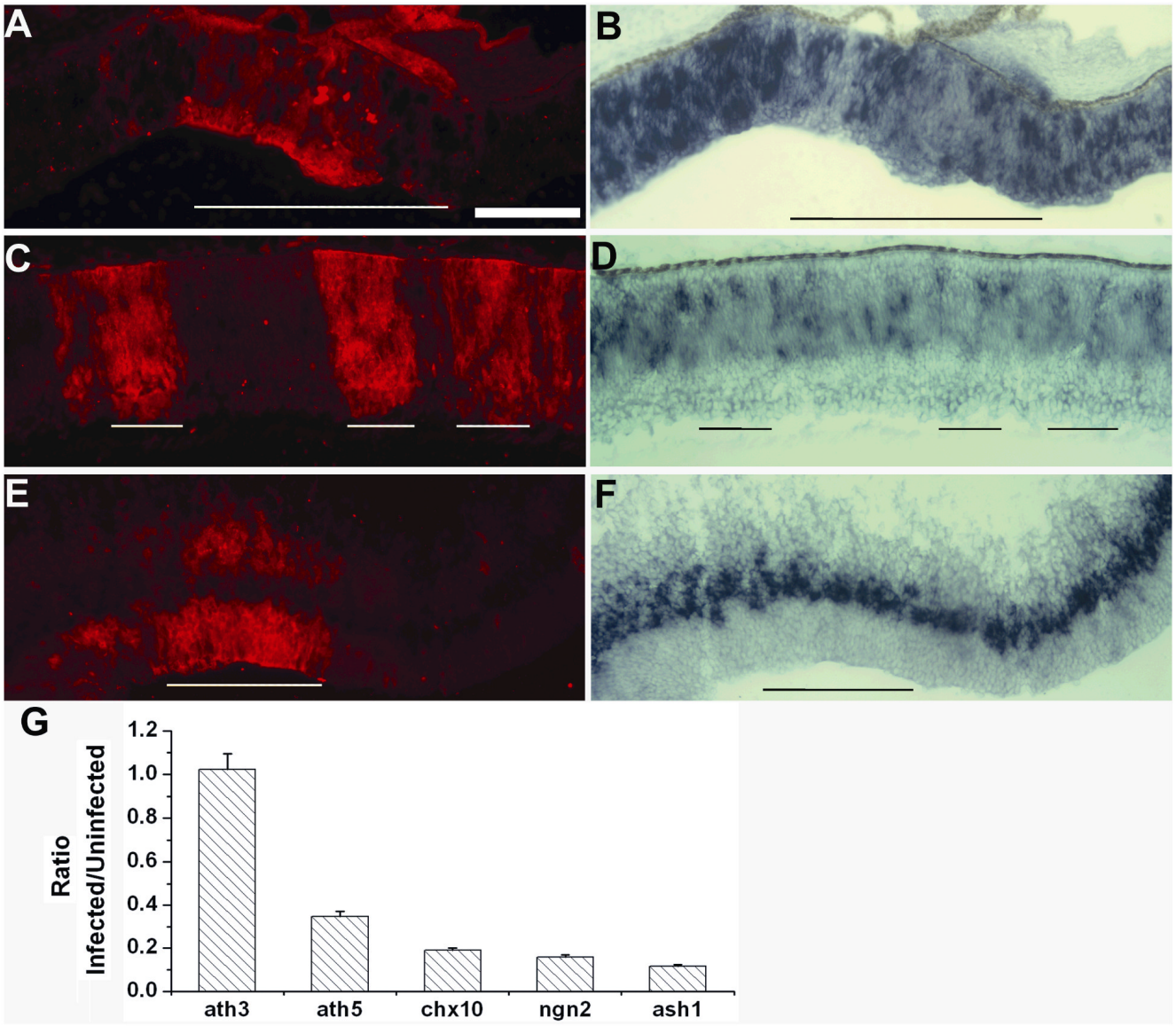

Fig. 5.

Down-regulation of *ngn2* expression in retinas infected with RCAS-ngn1. A–D: Double-labeling for RCAS viral protein p27 (A, C) and for *ngn2* mRNA (B, D) in E7.5 peripheral retina (A, B) and central retina (C, D). E, F: Double-labeling for viral protein p27 (E) and *ngn2* expression (F) in E7.5 brain partially infected with RCAS-ngn1. Infected regions are approximately underlined. Scale bar: 100 μm. G: Means and SDs of the ratio of the number of cells expressing each of the markers in infected regions over the number in uninfected regions.

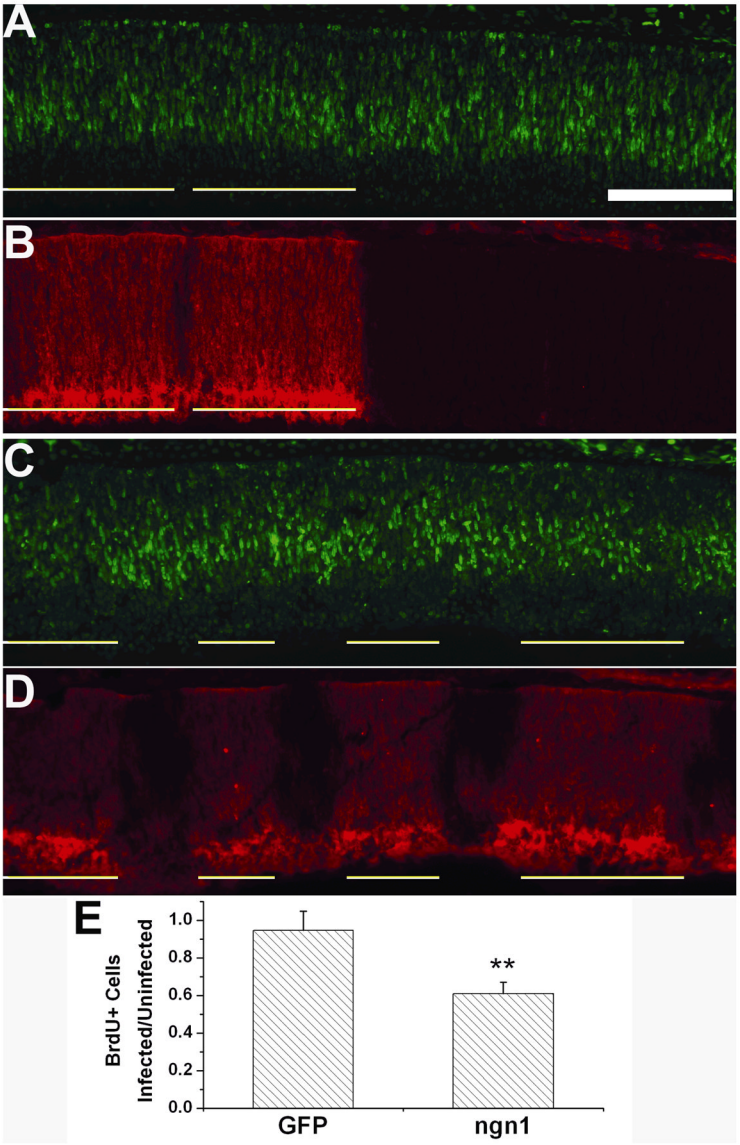

Fig. 6.

Analyses of cell proliferation with BrdU incorporation of retinas infected with RCAS-ngn1. A, B: Double-labeling for RCAS viral protein p27 (A) and for BrdU incorporation (B) of a control E7.5 retina with partial infection by RCAS-GFP. C, D: Double-labeling for the viral protein p27 (C) and for BrdU incorporation (D) of an E7.5 retina with partial infection by RCAS-ngn1. Infected regions are underlined. Scale bar (100 μm) applies to all panels.

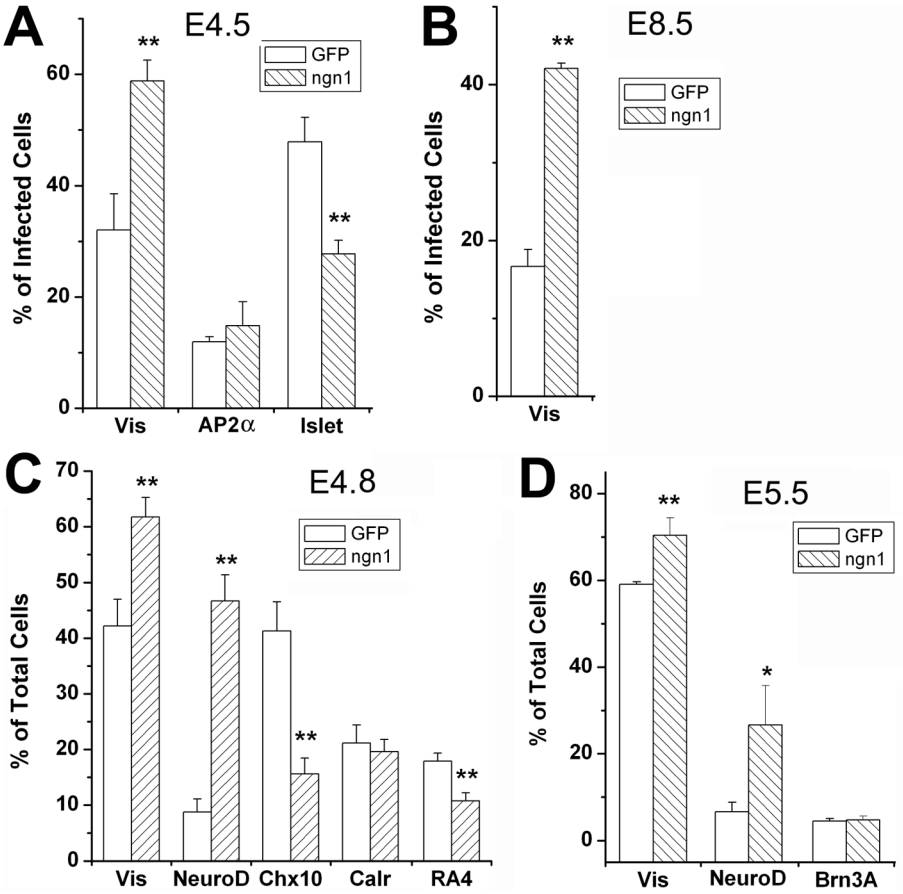

Fig. 7.

Quantitative analysis of alterations in retinal neurogenesis from *ngn1* overexpression in dissociated retinal cells. Shown are the means ± SDs of the calculated percentage of cells identified with each marker. Statistically significant differences from the control are shown at the 0.05 (*) and at 0.01 (**) levels.

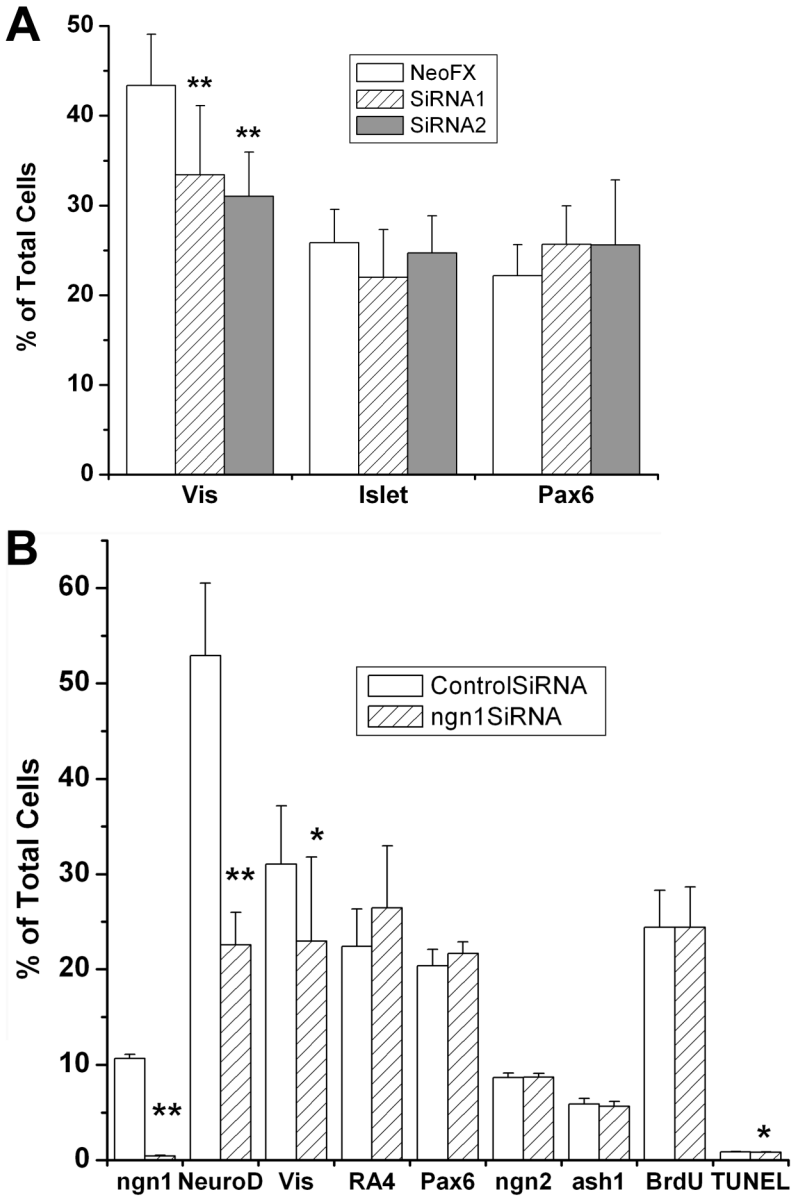

Fig. 8.

Effect of siRNA against *ngn1* on *ngn1* mRNA and on retinal cell populations. Shown are the means ± SDs of the percentage of cells identified with each marker among dissociated E4.5 retinal cells cultured for 2 (for data on ngn1, NeuroD, and TUNEL) or 4 days (for data on the other markers) under the treatments specified. Vis, visinin. Statistically significant differences from the control are shown at the 0.05 (*) and at 0.01 (**) levels.