

neurogenin2 elicits the genesis of retinal neurons from cultures of nonneural cells

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neurogenin2 (*ngn2*) encodes a basic helix–loop–helix transcription factor and plays an important role in neurogenesis from migratory neural crest cells. Its role in retinal development is poorly understood. We observed that in the developing chick retina, *ngn2* was expressed in a subpopulation of proliferating progenitor cells. Ectopic expression of *ngn2* in nonneural, retinal pigment epithelial cell culture triggered *de novo* generation of cells that expressed neural-specific markers and exhibited neuronal morphologies. Further molecular and morphological analyses showed that the main products of the induced neurogenesis were cells resembling young photoreceptor cells and cells resembling retinal ganglion cells. The generation of multiple cell types suggests that *ngn2* induces various retinal pathways. Thus, unlike in the peripheral nervous system where *ngn2* specifies one type of sensory neuron, *ngn2* in the retina is likely involved in a common step leading to different cellular pathways. Our finding that *ngn2* can instruct nonneural retinal pigment epithelial cells to differentiate toward retinal neurons demonstrates one possible way to induce *de novo* retinal neurogenesis.

The vertebrate retina contains five major types of neurons: photoreceptor, horizontal, bipolar, amacrine, and ganglion cells. Each cell type is functionally and morphologically distinct and resides at a stereotyped histological location. After receiving photons, photoreceptor cells initiate electrophysiological signals that are modulated and relayed by interneurons (horizontal, bipolar, and amacrine cells) to ganglion cells. Retinal ganglion cells send the information to the brain (1). Degeneration of the retina results in irreversible visual loss. Several common eye diseases, such as retinitis pigmentosa, age-related macular degeneration, and glaucoma, are caused by death of either photoreceptors or ganglion cells (2–4). Replacing the faulty retinal neurons with healthy ones might restore vision (5). Yet, inducing mature retinal neurons to regenerate remains an elusive task, because they, like other neurons, are terminally differentiated and will not reenter the cell cycle.

Elucidation of the molecular mechanisms underlying retinal development bears clinical implications as it might lead to genes capable of inducing the genesis of retinal neurons for tissue or cell replacement therapies. Studies with various experimental approaches have shown that the different types of retinal neurons are generated from common multipotent progenitor cells under the instruction of intrinsic and extrinsic factors (for a recent review, see ref. 6). Recent reports indicate that the intrinsic factors may include a number of basic helix–loop–helix (bHLH) genes with proneural activities, such as *ash-1* (7), *neurogenin1* (*ngn1*) (8), *ath3* (8, 9), *ath-5* (10–15), and *neuroD* (16–18).

ngn2 is a member of the *neurogenin* subfamily of bHLH genes homologous to the *Drosophila* proneural gene *atonal*. During mouse neurogenesis, *ngn2* and *ngn1* are expressed in distinct progenitor populations in the central and the peripheral nervous systems (19, 20). Their expression patterns partially overlap in some areas but are distinct in others. Targeted mutational analyses showed that *ngn1* is essential for the determination of neuronal precursors for proximal cranial sensory ganglia (21),

and *ngn2* is essential for the determination of precursors for epibranchial placode-derived sensory neurons (22). In the peripheral nervous system of the chick, *ngn1* and *ngn2* are expressed in a subset of neural crest cells early during crest cell migration (23, 24). *ngn2* specifies one type ($\text{trkB}^+/\text{trkC}^+$) of sensory neurons and *ngn1* specifies another (trkA^+), and both genes induce *neuroD* (23). Mouse and chick *ngn2* is expressed in the retina (14, 15), and the promoter/reporter assay indicates that *ngn2* can activate *ath5* (15). However, how *ngn2* participates in retinal neurogenesis remains largely unknown.

The retinal pigment epithelium (RPE) consists of nonneural cells in a monolayer structure that lies immediately outside the neural retina. Unlike retinal neurons, RPE cells from several species, including human, can reenter the cell cycle when stimulated. Furthermore, their progenies may, under appropriate conditions, differentiate into cell types other than RPE (25–27). One of the most intriguing phenomena reported is the transdifferentiation of RPE tissue into a neural retina *in vivo* (28–30) or *in vitro* (31), under the induction of basic fibroblast growth factor (bFGF). However, bFGF-induced transdifferentiation into a neural retina can occur only in young RPE up to embryonic day (E) 4.5 in chick (29, 31) and up to E13 in rat (27). Furthermore, bFGF has failed to induce neuronal transdifferentiation when RPE is cultured as dissociated cells (31).

We have recently observed that dissociated RPE cells derived from an E6 chick can be guided toward distinct pathways with various stimuli. For example, ectopic expression of *neuroD* triggers *de novo* production of photoreceptor cells (16, 17). In the presence of basic fibroblast growth factor, on the other hand, cultured RPE cells will transdifferentiate into cells that express RA4 (32), which is a marker for ganglion cells in the chick retina (33). Because it shares the same origin as the retina, it can reenter the cell cycle (i.e., can be cultured) and its progenies display developmental plasticity, RPE might serve as a source of cells to regenerate retinal neurons.

In this study, we examined the retinal expression of *ngn2* and tested whether *ngn2* could induce the genesis of retinal neurons from RPE cells. We found that during retinal neurogenesis in the chick, a subpopulation of proliferating progenitors expressed *ngn2*. When ectopically expressed in cultured RPE cells, *ngn2* elicited *de novo* generation of neuronal cells, which resembled young photoreceptor cells and young ganglion cells. We propose that *ngn2* in the retina, unlike in the peripheral nervous system, is likely to be involved in a common developmental event that precedes cell fate specification and can induce the genesis of different types of retinal neurons from cultured RPE cells.

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Abbreviations: RPE, retinal pigment epithelium; bHLH, basic helix–loop–helix; En, embryonic day *n*; PCNA, proliferating cell nuclear antigen; N-CAM, neural cell adhesion molecule; RCAS, replication-competent retrovirus; GFP, green fluorescent protein; IRBP, interphotoreceptor retinoid-binding protein.

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Materials and Methods

Cloning of Chick *ngn2*. The coding region of mouse *ngn1* (also called *neuroD3*) was PCR-amplified from mouse genomic DNA with primers (5'-TAC ACC ATG GCT GCC CCT TTG GAG ACC TG-3' and 5'-CTC TGG ATC CTT ACA AAG GCC TAG TGG TA-3') based on a published sequence (20, 34). After sequence verification, the cloned PCR product was used as a probe to screen a λ ZAP II (Stratagene) cDNA library constructed with mRNA isolated from an E8 chick brain. Three full-length primary clones were obtained; the longest clone contained a longer 3' untranslated sequence of *ngn2* (GenBank accession no. AF109014). Chick *ngn2* was also independently isolated by Perez *et al.* (24) and Matter-Sadzinski *et al.* (15).

In Situ Hybridization. To avoid potential cross hybridization with other bHLH genes, the coding sequence for the C' terminal nonconserved 61-aa and 3' untranslated sequence was used to synthesize digoxigenin-labeled antisense RNA probes (788 nucleotides long) against *ngn2*. Preparations of digoxigenin-labeled probes against other genes and the details of *in situ* hybridization with retinal cryosections have been described (16, 17). *In situ* hybridization of cultured cells was also as described (32). For double-labeling, dishes were subjected to *in situ* hybridization first, followed by immunocytochemistry.

Immunocytochemistry. MAb against microtubule associate proteins (MAP2) was purchased from Sigma (clone HM-2) and used at a 1:100 dilution. Polyclonal anti-AP2 Ab (1:500) was purchased from Santa Cruz Biotechnology. Affinity-purified anti-Calretinin (1:2000) was purchased from Chemicon. MAb against proliferating cell nuclear antigen (PCNA) was purchased from Dako and used at a dilution of 1:200. Three mAbs were obtained from the Developmental Studies Hybridoma Bank (Iowa University, Ames): Ab against neural cell adhesion molecule (NCAM) (clone 5e; 1:200; developed by U. Rutishauser), anti-BrdUrd (clone G3G4; 1:100; developed by S. J. Kaufman), and anti-Visinin (clone 7G4; 1:500; developed by C. Cepko). RA4 (1:500) was a gift from S. McLoon (Univ. of Minnesota, Minneapolis). Standard immunocytochemistry was performed. For double-labeling experiments, secondary Abs conjugated with fluorescent chromophore were used.

Pulse-Labeling of Chick Embryos with BrdUrd. BrdUrd (50 μ g) was dropped onto E7 chick embryos through a window in the shell. Four hours later, the eyes were fixed. Cryosections were subjected to *in situ* hybridization with antisense RNA probes against *ngn2*, followed by immunocytochemistry with a mAb against BrdUrd, as described (35).

Generation of Retrovirus Expressing *ngn2*. The coding region of chick *ngn2* was PCR-amplified with primers based on the sequence of the full-length primary clone and subcloned into shuttle vector *Clal2Nco*, from which a *Clal* fragment was inserted into proviral DNA vector replication-competent retrovirus (RCAS) (36). Recombinant viral DNA was introduced into chick embryonic fibroblast cells through transfection. Retrovirus particles were harvested as described (16). The titer of the concentrated viral stock was $1-2 \times 10^8$ pfu/ml.

RPE Transdifferentiation Assay. RPE tissues were dissected from E6 chick embryos, and dissociated RPE cells were cultured as described (16). After 3–4 days in culture, when $\approx 50\%$ confluence was reached, 5 μ l of the retrovirus stock was added to each 35-mm dish, and the culture was kept for an additional 8–12 days. At the end of culturing, cells in the dish were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min. After washing with PBS, the cells were subjected to immunocytochem-

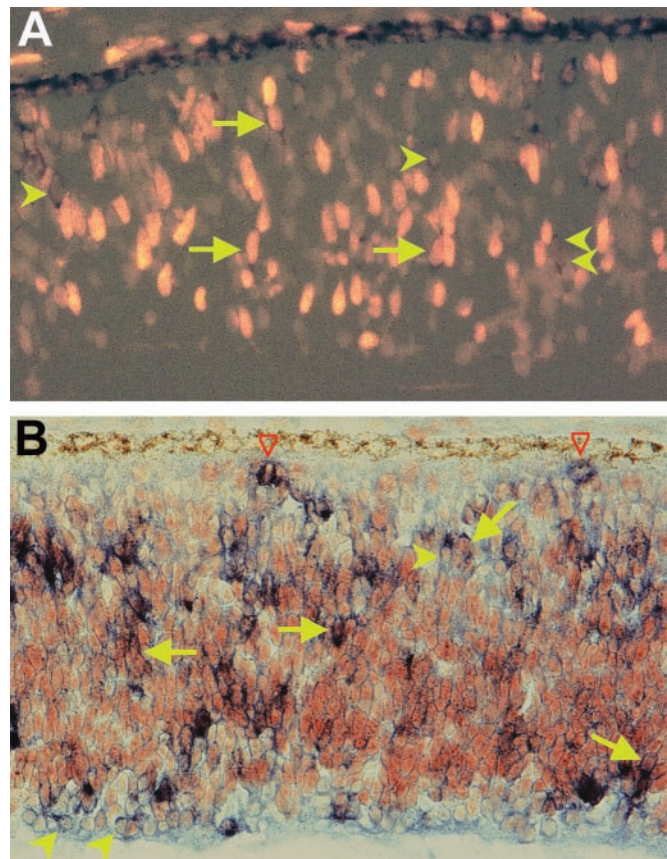


Fig. 1. Expression of *ngn2* in proliferating cells of E7 chick retinal neuroepithelium. (A) Doubling-labeling for *ngn2* mRNA (dark stains in the cytoplasm) and BrdUrd incorporation (bright nuclei). (B) Doubling-labeling for *ngn2* mRNA (blue stains in the cytoplasm) and PCNA immunoreactivity (red/brown stains of the nuclei). Arrows point to double-labeled cells. Arrowheads point to *ngn2*⁺/BrdUrd⁻ or *ngn2*⁺/PCNA⁻ cells. Red open arrowheads point to *ngn2*⁺ cells that were in M-phase of the cell cycle. Magnification: $\times 100$.

istry or *in situ* hybridization. All experiments were independently repeated at least 3 times in their entirety, from RPE isolation to cell labeling.

Results

Expression of *ngn2* in Proliferating Retinal Cells. The retinal neuroepithelium is a pseudostratified structure lacking a conspicuous demarcation between the ventricular zone, which contains proliferating cells, and the mantle zone, which contains differentiating neurons. Nevertheless, within the pseudostratified structure, young neurons accumulate in their prospective locations. In the developing chick retina, *ngn2* mRNA was detected in cells scattered across the retinal neuroepithelium. Double-labeling showed that cells expressing *ngn2* also incorporated BrdUrd (Fig. 1A) or expressed PCNA (Fig. 1B), indicating that proliferating neuroblasts expressed *ngn2*. Note that only a subpopulation of BrdUrd⁺ cells or PCNA⁺ cells contained detectable levels of *ngn2* mRNA. Some M-phase cells were also found to contain *ngn2* mRNA (open arrows in Fig. 1B).

De Novo Generation of Neural Cells in RPE Cultures Under the Induction of *ngn2*. The RPE cell culture system we recently developed (16) was used to study whether *ngn2* could induce *de novo* generation of retinal neurons. E6 chick RPE cells were dissociated and cultured for 2–4 days to reach about 50% confluence. At this point, RCAS expressing *ngn2*, or the green

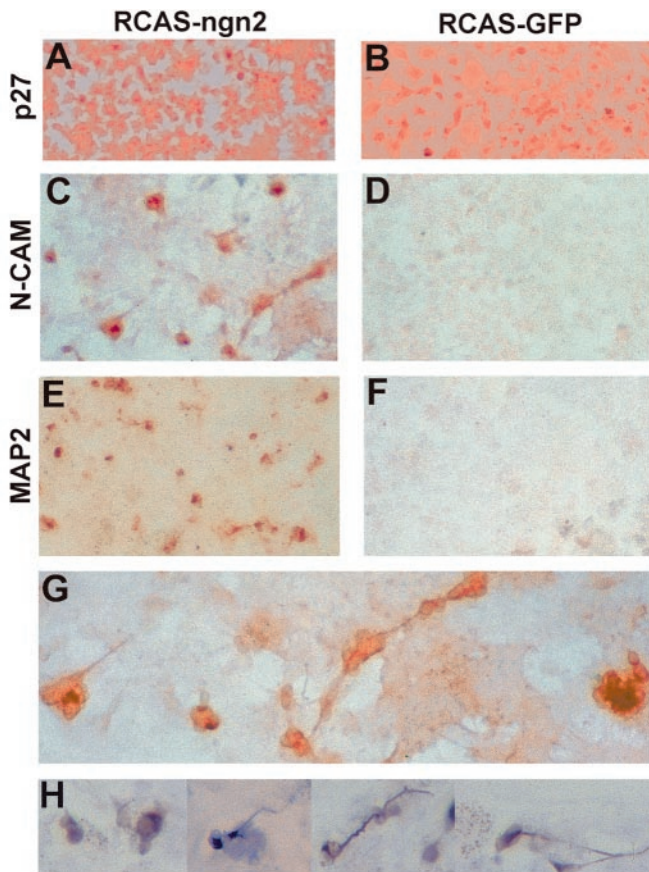


Fig. 2. *De novo* generation of neurons in RPE cell culture under the induction of *ngn2*. (A and B) Immunostaining of RPE culture infected with RCAS-*ngn2* (A) or RCAS-GFP (B) with anti-p27 Abs. (C and D) Immunostaining with anti-N-CAM Ab. (E and F) Immunostaining with anti-MAP2 Abs. (G) Higher magnification of C. (H) Individual MAP2⁺ cells or neural clusters at a higher magnification. Magnification: A–F, $\times 50$; G, $\times 100$; H, $\times 120$.

fluorescent protein (GFP), was added. Retroviral infection was monitored by immunocytochemistry with a specific Ab against a viral protein, p27. Four days after administration of the retrovirus, more than 80% of the cells in the dish became infected with the virus (Fig. 2 A and B).

To detect the presence of neural cells, RPE cultures were examined 8–12 days after administration of the retrovirus with mAbs against two general neural markers, N-CAM and MAP2. In dishes infected with RCAS-*ngn2*, tens of thousands per dish of 8 cm² were N-CAM⁺ and MAP2⁺ (Fig. 2 C and E). No such positive cells were present in control dishes infected with RCAS-GFP (Fig. 2 D and F). N-CAM⁺ cells were often found in clusters, and the clusters were sometimes interconnected by a long cellular process characteristic of neurons (Fig. 2G). Morphologically, both N-CAM⁺ and MAP2⁺ cells were neural-like (Fig. 2 G and H). Their morphologies were clearly different from the cuboidal-hexagonal morphology of nondividing RPE cells or the fibroblast-like morphology of actively dividing RPE cells.

Because bHLH proteins are known to form homo- or heterodimers, experimental manipulation of a bHLH gene expression always creates concerns about the specificity. In the RPE transdifferentiation assay, we performed ectopic expression of two other bHLH genes, *cNSCL1* and *cNSCL2*, and found neither could induce neural transdifferentiation (data not shown).

Presence of Young Photoreceptor Cells. To identify photoreceptor neurons in the RPE cultures, we examined the expression of

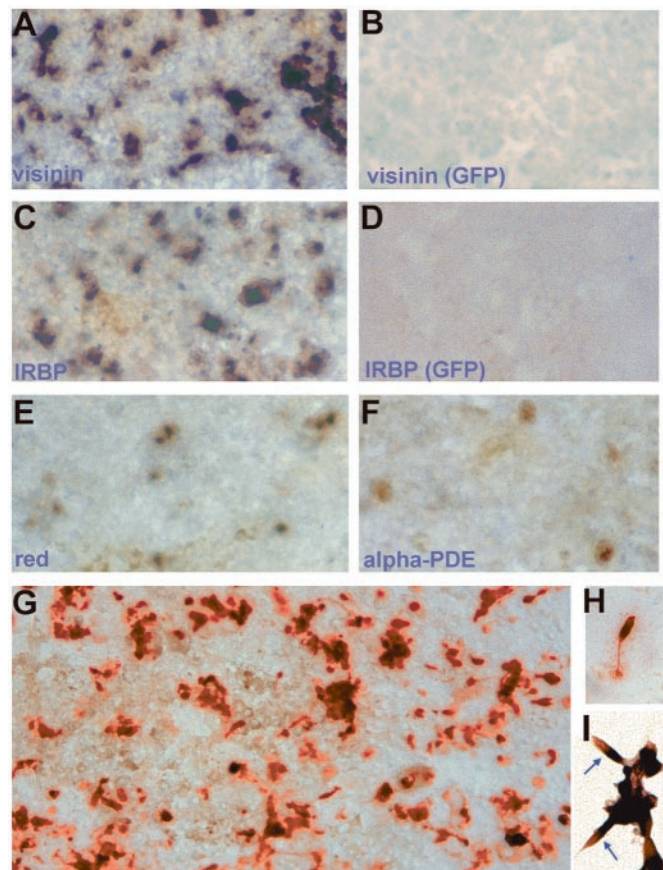


Fig. 3. Expression of photoreceptor-specific genes in RPE cultures under *ngn2* induction. (A, C, E, and F) *In situ* hybridization of E6 RPE culture infected with RCAS-*ngn2* with digoxigenin-labeled antisense RNA probes against *visinin* (A), IRBP (C), the red pigment gene (E), and α -phosphodiesterase (PDE) (F). (B and D) *In situ* hybridization of cultured E6 RPE cells infected with RCAS-GFP with antisense RNA probes against *visinin* (B) and IRBP (D). (G) Immunostaining of RPE culture infected with RCAS-*ngn2* with anti-Visinin protein. (H) A Visinin⁺ cell with cone photoreceptor morphology. (I) Double-labeling for IRBP mRNA (blue/purple) and Visinin protein (red). Arrows point to double-labeled cells. Magnification: A–G, $\times 50$; H and I, $\times 200$.

photoreceptor-specific genes and their cellular morphologies. We found tens of thousands (per dish) in RCAS-*ngn2*-infected cultures expressed *visinin* (Fig. 3 A and G), a gene encoding a calcium-binding protein present only in cone photoreceptor cells (37). These *visinin*-expressing cells were generated *de novo* under the induction of *ngn2*, because no such cells were present in any of the control dishes infected with RCAS-GFP (Fig. 3B). Interphotoreceptor retinoid-binding protein (IRBP) was also expressed in similar numbers of cells in dishes infected with RCAS-*ngn2* (Fig. 3C), but not in the control (Fig. 3D). In the retina, IRBP is expressed in photoreceptor cells and is involved in shuttling retinoids between photoreceptors and RPE cells. Expression of the red pigment gene, a hallmark of cone photoreceptor cells that sense red light, was also detected in RPE cultures forced to express *ngn2* (Fig. 3E). We also detected the expression of α -phosphodiesterase (PDE; Fig. 3F), which is involved in phototransduction. About 10–100-fold more cells expressed the early photoreceptor markers (*visinin* and IRBP) than the later photoreceptor markers (red pigment and α -PDE). This finding might be a result of different detection sensitivities or the result of *in vitro* conditions being unfavorable for photoreceptor maturation.

To better preserve cellular morphology, immunocytochem-

istry was used instead of *in situ* hybridization. Expression of *visinin* was detected with a mAb against Visinin protein. Like N-CAM⁺ cells, Visinin⁺ cells often appeared as clusters (Fig. 3G). Within each cluster, a large number of cells, but not all of the cells, were immunostained. The Visinin⁺ cells exhibited morphologies typical of young photoreceptor cells under culture conditions (38). Some cells developed an inner segment-like process at the apical end and an axonal process with terminal arborization at the basal end (Fig. 3H). Double-labeling showed that IRBP mRNA and Visinin protein were colocalized to the same cells, with the former restricted to the inner segment-like compartment and the latter distributed in the cell body (Fig. 3I).

Expression of Retinal Ganglion Cell Markers. We then asked whether the induction of the photoreceptor pathway was the sole downstream event of *ngn2* expression. RPE cultures infected with RCAS-*ngn2* were examined for the presence of other retinal neurons. To identify ganglion cells, we used both morphological criteria and molecular markers. Retinal ganglion cells typically have long axons and can be identified with mAb RA4 (33), or mAb against Calretinin (unpublished observations), or *cNSCL1*, which is transiently expressed in ganglion cells (39).

RA4⁺ were present in RPE cultures infected with RCAS-*ngn2* (Fig. 4A) but not in a control culture infected with RCAS-GFP (Table 1). Calretinin⁺ cells also were present in the experimental dishes (Fig. 4B) but not in the control. We also detected the expression of *cNSCL1* specifically in *ngn2*-expressing cultures (Fig. 4C). Many of the RA4⁺ and Calretinin⁺ cells displayed rather long cellular processes (Fig. 4A and B). Photomicrographs of RA4⁺ cells at a higher magnification (Fig. 4D) showed that these cells resembled young retinal ganglion cells under similar culture conditions. Double-labeling revealed that RA4 and Calretinin immunoreactivities were colocalized to the same cells (Fig. 4F–H).

Although cells reminiscent of photoreceptor cells often appeared as clusters, cells with ganglion cell traits were more physically separated. The morphologies of the two populations were also different: the former had short processes typical of young photoreceptor cells and the latter had long processes typical of ganglion cells, indicating that the two types of cells were distinct. We directly addressed the question of whether individual cells expressed both photoreceptor and ganglion cell markers. Double-labeling was carried out to determine whether cells coexpressed *visinin*, the most abundantly expressed photoreceptor marker among those tested, and RA4, the most abundantly expressed ganglion cell marker. Within a cluster, the two markers labeled different cells (Fig. 4E), further indicating that different types of neural cells were generated in RPE cultures infected with RCAS-*ngn2*.

Markers that label horizontal cells and amacrine cells were also used in the analysis. AP2 immunoreactivity and expression of *cNSCL2*, a bHLH gene homologous to *Drosophila atonal*, label horizontal and amacrine cells in the chick retina (40). Both markers were induced by *ngn2* in the RPE culture and they were not expressed in control cultures infected with RCAS-GFP (Table 1). Nonetheless, fewer cells expressed these markers, compared with the photoreceptor and ganglion cell markers (Table 1).

Induction of *neuroD*. In the chick retina, *neuroD* appears to be specifically expressed in photoreceptor cells and their precursors (ref. 16; unpublished data). Ectopic expression of *neuroD* triggers selective *de novo* generation of photoreceptor cells from RPE cell cultures (16, 17). We examined whether *ngn2*-induced RPE cell transdifferentiation into photoreceptor cells was mediated by *neuroD*, and whether there is a linear or circular

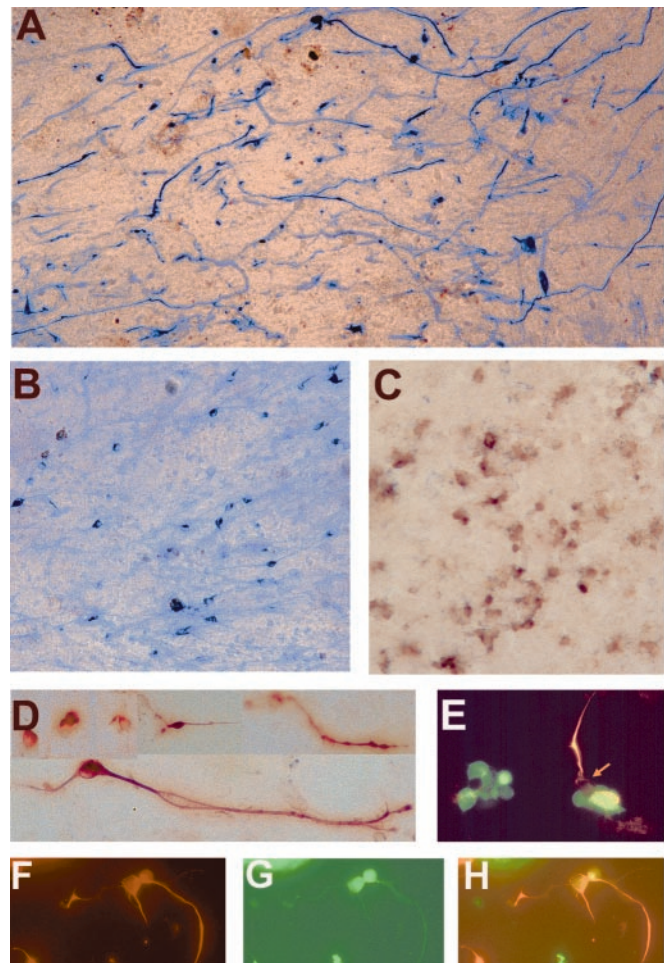


Fig. 4. Expression of ganglion cell markers in RPE cultures under *ngn2* induction. (A) Immunocytochemistry with RA4. (B) Immunocytochemistry with anti-Calretinin. (C) *In situ* hybridization with digoxigenin-labeled RNA probes for *cNSCL1*. (D) Higher magnification of RA4⁺ cells from *ngn2*-expressing RPE culture. (E) Double-labeling with RA4 (in red) and anti-Visinin Ab (in green). Arrow points to an RA4⁺/Visinin⁻ cell in a cluster of Visinin⁺ cells. (F–H) Double-labeling with RA4 (in red, F) and anti-Calretinin Ab (in green, G). H is a merge of F and G. Magnification: A–C, ×50; D and E, ×100; F–H, ×80.

relationship between *ngn2* and *neuroD* in our RPE culture system. We detected *ngn2* mRNA in cultures infected with RCAS-*ngn2* (Fig. 5A) but not in cultures infected with RCAS-*neuroD* (Fig. 5B) or RCAS-GFP (Fig. 5C), indicating that *neuroD* did not induce *ngn2* expression.

Expression of *neuroD* in the *ngn2* cultures was examined with affinity-purified Abs against the C-terminal nonconserved sequence of chick NeuroD (unpublished data). In control cultures infected with RCAS-GFP, no cells were found to be positive for NeuroD protein (Fig. 5F). In cultures infected with RCAS-*neuroD*, essentially all cells were stained with anti-NeuroD Ab (Fig. 5E). The Ab also labeled many cells in cultures infected with RCAS-*ngn2* (Fig. 5D), demonstrating that *ngn2* induced *neuroD* expression in cultured RPE cells.

Discussion

When ectopically expressed in cultured RPE cells, *ngn2* induced the expression of genes associated with photoreceptor production and differentiation (both general neuron markers and photoreceptor-specific markers). Furthermore, *ngn2* induced morphological changes from nonneuronal to photoreceptor-like

Table 1. Expression of various neural markers in RPE cell cultures infected with retrovirus expressing GFP or *ngn2*

| | Photoreceptor | | | | Ganglion | | | Horizontal and amacrine | | Neurons in general | |
|-------------------|---------------|------|-----|---------------|----------|--------|------------|-------------------------|--------|--------------------|------|
| | Visinin | IRBP | Red | α -PDE | RA4 | cNSCL1 | Calretinin | AP2 | cNSCL2 | N-CAM | MAP2 |
| RCAS-GFP | – | – | – | – | + | – | + | – | – | – | – |
| RCAS- <i>ngn2</i> | +++ | +++ | ++ | + | +++ | ++ | +++ | + | + | +++ | +++ |

No. of positive cells per dish (8 cm²) are indicated by “+” for hundreds, “++” for thousands, and “+++” for equal or more than tens of thousands or too many to count. The data presented were the average of data collected from at least three independent experiments with three dishes used/counted for each marker in one experiment. PDE, phosphodiesterase.

cells. These observations argue strongly that *ngn2* is capable of triggering a cascade of gene expression events leading to a photoreceptor phenotype.

Photoreceptor cells were not the only product of *ngn2*-induced neurogenesis in cell cultures derived from RPE. In fact, *de novo* generation of ganglion cells, judged by morphological and molecular criteria, was observed. In addition, markers of other types of retinal neurons (amacrine and/or horizontal cells) were also detected in RPE cultures ectopically expressing *ngn2*. These findings suggest that *ngn2* induced at least two, and probably more, retinal pathways.

The presence of nonphotoreceptor neurons indicated that *ngn2* exhibited a general proneural activity. However, this does not necessarily rule out the possibility that *ngn2* in the developing retina specifies one type of cells. Studies have shown that some proneural genes exhibit flexibility or genetic compensation, and often double-knockouts of proneural genes are needed to demonstrate phenotypic changes in neurogenesis (9). It is possible that when present alone in RPE cells, *ngn2* exerted functions that would otherwise be inhibited by other proneural genes present in the retina. Presently, little is known about how proneural

genes interact to regulate retinal neurogenesis. In any event, our observations indicate that *ngn2* alone could induce cells in an RPE culture to differentiate along at least two different neural pathways.

In the developing chick retina, *ngn2* was expressed in retinal neuroepithelium lacking a recognizable histological demarcation. Thus, it is difficult to judge, based on the spatial expression pattern, whether cells expressing *ngn2* would develop into a single cell type or multicell types. Expression of *ngn2* in BrdUrd⁺ or PCNA⁺ cells suggests that it may be involved in a developmental step before terminal mitosis. Considering the current concept that retinal cell fate specification is a postmitotic event (41–43), expression in proliferating cells suggests that *ngn2* is involved in a developmental event that precedes retinal cell fate specification. Thus, it is likely that *ngn2* plays a role in the genesis of retinal neurons in general, and not a single cell type in particular. Consistent with this scenario was our observation that *ngn2* led to the genesis of at least two types of retinal neurons from RPE cell cultures.

Apparently, the detailed molecular events of retinal neurogenesis are different from those of peripheral nervous system (PNS) neurogenesis. During PNS development, *ngn2* specifies one type of neuron and *ngn1* specifies another type. Both *ngn1* and *ngn2* induce the expression of *neuroD* and *NSCL1*, which are commonly expressed in both types of neurons (23). However, in the chick retina, *neuroD* and *NSCL1* are expressed in different types of cells (16, 39), and *neuroD* plays a role in specifying a photoreceptor cell fate (16). Furthermore, data in this report suggest that *ngn2* is likely to be involved in a general aspect of retinal neurogenesis, not the specification of a single retinal cell type.

An interesting and potentially important observation was the presence of neural clusters, a typical feature of neural stem cells, in RPE cultures ectopically expressing *ngn2*. Not all of the cells in a cluster were labeled with anti-Visinin Ab. Visinin[–] cells could be young rod cells or young nonphotoreceptor neurons. They could also be undifferentiated progenitor cells. This finding raises a possibility that *ngn2* might induce the generation of retinal stem cells from cultured RPE, which possibility warrants further investigation.

Various approaches have recently been explored to identify renewable sources of cells that have the potential to differentiate into particular types of neurons or neural precursors for cell-based therapies. The possibility of harnessing multipotent stem cells for neural transplantation has recently been demonstrated (44). Among the many possible cells to use, embryonic stem cells and neural stem cells, particularly retinal stem cells, hold promise as renewable sources of cells for generating retinal neurons. Our study showed that cultured RPE cells could be guided toward retinal neuron pathways by a bHLH gene, *ngn2*, offering a potential source of cells for generating retinal neurons.

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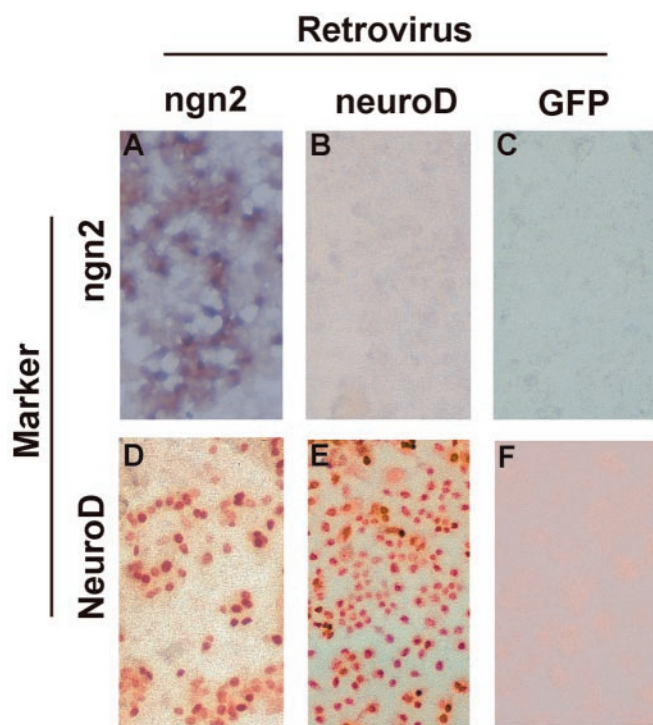


Fig. 5. Induction of *neuroD* by *ngn2*. (A–C) *In situ* hybridization with antisense RNA probes against *ngn2* in RPE cultures infected with RCAS-*ngn2* (A), RCAS-*neuroD* (B), or RCAS-GFP (C). (D–F) Immunostaining with anti-NeuroD Ab in RPE cultures infected with RCAS-*ngn2* (D), RCAS-*neuroD* (E), or RCAS-GFP (F). Magnification: $\times 50$.

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1. Dacheux, R. F. & Raviola, E. (2000) in *Principles and Practice of Ophthalmology*, eds. Albert, D. M. & Jacobiec, F. A. (Saunders, Philadelphia), pp. 1601–1624.
2. Stone, J. L., Barlow, W. E., Humayun, M. S., de Juan, E., Jr., & Milam, A. H. (1992) *Arch. Ophthalmol.* **110**, 1634–1639.
3. Curcio, C. A., Medeiros, N. E. & Millican, C. L. (1996) *Invest. Ophthalmol. Visual Sci.* **37**, 1236–1249.
4. Quigley, H. A., Dunkelberger, G. R. & Green, W. R. (1989) *Am. J. Ophthalmol.* **107**, 453–464.
5. Berson, E. L. & Jacobiec, F. A. (1999) *Ophthalmology* **106**, 445–446.
6. Livesey, F. J. & Cepko, C. L. (2001) *Nat. Rev. Neurosci.* **2**, 109–118.
7. Tomita, K., Ishibashi, M., Nakahara, K., Ang, S. L., Nakanishi, S., Guillemot, F. & Kageyama, R. (1996) *Neuron* **16**, 723–734.
8. Perron, M., Opdecamp, K., Butler, K., Harris, W. A. & Bellefroid, E. J. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 14996–15001.
9. Hatakeyama, J., Tomita, K., Inoue, T. & Kageyama, R. (2001) *Development (Cambridge, U.K.)* **128**, 1313–1322.
10. Wang, S. W., Kim, B. S., Ding, K., Wang, H., Sun, D., Johnson, R. L., Klein, W. H. & Gan, L. (2001) *Genes Dev.* **15**, 24–29.
11. Brown, N. L., Patel, S., Brzezinski, J. & Glaser, T. (2001) *Development (Cambridge, U.K.)* **128**, 2497–2508.
12. Liu, W., Mo, Z. & Xiang, M. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 1649–1654.
13. Hutcheson, D. A. & Vetter, M. L. (2001) *Dev. Biol.* **232**, 327–338.
14. Brown, N. L., Kanekar, S., Vetter, M. L., Tucker, P. K., Gemza, D. L. & Glaser, T. (1998) *Development (Cambridge, U.K.)* **125**, 4821–4833.
15. Matter-Sadzinski, L., Matter, J. M., Ong, M. T., Hernandez, J. & Ballivet, M. (2001) *Development (Cambridge, U.K.)* **128**, 217–231.
16. Yan, R.-T. & Wang, S.-Z. (1998) *J. Neurobiol.* **36**, 485–496.
17. Yan, R.-T. & Wang, S.-Z. (2000) *Neurosci. Lett.* **280**, 83–86.
18. Morrow, E. M., Furukawa, T., Lee, J. E. & Cepko, C. L. (1999) *Development (Cambridge, U.K.)* **126**, 23–36.
19. Sommer, L., Ma, Q. & Anderson, D. J. (1996) *Mol. Cell. Neurosci.* **8**, 221–241.
20. Ma, Q., Kintner, C. & Anderson, D. J. (1996) *Cell* **87**, 43–52.
21. Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J. L. & Anderson, D. J. (1998) *Neuron* **20**, 469–482.
22. Fode, C., Gradwohl, G., Morin, X., Dierich, A., LeMeur, M., Goridis, C. & Guillemot, F. (1998) *Neuron* **20**, 483–494.
23. Ma, Q., Fode, C., Guillemot, F. & Anderson, D. J. (1999) *Genes Dev.* **13**, 1717–1728.
24. Perez, S. E., Rebelo, S. & Anderson, D. J. (1999) *Development (Cambridge, U.K.)* **126**, 1715–1728.
25. Eguchi, G. (1986) in *Current Topics in Developmental Biology*, eds. Moscona, A. A. & Monroy, A. (Academic, New York), pp. 21–37.
26. Dutt, K., Scott, M., Sternberg, P. P., Linser, P. J. & Srinivasan, A. (1993) *DNA Cell Biol.* **12**, 667–673.
27. Zhao, S., Rizzolo, L. J. & Barnstable, C. J. (1997) *Int. Rev. Cytol.* **171**, 225–265.
28. Orts-Llorca, F. & Genis-Galvez, J. M. (1960) *Acta Anat.* **42**, 31–70.
29. Coulombre, J. L. & Coulombre, A. J. (1965) *Dev. Biol.* **12**, 79–92.
30. Park, C. M. & Hollenberg, M. J. (1989) *Dev. Biol.* **134**, 201–205.
31. Pittack, C., Jones, M. & Reh, T. A. (1991) *Development (Cambridge, U.K.)* **113**, 577–588.
32. Yan, R.-T. & Wang, S.-Z. (2000) *Visual Neurosci.* **17**, 157–164.
33. Waid, D. K. & McLoon, S. C. (1995) *Neuron* **14**, 117–124.
34. McCormick, M. B., Tamimi, R. M., Snider, L., Asakura, A., Bergstrom, D. & Tapscott, S. J. (1996) *Mol. Cell Biol.* **16**, 5792–5800.
35. Li, C.-M., Yan, R.-T. & Wang, S.-Z. (1999) *Dev. Dyn.* **215**, 238–247.
36. Hughes, S. H., Greenhouse, J. J., Petropoulos, C. J. & Suttrave, P. (1987) *J. Virol.* **61**, 3004–3012.
37. Yamagata, K., Goto, K., Kuo, C. H., Kondo, H. & Miki, N. (1990) *Neuron* **4**, 469–476.
38. Madreperla, S. A., Edidin, M. & Adler, R. (1989) *J. Cell Biol.* **109**, 1483–1493.
39. Li, C.-M., Yan, R.-T. & Wang, S.-Z. (1999) *Mol. Cell. Neurosci.* **14**, 17–27.
40. Li, C.-M., Yan, R.-T. & Wang, S.-Z. (2001) *Invest. Ophthalmol. Visual Sci.*, in press.
41. Ezzeddine, Z. D., Yang, X., DeChiara, T., Yancopoulos, G. & Cepko, C. L. (1997) *Development (Cambridge, U.K.)* **124**, 1055–1067.
42. Belliveau, M. J., Young, T. L. & Cepko, C. L. (2000) *J. Neurosci.* **20**, 2247–2254.
43. Adler, R. (2000) *Prog. Retina Eye Res.* **19**, 529–557.
44. Brustle, O., Jones, K. N., Learish, R. D., Karam, K., Choudhary, K., Wiestler, O. D., Duncan, I. D. & McKay, R. D. (1999) *Science* **285**, 754–756.