Adult Human RPE Can Be Activated into a Multipotent Stem Cell that Produces Mesenchymal Derivatives

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SUMMARY

The retinal pigment epithelium (RPE) is a monolayer of cells underlying and supporting the neural retina. It begins as a plastic tissue, capable, in some species, of generating lens and retina, but differentiates early in development and remains normally non-proliferative throughout life. Here we show that a subpopulation of adult human RPE cells can be activated in vitro to a self-renewing cell, the retinal pigment epithelial stem cell (RPESC) that loses RPE markers, proliferates extensively, and can redifferentiate into stable cobblestone RPE monolayers. Clonal studies demonstrate that RPESCs are multipotent and in defined conditions can generate both neural and mesenchymal progeny. This plasticity may explain human pathologies in which mesenchymal fates are seen in the eye, for example in proliferative vitreoretinopathy (PVR) and phthisis bulbi. This study establishes the RPESC as an accessible, human CNS-derived multipotent stem cell, useful for the study of fate choice, replacement therapy, and disease modeling.

INTRODUCTION

The RPE is a pigmented, single-layered, polarized epithelium located between the neural retina and the vascular choriocapillaris (Figures 1A–1C). It supports metabolic and cellular processes of retinal photoreceptors, including diurnal phagocytosis of the photoreceptor outer segments. Degeneration of the RPE, which occurs in diseases such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP), leads to death of the overlying photoreceptors and neural retina, resulting in vision loss. Because there are currently limited treatments available for these diseases, discovering novel ways to stimulate RPE repair is an important therapeutic goal.

During development, the RPE arises from the central nervous system (CNS) neuroepithelium. Bilateral outpocketings at the diencephalon form the optic anlage, and invagination of the optic vesicles produces a bilayered structure, with the inner layer forming the neural retina and the outer layer the RPE. While the neural retinal progenitor cells proliferate further to form the multi-layered retina, the RPE becomes postmitotic and differentiates early, around day 32–50 in humans (Mann, 1964; Rapaport et al., 1995), into a polarized, pigmented, single-layered epithelium that remains nonproliferative throughout life.

Interestingly, in some organisms, such as amphibians, injury can cause RPE cells to proliferate and regenerate both the RPE and other ocular tissues, including neural retina and even lens, demonstrating an inherent plasticity and ability to transdifferentiate. Plasticity is also seen in embryonic chick and mouse, as RPE can transdifferentiate into neural retina, but this potential is rapidly lost with maturity (reviewed in Lamba et al., 2008).

Human RPE plasticity is seen in pathological conditions; for example, it proliferates in proliferative vitreoretinopathy (PVR) and has been suggested to adopt mesenchymal fates in PVR and phthisical eye, although direct evidence that the RPE can generate mesenchymal progeny is lacking. Adult human RPE can express the neuronal marker TuJ1 in vitro, indicating an incipient potential to adopt different fates (Amemiya et al., 2004).

The basis of adult human RPE proliferative potential and plasticity is not fully understood. Retinal stem cells (RSCs) that can self-renew in vitro and produce retinal neurons and glia have been identified in the anterior ciliary epithelium, the iris pigmented epithelium in adult rodents and humans (Ahmad et al., 2000; Haruta et al., 2001; Tropepe et al., 2000; Cicero et al., 2009). However, to date, there has been no evidence for a stem-like cell from the adult human RPE. Here we identify a subpopulation of RPE cells that can be activated to self-renew and that exhibit multipotentiality, producing either stable RPE progeny or neural, osteo, chondro, or adipose-lineage mesenchymal progeny. These observations indicate that RPE has the capacity for self-repair under the correct circumstances and that RPE can proliferate and differentiate pathologically in others, capacities that are relevant to human diseases in which...
the RPE changes, proliferates, or degenerates, such as RP, PVR, and AMD.

RESULTS

Dormant Human RPE Cells Can Be Activated into a Self-Renewing Cell

To determine whether human RPE contains stem-like cells, RPE was isolated from adult human donor eyes obtained from eye banks (Figures 1A–1L). The anterior portion of the eye, containing the iris pigmented epithelium and the ciliary epithelium, which harbor retinal stem cells, was removed. From the remaining posterior eyecup, the neural retina was removed and the RPE isolated, then dissociated and plated in adherent conditions. Despite being normally nonproliferative in situ, these cells showed robust growth, doubling approximately once per 2 days. Remarkably, RPE cells from 99-year-old donors produced actively growing cultures, and the growth of RPE cells derived from young and elderly donors was similar (data not shown). Proliferation is stimulated by serum, and the cells could be passaged at least 6–8 times. Hence, the adult RPE, normally nonproliferative in situ and dormant for many decades, can be activated to display a robust capacity to divide upon exposure to appropriate growth conditions.

After achieving confluency, the resulting primary adherent cells at first plating (P0) slow proliferation and re-establish a cobblestone monolayer, expressing genes characteristic of RPE, at a lower level than native RPE tissue but higher than the long-established human RPE cell line ARPE19 (Figure 1M). In some culture conditions, the morphology of the cells is primarily fusiform, but growth in conditions optimized for cobblestone RPE growth (Blenkinsop et al., 2010), which is aided by isolating small RPE sheets by gentle dissociation (Figure 1F), produced cobblestone, polarized monolayers with typical RPE morphology that were stable for several months.

We characterized the native RPE and the RPE-derived proliferative cells for the expression of markers associated with early stem cells and reprogramming, including NANOG, OCT4, Sox2, SSEA4, KLF4, C-MYC, and Lin28. Interestingly, at all stages, high levels of C-MYC and KLF4 were seen in RPE cells compared to human embryonic stem cells (hESCs) or to fibroblasts or neural retina derived from the same human eye donors (Figure 2). The proliferative RPE-derived cells were positive for the surface marker SSEA4 (Figure 2A) but we did not detect immunostaining for OCT4 and NANOG, and negligible Sox2 (data not shown), findings that were verified by qPCR (Figure 2B).

Clonal, nonadherent sphere formation is an indication of stem cell presence within CNS tissue (Reynolds and Rietze, 2005). Adult RPE tissue was isolated, dissociated into single cells, and grown at clonal density in nonadherent conditions in knockout serum replacement (KSR) medium supplemented with FGF2. Primary spheres were visible after 4 days at a frequency of 1.5% of the initial plated cells (Figures 2C–2E), and the sphere cultures could be passaged at least three times, demonstrating self-renewal. However, this approach underestimates the number of cells capable of proliferating, because typically RPE grows more successfully when plated in adherent conditions, probably reflecting the importance of cell-cell interactions and cell adhesion for RPE cell growth. Therefore, we assessed self-renewal potential by cloning single, freshly isolated RPE cells, one cell per well, in adherent conditions in Terasaki wells that each hold approximately 10 μl of medium. The vast majority of single RPE cells did not divide or produced just 2–4 progeny. However, 10.6% of the cells divided and eventually filled the culture well with a confluent cobblestone monolayer. When these clones were passaged and plated again as single cells in Terasaki wells, 26.4% proliferated extensively to fill the entire well with a cobblestone RPE monolayer, demonstrating self-renewal in adherent conditions.

Human RPESCs Produce Neural and Mesenchymal but Not Liver Progeny

To examine their plasticity, RPE cobblestone monolayers (Figure 3A) were treated for 4 weeks with differentiation media that have been used previously to promote neural, adipocyte, chondrocyte, and osteogenic differentiation. The cells successfully differentiated and expressed markers for these cell types, assessed by histochemical stains, immunostaining, and qPCR (Figures 3C and 3D). Such markers were not present prior to differentiation (Figures S1C–S1F). In some cases, at the 4 week time point, the resulting cells maintained a pigmented cobblestone-like morphology, demonstrating a transitional form between the two phenotypes, a classic sign of cells transdifferentiating from one fate to another.

The same plasticity was exhibited by RPE grown as fusiform cultures, which are largely depigmented, and at different passages (P2–P5, Figure S2). The fact that both fusiform and cobblestone RPE exhibit this plasticity indicates that the acquisition of these fates does not depend upon cells undergoing an epithelial-mesenchymal transition. Furthermore, that both pigmented and unpigmented cells do so indicates that loss of pigment is not essential for trans-fating to occur. In amphibians in which the RPE is stimulated to transdifferentiate into neural retina, pigment loss accompanies the transition to retinal cells (Ikegami et al., 2002). However, in our experiments, pigment is diluted upon cell division, even before cells are placed in differentiation medium. For example, Movie S1 shows a freshly isolated human RPE cell dividing in RPE medium and splitting the pigment granules approximately evenly between the two daughter cells. This continues so that actively proliferating RPESCs have little pigment, although it is not a prerequisite for acquiring mesenchymal potential as evinced by examples of differentiated RPE-containing pigment (Figure S2).

When the RPE cells were grown in neural differentiation conditions, qPCR analysis showed that they markedly upregulated, by more than 1,000-fold, the neural progenitor marker Nestin and by approximately 90-fold the neuronal marker β-tubulin III. Interestingly, the cells also upregulated markers expressed in the early eye-field and forebrain: LHX2, Otx2, and RX. In contrast, the retinal progenitor markers CHX10 and Rhodopsin were unchanged and Pax6 actually declined. The neural differentiation medium we used was based on a medium that promotes retinal differentiation (Levine et al., 2000; Osakada et al., 2009; Spence et al., 2007). Thus it appears that neural differentiation encouraged the acquisition of an anterior/eye field neural progenitor phenotype, and the RPE marker Mitf was significantly suppressed, consistent with a fate change.

In contrast to this neural and mesenchymal potency, RPE cells subjected to protocols for endoderm differentiation did not
Figure 1. Isolation and Characterization of Human RPESCs
(A) Schematic of the adult human eye with the vertical dashed line indicating the incision.
(B and C) Phase-contrast photomicrograph (B) of a sagittal section of adult human retina and immunostaining with RPE65 (C) reveals the RPE layer.
(D) Schematic of the protocol for RPE culture; the first monolayer generated is designated P0.
(E) Dense collection of dissociated single cells and small sheet fragments after RPE dissociation.
(F) Gentle dissection of RPE results primarily in sheets of RPE.
(G) Aggregates seeded onto matrigel-coated plates in serum-containing medium expanding after 1 week in culture.
(H) After 2 months in culture, RPE form a monolayer of cells that exhibit morphology characteristic of native RPE cells.
(I–L) Immunofluorescence shows that the monolayers express the RPE markers, ZO-1, RPE65, CRALBP (cellular retinaldehyde binding protein) (green), and F-Actin and Bestrophin (Best1) (red).
display the panel of markers that would indicate successful differentiation into early endoderm (SOX17, FOXA2) or liver cells (AFP is not detected, although low expression of albumin is seen) (Figure S1A).

Production of mesenchymal progeny, which are typically mesoderm-associated phenotypes, from a CNS-derived cell was surprising. Although the evidence for transitional forms reduces the likelihood that mesenchymal phenotypes originated from a contaminating cell, the definitive test of multipotency is to take an individual RPE cell, expand it clonally, and test whether its progeny can differentiate into diverse cell types. Therefore, we used the clonal lines described above that were derived from primary human RPE, split these into different culture wells, and exposed them to differentiation media for adipocyte, chondrocyte, and bone differentiation, while maintaining other wells in control (RPE) medium for 4 weeks without passaging. We tested 17 nonadherent clones of which 14 successfully survived the differentiation procedures and all were found to be multipotent, producing RPE and mesenchymal derivatives (Figures 3E–3H). We also tested three of the adherent clonal lines, and again, all were found to produce RPE, adipocyte, chondrocyte, and bone lineage progeny. Moreover, this clonogenic experiment is a further demonstration of self-renewal: extensive proliferation from a single cell over a long time period with retention of multipotency has been used to demonstrate stem cells, e.g., in the skin and blood (Blanpain et al., 2004; Petzer et al., 1996; Piacibello et al., 1997).

To determine whether the RPE plasticity observed in vitro could be replicated in vivo, we implanted GFP-retroviral vector-labeled RPE monolayers into the chick chorioallantoic membrane (CAM), a common in vivo assay system to evaluate tumor formation and to investigate development. After 1 week, we observed the presence of vascularized growths (Figure 4A) that were dissected, cryo-sectioned, and stained. GFP + RPE-derived cells showed expression of the early definitive osteogenic differentiation marker Runx2 and of the mesenchymal marker smooth muscle actin (SMA) (Figure 4B), demonstrating that naive RPE can produce mesenchymal derivatives in vivo.

(K and L) Nuclei are labeled with 4′,6-diamidino-2-phenylindole (DAPI) (DNA, blue).
(M) qPCR analysis of RPE cell monolayers versus native RPE tissue (left) and ARPE-19 cells (right).
Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; BR, Bruch’s membrane; VC, vascular choriocapillaris. Scale bars represent 50 μm (B and C), × 20 magnification, 100 μm (E and F), 20 μm (G–M). Error bars are shown as the mean relative change ± SEM. See also Figure S1 and Movie S1.
Mesenchymal Potential Is a General Feature Shown by Different RPE Sources

It was possible that this plasticity was present in human adult RPE and not seen in other types of RPE cells. To test this, further experiments were performed on RPE cells obtained from a variety of sources (Figure S2). ARPE-19 cells, a transformed human line commonly used as an RPE model, showed clear evidence of differentiation into osteogenic, chondrogenic, adipogenic, and myogenic cells (Figure S2). We derived pure RPE cultures from pluripotent and karyotypically normal H9 hESCs (Idelson et al., 2009) and showed that they also had this capacity (Figure S2), as had fetal human RPE (Figure S2). Finally, bovine RPE behaved similarly, demonstrating that mesenchymal differentiation is a feature of RPE from different mammalian species (Figure S2). Interestingly, when comparing the plasticity across these different sources, we noticed that fetal RPE in general was more resistant to acquiring mesenchymal fates, perhaps reflecting an underlying stability (Figure S3). Particularly noteworthy, the human ESC-derived RPE cells were especially vulnerable to osteogenic culture conditions, a propensity that should be taken into account if these cells are to be used clinically for RPE replacement therapies.

DISCUSSION

These findings reveal a previously unappreciated plasticity of RPE. Placing isolated RPE into a variety of defined culture conditions releases their normal quiescence, stimulates self-renewal, and reveals multipotency, which defines the RPE stem cell
In summary, we have shown that RPE can be activated in vitro to a multipotent stem cell, the RPESC, which can be expanded to produce stable RPE or readily differentiated into mesenchymal lineages. It will be important to study the molecular basis of this mesenchymal potential to understand the basis of RPE metaplastic contribution to retinal pathologies. RPESCs can be expanded considerably even from elderly donors and could be valuable to generate stable RPE cells suitable for autologous or allogeneic replacement therapy and for producing in vitro models of RPE disease. Sophisticated protocols of differentiation toward specific neuron cell fates exist (Tucker et al., 2011) and future studies will explore the extent of RPE differentiation capacity. The fact that RPESCs can be readily stimulated to express features of anterior neural progenitor cells suggests that their potential might be broader than demonstrated here, and it will be worthwhile to investigate whether they can be advanced into other differentiated CNS phenotypes.

**EXPERIMENTAL PROCEDURES**

**Isolation and Culture of Human RPE Cells**

**Dissection and Cell Isolation**

Human eyes from 22- to 99-year-old donors were obtained from The Eye Bank for Sight Restoration, Inc. (New York, NY), the National Disease Research Interchange (NDRI) (Philadelphia, PA) (supported by NIH grant 5 U42 RR006042), and the Lions Eye Bank (Albany, NY). The eyes were cut at the ora serrata, the anterior segment was discarded, and the vitreous and retina were removed, exposing the RPE layer. RPE dissection and dissociation were performed as described previously (Burke et al., 1996; Maminishkis et al., 2006). Gentle trituration within the eyecup using care to maintain Bruch’s membrane yields a suspension of largely single RPE cells with minimal contamination by rod outer segments, blood, or other cell types.

**Dissection and Cobblestone Culture**

The eyecup was rinsed with sterile PBS and incubated with enzyme-free Hank’s-based cell dissociation buffer (GIBCO) for 10 min at 37°C. Gently, the dissociation buffer was removed and the eyecup filled with DMEM/F12 media supplemented with 10% FBS (GIBCO). Using a double bevel spoon blade (3.0 mm), small sheets (1 mm²) of RPE were removed from the Bruch’s membrane yields a suspension of largely single RPE cells with minimal contamination by rod outer segments, blood, or other cell types.
supplemented with THT (Taurine, Hydrocortisone, Triiodo-thyronin), and N1 (Sigma-Aldrich) (Maminishkis et al., 2006). Cells were incubated in a 37°C, 5% CO2 humidified incubator and the medium was replaced every 3 days. An epithelial monolayer is observed after 1 month of culture. Bovine RPE were dissected according to the protocol described above and cultured in RPE medium. ARPE19 (ATCC) cells were cultured in RPE medium. Human fetal RPE cells were provided by Dr. Sheldon Miller (National Eye Institute, National Institutes of Health, Bethesda, MD) and cultured in RPE medium, as described previously (Maminishkis et al., 2006).

Clonal Sphere Formation
Disassociated single cells from freshly isolated RPE were plated on ultra-low attachment 6-well plates (Corning, Costar) at 1,000 cells/plate in KSR medium (Knockout DMEM, 20% KO-SR, 10 mM MEM nonessential amino acids, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, penicillin-streptomycin solution [1:100], with medium changes every 3 days. After 10 days, cultures were assessed for neurosphere number. Neurospheres were defined as free-floating spheres of >40 μm diameter. All spheres in a given well were counted and results expressed as a percentage of the plated cells. To assess size and number, spheres were visualized with a Zeiss Axiovert 200 inverted microscope fitted with a Zeiss AxioCam MRm digital camera image capture system and analyzed with AxioVision 4.72 software (Carl Zeiss AG).

RPE Differentiation

Neurogenic Differentiation
Cells were grown in neural differentiation medium: DMEM medium (GIBCO), B-27, N2 (StemCell Technologies), 2 mM L-glutamine, penicillin-streptomycin solution [1:100], 1% antioxidant N-acetylcysteine (NAC), 1% Na-Pyruvate (GIBCO) with 10 ng/ml FGF2 (GIBCO), 100 ng/ml FGF8b and 100 ng/ml SHH (R&D Systems), and 2 μM all trans retinoic acid (RA, Sigma-Aldrich). Cells were fed every third day and maintained in this medium for 4 weeks. Osteogenic Differentiation
Cells were grown in wells coated with gelatin and treated with StemPro osteogenesis medium ( Gibco) for 4 weeks, with feeding every third day. Chondrogenic Differentiation
Cells were grown in wells coated with gelatin and treated with StemPro chondrogenesis differentiation basal medium (GIBCO) for 4 weeks, with feeding every third day.

Adipogenic Differentiation
Cells were treated with adipogenic differentiation medium Mesencult MSC Human Basal Medium with adipogenic stimulatory supplements (StemCell Technologies) for 4 weeks with feeding every third day. Myogenic Differentiation
Cells were treated with myogenic differentiation medium Mesencult MSC Human Basal Medium with 20% KO-SR, 10 mM MEM nonessential amino acids, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, B-27, N2 supplement (StemCell Technologies) with 20 ng/ml FGF2 (GIBCO), 50 ng/ml BMP4, 50 ng/ml BMP2 (R&D Systems) for 4 weeks with feeding every 2 days.

Establishing Clonal Primary RPE Lines
Freshly isolated RPE or RPE P0 monolayers were dissociated to single cells and seeded at clonal density, 1 x 105 cells per 100 mm Petri dish coated with 5 μg/ml fibronectin and 1 μg/ml laminin (GIBCO), and cultured in KSR medium, supplemented with 10 ng/ml FGF2 (GIBCO). After 8 days, individual clones were isolated with a cloning cylinder, and each transferred to a single well of a 96-well plate and cultured in RPE medium and gradually expanded into 24-well plates for differentiation procedures.

Retroviral Infection and In Vivo Transplantation
pMX-EGFP retroviral vector was used to transduce RPE cultures. After transduction, RPE cells were dissociated and cultured as aggregates for 1 day. 1 x 106 cells/CAM were inoculated onto the CAMs of White Leghorn chick embryos (Stage 36 HH) (Charles River laboratories) and grown for 7 days (Stage 43-44 HH). Masses were dissected, embedded in OCT, then cryostat sectioned into 12 μm sections. For immunohistochemical analysis, sections were fixed with 4% paraformaldehyde, bleached with KMnO4 to remove autofluorescence resulting from pigment, and immunostained.

hESC Differentiation into RPE
Differentiation of hESCs (HH) to RPE cells followed a previously described protocol (I detalson et al., 2009). After 4 weeks of differentiation, pigmented areas were isolated, triturated, plated on matrigel-precoated 48-well plates (Corning, Costar), and cultured in RPE medium.

Human MSC Culture
Human MSCs were cultured in Mesencult MSC Human Basal Medium (StemCell Technologies), supplemented with MSC growth kit (ATCC) and penicillin-streptomycin solution (1:100).

Cytochemistry

Mineralization Assay
Osteogenic differentiation indicated by mineralization of extracellular matrix and calcium deposits was revealed by Alizarin Red S staining. Cells were fixed with 4% PFA for 30 min. After rinsing in distilled water, cells were stained with 40 mM Alizarin Red S (Sigma-Aldrich) solution at pH 4.2, rinsed in distilled water, and washed in Tris-buffered saline (TBS) for 15 min to remove nonspecific stain. Aiccan Blue Staining
Chondrocytes were revealed by Aiccan Blue staining, which detects the synthesis of glycosamminglycans. Cells were fixed with 4% PFA for 30 min and washed three times with PBS, then incubated with 1% Aiccan Blue (Sigma-Aldrich) solution in 3% acetic acid at pH 2.5 for 30 min. To remove any nonspecific stain, the cultures were rinsed once with 3% acetic acid and then with TBS for 15 min.

Oil Red O Staining
Adipocytes were identified by production of lipid droplets, detected by staining with Oil Red O. Cells were washed with PBS and, without any fixation, directly overlaid with 0.18% Oil Red O dye/50% 2-propanol (Sigma-Aldrich) solution for 15 min then rinsed in distilled water.

Immunocytochemistry
Adult human eyes and cultured RPE monolayers were fixed and processed for immunostaining as described (De et al., 2007) with antibodies shown in Table S1.

Quantitative PCR
Total RNA was extracted with the RNeasy mini kit (QIAGEN) and reverse-transcribed with SuperScriptIII First-Strand Synthesis System (Invitrogen). Real-time RT-PCR was carried out with SYBR Green PCR mix (Applied Biosystem) and run on an Applied Biosystems 7900 instrument in 20 μl reaction volumes containing 10 μl of SYBR Green 2 × master mix. Reactions were carried out in triplicate with -RT controls and CT values obtained by averaging the results. The relative differences (n-fold) in gene expression were normalized to the level of a housekeeping gene cyclophilin (to determine ΔCT values), and designated controls as a reference (to determine ΔΔCT values). Data were analyzed for significant differences with a paired t test. Data are shown as the mean relative change ± SEM of the n separate qPCR amplification reactions. The primer sequences, annealing temperatures, and product size are shown in Table S4.

Flow Cytometry
Adult human RPESC cultures or MSCs were tretinized, incubated with human STRO-1 antibody (R&D System) for 30 min at room temperature, washed, then incubated with Cy5-conjugated goat anti-mouse IgM (Jackson Immunoresearch) for 20 min at room temperature. Adult human RPESC cultures or MSCs were incubated with APC-CD90 and FITC-CD105 for 30 min at room temperature. Cells were analyzed on a FACS Aria 2 Cell Sorter (BD Biosciences) with 10,000 events acquired for each sample. Data were analyzed with FlowJo software.

Time-Lapse Recording
RPE cells were dissected from donor globes, then plated at a density of 5,000 cells/well in 24-well plates coated with 5 μg/cm2 human extracellular matrix (BD Biosciences) and incubated at 37°C with 5% CO2 for 24 hr to allow them to attach. Time-lapse images were taken every 10 min at 10x magnification on an inverted Zeiss microscope with a Pecon Incubator XL S1. Software used for the recording and analysis was Zeiss Axiovision version 4.8.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, two tables, and one movie and can be found with this article online at doi:10.1016/j.stem.2011.11.018.

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