The Retinal Pigment Epithelium: a Convenient Source of New Photoreceptor cells?

Shu-Zhen Wang, PhD, Run-Tao Yan, PhD

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

Recent success in restoring visual function through photoreceptor replacement in mouse models of photoreceptor degeneration intensifies the need to generate or regenerate photoreceptor cells for the ultimate goal of using cell replacement therapy for blindness caused by photoreceptor degeneration. 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 (iPS) cells to generate transplantable cells. Nonetheless, naturally occurring regeneration, such as wound healing, involves awakening cells at or near a wound site to produce new cells needed to heal the wound. Here we discuss the possibility of tweaking an ocular tissue, the retinal pigment epithelium (RPE), to produce photoreceptor cells in situ in the eye. Unlike the neural retina, the RPE in adult mammals maintains cell proliferation capability. Furthermore, progeny cells from RPE proliferation may differentiate into cells other than RPE. The combination of proliferation and plasticity opens a question of whether they could be channeled by a regulatory gene with pro-photoreceptor activity towards photoreceptor production. Studies using embryonic chick and transgenic mouse showed that indeed photoreceptor-like cells were produced in culture and in vivo in the eye using gene-directed reprogramming of RPE cells, supporting the feasibility of using the RPE as a convenient source of new photoreceptor cells for in situ retinal repair without involving cell transplantation.

Keywords: Photoreceptor; Regeneration; Replacement; Retina; Retinal Pigment Epithelium

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Correspondence to: Shu-Zhen Wang, PhD. Department of Ophthalmology, University of Alabama at Birmingham School of Medicine, 1670 University Boulevard, VH444, Birmingham, AL 35294 0199, USA; Tel: +1 205 996 8678 Fax: +1 205 934 3425; email: szwang@uab.edu, rtyan@uab.edu

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INTRODUCTION

Photoreceptors are the primary neurons in the vertebrate retina and their degeneration underlies many forms of visual impairment. Studies using mouse models of photoreceptor degeneration have demonstrated successful rescue or restoration of visual function through transplantation of photoreceptor cells harvested from young retina or generated in vitro from mouse stem cells.[1-3] This has generated great excitement in the vision community and in the general public. At the same time, it has heightened the scientific and societal importance of defining a reliable source of new photoreceptor cells.

Current research on deriving new photoreceptors for replacement, as regenerative medicine in general, centers on using embryonic stem cells and induced pluripotent stem (iPS) cells to generate transplantable cells.[4-10] Great strikes have been made towards the ultimate goal of cell replacement using photoreceptor

cells derived from the patient's own somatic cells through iPS cell technology. Nonetheless, nature uses a different approach to repair an injury. It begins with awakening adult cells at the site of a wound to proliferate, followed by activating/reactivating cell differentiation programs to produce the desired cells. If an in vivo regeneration mechanism could be employed to produce new photoreceptor cells, then photoreceptor replacement could be attainable without cell transplantation and associated risks and complications.

CHALLENGES FACING PHOTORECEPTOR REGENERATION IN THE MATURE MAMMALIAN RETINA

Injuries induce photoreceptor regeneration from retinal stem cells residing at the ciliary margin in teleost fish eyes.[11] However, this regeneration mechanism seems lacking in mammals.[12,13] The previously reported presence of retinal stem cells in adult ciliary epithelium of the mammalian eye[14,15] has been contested.[16,17] A recent study reported the presence of multipotent stem cells in the retina of 4-8 weeks old mice that could be expanded in vitro for over 35 passages and produced different types of cells including functional photoreceptor cells.[18] It would be interesting and important to demonstrate the presence of multipotent retinal stem cells in well matured and aged mice. Muller glia in various species, including mammals, possess certain properties of progenitor cells, but their ability to efficiently give rise to photoreceptors needs to be demonstrated.[19-28]

ALTERNATIVE OCULAR TISSUES AS A SOURCE OF NEW PHOTORECEPTORS

The rather disappointing outcomes from exploring the neural retina for photoreceptor regeneration, along with the discoveries of multipotent stem cells from various tissues in adult mammals, have spurred interests in non-neural tissues of the eye for photoreceptor genesis. Alternative sources being examined include the iris pigment epithelium,[29-34] the ciliary body,[14,15,32,35] the limbal epithelium,[36] and the retinal pigment epithelium (RPE). Excluding the RPE, none of the alternatives give rise to a significant number of, if any, photoreceptor-like cells. This may reflect the biological nature of these tissues; it may also stem from the used approaches, as few of the studies used a regulatory gene with pro-photoreceptor activity to steer uncommitted cells toward the photoreceptor path or to initiate photoreceptor differentiation program in the otherwise non-neural cells.

ATTRACTION OF THE RPE

The anatomical location places the RPE at an ideal position for providing new photoreceptors to repopulate a damaged retina. The key question upfront is whether or not the RPE is biologically amenable to a reprogramming scheme to give rise to photoreceptor cells. Developmentally, the RPE and the neural retina originate from the same structure, i.e. the optic vesicle. This common origin may facilitate fate changes. Indeed, classic experiments have revealed an intriguing phenomenon, RPE becoming a neural retina, referred to as RPE transdifferentiation. In very young chick embryos, physically separating the RPE from the neural retina,[37] or surgically removing most of the neural retina,[38] results in the RPE developing into a neural retina. Subsequent investigation led to the discovery of bFGF as a stimulus for this phenomenal transdifferentiation.[39] RPE-to-neural retina transdifferentiation also occurs in amphibians.[40-42] In culture, rodent RPE from young embryos has been shown to undergo RPE-to-retina transdifferentiation.[43,44] Mutations in regulatory factors involved in regulating RPE versus retinal fate or disruptions of bone morphogenetic protein (BMP) and Wnt (Int/wingless) signaling pathways can result in ventral RPE transdifferentiation into neural retina.[45-52] Notably, this RPE-to-retina transdifferentiation results in the RPE to be no longer present.

The RPE plays important roles in the well-being and function of photoreceptor cells and the retina as a whole. Dysfunctional RPE is believed to be an underlying pathological condition in age-related macular degeneration, a leading cause of blindness in the elderly in

developed countries. Adult mammalian RPE is well-known for two biological properties: proliferation and plasticity. In the mature eye, most cells in the RPE remain quiescent, except a small population in the periphery where cell proliferation has been observed.[53] However, RPE cells can re-enter the cell cycle to proliferate upon retinal detachment,[54-56] when stimulated physically,[57] or under disease conditions.[60-62] This proliferative response may result in RPE regeneration/wound healing.[61-65] It may also lead to proliferative retinopathy when progeny cells from RPE proliferation differentiate into cells with tractional force causing retinal detachment.[66] On the other hand, these very traits of proliferation and plasticity raise an intriguing possibility of exploring the RPE as a source of new photoreceptor cells.

With mounting knowledge on the regulatory guidance of photoreceptor production during retinal development, an alternative approach emerged to produce new photoreceptor cells, i.e. reprogramming the RPE by genes with pro-photoreceptor activities, to channel RPE proliferation and plasticity towards photoreceptor production.

RPE-TO-PHOTORECEPTOR REPROGRAMMING IN EMBRYONIC CHICK CELL CULTURE

The feasibility of channelling RPE proliferation and plasticity to photoreceptor production using genes that steer unspecified cells toward the path of differentiating into photoreceptors was first tested with the chick system, taking advantage of abundant RPE tissue from chick embryos and readily achievable wide-spread gene transduction from replication-competent retrovirus RCAS. Dissociated RPE cell culture was established from chick embryos at day 6 (E6) and thereafter, stages at which the RPE has already lost its competence to undergo the classic RPE-to-retina transdifferentiation. Cells in the primary RPE cell culture were then infected with RCAS expressing a gene with pro-photoreceptor activities. Using this system, over 20 regulatory genes shown or implicated to be important for the development of the eye, the retina, and/or photoreceptor cells have been screened for activities to guide cultured RPE cells to the path of differentiating towards photoreceptors.[67] The screening identified *neurogenin1* (*ngn1*) and *ngn3* as the two top-performers for eliciting RPE-to-photoreceptor reprogramming.[67] Both genes induced the production of neural clusters from otherwise monolayer RPE cell cultures (Figures 1A and B)[67] and de novo generation of large numbers (as high as 80% of the cells present in the culture) of cells (referred to as reprogrammed cells) positive for photoreceptor protein visinin (Figure 1C).[67]

Molecularly, the reprogrammed cells expressed transcription factors *crx*, *nr2e3*, *raxL*, *RXRγ*, and *neuroD*, which participate in initiating the photoreceptor differentiation program. Reprogrammed cells also expressed components of phototransduction, including red opsin, the α-subunit of CNG channels, and cone α-transducin. Red opsin$^+$ cells displayed dot-like immunostaining at the apices of the cells (Figures 1F and G),[67] reminiscent of that in the retina, indicating proper localization of red opsin in the reprogrammed cells.

Morphologically, in contrast to the hexagonal RPE cells, visinin$^+$ cells resembled young photoreceptor cells, with an elongated cell body, an axon-like process, an inner segment-like compartment, and a lipid droplet-like structural feature (Figure 1D and E).[67] Electron microscopy showed that reprogrammed cells developed a cellular compartment rich in mitochondria, resembling the inner segment of photoreceptor cells.[67] On the apex of the inner segment-like structure, reprogrammed cells displayed ciliary expansions, reminiscent of the developing outer segments of retinal photoreceptors in E17 eye or in culture.

Physiologically, light response and visual recovery are two hallmarks of photoreceptors. Fluorescent calcium imaging showed that reprogrammed cells exhibited both hallmarks: they responded to light by decreasing their Ca^{2+} levels (Figure 2) and responded to 9-cis-retinal by increasing their Ca^{2+} levels (Figure 3).[67] These results indicate that reprogrammed cells were able to develop advanced photoreceptor traits at molecular, structural, and physiological levels.

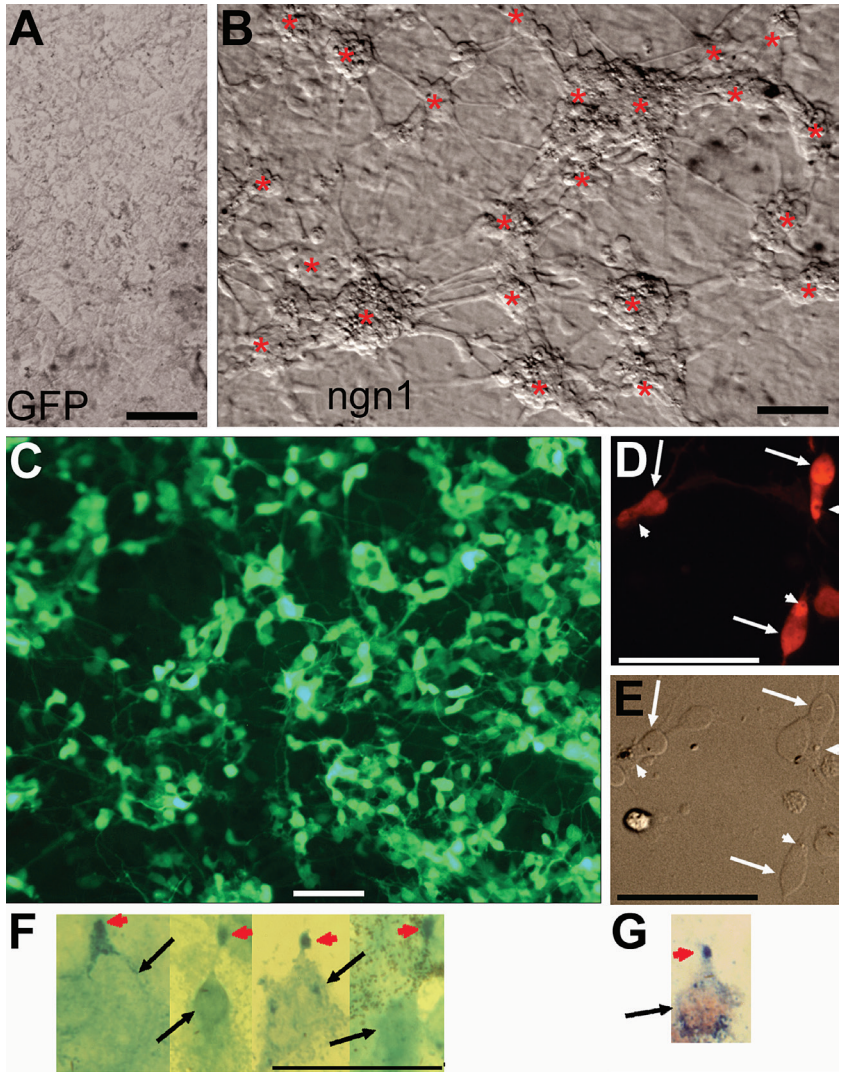

Figure 1. Photoreceptor-like cells in reprogrammed RPE cell culture derived from day 6 chick embryos. A: Bright field view of a control culture infected with RCAS-GFP. B: Bright field view of *ngn1* reprogrammed culture (infected with RCAS-ngn1) displaying neuron-like clusters (*), which were absent in the control (A). C: Epi-fluorescence view of reprogrammed culture immunostained for photoreceptor protein visinin. D, E: Morphologies of visinin$^+$ cells viewed with bright field (E) and epi-fluorescence (D). Arrows point to the cell body and arrowheads point to a structural feature reminiscent of the lipid-droplet typically present in chick photoreceptors. F: Morphologies of red opsin$^+$ cells in *ngn1* reprogrammed culture. Arrows point to cell bodies. Arrowheads point to cells' apices decorated by anti-red opsin immunostaining. G: A cell double-labeled for visinin (in red) and red opsin (in blue) in *ngn1* reprogrammed culture. Scale bars: 50 μm.
[Data are from the authors' laboratories].
[Modified from Yan et al., 2010. Originally published in *Journal of Comparative Neurology*; DOI 10.1002/cne.22236].[67]

IN OVO RPE-TO-PHOTORECEPTOR REPROGRAMMING IN EMBRYONIC CHICK EYE

To be a convenient source of new photoreceptor cells, RPE in the eye needs to be responsive to the reprogram scheme. In E7.5 chick eyes, either the wild type or those infected with control retrovirus RCAS expressing GFP (RCAS-GFP), developing photoreceptor cells positive for visinin were confined within the neural retina at the prospective location of the outer nuclear layer (ONL), and the RPE layer lacked visinin$^+$ cells (Figures 4A and B).[68] In embryonic chick eyes infected with retrovirus RCAS-ngn3, the RPE layer contained visinin$^+$

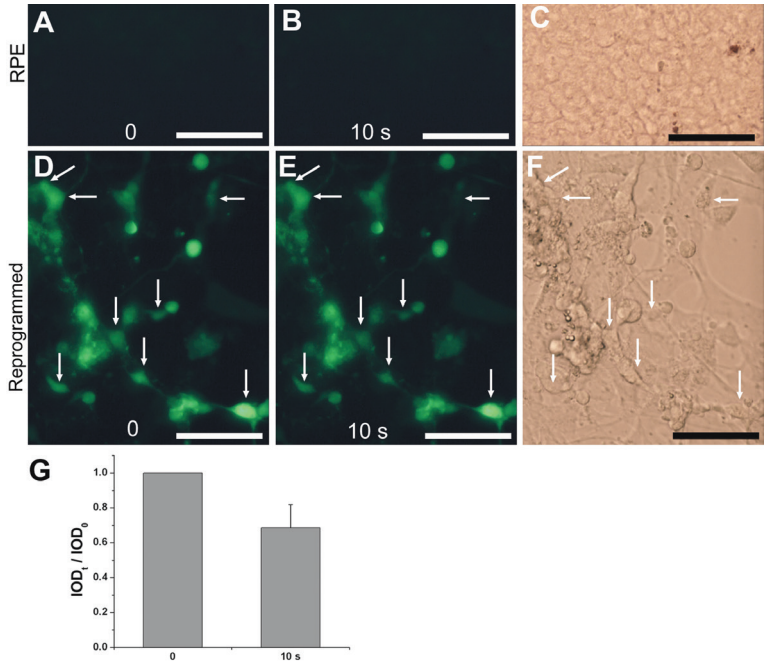

Figure 2. Light responses of reprogrammed cells examined with Ca^{2+} imaging. A, B: Images before (A) and after 10 seconds (10 s, B) of light exposure of a control RPE cell culture. C: A bright field view of the control culture. D, E: Images of a reprogrammed culture before (D) and after 10 seconds (E) of light exposure. F: A bright field view of the reprogrammed culture. Arrows: cells with noticeable reductions in fluorescence intensities. G: Means and SDs of calculated integrated optical density (IOD) ratios (IOD_t/ IOD_0) of cell's fluorescent intensity. Scale bars: 50 µm
[Data from the authors' laboratories].
[Modified from Yan et al., 2010. Originally published in *Journal of Comparative Neurology*; DOI 10.1002/cne.22236].[67]

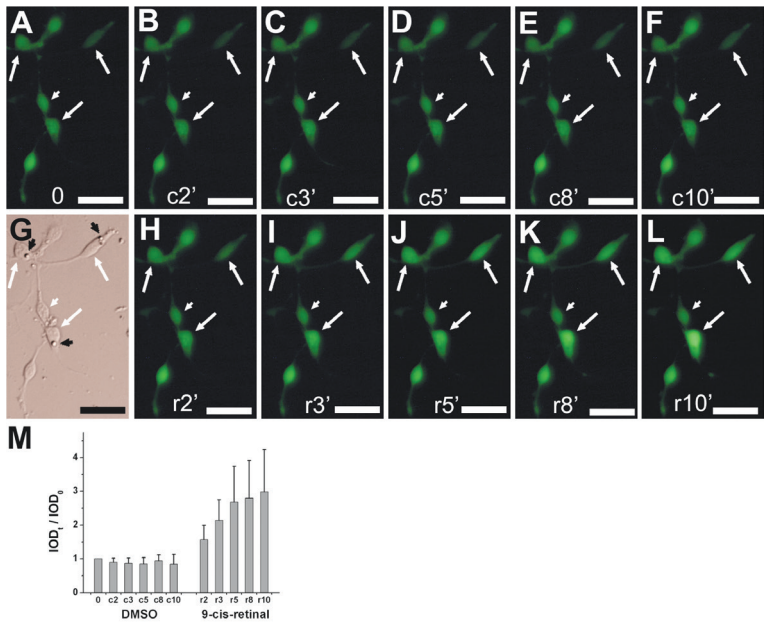

Figure 3. Response to 9-cis-retinal by reprogrammed cells examined with Ca^{2+} imaging. A-L: Images of reprogrammed cells after light bleaching (A) and at the indicated number of minutes (2'-10') after sequential administration of vehicle control (c, B-F), replacement of medium (G), and then 9-cis-retinal (r, H-L). Arrows: cells showing increases in fluorescence intensity. Arrowheads: cells lacking such an increase. M: Means and SDs of calculated integrated optical density (IOD) ratios (IOD_t/IOD_0) of cell's fluorescent intensity. Scale bars: 20 µm.
[Data are from the authors' laboratories].
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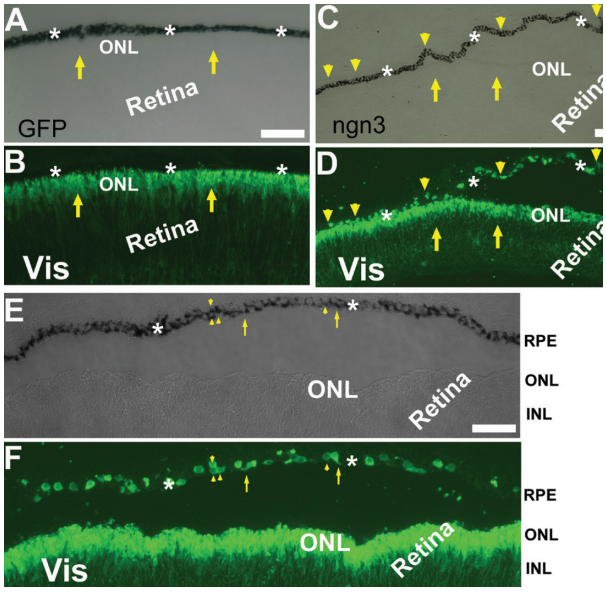

Figure 4. Visinin$^+$ cells in the RPE layer in chick embryos infected with RCAS expressing *ngn3* (RCAS-ngn3). A, B: Bright-field (A) and epi-fluorescence of immunostaining for visinin (Vis, B) of an E7.5 control eye infected with RCAS-GFP. C, D: Bright-field (C) and epi-fluorescence of visinin immunostaining (D) of an E7.5 eye infected with RCAS-ngn3. E, F: Bright-field (E) and epi-fluorescence of visinin immunostaining (F) of a detached region in E7.5 eye infected with RCAS-ngn3. Arrows point to visinin$^+$ cells of the retina, except in E,F, where arrows point to visinin$^+$ cells with a neural-like process in the RPE layer. Arrowheads point to visinin$^+$ cells in the RPE layers.
ONL, outer nuclear layer; INL, inner nuclear layer
[Data are from the authors' laboratories].
[Modified from Li et al., 2010. Originally published in *Investigative Ophthalmology & Visual Science*; DOI:10.1167/iovs.09-3822].[68]

cells. This was unequivocally observed in regions where the RPE and the retina were separated (Figures 4C and D).[68] The visinin$^+$ cells accounted for ~37.2±4.3% of cells in the RPE layer. A thin process could be seen on some of the visinin$^+$ cells (arrows in Figures 4E and F), indicating they were morphologically more similar to neurons than to RPE cells, while other visinin$^+$ cells were more RPE-like. Some of the visinin$^+$ cells in the RPE layer retained dark pigmentation typical of RPE cells (arrowheads in Figures 4E and F). In addition, visinin$^+$ cells in the RPE predated their presence in the retina.[68] Thus visinin$^+$ cells in the PRE layer likely originated from the RPE and possibly were in transitional stages in RPE-to-photoreceptor switching process.

RPE-TO-PHOTORECEPTOR REPROGRAMMING IN TRANSGENIC MOUSE

The above results provide compelling evidence for the feasibility of reprogramming embryonic chick RPE to produce photoreceptors. Nonetheless, the study used the chick, a non-mammalian vertebrate that is evolutionally more ancient and may manifest phenotypical changes that are lacking in mammals after comparable experimental manipulations. To move forward towards the ultimate therapeutic goal of inducing in situ photoreceptor generation, transgenic mice were created with a DNA construct that would express *ngn1* under the control of RPE *bestrophin1* (*VMD2*) promoter[69] or *ngn3* under the control of *RPE65* promoter.[70] The logic was to use *ngn1/ngn3* to induce in mouse RPE cells the expression of genes that initiate photoreceptor differentiation and suppress the expression of RPE genes, including the transgene itself from the RPE promoter (Figure 5). This would emulate transient *ngn1/ngn3* expression in the developing neural retina.[71,72] Continuation of the process would lead to the production of mature photoreceptor cells.

Transgenic animals of P_{VMD2}-ngn1 or P_{RPE65}-ngn3 showed varied degrees of phenotype manifestation. A pronounced phenotypical change was the presence of photoreceptor-like (PR-L) cells in the subretinal space (Figure 6).[73]

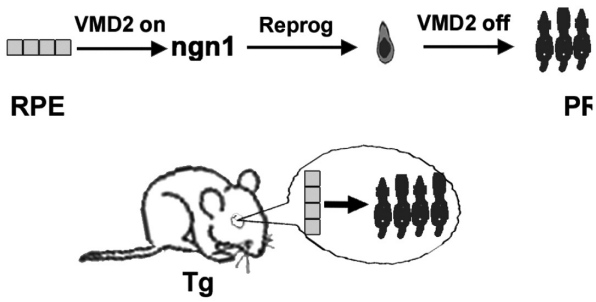

Figure 5. Diagram of the experimental scheme to induce RPE-to-photoreceptor reprogramming by *ngn1* in the mouse eye.
Reprog, reprogramming; PR, photoreceptor; Tg, transgenic mouse.
[The diagram originated from the authors' laboratories].
[Modified from Yan et al., 2013. Originally published in *Investigative Ophthalmology & Visual Science*; DOI:10.1167/iovs.13-11936].[73]

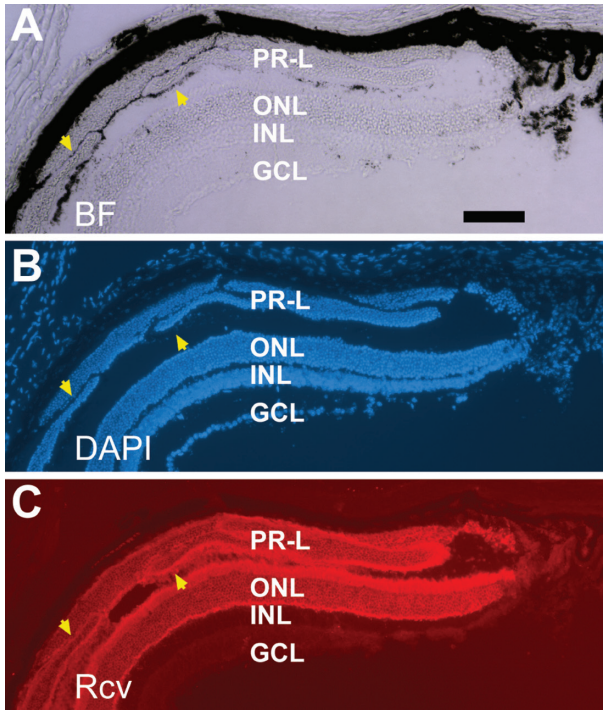

Figure 6. Recoverin$^+$ cells in the subretinal space in a 2.5-month-old P_{RPE65}-ngn3 transgenic mouse. Shown are views of the same sample under bright field (BF, A), counterstaining with nuclear dye (DAPI, B), and immunostaining for photoreceptor protein recoverin (Rcv, C). Arrowheads point to darkly pigmented tissue associated with, as well as demarcating different domains of, recoverin$^+$ (PR-L) cells.
ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer
Scale bar, 100 µm, applies to all panels.
[Data are from the authors' laboratories].
[Modified from Yan et al., 2013. Originally published in *Investigative Ophthalmology & Visual Science*; DOI:10.1167/iovs.13-11936].[73]

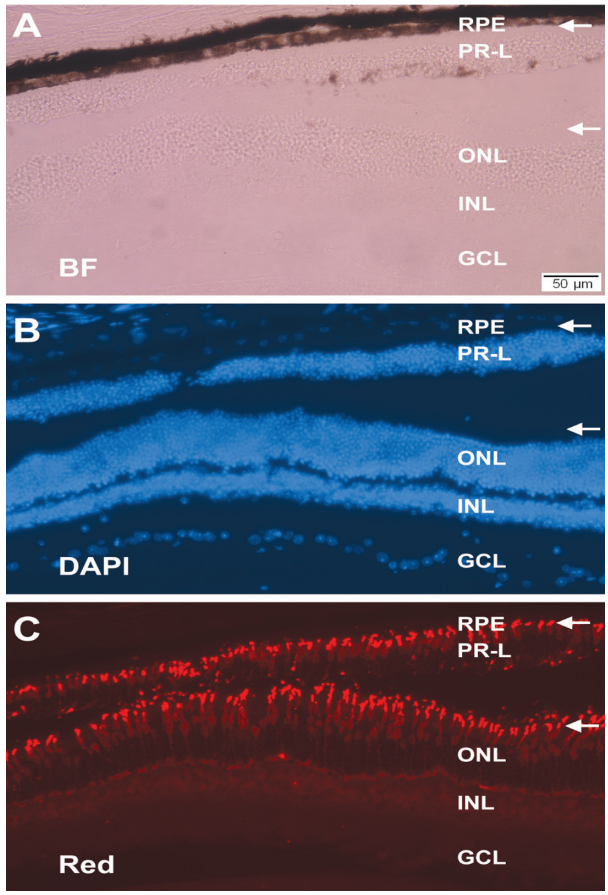

Figure 7. Structural resemblance of PR-L cells to photoreceptors and the preservation of the RPE. Shown are histology and immunohistology of the retina from a 1-year-old P_{RPE65}-ngn3 transgenic mouse under bright field view (A), DAPI counterstaining (B), or immunostaining for red opsin (C). In the 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.
ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 50 µm, applies to all panels.
[Data are from the authors' laboratories].
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Immunohistologically and morphologically these cells appeared similar to those in the ONL. They displayed outer segments discernible with bright-field microscopy, decorated by anti-red opsin and anti-rhodopsin immunostaining (Figure 7), and containing stacks of electron-dense disc membrane that constitutes the outer segments of photoreceptors.[73]

The very approach of reprogramming the RPE to give rise to photoreceptor cells inherently raises a concern of whether new photoreceptor cells would be produced at the expense of the PRE, an undesirable outcome as the RPE plays essential roles in maintaining the health of the retina, particularly of photoreceptors. This concern was eased by the presence of the monolayer RPE in eyes with PR-L cells (Figure 7)[73] and at the place with cells seemingly en route RPE-to-photoreceptor transition.[73] It appears 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.[62] RPE wound healing has also been reported in aged-related macular

degeneration patients after debridement of defective RPE monolayer.[65] RPE repair/wound healing initially involves cell sliding migration and subsequently cell proliferation[61] and may require the presence of neural retina for the new RPE to structurally and functionally mature.[63]

A question highly relevant to the potential application of the reprogramming scheme to photoreceptor generation is whether the RPE in an aging mouse eye would be responsive to the gene-directed reprogramming. Cells seemingly en route RPE-to-photoreceptor transition were present in the subretinal space in a 9-month-old P_{VMD2}-ngn1 animal.[73] Dark pigment granules were present in these cells, as in cells of the adjacent, monolayer RPE. Yet, these transitional cells were positive for recoverin, a photoreceptor protein involved in phototransduction, and displayed an elongated cell body typical of young photoreceptor cells. Distal to the domain of the transitional cells was a well-defined layer of PR-L cells, which no longer displayed conspicuous RPE marks.[73] These observations suggest that the aging RPE in the 9-month-old animal was responsive to the reprograming scheme. Additionally, there were recoverin$^+$/BrdU$^+$ cells in eyecup explants derived from a 6-month-old transgenic mouse, indicating that the eyecup from the animal that was well into adulthood was able to give birth to PR-L cells in vitro.[73]

In conclusion, results from experiments using chick and mouse systems provided support to the biological feasibility of using gene-directed reprogramming of the RPE to produce new photoreceptor cells in situ in the eye. If applicable to the human eye, gene-directed reprogramming may offer a tantalizing prospect of using the RPE as a convenient source of new photoreceptor cells for retinal repair in situ, without involving cell transplantation and its associated risks and complications. However, enticing as it may seem, it is imperative to carry out rigorous studies to address many issues important to both basic science and potential clinical applications.

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Conflicts of Interest

None.

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