Photoreceptor-Like Cells in Transgenic Mouse Eye

Run-Tao Yan,¹ Xiumei Li,^{1,2} and Shu-Zhen Wang¹

¹Department of Ophthalmology, University of Alabama at Birmingham School of Medicine, Birmingham, Alabama

²Guangzhou First People's Hospital, Department of Ophthalmology of Nansha Central Hospital, Guangzhou, Guangdong, China

Correspondence: Run-Tao Yan, 1670 University Boulevard, VH 444, Birmingham, AL 35294-0019; rtyan@uab.edu.

Shu-Zhen Wang, 1670 University Boulevard, VH 444, Birmingham, AL 35294-0019; szwang@uab.edu.

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PURPOSE. Recent success of rescuing vision by photoreceptor replacement in mouse models of photoreceptor degeneration intensifies the need to identify approaches to generate photoreceptors cells for future replacement therapies. We explored the possibility of whether in the mouse eye photoreceptor-like cells could arise from the RPE experimentally manipulated to express a regulatory gene participating in transcriptional networks leading to photoreceptor genesis during retinal development.

METHODS. Transgenic mice were generated with a DNA construct that would express *neurogenin1* from RPE *bestrophin-1* promoter or *neurogenin3* from *RPE65* promoter. Transgenic mice were examined with histology and immunohistology for the presence of photoreceptor-like cells and for the presence of cells that might represent transitional stages in RPE-to-photoreceptor reprogramming. Explant culture of “sclera+choroid+RPE” eyecup was used to examine whether cells with photoreceptor traits could arise from the eyecup derived from transgenic mice.

RESULTS. Transgenic animals showed varied degrees of phenotype manifestation. Approximately 60% of offspring from ~50% of founders contained photoreceptor-like cells in the subretinal space. These cells expressed photoreceptor proteins recoverin, red opsin, and rhodopsin, and displayed morphologic similarities to photoreceptors. In these eyes, the RPE was maintained. Cells seemingly amid RPE-to-photoreceptor transformation were observed in young and aged mice, suggesting old animals were responsive to the reprogramming scheme. De novo generation of photoreceptor-like cells was detected in “sclera+choroid+RPE” eyecup explants derived from adult animals.

CONCLUSIONS. Our results point to a potential way to generate photoreceptor cells in situ in adult mammalian eyes.

Keywords: photoreceptors, regeneration, reprogramming, mammalian retina

Photoreceptors in the vertebrate retina are primary neurons responsible for converting photons into electrophysiological signals through a phototransduction process initiated by visual pigments, which are opsin proteins covalently linked to chromophore 11-*cis* retinal.¹ Photoreceptor degeneration leads to blindness. One promising therapy on the scientific horizon is photoreceptor replacement.^{2,3} While tantalizing, applying this technology to humans requires first to identify a viable source of differentiating photoreceptors or their precursors.^{4,5} Current research on deriving new photoreceptors for replacement, as regenerative medicine in general, focuses on the use of embryonic stem cells and induced pluripotent stem cells to produce transplantable cells. Nonetheless, naturally occurring regeneration involves awakening cells at the site of an injury/insult to produce new cells for repair. If this natural approach could be adopted for photoreceptor generation, then photoreceptor replacement might be accomplished without cell transplantation and associated risks and complications.

In the mammalian eye, photoreceptors constitute the outer nuclear layer (ONL) of the retina and the ONL lies against a nonneural, epithelial tissue—the RPE. This anatomical location makes the RPE attractive for in situ photoreceptor repopulation, provided the RPE being programmable to produce photoreceptor cells. Biologically, the RPE is capable of two fundamental processes that could be channeled toward photoreceptor

generation: cell proliferation and cell fate plasticity. In the mature eye, most cells in the RPE remain quiescent, with the exception of a small population in the periphery.⁶ However, under disease conditions,^{7–9} with retinal detachment,^{10–12} or when stimulated physically,¹³ RPE cells in mammalian eyes reenter the cell cycle to proliferate. A recent study showed that even the RPE from a 99-year-old person contained cells capable of proliferation in culture.¹⁴ Progeny cells from RPE proliferation may differentiate into cells other than RPE, resulting in transdifferentiation. In a clinical setting, RPE's proliferative response may cause vision loss from retinal detachment due to the progeny cells differentiating into cells with tractional force.¹⁵

The demonstration¹⁶ of highly efficient RPE-to-photoreceptor reprogramming of embryonic chick RPE cells by *neurognin1* (*ngn1*) and *ngn3* prompted an exploration of whether the mammalian RPE's natural capabilities of proliferation and plasticity could be guided by *ngn1* or *ngn3* into photoreceptor generation in the mouse eye. For this we created transgenic mice with a DNA cassette expressing human *ngn1* from the promoter of RPE gene *bestrophin-1*^{17,18} or expressing human *ngn3* from *RPE65* promoter.^{19–21} Here we report photoreceptor-like (PR-L) cells in transgenic mouse eyes and de novo generation of PR-L cells in “sclera+choroid+RPE” explants. Perhaps more importantly, cells seemingly in RPE-to-photoreceptor transformation process were detected in young and old

animals. Notably, RPE was maintained in eyes with photoreceptor-like cells. The results support the biological feasibility of exploring RPE as a source of photoreceptor cells in situ in adult mammalian eyes.

METHODS

Generation of Transgenic Mice

All experimental procedures involving the use of animals adhered to the ARVO Statement of the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

The coding regions of human *ngn1* (NCBI accession # NM_006161) and human *ngn3* (NCBI accession # NM_020999) and the sequences of mouse P_{RPE65} (−737 to 0)²¹ and P_{VMD2} (−585 to +38)¹⁸ were PCR amplified, cloned into pGEM-T, and their sequences verified. P_{RPE65} (or P_{VMD2}) was inserted into pCI (Promega Corporation, Fitchburg, WI) replacing P_{CMV} to drive transgene expression in RPE cells. Human *ngn1* was inserted into pCI- P_{VMD2} to generate pCI- P_{VMD2} -*ngn1*, and human *ngn3* was inserted into pCI- P_{RPE65} to generate pCI- P_{RPE65} -*ngn3*. Linearized and purified DNA was provided to the University of Alabama at Birmingham Transgenic Facility, where microinjection, implantation, and derivation of potential founders were carried out by the facility staff.

Potential founders were screened by PCR genotyping with DNA from tail snips, using various combinations of primers corresponding to the sequences in the intron of pCI or the transgene. Each PCR (38 cycles) used 200 ng of tail DNA. DNA from wild-type mice was used as a negative control. Either diluted plasmid DNA or wild-type DNA spiked with 10 pg of pCI- P_{VMD2} -*ngn1* (or pCI- P_{RPE65} -*ngn3*) DNA was used as a positive control. Gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward primer of 5' gcc aag tat gat tac atc aag 3' and reverse primer of 5' cag gaa atg atg ttg aga aag 3') was used as an internal control. Screening for P_{VMD2} -*ngn1* transgenic animals used forward primer of 5' agg agc cgg cgc gtc aag 3' and reverse primer of 5' tca gag agc ggg gag gc 3' of the transgene. Screening for P_{RPE65} -*ngn3* animals used primers located in the intron (5' gca ctg gcc agg taa gta tca a 3') and the 5' of *ngn3* (5' ccg agg gtt gag gcg tca t 3').

Electroporation of Chick RPE Cells

Eye cup (sclera+choroid+RPE) was isolated from day 13 chick embryos and divided into 4 pieces. One piece of the tissue was placed vertically into a 2-mm gap cuvette (Electroporation Cuvettes Plus; Harvard Apparatus, Holliston, MA) with 25 μ L of pCI- P_{VMD2} -*ngn1* DNA in 50 μ L PBS. The electrical current was delivered with an electroporator system (BTX 830 Electro Square Porator; Harvard Apparatus) at a setting of 45 V for 45 ms repeating 5 times with a 500-ms interval. The electroporated tissue was then cultured as explant using a permeable support (Transwell; Corning Inc., Lowell, MA) as described.²² After 4 days in culture as explant, the tissue was trypsinized and seeded into a polyornithine-treated 24-well plate and cultured as dissociated cells for 4 days, followed by immunocytochemical analysis with a monoclonal antibody against visinin.²³

Immunohistology

Eyes were enucleated from mice at postnatal days 5 through 8 (P5–P8; $n = 9$); P14 to P20 ($n = 18$); 3 weeks to 3 months ($n = 31$); 4 to 5 months ($n = 6$); and 9 to 15 months ($n = 25$). Eyes were fixed with ice-cold 4% paraformaldehyde in 0.1 M

phosphate buffer (pH 7.2) for 3 hours, followed by infusion with 20% sucrose overnight at 4°C and ratio of OCT to sucrose (2:1) for 30 minutes at room temperature before flash-freeze in ratio of OCT to sucrose (2:1) with liquid nitrogen. Frozen samples were kept at −80°C. Cryosections of 10 to 12 μ m were collected onto Superfrost Plus slides (Fisher Scientific, Waltham, MA). Standard procedure of immunohistochemistry was followed. Briefly, after a blocking step with 2% blocking agent (Roche, Basel, Switzerland) in PBS containing 0.25% Triton X-100 (PBST), the slides were incubated at 4°C overnight with these primary antibodies (in PBST): monoclonal antibody against rhodopsin (1:200 dilution; Chemicon/Millipore, Billerica, MA) and polyclonal antibodies against recoverin (1:200 dilution; Chemicon/Millipore) and red opsin (1:200 dilution; Chemicon/Millipore). Secondary antibodies used included Alex 594-conjugated goat-anti-rabbit, Alex 594-conjugated goat-anti-mouse, Alex 488-conjugated goat-anti-rabbit, and Alex 488-conjugated goat-anti-mouse (1:200 dilutions; Invitrogen). Specificity of the immunostaining was confirmed by the anatomical locations of positive cells in normal retinal sections.

Electron Microscopy

Frozen eyes were cryosectioned at 10 to 12 μ m until PR-L cells appeared in the sample. The remaining, frozen tissue was quickly thawed in 37°C fixation solution (4% paraformaldehyde plus 0.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2), followed by fixation at room temperature for 4 hours. At this point, the tissue, along with information on the desirable tissue orientation for embedding, was transferred to the Imaging Center of Department of Ophthalmology, University of Alabama at Birmingham, where standard electron microscopy was carried out by center staff.

Mouse Eyecup Explant Culture

Eyes were enucleated from P0, P12, P49, and 6-month-old mice, and residual fat and extraocular muscle tissues were removed as much as possible. A circumferential incision was made along the ora serrata to rid of the cornea and the iris, and the eye cup (sclera+choroid+RPE) was separated from the rest of the eyeball (lens+retina+vitreous). The isolated eye cup was then notched at 4 to 5 places and then placed onto the membrane of a permeable support (Corning Inc.) with the sclera facing the membrane. The permeable support (Corning Inc.) with multiple eyecups of littermates was placed into a well of a 6-well plate. The eyecup was cultured with 1.5 mL knockout DMEM (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum with or without 4 μ g/mL BrdU. Culture medium was replaced every 2 days. Explants derived from P0 and P49 mice were cultured without BrdU. Explants derived from P12 mice were cultured for 7 days without BrdU and then 6 days in the presence of BrdU. Explants derived from 6-month-old mice were cultured with BrdU present throughout the culture period. After 1 to 2 weeks in culture, eyecups were fixed by immersing the permeable support (Corning Inc.) in ice-cold 4% paraformaldehyde for 1 hour, followed by cryoprotection with 20% sucrose overnight at 4°C and ratio of OCT to sucrose (2:1) for 30 minutes at room temperature. Eyecups along with the permeable support membrane (Corning Inc.) were excised and those from the same membrane (Corning Inc.) were stacked together in a sample mold and flash frozen with liquid nitrogen.

Double Immunocytochemistry

To detect recoverin protein and BrdU incorporation, cryosections of eyecup explants (10–12 μ m) were first subjected to

the following treatment: (1) PBST/5 N HCl (50:50) for 1 hour; (2) 1 M Tris-HCl, pH 7.5, for 10 minutes; (3) two washes with PBST, 10 minutes each; and (4) 2% blocking agent for 30 minutes. The sample was then incubated simultaneously with monoclonal anti-BrdU antibody (1:200 dilution; Developmental Hybridoma Bank, Iowa City, IA) and polyclonal anti-recoverin antibody (1:200) overnight at 4°C, followed by standard steps for remaining part of immunocytochemistry.

Detection of Dying Cells

Retinal cryosections of P_{VMD2} -*ngn1* mice of P40 (two animals, 18 retinal sections), 2-month-old (two animals, 37 sections); 9-month-old (two animals, 31 sections); and 15-month-old (one animal, 15 sections) were examined for the presence of dying cells by the TUNEL method using the cell death detection kit (In Situ Cell Death Detection Kit; Roche) following the manufacturer's instructions. Similarly prepared/processed cryosections of RCAS-GFP infected, embryonic day 10 chick retina, which was previous shown to have a significant number of TUNEL⁺ cells,²⁴ were included as positive control in the TUNEL assay.

RESULTS

Transgenic Mice

The rationale of the transgenic approach was to use *ngn1* or *ngn3* to turn on genes that initiate a cascade of events leading to photoreceptor differentiation and maturation and that suppress the expression of RPE genes, including the transgene itself from the RPE promoter (Fig. 1A). This would emulate transient *ngn1*, or *ngn3*, expression in the developing neural retina.^{25,26} Continuation of the process would lead to the production of mature photoreceptors. DNA of pCI- P_{VMD2} -*ngn1* and pCI- P_{RPE65} -*ngn3* was used to create transgenic mice (Fig. 1B).

To examine whether the level of transgene expression from the DNA construct would be sufficient for biological activity, pCI- P_{VMD2} -*ngn1* was tested with embryonic chick RPE, which previous studies have shown to be amenable to *ngn1* induced RPE-to-photoreceptor reprogramming.^{16,22} Electroporation of pCI- P_{VMD2} -*ngn1* DNA into chick RPE cells obtained from day 13 embryos resulted in the generation of cells positive for photoreceptor protein visinin (Fig. 1C), indicating that *ngn1* expression from the cassette was biologically effective.

PCR genotyping of tail DNA identified five founders for P_{VMD2} -*ngn1* and 7 for P_{RPE65} -*ngn3* (Fig. 1D).

We have attempted, but in vain, to detect the transgene expression with antibodies from different sources and with in situ hybridization. The very experimental design might be one of the potentially multiple causes for the unfruitful effort. Notionally, the transgene expression could reach a substantial level beginning P10 from P_{VMD2} ¹⁷ and P4 from P_{RPE65} RPE,¹⁹ when the RPE cells already contain pigmented granules that would mask detection signals, and the transgene expression would diminish in cells undergoing, or having undergone, RPE-to-photoreceptor reprogramming.

Photoreceptor-Like Cells in Transgenic Mice

Transgenic mice were examined for retinal alterations with histology and immunohistology. Offspring from three of the five P_{VMD2} -*ngn1* founders and offspring from three of the seven P_{RPE65} -*ngn3* founders displayed either no apparent phenotypic alterations (four out of six combined) or a mild change observed as a small piece of RPE "branching" off the main RPE toward the retina without noticeable changes in retinal

FIGURE 1. Generating transgenic mice expressing *ngn1* (or *ngn3*) in the RPE. (A) Diagram of the experimental scheme. (B) Diagram of the DNA construct. (C) Testing result of whether the level of transgene expression from the DNA construct, pCI- P_{VMD2} -*ngn1*, was sufficient for biological activity. Shown are images of immunostaining for photoreceptor protein visinin of E13 chick RPE receiving no treatment (nt) or electroporation with pCI- P_{VMD2} -*ngn1* (P_{VMD2} -*ngn1*). Arrows point to visinin⁺ cells. Note that the antivisinin immunostaining was in blue, while the pigment granules of the RPE appeared as rusty-brown. (D) PCR genotyping of 7 P_{RPE65} -*ngn3* founders (Fd 1-Fd 7). Int, intron; Reprog, reprogramming; PR, photoreceptor; Wt, wild-type mouse DNA; pls: diluted pCI- P_{RPE65} -*ngn3* DNA; Int-N3, PCR product spanning the intron and *ngn3*; GAPDH, PCR product of glyceraldehyde-3-phosphate dehydrogenase. Asterisk marks the 4 P_{RPE65} -*ngn3* founders whose offspring showed recoverin⁺ cells in the subretinal space. Scale bar: 50 μ m (C).

histology (two out of six). Offspring from the other founders (two of the five P_{VMD2} -*ngn1* and four of the seven P_{RPE65} -*ngn3*) showed PR-L cells recognized by immunostaining for recoverin, a photoreceptor protein involved in phototransduction, in the subretinal space.

The regions with recoverin⁺ cells contained darkly pigmented tissue (presumably of RPE origin) surrounding and demarcating the subretinal recoverin⁺ cells and their different domains (Figs. 2A-C). Subretinal recoverin⁺ cells were detected more often in the peripheral than in the central regions; in the latter case, the subretinal recoverin⁺ cells mostly were detected in the vicinity of the optic nerve head. The thickness of the zone occupied by subretinal recoverin⁺ cells varied, from comparable with the ONL to consisting of only a single layer of cells. There seemed no clear correlation between the amount of the subretinal recoverin⁺ cells and the age of the mice: various amounts were observed among mice of same age and varied amounts were observed among different histological locations of the same eye. Subretinal recoverin⁺ cells were not observed in every examined offspring derived from the same founders. Among 33 P_{RPE65} -*ngn3* animals between P5 and P75, 23 contained subretinal recoverin⁺ cells. Subretinal recoverin⁺ cells were observed in 9 out of 15 P_{VMD2} -*ngn1* animals aging between P14 and P75.

FIGURE 2. Recoverin⁺ cells in the subretinal space in a 2.5-month-old $P_{RPE65}\text{-ngn3}$ mouse. Shown are views of the same sample under bright-field (BF, [A]), counterstaining with nuclear dye DAPI (B), and immunostaining for photoreceptor protein recoverin (Rcv, [C]). Arrowheads point to darkly pigmented tissue associated with, as well as demarcating different domains of, recoverin⁺ (PR-L) cells. Scale bar: 100 μm (A–C).

Immunohistochemistry for opsins, hallmark proteins of photoreceptors, coupled with morphology, and electron microscopy were used to compare and contrast the subretinal cells with the ONL cells. Like the bonafide photoreceptors in the ONL, the subretinal cells displayed outer segments discernible with light microscopy and with epifluorescent microscopy as being decorated by antirhodopsin (Fig. 3C) or antired opsin (Fig. 3D) immunostaining. Hence, they were referred to as photoreceptor-like (PR-L) cells. Unlike those in the ONL, the PR-L cells displayed varied orientations in respect to the RPE. Some were opposing their counterparts in the ONL (Figs. 3A–D), and some were in the same orientation, with their apices (outer segments) point toward the RPE (Figs. 3E–G). Electron microscopy showed that both ONL cells and PR-L cells displayed stacks of electron-dense disc membrane that constitutes the outer segments of photoreceptors (Figs. 3H–J).

Subretinal recoverin⁺ cells were detected at P5, a time when the birth of photoreceptor cells is tapering off.²⁷ The developing retina at this stage consists of an emerging photoreceptor (PR) layer with differentiating photoreceptor cells (recoverin⁺), a discernible ganglion cell layer (GCL), and a thick layer composed of progenitor cells and differentiating inner nuclear layer (INL) cells. In a P5 $P_{RPE65}\text{-ngn3}$ eye, a “pocket” of subretinal recoverin⁺ cells demarcated by pigmented tissue was present at the periphery (Figs. 4A–C). At another location of the same eye, there were discrete domains of subretinal recoverin⁺ cells and multiple pieces of pigmented tissue (Figs. 4D–J). Some of the subretinal recoverin⁺ cells were adjacent to, or within, the RPE layer (Figs. 4K–N).

The very experiment design that aimed at reprogramming the RPE to give rise to photoreceptor cells inherently raised a concern of whether the PR-L cells were generated at the expense of the single-layered PRE. We observed that the RPE remained in transgenic eyes containing PR-L cells (Fig. 3E). Furthermore, a normal-looking RPE was present at places

where the subretinal space contained a layer of cells seemingly amid RPE-to-photoreceptor transition (Figs. 5D–F).

Cells in RPE-to-Photoreceptor Transition in Young and Old Mice

Despite the use of an RPE promoter to target transgene expression in mature RPE, the PR-L cells might not have originated, at least not exclusively, from the RPE, as the traditional transgenic approach used in this study would not ensure strict regulation, both temporally and spatially, of the transgene expression. To determine whether the RPE was indeed one—although not necessarily the only—source of the PR-L cells in the transgenic mouse eye, we first examined eyes for the presence of cells that might represent transitional stages in RPE-to-photoreceptor reprogramming.

In an 8-week-old (adult) $P_{VMD2}\text{-ngn1}$ mouse, discrete cells in the RPE were recoverin⁺ (Figs. 5A–C). Morphologically, these recoverin⁺ cells were more similar to RPE cells than to photoreceptor cells. In addition to their pigment granules, which otherwise are typically present in RPE cells, these recoverin⁺ cells displayed a nucleus that appeared: (1) round (as in RPE cells) versus elongated (as in photoreceptors); (2) large versus compact; and (3) contained several (five to seven) discrete, brightly fluorescent spots of DAPI staining (Figs. 5B, 5E).

In a 9-month-old (aged) $P_{VMD2}\text{-ngn1}$ animal, a layer of cells reminiscent of being in early stages of RPE-to-photoreceptor transition was present in the subretinal space (Figs. 5D–F). Cells in this layer showed pigment granules, as seen in cells of the adjacent, monolayer RPE (Fig. 5D). Yet, those emerging PR-L cells were recoverin⁺ and displayed an elongated cell body. At the tip (to the right; Figs. 5D–F) of this domain of transitional cells, there was a well-defined layer of PR-L cells, which no longer were displaying conspicuous RPE marks.

The presence of transitional cells in both young and old adult, along with the variations in the thickness of the zone occupied by the PR-L cells, promoted a question that whether PR-L cells were constantly produced and then underwent degeneration. TUNEL assay was carried out on retinal sections of $P_{VMD2}\text{-ngn1}$ mice at the age of P40 ($n = 2$); 2 months ($n = 2$); 9 months ($n = 2$); and 15 months ($n = 1$). Similarly processed embryonic chick retina infected with RCAS-GFP²⁴ was included in the assay as positive control. The 14 retinal sections of the positive control had an average of 13.8 TUNEL⁺ cells in the area equivalent to one cross-section of the mouse retina. No TUNEL⁺ cells were detected in the retina of the P40 $P_{VMD2}\text{-ngn1}$ mice (18 retinal sections, three of which contained PR-L cells); the 2-month-old $P_{VMD2}\text{-ngn1}$ mice (37 retinal sections, 23 of which contained PR-L cells); the 9-month-old mice (total of 31 retinal sections, 10 of which contained PR-L cells); and the 15-month-old mouse (total of 15 retinal sections, three of which contained PR-L cells) with the exception of two TUNEL⁺ cells in the RGC in this old animal.

Photoreceptor De Novo Genesis in Explant Culture

We then used explant culture of “sclera+choroid+RPE” eyecup (without the neural retina) to examine whether cells with photoreceptor traits could arise from the eyecup derived from transgenic mice at the age of P0 (Figs. 6A–C), P12 (Figs. 6D–F), or P48 (Figs. 6G–j). Cells positive for recoverin (Figs. 6C, 6F); rhodopsin (Fig. 6G); and red opsin (Fig. 6j) were detected in the explant. Most of the cells exhibited a compacted cell body and lacked the pigment granules of the RPE. Yet, cells in an RPE-to-photoreceptor transitional stage were detected. The transitional cell contained pigment granules (arrow, Figs. 6A–

FIGURE 3. Structural resemblance of PR-L cells to photoreceptors and the preservation of the RPE. (A–D) Rhodopsin (Rho, [C]) and red opsin (Red, [D]) immunopositive apices (*white arrows*) of the PR-L cells and the ONL cells of a 2.5-month-old $P_{RPE65}\text{-ngn3}$ mouse. Note that the PR-L cells and the ONL cells were in opposite orientation. (A, B) bright-field view (A) and counterstaining with DAPI (B) of the same sample. (E–G) Histology and immunohistochemistry of the retina from a 1-year-old $P_{RPE65}\text{-ngn3}$ mouse under bright-field view (E), DAPI counterstaining (F), or immunostaining for red opsin (G). In the aging animal, the single-layered RPE and a layer of PR-L cells were apparent. *White arrows* point to the immunostained outer

segments of red cones in the ONL and the apices of similarly oriented PR-L cells. (H-J) Ultra structural similarity between PR-L cells and ONL cells. (H) Histology of a region with PR-L cells in the eye of a 6-month-old $P_{RPE65}\text{-ngn3}$. *Black arrowhead* points to a line of pigmented tissue between the PR-L cells and the ONL. (I) Electron microphotograph of a region adjacent to that shown in (H). (J) Higher magnification of (I). *Yellow arrowheads* point to the reference point in (I, J), as well as the line of pigmented tissue between the PR-L cells and the ONL (H). *Blue arrows* point to stacks of discs of the PR-L cells, and *purple arrows* point to stacks of discs of the ONL cells. Note that the discs seemed imperfectly stacked; this may reflect a fixation artifact and/or an adverse effect on photoreceptor wellbeing from the presence of two layers of photoreceptor cells. *Scale bars*: 50 μm (A-H), 10 μm (I), 2 μm (J).

C) and displayed an elongated, recoverin⁺ apical process (arrowhead, Figs. 6A-C) of a young photoreceptor.

To determine that the PR-L cells were not retinal photoreceptor cells being carried with the eyecup during dissection, but instead were generated from the explant in

vitro, BrdU was added to the culture medium. Double-labeled cells, BrdU⁺/recoverin⁺, were detected in the explant derived from P12 transgenic mice (Figs. 7A-F), indicating that indeed they were generated de novo from the explant. Eyecup explants from 6-month-old transgenic mice also contained

FIGURE 4. Subretinal recoverin⁺ cells in the developing retina of a P5 $P_{RPE65}\text{-ngn3}$ mouse. (A-C) A cross-section of the eye viewed by bright field (A), DAPI nuclear staining (B), and antirecoverin immunostaining (C). *Arrows* point to the area with subretinal recoverin⁺ cells separated from the retina by pigmented tissue. (D-F) Another section from the same eye with multiple domains of subretinal recoverin⁺ cells and multiple pieces of pigmented tissue. (G-N) Higher magnifications of (D-F). A merge of bright field (G, K) and antirecoverin (I, M) images are provided (J, N), respectively) for colocalizing the pigmented tissue and recoverin⁺ cells. *Arrows* in (K-N) point to recoverin⁺ in close proximity to, or within, the RPE layer. *Scale bars*: 200 μm (A-L).

FIGURE 5. Cells displaying both RPE and photoreceptor phenotypes. (A–C) Recoverin⁺ cells in the RPE layer and with RPE cell morphology (arrows) in an 8-week-old (8 wks) P_{VMD2}-ngn1 mouse. (D–F) Recoverin⁺ cells with an elongated cell body and with pigment granules (arrowheads) in what appeared to be cells in early stages of RPE-to-photoreceptor transition in a 9-month-old (9 mons) P_{VMD2}-ngn1 mouse. The monolayer RPE was clearly visible. Arrowheads point to RPE cells negative for recoverin. Scale bar: 50 μm (A–F).

BrdU⁺/recoverin⁺ cells (Figs. 7G–J). No pigment granules were apparent in the BrdU⁺/recoverin⁺ double-labeled cells, consistent with their originating from RPE cells that had gone through cell proliferation, a process often resulting in the disappearance of pigment granules.

DISCUSSION

Aiming to elicit photoreceptor generation in situ in the mouse eye, we explored gene-directed reprogramming of the RPE by creating transgenic mice of P_{VMD2}-ngn1 and of P_{RPE65}-ngn3. Degrees of phenotype manifestation varied among transgenic lines, as well as among offspring of the same line. The variation could be attributed to heterogeneity in transgene expression from stochastic fluctuations in gene expression^{28–30} and from the limited promoter sequence insufficient for ensuring specificity and/or consistent levels of transgene expression.

FIGURE 6. Cells expressing photoreceptor markers in eye cup (sclera+choroid+RPE) explants derived from neonatal and adult mice. (A–C) A recoverin⁺ cell with pigment granules in an explant derived from a P0 P_{RPE65}-ngn3 mouse and cultured for 7 days. Arrow points to cell's pigment granules, and arrowhead points to its recoverin⁺ apical side. Merges of the different views (A+B, B+C, A+C) were provided to facilitate viewing cell's "mixed" features. (D–F) Recoverin⁺ cells (arrows) in an explant derived from a P12 P_{RPE65}-ngn3 mouse and cultured for 13 days. (G–J) Cells positive for Rho (arrows) and Red (arrowhead) in an explant derived from a 7-week-old P_{RPE65}-ngn3 mouse and cultured for 12 days. Scale bar: 50 μm (A–J).

A pronounced phenotype was the presence of PR-L cells in the subretinal space. The amount of PR-L cells varied, possibly due to variation in cell production, migration, and death, as well as heterogeneity in transgene expression. TUNEL assay did not detect positive cells in places occupied by PR-L cells or in

FIGURE 7. De novo genesis of recoverin⁺ cells in eyecup (sclera+choroid+RPE) explants derived from young and old mice. (A–F) Eyecup explant from a P12 $P_{RPE65-ngn3}$ mouse and cultured for 13 days with BrdU absent in the first 7 days but present in the last 6 days, viewed under: (A) bright field, (B) DAPI counterstaining, (C) anti-recoverin immunostaining, (D) anti-BrdU immunostaining, (E) merge of (B, D), and (F) merge of (C, D). (G–J) Eyecup explant from a 6-month-old $P_{VMD2-ngn1}$ mouse cultured for 13 days in the presence of BrdU, viewed under: (G) bright field, (H) anti-BrdU immunostaining, (I) anti-recoverin immunostaining, (J) and merge of (H, I). Arrows point to double-labeled (recoverin⁺/BrdU⁺) cells. Arrowheads point to recoverin⁺, BrdU[−] cells. Scale bar: 50 μ m (A–J).

the ONL, suggesting cell death was either an insignificant factor or occurring at a very low frequency/rate undetectable by the TUNEL assay. The variations in the amount of PR-L cells among different histological locations of the same eye bring forth a question of whether certain anatomical locations were more reactive to the experimental manipulations than others. PR-L cells were often observed in the peripheral regions and occasionally in the area immediately adjacent to optic nerve head. It has been shown that the periphery RPE contains proliferating cells.⁶ It is possible that those naturally proliferating cells were more susceptible to the gene-directed reprogramming. The RPE at the area of optic nerve head transitions into part of the optic nerve structure, and this might render the RPE at the region somewhat more responsive to reprogramming. If and when experimentally confirmed and defined, the specific anatomic location may become the target for delivering an inductive factor with a vehicle more applicable to human use than the traditional transgenic approach.

Despite the design that aimed at transgene expression in the RPE, the source of the PR-L cells may not be simply attributed to the RPE, because the transgenic approach used in this study would not ensure strict regulation, both temporally and spatially, of the transgene expression. Thus, various tissues possibly had participated in the generation of the PR-L cells. Support to RPE being one—although not necessarily the only—source of the PR-L cells came from results of two sets of experiments: (1) Eyes from transgenic animals contained cells displaying characteristics suitable for being in transitional stages in RPE-to-photoreceptor reprogramming. Recoverin⁺

cells were founded (a) within the RPE layer, and (b) in a layer of darkly pigmented cells adjacent to the RPE, in addition to (c) in territories occupied by well-defined PR-L cells expressing hallmark proteins and displaying feature morphologies of photoreceptors. Further, cells in the three groups displayed gradual changes of RPE-to-photoreceptor, from overtly RPE-like in group A, to bearing marks of both RPE and photoreceptor in group B, to utterly PR-L in group C. (2) Cultured eyecups (without the neural retina) contained cells expressing photoreceptor proteins and exhibiting photoreceptor morphologies. These cells incorporated BrdU while in culture and, thus, were likely derived from the explant and not from contaminating retinal tissue/cells. Other observations seem also in favor of the RPE being a source of the PR-L cells. The PR-L cells were always physically associated with pigmented tissue (presumably of RPE origin) that demarcated the PR-L cells and their domains, implicative of a lineage relationship. Some of the PR-L cells were in reverse orientation in reference to retinal photoreceptors, consistent with the classic observations in amphibians and embryonic chick that RPE-derived retina (through trans-differentiation) exhibits a reverse orientation with its ONL facing the vitreous. Notably, some PR-L cells were in the same orientation as those ONL cells, raising a question of whether the orientation of PR-L cells was adaptive or was determined by the apical-basal orientation of the RPE that produced the PR-L cells.

One critical issue in the potential application of using the RPE for photoreceptor generation is whether the new cells were produced at the expense of the RPE, an undesirable outcome as the RPE plays essential roles in maintaining the

health of the retina, particularly of photoreceptors. This scenario was ruled out by the presence of the monolayer RPE in eyes with PR-L cells and at the place with cells seemingly en route of RPE-to-photoreceptor transition. It seems that the RPE might have regenerated itself, after some of its cells had taken on the route to becoming PR-L cells. Self-regeneration or wound healing of the mammalian RPE is well documented. After experimental RPE debridement in the pig eye, the RPE heals.³¹ RPE wound healing has also been reported in aged-related macular degeneration patients after debridement of defective RPE monolayer.³² RPE repair/wound healing initially involves cell sliding migration and subsequently cell proliferation³³ and may require the presence of neural retina for the new RPE to structurally and functionally mature.³⁴ Nonetheless, it is unclear at the present time whether RPE proliferation and regeneration in our transgenic mice stemmed from RPE's natural capacity, from the expression of the transgene, or a combination of the two. *ngn1* and *ngn3* are generally believed to suppress Notch/Delta and activate cell differentiation. Likewise, it remains to be determined whether and how the ability to regenerate RPE was coupled to the ability to generate PR-L cells in the mice.

A question highly relevant to the potential application of our theme of photoreceptor generation is whether the RPE in an aging mammalian eye would be responsive to the gene-directed reprogramming. The presence in the eye from a 9-month-old mouse of cells bearing marks of being en route RPE-to-photoreceptor transition suggests that the aging RPE was responsive. Additionally, the presence of recoverin⁺/BrdU⁺ cells in eyecup explants derived from a 6-month-old mouse indicates that the eyecup from mice well into adulthood was able to give birth to PR-L cells in vitro.

Together the data presented in this report support the prospect of exploring gene-directed reprogramming of the RPE for photoreceptor generation in situ in the mammalian eye for cell replacement. At the same time, they raise many questions, some of which important to clinical implications. For example, the indication of RPE-to-photoreceptor transition occurring in both young and aging mice raises a question of whether new PR-L cells were being continuously generated. Continuous generation of new PR-L cells could be beneficial under certain circumstance, but raises the concern of tumorigenesis. If new cells had been continuously generated at significant rate, there might have been more PR-L cells in older mice than in younger ones as a result of cell accumulation. However, there was no clear correlation between age and the amount of PR-L cells. The lack of PR-L cell accumulation in aged mice might not simply due to death of new, continuously generated PR-L cells, as no TUNEL⁺ cells were observed. One plausible scenario is that the RPE-to-photoreceptor reprogramming was a noncontinuous event and had commenced in different animals at different ages due to either stochastic mechanisms^{28–30} or heterogeneities in the spatial/temporal expression of the transgene. In the latter case, an approach to ensure temporally and spatially specific expression of the transgene needs to be used in future investigations.

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