Using neurogenin to Reprogram Chick RPE to Produce Photoreceptor-like Neurons

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PURPOSE. One potential therapy for vision loss from photoreceptor degeneration is cell replacement, but this approach presents a need for photoreceptor cells. This study explores whether the retinal pigment epithelium (RPE) could be a convenient source of developing photoreceptors.

METHODS. The RPE of chick embryos was subjected to reprogramming by proneural genes neurogenin (ngn1) and ngn3. The genes were introduced into the RPE through retrovirus RCAS-mediated transduction, with the virus microinjected into the eye or added to retinal pigment epithelial explant culture. The genes were introduced into the RPE through retrovirus RCAS-mediated transduction, with the virus microinjected into the eye or added to retinal pigment epithelial explant culture. The retinal pigment epithelia were then analyzed for photoreceptor traits.

RESULTS. In chick embryos infected with retrovirus RCAS-expressing ngn3 (RCAS-ngn3), the photoreceptor gene visinin (the equivalent of mammalian recoverin) was expressed in cells of the retinal pigment epithelial layer. When isolated and cultured as explants, retinal pigment epithelial tissues from embryos infected with RCAS-ngn3 or RCAS-ngn1 gave rise to layers of visinin-positive cells. These reprogrammed cells expressed genes of phototransduction and synapses, such as red opsin, the a-subunit of cone transducin, SNAP-25, and PSD-95. Reprogramming occurred with retinal pigment epithelial explants derived from virally infected embryos and with retinal pigment epithelial explants derived from normal embryos, with the recombinant viruses added at the onset of the explant culture. In addition, reprogramming took place in retinal pigment epithelial explants from both young and old embryos, from embryonic day (E6) to E18, when the visual system becomes functional in the chick.

CONCLUSIONS. The results support the prospect of exploring the RPE as a convenient source of developing photoreceptors for in situ cell replacement. (Invest Ophthalmol Vis Sci. 2010;51: 516–525) DOI:10.1167/iovs.09-3822

Photoreceptor death underlies many forms of visual impairment. Because they are terminally differentiated and do not reenter the cell cycle for regeneration, photoreceptors lost due to various causes cannot be replenished; this loss leads to permanent blindness. The reduced quality of life for people with severe vision loss has spurred a spectrum of investigations ranging from photoreceptor rescue1–6 to photoreceptor replacement.7,8 The recent demonstration of successful photoreceptor transplantation in blind mice9 highlights the importance of finding a source of developing photoreceptors for cell replacement therapies.10 In recent years, stem cells (brain, retinal, and embryonic) have been examined for their capacity to produce photoreceptor cells.11–16 Nonetheless, interest in novel, perhaps provocative, sources of developing photoreceptors remains high.17

The photoreceptor layer of the vertebrate retina lies immediately next to a darkly pigmented, transporting epithelium—the retinal pigment epithelium (RPE)—which forms the outer blood-retinal barrier and regulates retinal physiology. Developmentally, the nonneural RPE and the neural retina originate from the same structure, the optic vesicle. Immigration of the optic vesicle forms a double-layered optic cup and an anatomic separation of the RPE and the retina: cells in the outer layer of the optic cup form the RPE, and cells in the inner layer constitute the retina. Early embryonic chick RPE can be induced to transdifferentiate into a neural retina in vivo and in vitro by fibroblast growth factors.18 However, this RPE-to-retina transdifferentiation no longer occurs after embryonic day (E)4.5, nor does it occur with dissociated retinal pigment epithelial cells.19 Rat RPE and mouse RPE isolated from young embryos can also undergo similar transdifferentiation.20,21

Recent reports show that specific genes encoding certain transcription factors can reprogram dissociated retinal pigment epithelial cells from E6 chick to differentiate toward retinal neurons.22–26 A screening of more than 20 transcription factors known or implicated to regulate the formation of the eye, the retina, and the photoreceptors has identified proneural gene neurogenin (ngn)1 as the most potent (in an order of ngn1 ≳ ngn3 ≳ ngn2 ≳ neuroD1)27 in inducing retinal pigment epithelial progeny cells propagated in vitro to express the photoreceptor marker visinin. Visinin is a calcium-binding protein present in photoreceptor cells in the chick retina and is considered an equivalent of mammalian recoverin.28 Reprogramming of a dissociated retinal pigment epithelial cell culture by ngn1 gives rise to cells that display photoreceptor morphologies and express an array of photoreceptor genes, including transcription factors key to photoreceptor differentiation and components of the phototransduction pathway.27 Furthermore, reprogrammed cells exhibit physiological properties typical of photoreceptor cells: they respond to light by decreasing their cellular free calcium (Ca2+) levels, and, after light bleaching, they respond to 9-cis-retinal by increasing their intracellular Ca2+ levels. The presence of these advanced physiological traits raises the possibility of producing functional photoreceptors from retinal pigment epithelial reprogramming.

In the mature eye, retinal pigment epithelial cells remain quiescent under normal conditions. Under some pathologic conditions or when stimulated physically (such as by physical
triggers that occur during surgery), retinal pigment epithelial cells reenter the cell cycle and proliferate. This proliferative response is an undesirable side effect of surgery because progeny cells may differentiate into cells with tractional force causing retinal detachment and leading to visual impairment. However, if the RPE can be reprogrammed to produce layers of developing photoreceptor cells, it would then become a convenient source of photoreceptors for replacement in situ.

In this study, we used ngn1 and ngn3 to reprogram retinal pigment epithelial tissue to produce cells with photoreceptor properties. We report that visinin-positive cells were abundant in the retinal pigment epithelial layer in chick embryos infected with RCAS retrovirus expressing ngn3 (RCAS-ngn3). In explant culture, retinal pigment epithelial tissues from embryos infected with RCAS-ngn3 and RCAS-ngn1 gave rise to layers of photoreceptor-like cells. These cells exhibited advanced photoreceptor differentiation, including the expression of proteins involved in phototransduction and synapses. The reprogramming occurred with RPE isolated from embryos of different ages, from E5 to E12. Further, reprogramming also took place when the recombinant virus was added to the established explant cultures of RPE from embryos near hatching (E18). These results support the prospect of exploring the RPE as a convenient source of developing photoreceptors for future cell replacement studies.

**MATERIALS AND METHODS**

**Chick Embryos**

Fertilized, pathogen-free White Leghorn chicken eggs were purchased from Spafas (Preston, CT) and incubated in a Petersime incubator (Gettysburg, PA). All use of animals adhered to the procedures and policies set by the Institutional Animal Use and Care Committee at the University of Alabama at Birmingham.

**Infection of Embryonic Chick Eyes with RCAS Viruses**

Retrovirus RCAS-expressing ngn1 (RCAS-ngn1),27 RCAS-ngn3,30 RCAS-ashl,32 and RCAS-GFP31 were produced as described. Concentrated virus (0.5–2 × 106 pfu/mL) was microinjected into the subretinal space of E2.5 chick eyes as described.32 The chick embryos were further incubated for various lengths of time before RPE was isolated for explant culture or their eyes were fixed for analysis.

PCR was used to generate deletion constructs of ngn3. The N’ terminal 62 amino acids was omitted in Ngn3AN, and the C’ terminal 41 residues was omitted in Ngn3AC. To generate a repression construct, the repressor domain of Drosophila Engrailed (En) was added, in frame, to the N-terminus of Ngn3, as described.32 All constructs were sequenced for verification. The DNA constructs were first subcloned into shuttle vector ClaI2Nco and then into proviral DNA vector RCAS. The final cloning products were also sequence verified to rule out frame-shift or non-sense mutations. Concentrated retroviruses expressing the mutation constructs were produced as described.31

**Retinal Pigment Epithelial Explant Culture**

The RPE was isolated from chick embryos of different ages, from E6 to E18. Briefly, the eye was enucleated and cleared of all extraocular tissue. Subsequently, the sclera and the cornea were removed. The RPE was then circumferentially separated from ciliary pigment epithelium just behind the ora serrata. After the removal of the lens, the vitreous, and the retina, the RPE was cut with four radial-relaxing incisions. The clover-shaped retinal pigment epithelial sheet was then placed into an insert (Transwell; Corning, Corning, NY), with the Bruchs membrane side facing the polycarbonate membrane of the insert. Retinal pigment epithelial tissues were cultured with the insert hanging in the well of a six-well plate. Each well contained 1.5 mL knockout Dulbecco’s modified Eagle’s medium supplemented with serum replacement (Invitrogen, Carlsbad, CA). Excess liquid was removed from the membrane to prevent dislodging of the retinal pigment epithelial sheet from the membrane. Culture medium was changed every other day.

At various times during culture, retinal pigment epithelial explants were fixed with ice-cold 4% paraformaldehyde for 1 hour, cryoprotected with 20% sucrose for 2 hours at 4°C and for 1 hour in OCT/20% sucrose (2:1), embedded in OCT/sucrose mixture, and frozen with liquid nitrogen. Cryosections measuring 5 to 10 μm were collected for immunohistochemistry and in situ hybridization.

For BrdU analysis, 6 μL BrdU solution (1 μg/μL in HBSS) was added to each well with 1.5 mL medium for 48 hours before fixation. Cryosections were used in immunocytochemistry to detect BrdU incorporation. For double labeling, cryosections of peripheral retina were fixed with digoxigenin (dig)-labeled anti-visinin RNA probes and then to anti-BrdU immunostaining, as previously described.53

**Immunohistochemistry**

Anti-visinin (7G4, 1:500; developed by Constance Cepko, Harvard University, Cambridge, MA) and anti-BrdU (G3G4, 1:100; developed by Stephen J. Kaufman, University of Illinois, Urbana, IL) were obtained from the Developmental Studies Hybridoma Bank (Iowa University, Iowa City, IA). Other antibodies were purchased from commercial sources: polyclonal anti-API2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal PSD-95 (7E3-1BB; 1:200; Sigma, St. Louis, MO), polyclonal SNAP-25 (1:200; Sigma), polyclonal antibody against calretinin (1:500; Chemicon, Temeacula, CA), polyclonal anti-red opsin (1:200; Chemicon), and polyclonal antibody against viral protein P27 (1:500; Spafas, Norwich, CT). Monoclonal antibody against chick NeuRoD (CND2, 1:20) was developed in our laboratory. Standard immunohistochemistry was carried out with fluorophore-conjugated secondary antibodies goat anti-rabbit Alexa Fluor 546 and goat anti-mouse Alexa Fluor 488 (Invitrogen, Carlsbad, CA).

**In Situ Hybridization**

Dig-labeled antisense RNA probes were prepared as previously described for visinin and chx10 and for interphotoreceptor retinoid binding protein (IRBP), rhodopsin, and green opsin.32 To synthesize antisense RNA probe against the chick mmp115 mRNA, a 1025 bp fragment of the coding region was PCR amplified using primers cagcagagcattc and tattagcggctgata, described according to publicly available sequence information (NCBI accession no. NM_205112). After cloning into pGEM-T (Promega, Madison, WI) and sequence verification, linearized plasmid DNA harboring the mmp115 DNA fragment was used to synthesize Dig-labeled antisense RNA (Genius kit; Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions. To improve the visualization of in situ signals, bleaching of retinal pigment epithelial pigments was carried out. Briefly, at the conclusion of in situ hybridization, the specimens on the slide was subjected to the following treatment: 0.25% potassium permanganate (KMN04) for 20 minutes, three rinses with PBS, and the slide was subjected to the following treatment: 0.25% potassium permanganate (KMN04) for 5 minutes before mounting.

**RT-PCR**

Total RNAs were isolated from dissociated retinal pigment epithelial cell cultures infected with RCAS-ngn1, RCAS-ngn3, or RCAS-GFP using a kit (RNeasy Protect mini kit; Qiagen, Valencia, CA). First-strand cDNA was synthesized with a cDNA synthesis kit (Ambion, Austin, TX) using oligo-dT as the primer. After a 10-fold dilution, 1 μL first-strand cDNA was used as the template in each 30-μL PCR reaction. The small ribosomal protein 17 (s17) was used as an internal control to normalize the amount of cDNA in each sample with 20 cycles of amplification.26 Mlf was amplified by 30 cycles with primers of ccgctgctgctgac and gatgatgctgtcctcct (GenBank accession no. D88365). Mmp115 was amplified by 25 cycles with primers of cacagctggaggagac and cacgctggaggagac and...
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layer contained visinin-positive cells. In control E7.5 eyes,
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eye, we examined embryos for the expression of visinin in the
retinal pigment epithelial layer. We observed that in embryos
had received RCAS-ngn3, the retinal pigment epithelial
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positive cells were confined within the neural retina at the
prospective location of the outer nuclear layer (ONL) and were
absent in the RPE (Figs. 1A, 1B). In eyes infected with RCAS-
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prospective ONL (Figs. 1C, 1D). Visinin-positive cells in the
RPE were unequivocally observed in regions in which the RPE
and the retina were separated (Figs. 1C, 1D). By scoring the
cell numbers at detached areas from five retinal sections, we
calculated that approximately 37.2% ± 4.3% of the cells in the
retinal pigment epithelial layer were visinin-positive. Morpho-
logically, some of the visinin-positive cells displayed a thin
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Some of the visinin-positive cells in the retinal pigment
epithelial layer retained dark pigmentation typical of retinal
pigment epithelial cells (Figs. 1G, 1H, arrowheads), indicating
that those visinin-positive cells likely originated from retinal
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tors from the retina. To rule out the possibility that those
visinin-positive cells with residual pigments were retinal pig-
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tor cells, we examined the eye at early developmental stages,
when retinal photoreceptor cells had not yet emerged. In
experimental eyes at E5.5, when the neural retina either had
fewer (Figs. 1I–K) or lacked visinin-positive cells (Figs. 1L–N),
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pigment epithelial layer predate those in the neural retina.
Whether the residual pigments in those visinin-positive cells
would disappear later as photoreceptor differentiation became
more pronounced could not be examined directly because of
the early death of the experimental embryos.

In E7.5 chick eyes infected with RCAS-ngn1, a few visinin-
positive cells were present in the retinal pigment epithelial
layer, despite 
gn1 having the highest activity in vitro.27 Dou-
ble labeling for viral protein p27 ruled out the possibility that
the lack of visinin expression in the RPE was caused by lack of
RCAS-ngn1 infection (Figs. 2A–C). In eyes infected with RCAS-
ash1, no visinin-positive cells were observed in the retinal
pigment epithelial layer (Figs. 2D–F). These results suggested
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RESULTS

Visinin Expression in the RPE In Ovo

Previously, we screened a large number of genes/factors for
the ability to reprogram cells in a dissociated retinal pigment
epithelial cell culture to express photoreceptor marker visinin.
The screening identified ngn1 as the most proficient gene in an
order of ngn1 ≥ ngn3 > ngn2 > neuralD > ash1 > ath5.27

To explore whether reprogramming could occur in ovo in the
eye, we examined embryos for the expression of visinin in the
retinal pigment epithelial layer. We observed that in embryos
that had received RCAS-ngn3, the retinal pigment epithelial
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Few or no visinin-positive cells in the retinal pigment epithelial layer in chick embryos infected with RCAS expressing ngn1, ash1, or mutation constructs of ngn3. (A–C) Bright-field (A) and epifluorescence of immunostaining for viral protein p27 (B) and visinin (Vis; C) of an E7.5 eye infected with RCAS-ngn1. Arrowhead: visinin-positive cell in the retinal pigment epithelial layer. (D–F) Bright-field (D) and epifluorescence of immunostaining for p27 (E) and visinin (F) of an E7.5 eye infected with RCAS-ash1. (G–I) Bright-field (G) and epifluorescence of immunostaining for p27 (H) and visinin (I) of an E7.5 eye infected with RCAS-ngn3ΔN. (J–L) Bright-field (J) and epifluorescence of immunostaining for p27 (K) and visinin (L) of an E7.5 eye infected with RCAS-ngn3ΔC. (M–O) Bright-field (M) and epifluorescence of immunostaining for p27 (N) and visinin (O) of an E7.5 eye infected with RCAS-En-ngn3. (P) Diagram of the regions contained in the mutation constructs of Ngn3. Asterisks: retinal pigment epithelial layer. Scale bar, 50 μm (applies to all images).

**Induction of Visinin in Retinal Pigment Epithelial Explants**

To examine whether the number of visinin-positive cells in animals infected by RCAS-ngn1 could be increased by a physical separation of the RPE from the retina, we injected sodium hyaluronate into the subretinal space at E4 and again at E5. Sodium hyaluronate solution has been used in a number of studies of experimental retinal detachment.34–36 Retinal detachment was evident in E7.5 eyes, but no increases in the number of visinin-positive cells in the retinal pigment epithelial layer were observed (data not shown).

We then tested whether subjecting RPE to explant culture would unleash its potential to generate visinin-positive cells. E6 and E7.5 retinal pigment epithelia were isolated and cultured as explants. After 8 days in vitro (8 DIV), the control retinal pigment epithelia from embryos infected with RCAS-GFP contained no visinin-positive cells. As expected from the in ovo results, visinin-positive cells were present in explants from embryos infected with RCAS-ngn3. Nonetheless, unlike in ovo, in which visinin-positive cells were more or less embedded in the monolayered RPE, retinal pigment epithelial explants gave rise to layers of visinin-positive cells (Figs. 3C, 3D). Layers of visinin-positive cells were also produced in explants from embryos infected with RCAS-ngn1 (Figs. 3E, 3F).

To address whether those visinin-positive cells arose during the explant culture process, we analyzed the explants at different times in culture. At the onset (4 hours in vitro), retinal pigment epithelial explants from embryos infected with RCAS-ngn3 contained a significant number of visinin-positive cells (Figs. 3G, 3H), whereas retinal pigment epithelial explants from those infected with RCAS-ngn1 contained only a few visinin-positive cells, similar to their in ovo counterparts. After 5 DIV, however, a large number of visinin-positive cells had accumulated in retinal pigment epithelial explants from both experimental samples (Figs. 3K–N).

**Expression of Photoreceptor Genes**

The presence of visinin-positive cells in the retinal pigment epithelial explants could reflect a single gene induction or a reprogramming process that might lead to differentiation into photoreceptor cells. To address this question, we analyzed retinal pigment epithelial explants for the expression of genes involved in regulating photoreceptor development or in photoreceptor function, coupled with morphologic examination. Retinal pigment epithelial explants from embryos infected with RCAS-ngn1 were used in this analysis because other experiments showed adverse effects on cell survival from RCAS-ngn3. Published studies have shown that neuroD, a proneural bHLH transcription factor gene, plays an instrumental role in photoreceptor development and survival.31,32,37,38 Immunostaining with a specific antibody detected NeuroD protein in more than 80% of the cells in retinal pigment epithelial explants from embryos infected with RCAS-ngn1 (Figs. 4C, 4D), whereas no NeuroD-positive cells were detected in the control infected with RCAS-GFP (Figs. 4A, 4B).

Double labeling was carried out to determine whether those layers of visinin-positive cells also expressed genes associated with photoreceptor function and expressed during photoreceptor maturation. Within the layers of visinin-positive cells, red opsin was expressed in explants cultured for 8 days
or longer. In 16-DIV explants, 53.7% ± 6.6% (calculated from five sections) of the cells in the visinin-positive layers were also red opsin-positive (Figs. 4E–J). Furthermore, the red opsin immunostaining in most cases gave a "short-rod" or "dot-like" appearance. This pattern of immunostaining is typically observed with photoreceptors in the retina, in which red opsin is concentrated in the outer segment (Figs. 4G, 4J, insets). Notably, mislocalization of red opsin to cytoplasm was apparent (Figs. 4J, 4P), particularly in explants with shorter culture times (data not shown).

Cells of the retinal pigment epithelial explants also expressed proteins associated with ribbon synapses. Double labeling showed expression of synaptosomal-associated protein 25 kDa (SNAP-25) within the layers of visinin-positive cells (Figs. 4K–N). In the retina, SNAP-25 is present at the membranes of photoreceptor somata and in the terminals of photoreceptor cells. Another ribbon synapse-associated protein, postsynaptic density protein PSD-95, was also expressed in the retinal pigment epithelial explants. In the retina, PSD-95 is most prominent in the outer plexiform layer on the axon terminals of rods and cones (the rod spherules and cone pedicles). In the retinal pigment epithelial explants, PSD-95 was present in the axon-like processes of a red opsin-positive

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**Figure 3.** Visinin-positive cells emerging from retinal pigment epithelial explants derived from embryos infected with RCAS-ngn3 or RCAS-ngn1. (A–F) Retinal pigment epithelial explants derived from E6 embryos infected with RCAS-GFP (A, B), RCAS-ngn3 (C, D), or RCAS-ngn1 (E, F), cultured for 8 days (8 DIV), and analyzed for visinin expression. Shown are bright-field views (A, C, E) and epifluorescence of visinin immunostaining (B, D, F). (G–J) Retinal pigment epithelial explants derived from E7.5 embryos infected with RCAS-ngn3 (G, H) or RCAS-ngn1 (I, J) after 4 hours in culture (4 HIV). Bright-field views (G, I) and epifluorescence (H, J) of visinin immunostaining. (K–N) Retinal pigment epithelial explants derived from E7.5 embryos infected with RCAS-ngn3 (K, L) or RCAS-ngn1 (M, N) after 3 DIV. Bright-field views (K, M) and epifluorescence (L, N) of visinin immunostaining. Asterisks: membrane of the culture insert. Scale bar, 50 μm (applies to all images).

**Figure 4.** Expression of genes associated with advanced photoreceptor differentiation in 16 DIV explants derived from E7.5 embryos infected with RCAS-ngn1. (A–D) Detection of neuroD expression in GFP control (A, B) and experimental retinal pigment epithelial explant (C, D). Shown are bright-field views (A, C) and epifluorescence (B, D) of immunostaining for NeuroD. (E–J) Double labeling of the experimental explant for visinin (F, I) and red opsin (G, J). Bright-field views (E, H), (I–J) Higher magnification views of double labeling of the experimental explant for visinin (F) and red opsin (G, J). (G, J, Insets) Images of E18 retina immunostained for red opsin. (K–N) Double labeling of the experimental explant for visinin (L) and SNAP-25 (M). Bright-field view (K) and merged view (N) of L and M. (O–R) Double labeling of the experimental explant for red opsin (P) and PSD-95 (Q). Bright-field view (O) and merged view (R) of P and Q. (S–U) In situ hybridization to detect the expression of a subunit of cone transducin (alt, S), irbp (T), and chx10 (U). Scale bars, 50 μm.
cell (Figs. 4O–R). In situ hybridization detected the expression of additional photoreceptor genes, including the α-subunit of transducin (alt; Fig. 4S), irbp (Fig. 4T), green opsin (data not shown), and rhodopsin (data not shown). Additionally, expression of chx10, a homeobox gene important for retinal progenitor cell development, was also detected (Fig. 4U). None of these genes was expressed in control retinal pigment epithelial explants derived from embryos infected with RCAS-GFP (data not shown).

**Induction of Visinin in Retinal Pigment Epithelial Explants from Older Embryos**

One important issue in using the RPE as a source of new photoreceptor cells is whether mature RPE is amendable to reprogramming. To address this issue, we tested RPE at various stages of development and maturation. Differentiation of the chick RPE becomes evident with the synthesis of melanin at E4. With subsequent increases in melanin (or pigmentation) and minor changes in morphologies, the RPE continues its molecular maturation. Tight junctions form on E7 and become functional between E10 and E12. At E16, the β1 subunit of integrin localizes to the basal membrane, characteristic of mature chick RPE. To determine whether RPE at late phases of differentiation and maturation could also give rise to visinin-positive cells, we cultured retinal pigment epithelial explants from older embryos. We observed layers of visinin-positive cells generated from retinal pigment epithelial explants from E12 embryos that had received RCAS-ngn1 through microinjection at E2.5 (Figs. 5A, 5B). Visinin-positive cells were also produced from explant from embryos as old as E18.

To rule out the possibility that the visinin-positive cells in the E12 explants resulted from a reprogramming event that had occurred during earlier developmental phases, as the RCAS-ngn1 virus was administered to the embryos on E2.5, we tested E12 retinal pigment epithelial explants from normal embryos, with RCAS-ngn1, RCAS-ngn3, or RCAS-GFP added at the onset of culture. In the control (RCAS-GFP), no visinin-positive cells were present (Figs. 5C–E). In explant culture with RCAS-ngn1 added, no visinin-positive cells were visible after 2 DIV (Figs. 5F–H) because of poor or limited viral infection (Fig. 5G). Visinin-positive cells were abundantly present after 8 DIV (Figs. 5I–K) because viral infection became widespread in the culture by this time (Fig. 5J). Visinin-positive cells were also abundantly detected in explants of E18 retinal pigment epithelia with RCAS-ngn3 added to the medium at the onset of the culture (Figs. 5L, 5M).

**Suppression of Retinal Pigment Epithelial Gene**

The presence of layers of visinin-positive cells in the retinal pigment epithelial explants implied that cell proliferation might have occurred during the culture process. Indeed, during the first 2 DIV, many cells in the GFP control (Figs. 6A, 6B) and in the experimental explant (Figs. 6C, 6D) incorporated BrdU. During this time, some of the BrdU-positive cells began visinin expression (Figs. 6F, 6G).

Regions in retinal pigment epithelial explants with layers of visinin-positive cells lacked the normal dark pigmentation of the RPE. To address whether the induction of photoreceptor gene expression was accompanied by suppression of retinal pigment epithelial genes, we compared the expression of retinal pigment epithelial gene mmp115 (revealed by in situ hybridization) with the expression of visinin, using serial sections. Mmp115 encodes a melanosomal matrix protein and plays a regulatory role in retinal pigment epithelial development. In the control retinal pigment epithelial explants, mmp115 mRNA was detected in essentially all cells (Figs. 6H, 6I, 6J, 6K). During this period (Figs. 6L, 6M), mmp115 expression was still detectable, particularly in the basal region of the epithelium. In the visinin-positive cells, mmp115 expression was detected in a subset of cells. This suggests that mmp115 expression is not completely suppressed in the visinin-positive cells.

**FIGURE 5.** Visinin-positive cells in retinal pigment epithelial explants derived from older (E12 and E18) embryos. (A, B) A 30-DIV retinal pigment epithelial explant derived from an E12 embryo infected with RCAS-ngn1. (A) Bright-field views and (B) epifluorescence of visinin (Vis) immunostaining. (C–E) An 8-DIV retinal pigment epithelial explant derived from a normal E12 embryo, with RCAS-GFP added to the culture medium at the onset of culture. (C) Bright-field views and epifluorescence of immunostaining for viral protein (D) p27 and (E) visinin. (F–H) A 2-DIV retinal pigment epithelial explant derived from a normal E12 embryo, with RCAS-ngn1 added to the culture medium at the onset of culture. (F) Bright-field views and epifluorescence of immunostaining for (G) p27 and (H) visinin. (I–K) An 8-DIV retinal pigment epithelial explant derived from a normal E12 embryo, with RCAS-ngn1 added to the culture medium at the onset of culture. (I) Bright-field views and epifluorescence of immunostaining for (J) p27 and (K) visinin. (L, M) A 15-DIV retinal pigment epithelial explant derived from a normal E12 embryo, with RCAS-ngn3 added to the culture medium at the onset of the culture. (L) Bright-field views and (M) epifluorescence of visinin immunostaining. Asterisk: membrane of the culture insert. Scale bar, 50 μm (applies to all images).
Detecting gene expression in darkly pigmented retinal pigment epithelial cells requires a postdetection bleaching treatment to reveal the detection signals. This bleaching treatment, however, often adversely affects the quality of detection signals. Therefore, we used RT-PCR to analyze changes in the expression of retinal pigment epithelial genes after reprogramming with ngn1 and ngn3. We found that the expression of mife, a gene vital to maintaining retinal pigment epithelial properties, became undetectable in E6 retinal pigment epithelial cell cultures infected with RCAS-ngn1 or RCAS-ngn3 (Fig. 6M). The expression of mmp115 was undetectable in an RCAS-ngn3 sample, and a low level of mmp115 expression was detected in the RCAS-ngn1 sample (Fig. 6M). Statistical analysis of the IOD showed the levels of mmp115 expression in the RCAS-GFP control was approximately 2.5-fold that in the ngn1 sample (Fig. 6N).

**DISCUSSION**

Because of the anatomic proximity of the RPE to photoreceptors, RPE could be a convenient source of new photoreceptors to replace faulty ones. A previous study has shown that a proneural gene involved in photoreceptor development can reprogram cultures of dissociated retinal pigment epithelial cells to give rise to cells that resemble developing photoreceptor cells at the molecular, cellular, and physiological levels.24,43 The present study examines whether retinal pigment epithelial tissue is amendable to such reprogramming. Retinal pigment epithelial tissue explants infected with RCAS-ngn1 or RCAS-ngn3 gave rise to layers of photoreceptor-like cells. Induction of the photoreceptor marker visinin also occurred in the retinal pigment epithelial layer of the eye infected with RCAS-ngn3. These results support the prospect of using the RPE to repopulate the retina with photoreceptor degeneration. The prospect is further supported by the results showing retinal pigment epithelial capacity to proliferate and plasticity even at advanced stages of maturation. BrdU incorporation analyses showed cell proliferation in retinal pigment epithelial explants. Cell proliferation may be the underlying cellular mechanism of the production of multilayers of visinin-positive cells in retinal pigment epithelial explants subjected to reprogramming with ngn1 or ngn3. In addition, reprogramming of RPE by ngn1 or ngn3 was not restricted to RPE of young embryos. RPE from nearly hatched embryos (e.g., E18) was also amenable to reprogramming.

In reprogrammed retinal pigment epithelial explants, more cells expressed visinin than red opsin, proteins representing, respectively, early and late phases of photoreceptor differentiation. This was expected because the culture conditions were not considered optimal for photoreceptor maturation, resulting in early genes expressed in more cells than late genes. Cellular localizations of red opsin showed similarity to those of retinal photoreceptor cells. At the same time, mislocalization was apparent. This is attributed to the in vitro system; opsin mislocalization occurs with cultured retinal photoreceptor cells24 or in photoreceptors detached from the RPE.55,43

In the eyes infected with RCAS-ngn3, the retinal pigment epithelial layer contained visinin-positive cells. Because their presence in the RPE predated their presence in the retina and some of them maintained residual pigments, these visinin-positive cells were likely produced in situ in the RPE and were not migrated retinal photoreceptor cells or retinal pigment epithelial cells that had phagocytosed retinal photoreceptor cells. Thus, ngn3 can induce photoreceptor gene expression in the context of retinal pigment epithelial cells in the eye. The presence of residual pigment in some of visinin-positive cells may be attributed to cells at initial steps in the reprogramming process.
process (i.e., at a transitional stage in the RPE→photoreceptor process). Photoreceptor differentiation and retinal pigment epithelial regression are expected to become more pronounced as the process unrolls. Before they reach a certain point in the process, cells may exhibit mixed traits. Direct demonstration of a complete loss of pigment in visinin-positive cells in the retinal pigment epithelial layer was hampered by the embryonic death of the experimental embryos before E8. Nonetheless, it is indirectly supported by the results from experiments with retinal pigment epithelial explants. In retinal pigment epithelial explants of more than 6 DIV, visinin-positive cells contained no such residual pigments.

The retinal pigment epithelial layer in animals infected with RCAS-ngn1 contained far fewer visinin-positive cells than those infected with RCAS-ngn3. Apparently, in vivo reprogramming could be initiated by ngn3, but only to a limited extent by ngn1. The differential effects may reflect differences in the repertoire of downstream genes between ngn3 and ngn1. It is possible that during development, retinal photoreceptor cells send the overlying retinal pigment epithelial inhibitory signals, preventing them from erroneously giving rise to photoreceptor cells. This inhibition could be effectively offset by ngn3 (or one of its downstream genes), but not by ngn1 (or its downstream genes). Consistent with the “inhibitory” scenario, once in explant culture, RPE can be effectively reprogrammed by both ngn1 and ngn3 to give rise to cells resembling photoreceptor cells. Whether the inhibition diminishes with photoreceptor degeneration is an important question and must be rigorously investigated.

A proneural gene participating in the genetic pathway leading to photoreceptor production is a key ingredient in the approach of producing developing photoreceptor cells by reprogramming the RPE. Our results show that both ngn1 and ngn3 were effective in inducing the process. Published data suggest that ngn1 directly participates in photoreceptor production. On the other hand, ngn3 promotes early neurogenesis by expanding the ganglion population at the expense of amacrine cells during chick retinal neurogenesis. On the surface, our results from retinal pigment epithelial experiments using ngn3 seem inconsistent with the ganglion-promoting activity of ngn3 in the retina. However, the differences are reconcilable considering that ngn1 is one of the downstream genes of ngn3. This ngn3→ngn1 genetic relationship enables ngn3 to indirectly induce photoreceptor genesis by inducing ngn1 expression. In addition, it is generally known that gene function can be cell context dependent. Ngf3 indirect induction of photoreceptor genesis could become conspicuous in retinal pigment epithelial cells, which lack the expression of the many bHLH genes that cross-regulate one another in the developing retina. Identifying a viable source, within the eye, of developing photoreceptor cells will facilitate the development of autologous cell-replacement therapies. To this end, various tissues or cells within the eye have been investigated. In fish and in chick, injuries induce the formation of new retinal neurons, including photoreceptors, from progenitor/stem cells at the ciliary marginal zone. However, the mammalian retina seems to lack such a regeneration mechanism. Müller glia in various species, including mammals, have recently been shown to have certain retinal progenitor cell properties, but their capabilities to efficiently give rise to photoreceptors remain to be demonstrated. “Alternative” sources being explored include the iris pigment epithelium, the ciliary body, and the limbal epithelium. Of note, early reports showed the ciliary epithelium (CE) containing retinal stem cells, but a recent study indicates that CE-derived spheres consist of proliferating pigmented CE cells rather than retinal stem cells. Thus far, experiments with these alternative sources produce minute amounts, if any, of photoreceptor-like cells. The low yield may reflect the biological nature of these tissues. It may also stem from the experimental approaches used because few of the studies used a pro-photoreceptor gene (e.g., ngn1) to prime the cells to unravel the photoreceptor differentiation program.

In contrast, our approach of coaxing RPE plasticity with a proneural gene participating in the genetic pathway leading to photoreceptor genesis in the retina yields massive amounts of photoreceptor-like cells. This study shows that the retinal pigment epithelial tissue can be reprogrammed to produce layers of cells expressing photoreceptor genes, and the reprogramming event can take place in the eye and in explants of young and mature RPE. Together with published data on the development of advanced photoreceptor traits, including responses to light and to 9-cis retinal, results reported here highlight the prospect of reprogramming the RPE with a pro-photoreceptor gene as a viable approach to produce new photoreceptor cells to repopulate the retina undergoing photoreceptor degeneration.

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References


RPE as a Convenient Source of Photoreceptors


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