Retinoid Uptake, Processing, and Secretion in Human iPS-RPE Support the Visual Cycle

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Purpose. Retinal pigmented epithelium derived from human induced pluripotent stem (iPS) cells (iPS RPE) may be a source of cells for transplantation. For this reason, it is essential to determine the functional competence of iPS RPE. One key role of the RPE is uptake and processing of retinoids via the visual cycle. The purpose of this study is to investigate the expression of visual cycle proteins and the functional ability of the visual cycle in iPS RPE.

Methods. iPS RPE was derived from human iPS cells. Immunocytochemistry, RT PCR, and Western blot analysis were used to detect expression of RPE genes lecithin retinol acyl transferase (*LRAT*), *RPE65*, cellular retinaldehyde binding protein (*CRALBP*), and pigment epithelium derived factor (*PEDF*). All *trans* retinol was delivered to cultured cells or whole cell homogenate to assess the ability of the iPS RPE to process retinoids.

Results. Cultured iPS RPE expresses visual cycle genes *LRAT*, *CRALBP*, and *RPE65*. After incubation with all *trans* retinol, iPS RPE synthesized up to 2942 ± 551 pmol/mg protein all *trans* retinyl esters. Inhibition of LRAT with N ethylmaleimide (NEM) prevented retinyl ester synthesis. Significantly, after incubation with all *trans* retinol, iPS RPE released 188 ± 88 pmol/mg protein 11 *cis* retinaldehyde into the culture media.

Conclusions. iPS RPE develops classic RPE characteristics and maintains expression of visual cycle proteins. The results of this study confirm that iPS RPE possesses the machinery to process retinoids for support of visual pigment regeneration. Inhibition of all *trans* retinyl ester accumulation by NEM confirms LRAT is active in iPS RPE. Finally, the detection of 11 *cis* retinaldehyde in the culture medium demonstrates the cells' ability to process retinoids through the visual cycle. This study demonstrates expression of key visual cycle machinery and complete visual cycle activity in iPS RPE.

Keywords: induced pluripotent stem cell, retinal pigment epithelium, visual cycle

The RPE is a pigmented monolayer of cells located between the photoreceptors and the choroid. This monolayer of cells serves in multiple roles that are all essential to visual function, including absorption of scattered light, maintenance of the blood retinal barrier, transport of molecules between the choroid and neural retina, phagocytosis of photoreceptor outer segments, and the processing of retinoids in the visual cycle.[1–4] The process of vision is initiated when phototransduction is activated by absorption of light energy by chromophores in the photoreceptors, causing a conformational change in which 11 *cis* retinaldehyde in rhodopsin is photoisomerized to all *trans* retinaldehyde. After activation of the phototransduction cascade, the all *trans* retinaldehyde enters a retinoid regeneration process known as the visual cycle. In this process, all *trans* retinaldehyde is reduced to all *trans* retinol within the photoreceptors and then transported to the RPE, where it is esterified by lecithin retinol acyl transferase (LRAT).[5–9] The all *trans* retinyl ester product is then isomerized by RPE65 and hydrolyzed to release 11 *cis* retinol[10–12]; 11 *cis* retinol is then oxidized by 11 *cis* retinol dehydrogenase into 11 *cis* retinaldehyde and transported back to the photoreceptors to be incorporated into opsin, making rhodopsin (Fig. 1).[13–19] The cycling of retinoids between the photoreceptors and RPE provides a mechanism for regeneration of 11 *cis* retinal needed for light perception.[20,21]

Dysfunction or degeneration of the RPE has been implicated in many diseases leading to impairment or loss of vision. Age related macular degeneration, Leber's congenital amaurosis (LCA), and other retinal dystrophies are causes of blindness with retinal pathology.[22–25] Additionally, trauma or exposure to intense light can damage the RPE, leading to visual impairment.[26–29] The eye is a complex organ that regenerates poorly following damage, and the retina itself is a complex tissue composed of multiple cell types.[29] The recent development of technology to derive differentiated cell types from iPS cells has brought the possibility of patient specific regenerative medicine closer to reality.[30,31] Several groups have developed protocols for the induction of RPE from both human embryonic stem (ES) cells and iPS cells.[32–36] In fact, recent clinical trials in humans have demonstrated the safety and tolerability of subretinal transplantation of stem cell derived RPE.[36–38] However, before therapies designed to replace damaged RPE and restore visual function can be successful, the ability of the iPS RPE to support visual pigment regeneration must be confirmed. Therefore, the primary aim of this study was to analyze the visual cycle in iPS RPE. We report that iPS RPE exhibits classic

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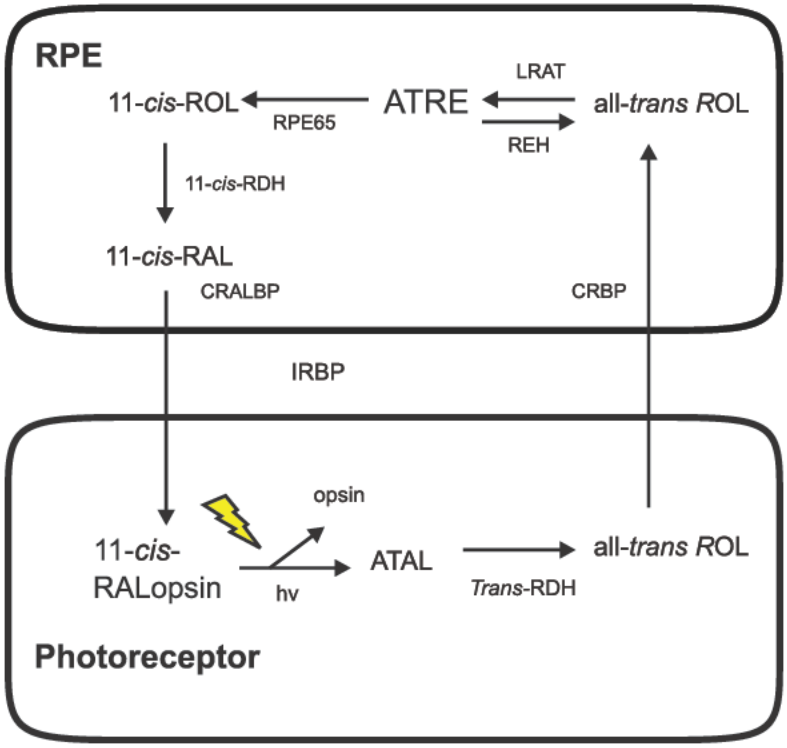

Figure 1. Flow of retinoids between RPE and photoreceptors in the visual cycle. Photoreceptors depend on the RPE for retinoid processing to maintain rhodopsin regeneration and visual sensitivity. 11 *cis* ROL, 11 *cis* retinol; ATRE, all *trans* retinyl ester; all *trans* ROL, all *trans* retinol; 11 *cis* RAL, 11 cis retinal; CRBP, cellular retinol binding protein; REH, retinyl ester hydrolase; hv, photon energy; ATAL, all *trans* retinaldehyde. Adapted with permission from Muniz A, Villazana Espinoza ET, Hatch AL, Trevino SG, Allen DM, Tsin ATC. A novel cone visual cycle in the cone dominated retina. *Exp Eye Res*. 2007;85:175 184.[19] Copyright 2007 Elsevier.

RPE morphology and expresses key visual cycle proteins RPE65, LRAT, and cellular retinaldehyde binding protein (CRALBP). Furthermore, we report visual cycle activity in these cells as indicated by their ability to uptake all *trans* retinol, synthesize retinyl esters, and release 11 *cis* retinalde hyde into the culture media. These findings demonstrate that iPS RPE possesses the enzyme machinery and activity to support visual pigment regeneration.

Methods

Culture and Differentiation of iPS Cells

Human iPS cells (IMR 90 1;WiCell Research Institute, Madison, WI) were cultured on Matrigel coated (BD Biosciences, San Jose, CA) six well plates and maintained in mTeSR1 medium (Stem Cell Technologies, Vancouver, BC, Canada). The medium was changed daily until cells were ready for passage. To initiate the differentiation protocol, the mTeSR1 medium was replaced with differentiation medium consisting of 10% Knockout serum replacement (Life Technologies, Grand Island, NY), 0.1 mM β mercaptoethanol, 0.1 mM nonessential amino acids, 2 mM glutamine, and 10 μg/mL gentamicin Dulbecco's modified Eagle's medium (DMEM)/F12. Half of the differenti ation medium was changed every other day. Pigmented foci composed of RPE appeared, and the foci were allowed to grow large enough to be manually dissected out of the culture. Pigmented iPS RPE colonies were pooled, and a single cell suspension was prepared with 0.25% trypsin. The enriched iPS RPE was then seeded and cultured in fetal RPE media composed of MEM, N1 supplement, glutamine, nonessential amino acids, taurine 0.25 mg/mL, hydrocortisone 10 ng/mL, triiodothyronine 13 ng/mL, and[39] 15% fetal bovine serum (FBS). The seeding density at each passage after enrichment was 1×10^5 cells/cm^2. Cells were allowed to grow until approximately 80% confluent and split accordingly. For experiments, iPS RPE at passages five and six were cultured in T75 flasks containing 10 mL fetal RPE media for up to 6 months prior to the experiment. The culture media was changed every 2 to 3 days.

Total RNA Extraction and RT-PCR

Gene expression was analyzed by RT PCR of total RNA extracted with RNeasy plus mini kit (Qiagen, Valencia, CA) from human iPS IMR90 1 cells and iPS RPE. Reverse transcrip tion was performed with High Capacity RNA to cDNA kit (Applied Biosystems, Carlsbad, CA). cDNAs were subjected to PCR to amplify target genes. PCR was performed with PCR Master Mix 2× (Promega, Madison, WI). PCR products were run on 1% agarose gels and visualized with ethidium bromide. *GAPDH* was used as a housekeeping gene to account for control differences in RNA quantity between samples.

Preparation of Cell Homogenates

Cell homogenates were prepared in 20 mM HEPES buffer pH 7.4, 150 mM NaCl, with EDTA free complete protease inhibitors (Roche, Indianapolis, IN). To remove cell debris, the homogenate was spun at 1000*g* for 10 minutes at 4°C. Protein concentration was determined using the Bradford protein assay (BioRad, Hercules, CA) with BSA as standards. Homogenates were snap frozen in liquid nitrogen and stored at

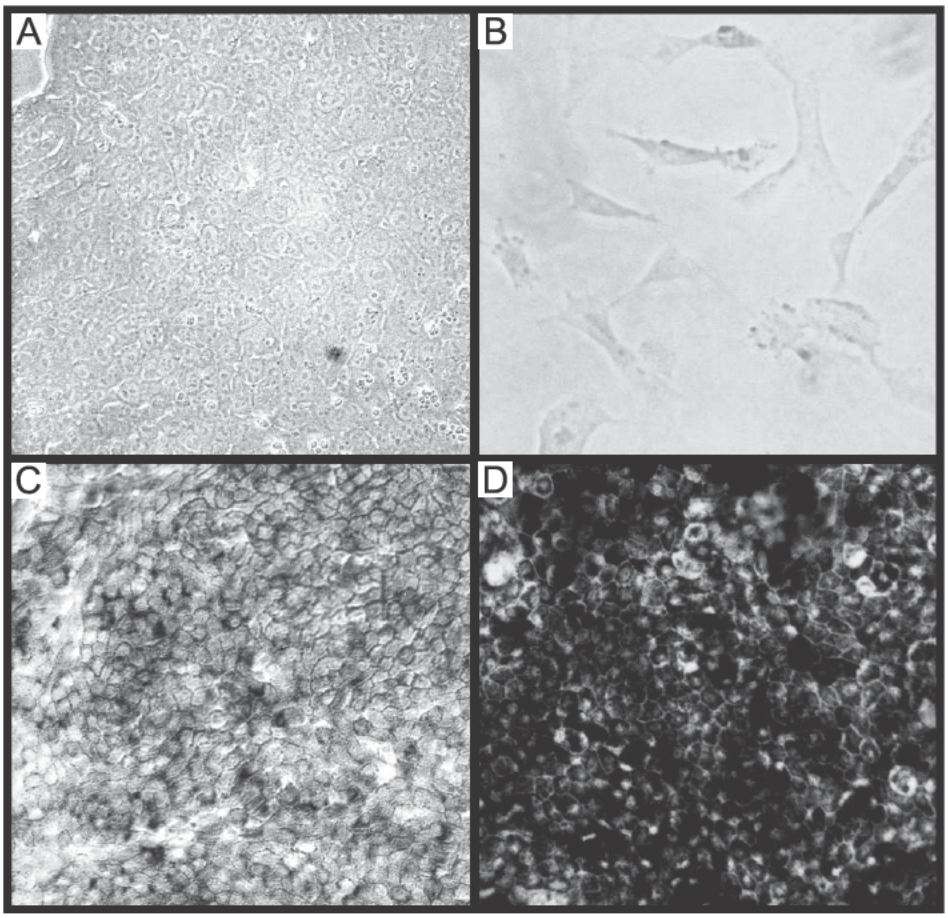

FIGURE 2. Brightfield images of cultured iPS cells and iPS RPE. (A) iPS cells prior to differentiation. The cells in the colonies maintain typical round pluripotent stem cell morphology. (B) After passage, iPS RPE cells revert to fibroblastic morphology, losing their classic hexagonal RPE morphology and pigmentation. (C) iPS RPE passage 6 regained hexagonal morphology and pigment within 4 weeks after passage. (D) Highly pigmented iPS RPE passage 6 after 6 months in culture. Magnification ×200.

80°C until used for Western blot analysis and in vitro retinyl ester synthesis assays.

Western Blot Analysis

For Western blot analysis, the iPS RPE was cultured for 6 months prior to preparation of whole cell homogenate. The homogenate was prepared and stored at 80°C until used. For detection of RPE65 and CRALBP, 100 µg cell homogenates from iPS cells and iPS RPE were fractionated by SDS PAGE on a 4% 12% gradient acrylamide gel. For LRAT detection, 160 µg cell homogenates was used. For a positive control for RPE65 and CRALBP proteins, RPE was collected from freshly explanted bovine eyes and homogenized as described above. For a positive control for LRAT, freshly explanted rat liver was homogenized as described above. Precision Plus Protein standards (161 0375, BioRad) were used as molecular weight markers. The proteins were then transferred to PVDF membranes and probed with RPE65 (1:5000, MAB5428; Millipore, Billerica, MA), CRALBP (1:5000, MA1813; Thermo Fisher, Waltham, MA), and LRAT antibodies (1:1000, Ab137304; Abcam, Cambridge, MA), followed by infrared dye 800 CW conjugated secondary antibody (1:15,000, C10207 03; LI COR, Lincoln, NE). Antibody labeling and molecular weight markers were visualized using an Odyssey Infrared Imager (LI COR) at 800 and 700 nm emission wavelengths, respectively.

Immunocytochemistry

iPS and iPS RPE were cultured on fibronectin coated inserts (Nunc, Rochester, NY). The cells were washed with PBS and fixed at room temperature in 4% paraformaldehyde for 10 minutes. Immunocytochemistry was performed using standard procedures with RPE65 antibody (1:250, MAB5428; Millipore) or CRALBP antibody (1:100, MA1 813; Thermo Fisher) applied overnight at 4°C. Goat anti mouse IgG Alexa Fluor 568 from Molecular Probes (1:50, cat. No. A11031; Life Technologies), was used as the secondary antibody.

Microscopy

Brightfield images shown in Figure 2 were acquired with an Olympus CK2 microscope (Olympus Life Science, Center Valley, PA) using the ×20 objective. Brightfield and immuno fluorescence images shown in Figure 3 were acquired with an Olympus BX3 microscope equipped with a DP73 17.28 megapixel digital color camera using a ×60 oil immersion objective (Olympus Life Science).

Exogenous Retinol Uptake and Processing by iPS-RPE and iPS Cells

To prevent photoisomerization, all experiments involving retinoids were performed in a dark room under dim red light. To prevent thermal isomerization and oxidation, all retinoid

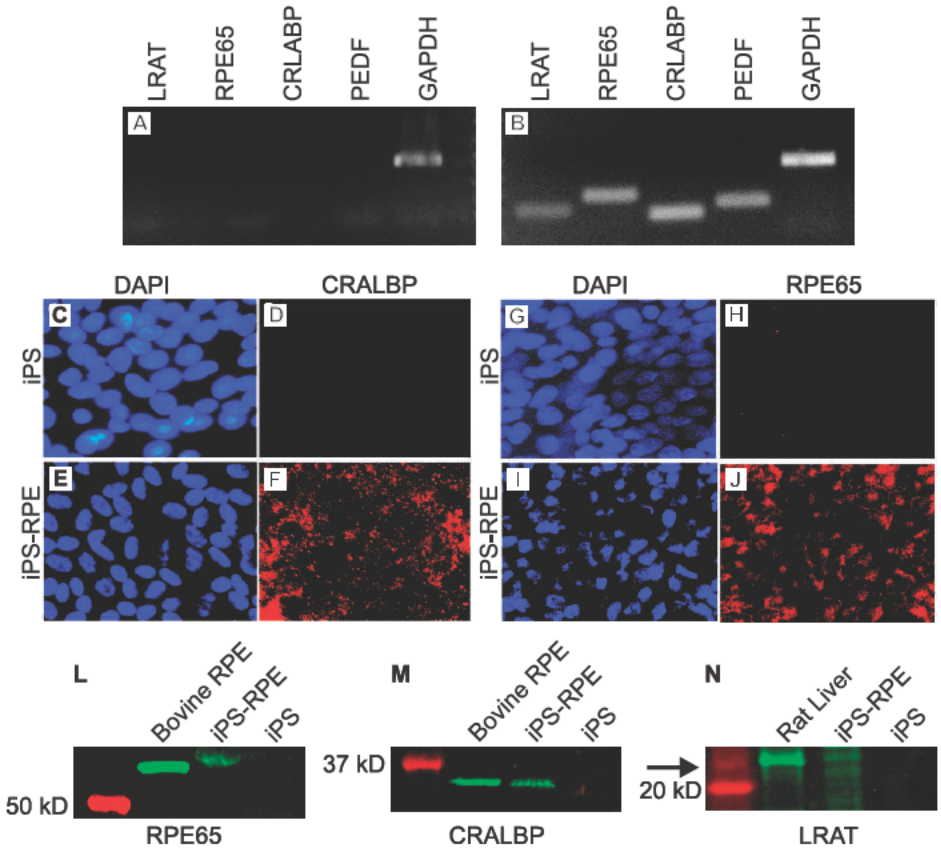

FIGURE 3. Expression of RPE genes in iPS RPE. Transcripts for LRAT, RPE65, CRALBP, and PEDF were analyzed by RTPCR. Gene expression was not detected in iPS cells cultured in nondifferentiation conditions (**A**), while iPS RPE (**B**) showed expression of all analyzed RPE genes after 4 weeks in culture. GAPDH was included as a control. Visual cycle proteins CRALBP (**D**, **F**) and RPE65 (**H**, **J**) were detected by immunocytochemistry in iPS RPE (**F**, **J**) after 5 weeks in culture, but not iPS cells cultured in nondifferentiation conditions (**D**, **H**). DAPI labeling of iPS cells is shown in **C** and **G**. DAPI labeling of iPS RPE is shown in **E** and **I**. Western blot detection of CRALBP, RPE65, and LRAT (indicated by *arrow*) further confirms protein expression at the expected molecular weights (35 kD, 65 kD, and 25 kD, respectively) in **L**, **M**, and **N**.

extracts were argon capped and maintained on ice until HPLC analysis. To study the processing of exogenous all *trans* retinol, iPS cells and iPS RPE were serum starved for 8 hours followed by a 24 hour incubation with 10 µM all *trans* retinol (Sigma Aldrich, St. Louis, MO) diluted in 2% BSA (fatty acid free) in MEM (Life Technologies) either with or without 15% FBS. To account for endogenous retinoids, cells incubated without all *trans* retinol were included as controls. The cells were washed, harvested, homogenized in Tris buffer pH 7.5 on ice, and all retinoids were extracted 3 times with 2 mL hexane. The culture media were also collected and retinoids extracted for HPLC analysis. In these experiments, *cis* and *trans* retinoids were separated by HPLC using gradient elution in a Waters System equipped with a Zorba Rx sil 5 µm 4.6 × 250 mm column and a 2 mL/min flow rate (Waters Corp., Milford, MA). Gradient (0.2% 10%) elution was achieved by maintaining 0.2% dioxane/hexane (D/H) flow for 9 minutes after which a linear gradient was applied to reach 10% D/H at minute 15. This was then maintained for 9 minutes and allowed to return to 0.2% D/H at minute 25; 0.2% D/H was then maintained until the end of the HPLC run. Retinyl esters and aldehydes were analyzed at an absorbance wavelength of 325 and 365 nm, respectively. All experiments were performed in triplicate, and retinoids were identified by comparison to retention time and absorbance spectrum of authentic retinoid standards.

In order to better resolve retinaldehyde isomers and clearly distinguish between 13 *cis* retinaldehyde and 11 *cis* retinaldehyde, a separate experiment was conducted. In this case, retinoids from the media fractions were resolved using 3% tert butyl methyl ether in hexane isocratic elution[40] on a Waters HPLC System equipped with a RESTEK Pinnacle II Silica 3 µM 250 × 4.6 mm column at a flow rate of 1 mL/min. The peak of interest was collected immediately after HPLC separation, combined with authentic 11 *cis* retinaldehyde standard, and analyzed by the same isocratic HPLC setup.

Preparation of Retinoid Standards

The standards for all *trans* retinyl palmitate, all *trans* retinol, all *trans* retinaldehyde, 9 *cis* retinaldehyde, and 13 *cis* retinaldehyde were obtained from Sigma Aldrich. The 11 *cis* retinaldehyde standard was a kind gift from Rosalie Crouch. The following retinoids were prepared according to established methods.[41,42] Briefly, 11 *cis* retinol was synthesized in the laboratory by sodium borohydride reduction of 11 *cis* retinaldehyde. Following sodium borohydride reduction, 11 *cis* retinol was purified by HPLC on a Zorba RX sil 5 µm 4.6 × 250 mm column using 10% dioxane/hexane at 1 mL/min flow rate. Purified 11 *cis* retinol was argon capped and stored at 20°C until use. The 11 *cis* retinyl palmitate standard was synthesized in the laboratory by acylation of 11 *cis* retinol. Following acylation, the 11 *cis* retinyl palmitate was purified by HPLC on a Zorba RX sil 5 µm 4.6 × 250 mm column using 0.2% D/H at 1 mL/min flow rate. Purified 11 *cis* retinyl palmitate was argon capped and stored at 20°C until use. For experiments, the

retinoid standards were pooled and run under the same HPLC conditions as the experimental samples.

In vitro retinyl ester synthesis assay: iPS RPE was cultured for 4 weeks after passage, harvested and homogenized in HEPES lysis buffer. Homogenates were exposed to UV light (wavelength 365 nm, lamp model # EN 140L; Spectrolin, Westbury, NY) for 5 minutes to destroy endogenous retinoids. After UV exposure, 100, 250, and 500 μg whole cell homogenates were incubated with 10 μM all *trans* retinol in 2% BSA in HEPES buffer pH 7.4, 150 mM NaCl at 37°C for 1 hour. The total reaction volume was 400 μL. The reaction was stopped by addition of 1 mL ice cold methanol, followed by retinoid extraction for HPLC analysis as described above. A 500 μg quantity of whole cell homogenate, incubated without all *trans* retinol, was included to control for endogenous retinoids. All experiments were performed in triplicate.

Inhibition of Retinyl Ester Synthesis by N-ethylmaleimide (NEM)

To determine the nature of the retinyl ester synthesis in iPS RPE, 500 μg aliquots of cell homogenate were incubated with 10 μM all *trans* retinol 2% BSA as described above, except with or without 20 μM NEM (Sigma, St. Louis, MO)[43] dissolved in ethanol. After 1 hour, the reaction was stopped with 1 mL ice cold methanol. Retinoids were hexane extracted and analyzed by gradient HPLC as described above. All experiments were performed in triplicate.

Statistical Analysis

Data were analyzed by the two tailed Student's *t* test with $P < 0.05$ considered to be significant.

Results

Differentiation and Subculture of iPS-RPE

iPS RPE was derived from iPS cells and enriched according to previously described methods.[34] Briefly, human iPS cells (Fig. 2A) were cultured on Matrigel coated six well plates and maintained in mTeSR1 medium. To initiate the differentiation protocol, the mTeSR1 medium was replaced with differentiation medium. Small pigmented colonies first appeared between 25 and 30 days in differentiation medium. The pigmented colonies were manually dissected out of the culture between 40 and 50 days after initiation of the differentiation protocol. The excised colonies were trypsinized to prepare single cell suspensions and reseeded onto Matrigel coated plates. Upon reseeding, the putative iPS RPE lost pigmentation and hexagonal morphology, and became fibroblastic (Fig. 2B). Consistent with earlier observations, classic hexagonal morphology and pigment were reestablished as the cells reached confluence.[32,34,44] iPS RPE consistently regained hexagonal morphology and pigment through passage 6 (Figs. 2C, 2D).

Expression of RPE and Visual Cycle Genes in iPS-RPE

RPE65, *LRAT*, and *CRALBP* are critical components of the visual cycle. These genes are important biomarkers of RPE differentiation and facilitate the processing of retinoids. Pigment epithelium derived factor (*PEDF*) is a characteristic gene expressed by RPE. As shown in Figure 3A, *LRAT*, *RPE65*, *CRALBP*, and *PEDF* gene transcripts were not detected by RT PCR in iPS cells but were detectable in the iPS RPE (Fig. 3B) after 1 month in culture. In addition, the majority of the iPS RPE was immunoreactive for *CRALBP* (Fig. 3F) and *RPE65* (Fig. 3J) after 5 weeks of culture, while the iPS cells were negative for *CRALBP* (Fig. 3D) and *RPE65* (Fig. 3H) expression. The protein expression of CRALBP, RPE65, and LRAT in iPS RPE is further supported by the Western blot results shown in Figures 3L, 3M, and 3N.

Retinyl Ester Synthesis in iPS-RPE and iPS Cultures

Uptake and processing of all *trans* retinol to produce 11 *cis* retinaldehyde are signature functions of the RPE in vivo. Conversion of all *trans* retinol to 11 *cis* retinaldehyde is a multi enzymatic process (Fig. 1) and, as shown in Figure 3, iPS RPE expresses several essential proteins required for the processing of retinoids. BSA and FBS have been shown to facilitate delivery of all *trans* retinol to cells[45,46]; therefore, to evaluate the functional ability of the visual cycle proteins in iPS RPE, cells cultured for 4 weeks were incubated with 10 μM all *trans* retinol delivered in either 2% BSA (fatty acid free) or 2% BSA plus 15% FBS. After a 24 hour incubation period to allow uptake and processing of all *trans* retinol, the cells were homogenized, and intracellular retinoids were extracted for HPLC analysis. As the results in Figure 4C show, a peak with the same retention time of 6.58 minutes and absorbance spectrum as the all *trans* retinyl palmitate standard was detected in the iPS RPE after incubation with all *trans* retinol. iPS RPE was able to uptake all *trans* retinol and synthesize all *trans* retinyl esters in the form of all *trans* retinyl palmitate. Quantification by comparison with authentic retinoid standard curves determined that 1724 ± 673 pmol all *trans* retinyl palmitate/mg total cell protein and 2942 ± 551 pmol/mg were detected in cells incubated with 2% BSA or 2% BSA plus 15% FBS, respectively, while only 20 ± 5 pmol/mg was detected in iPS RPE that was not incubated with all *trans* retinol (Fig. 4D). The amount of all *trans* retinyl palmitate detected in samples treated with all *trans* retinol and BSA alone approached statistical significance ($P = 0.06$). Uptake and processing of all *trans* retinol was more efficient in the presence of both BSA and FBS as indicated by the statistically significant higher amount of all *trans* retinyl palmitate detected in these samples ($P < 0.05$). Control iPS cells showed relatively small amounts of all *trans* retinyl ester synthesis from all *trans* retinol (Fig. 4B). Because of the absence of *LRAT* transcript and protein in our PCR and Western blot analysis of iPS cells (Fig. 3), retinyl ester synthesis in iPS cells is likely owing to the activity of a separate retinyl ester synthase, Acyl CoA retinyl acyl transferase (ARAT), which has been observed in multiple tissues.[47] The intracellular detection of all *trans* retinyl palmitate indicates that the iPS RPE had internalized and esterified all *trans* retinol.

Retinyl ester synthesis in vitro: An in vitro retinyl ester synthesis assay was conducted to study the protein dependence of retinyl ester synthesis in the iPS RPE. All *trans* retinyl palmitate is an ester that is a storage form of vitamin A in RPE. Increasing amounts of iPS RPE homogenate were incubated with 10 μM all *trans* retinol in 2% BSA for 1 hour. Retinoids were extracted for HPLC analysis, and as seen in Figure 5, peaks with corresponding retention time and spectrum of the authentic all *trans* retinyl palmitate standard peak were detected in the iPS RPE samples after incubation with all *trans* retinol. The amounts of all *trans* retinyl palmitate detected increased in accord with increasing amounts of cell homogenate (Fig. 5D). More than 140 pmol all *trans* retinyl palmitate was detected in the 500 μg cell homogenate sample, while all *trans* retinyl palmitate was undetectable in the control samples containing the same amount of iPS RPE cell homogenate incubated without all *trans* retinol.

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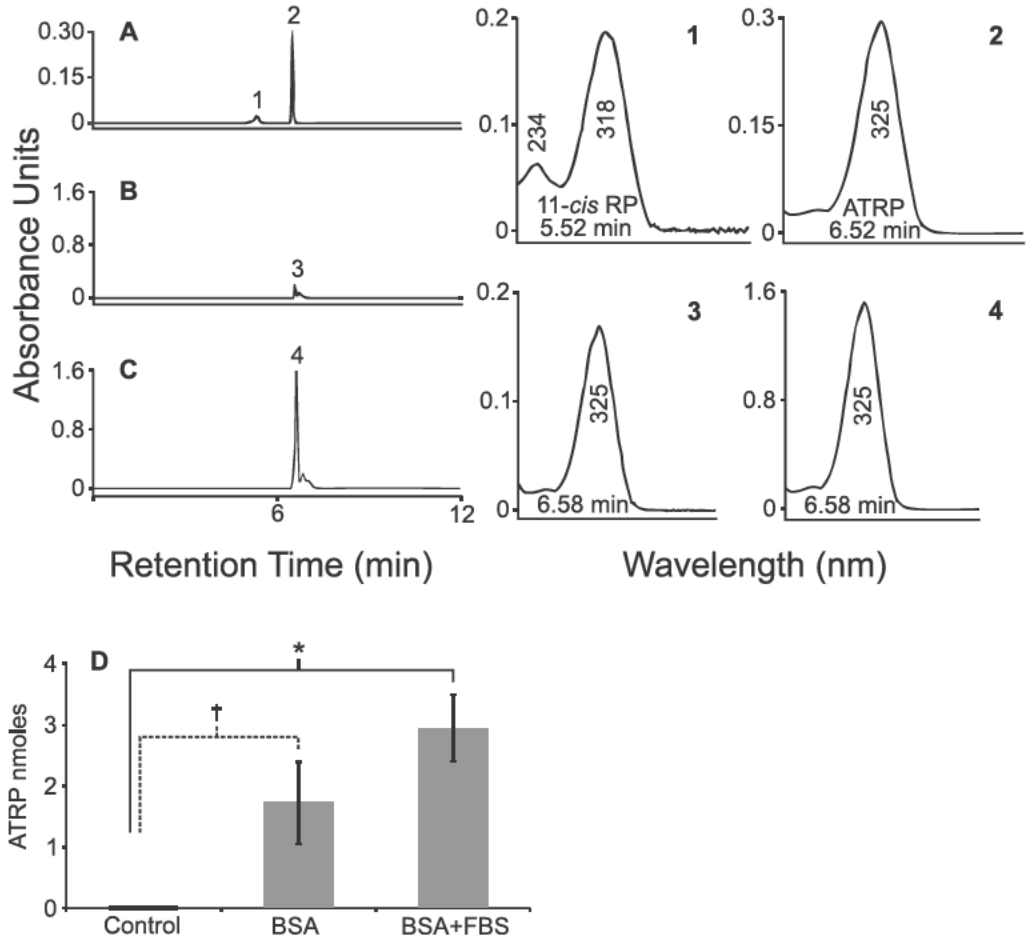

Figure 4. Retinyl ester synthesis in iPS RPE cultures. iPS and iPS RPE cells cultured for 4 weeks were incubated with 10 μm all *trans* retinol for 24 hours. Retinoids were extracted from the cultured cells for analysis by gradient HPLC. (**A**) Chromatogram of authentic retinyl ester standards. *Peak 1*, 11 *cis* retinyl palmitate; *Peak 2*, all *trans* retinyl palmitate. (**B**) Chromatogram of retinoids extracted from iPS cell cultures incubated without 10 μM all *trans* retinol. (**C**) Chromatogram of retinoids extracted from iPS RPE cultures incubated with 10 μM all-*trans* retinol. On the *right* are the absorbance spectra corresponding to the retinyl ester standard and experimental peaks; the peaks corresponding to all *trans* retinyl palmitate are indicated by 2, 3, and 4, respectively. All retinoids were identified by comparison with retention time and absorbance spectra of authentic retinoid standards and quantified by retinoid standard curves. (**D**) Chart representing all *trans* retinyl palmitate extracted from iPS RPE cells treated for 24 hours with 10 μM all *trans* retinol delivered either with 2% BSA or with 2% BSA plus 15% FBS. Retinoids were extracted and analyzed by HPLC. The iPS RPE in the treatment groups with BSA and BSA + FBS synthesized all *trans* retinyl palmitate in the amounts of 1724 ± 673 pmol/mg protein and 2942 ± 551 pmol/mg protein, respectively. Only trace amounts of all *trans* retinyl palmitate (20 ± 5 pmol/mg protein) were detectable in control iPS RPE cells incubated without all *trans* retinol. Monitoring λ 325. Data are expressed as mean ± SEM. *$P < 0.05$ and †P 0.06

Inhibition of Retinyl Ester Synthesis by NEM

The results of both the in cellulo and in vitro assays described in Figures 4 and 5 indicate that iPS RPE is capable of synthesizing all *trans* retinyl esters from all *trans* retinol after 4 weeks in culture. LRAT and ARAT are the two enzymes that have been shown to facilitate ester synthesis in RPE.[5,47] To determine if LRAT, the main enzyme responsible for all *trans* retinyl ester synthesis in the visual cycle, is active in iPS RPE, iPS RPE homogenates were incubated with all *trans* retinol for 1 hour with or without 20 μM NEM, a potent inhibitor of LRAT activity.[43,48] Retinoids were extracted for HPLC analysis, and as shown in Figure 5B, all *trans* retinyl esters were detected in cell homogenate incubated with all *trans* retinol. The results shown in Figures 5C and 5D demonstrate that in the presence of NEM, synthesis was reduced by 90%. These results indicate that iPS RPE possesses an active LRAT enzyme that is the main contributor to the all *trans* retinyl ester pool. In agreement with previous reports, ARAT activity may be responsible for the residual 10% of ester synthesis occurring during LRAT inhibition.[47]

Synthesis and Secretion of 11-cis Retinaldehyde by iPS-RPE

In vivo, 11 *cis* retinaldehyde is released into the interphoto receptor matrix (IPM) by the RPE. Secretion of 11 *cis* retinaldehyde into the culture media would prove that the visual cycle in iPS RPE is functional and complete. Therefore, to confirm that the visual cycle in iPS RPE is fully functional, iPS RPE cultured for up to 6 months were incubated with all *trans* retinol for 24 hours. The culture media were collected, and retinoids were extracted. As shown in Figure 6D, a peak corresponding to the retention time of 14.90 minutes and absorbance spectra for 11 *cis* retinaldehyde was detected in the culture media extract from iPS RPE after 24 hour incubation with all *trans* retinol.[49–51] By comparison with the standard curve, the amount of 11 *cis* retinaldehyde was determined to be 188 ± 88 pmol 11 *cis* retinaldehyde/mg iPS RPE protein. Peaks with retention times corresponding to the retention time of 9 *cis* and all *trans* retinaldehyde were also detected in the culture media of iPS RPE (Fig. 6D) and iPS (Fig.

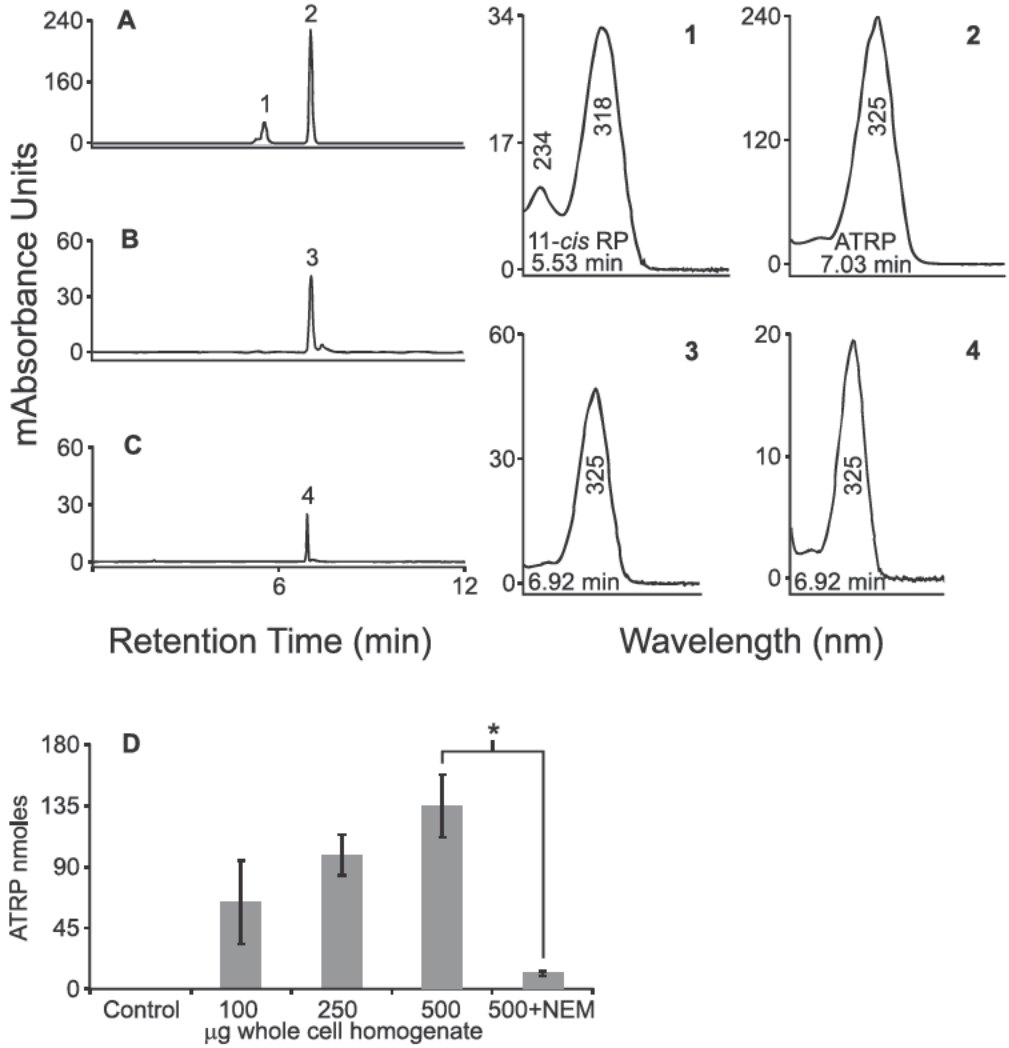

FIGURE 5. LRAT facilitates synthesis of all *trans* retinyl ester synthesis in iPS RPE. Whole cell homogenate from iPS RPE cultured for 4 weeks was incubated for 1 hour with all *trans* retinol in the presence or absence of NEM. Retinoids were extracted for gradient HPLC analysis. (**A**) Chromatogram of authentic retinyl ester standards. *Peak 1*, 11 *cis* retinyl palmitate; *Peak 2*, all *trans* retinyl palmitate. (**B**) Chromatogram of retinyl esters (*Peak 3*) extracted from 500 μg iPS RPE homogenate incubated with 10 μM ATOL. (**C**) Chromatogram of retinyl esters (*Peak 4*) extracted from 500 μg iPS RPE homogenate incubated with 10 μM ATOL and NEM. Note the reduction of all *trans* retinyl palmitate (*Peak 4*) in the presence of NEM, a specific LRAT inhibitor. On the *right* are the absorbance spectra corresponding to the retinyl esters, respectively. (**D**) Increasing total amounts (100, 250, and 500 μg) of iPS RPE homogenate protein were incubated for 1 hour with 10 μM all-*trans* retinol with or without NEM. The *bar graph* indicates synthesis of all *trans* retinyl palmitate increased as the amount of iPS RPE homogenate protein increased. As shown, chemical inhibition with NEM reduced the synthesis of all *trans* retinyl palmitate by 90%. Retinoids were not detected in iPS RPE homogenate controls incubated without all *trans* retinol. All retinoids were identified by comparison with retention time and absorbance spectra of authentic retinoid standards and quantified by retinoid standard curves. Monitoring λ 325. Data are expressed as mean ± SEM. $^*P < 0.05$.

6C) cells. The second peak in Figure 6C did not correspond to the retention time for 11 *cis* retinaldehyde. Furthermore, the absorbance spectrum of this peak did not match the absorbance spectrum for 11 *cis* retinaldehyde (data not shown), indicating that the iPS cells did not produce 11 *cis* retinaldehyde after incubation with all *trans* retinol.

The separation profile of four retinaldehyde isomers depicted in Figure 6A shows that 13 *cis* and 11 *cis* retinalde hyde were not clearly resolved utilizing the gradient elution system. Thus, in order to confirm the identity of the retinaldehydes in the culture media of iPS RPE, a separate experiment was conducted. HPLC analysis was performed by isocratic elution using 3% tert butyl methyl ether in hexane. As the separation profile shown in Figure 7A demonstrates, this method of analysis enabled clear separation between 13 *cis* and 11 *cis* retinaldehyde. For this experiment, iPS RPE was cultured for 5 months. The 5 month old cell cultures were then incubated for 24 hours with all *trans* retinol before the culture media were collected for analysis. Of importance, the media collected from the iPS RPE after 24 hour incubation with all *trans* retinol show a peak (Fig. 7C) with the same retention time of 13.99 minutes and absorbance spectrum as 11 *cis* retinaldehyde standard (Fig. 7D). To further confirm the identity of the peak from the culture media of iPS RPE, the peak of interest (*) was collected (Fig. 7C) and supplemented with authentic 11 *cis* retinaldehyde, then chromatographed. The combined culture media and authentic 11 *cis* retinalde hyde resulted in a peak with a retention time of 13.97 minutes (Fig. 7E). The absorbance spectrum of this peak is identical to the absorbance spectrum for the 11 *cis* retinaldehyde standard alone, proving that the peak detected in the culture media is in fact 11 *cis* retinaldehyde. The synthesis and secretion of 11 *cis*

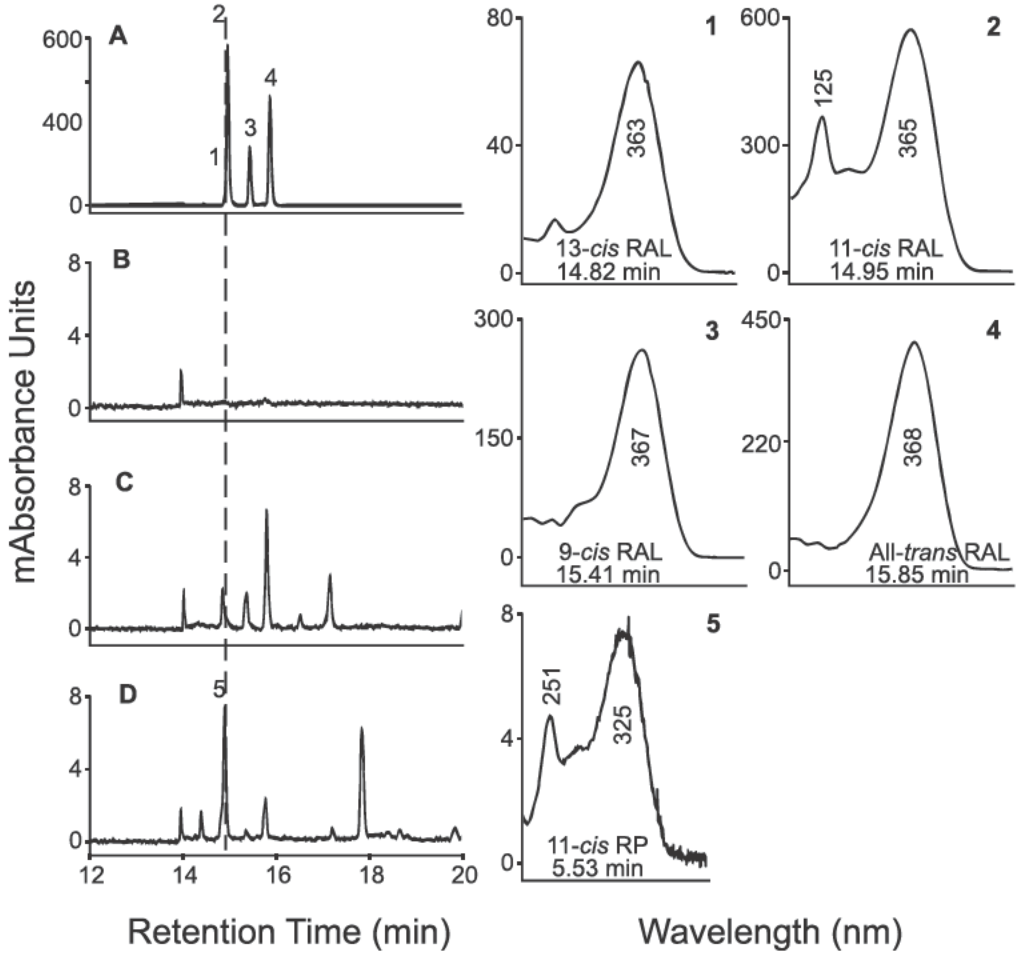

Figure 6. Cultured iPS RPE synthesize and release retinaldehydes from ATOL. iPS and iPS RPE cells cultured for 6 months were incubated with 10 μm all *trans* retinol for 24 hours. Retinoids were extracted from the culture media for analysis by gradient HPLC. (**A**) Chromatogram for authentic retinaldehyde standards. The retinaldehydes standards are identified as follows: *Peak 1*, 13 *cis* retinaldehyde; *Peak 2*, 11 *cis* retinaldehyde; *Peak 3*, 9 *cis* retinaldehyde; *Peak 4*, all *trans* retinaldehyde. *Insets to the right* are representative spectra for each retinaldehyde standard. (**B**) Chromatogram for culture media extract from IPS RPE cells incubated without all *trans* retinol. (**C**) Chromatogram for culture media extract from iPS cells incubated with all *trans* retinol. The peak that appeared just before the peak for 11 *cis* retinaldehyde did not correspond to any known retinoids, therefore the absorbance spectrum is not included. (**D**) Chromatogram for culture media extract from iPS RPE cells incubated with all *trans* retinol. Peaks corresponding to the retention time of 11 *cis* retinaldehyde, 9 *cis* retinaldehyde, and all *trans* retinaldehyde were detected in the iPS RPE media. *Peak 5* has a retention time of 14.9 minutes that correlates with the retention time of 11 *cis* retinaldehyde in the standard run shown by *Peak 2* in **A**. The absorbance spectrum[49 51] for this peak further indicates the presence of 11 *cis* retinaldehyde in the culture media. Quantification of 11 *cis* retinaldehyde peak results in 188 ± 88 pmol/mg of iPS RPE protein. Monitoring λ 365.

retinaldehyde from all *trans* retinol into the culture media verify that iPS RPE can uptake, process, and secrete retinoids that are required for the regeneration of visual pigment.

Discussion

The recent development of iPS cell technology has made the means for personalized regenerative medicine possible.[31] iPS cells are a potential source of healthy tissue including RPE. RPE derived from iPS cells would have several advantages over other cell sources. First, they would be derived directly from the patient, thus eliminating any possible adverse immune respons es. Second, iPS cells circumvent the valid ethical issues associated with human ES cells. Third, iPS cells can be expanded in vitro and induced to differentiate into RPE, providing a sufficient number of cells for experimental and therapeutic applications.

Over the last few years, RPE derived from iPS cells have been described.[33–35,52] However, direct evidence that these cells can support visual pigment regeneration has not been available. In this report we demonstrate differentiation of iPS cells into RPE. When observed by brightfield microscopy, our iPS RPE become fibroblastic, lose pigmentation early in the culture, and regain classic RPE polygonal morphology and pigment within 4 weeks (Fig. 2). We confirmed the expression of RPE markers RPE65, CRALBP, LRAT, and PEDF by RTPCR and demonstrated the presence of RPE65 and CRALBP in iPS RPE cultures by immunocytochemistry for cellular localization. In addition, we confirmed the expression of RPE65, CRALBP, and LRAT proteins in iPS RPE by Western blot analysis (Figs. 3L, 3M, 3N). Of importance, RPE65 protein was detected in passage 6 iPS RPE after 5 weeks in culture. This is in contrast to previous reports in which RPE65 transcripts were detectable in 1 month old cultures, but protein was only evident in passage 0 cells after 8 months in culture.[34] In RPE derived from human ES cells, *RPE65* mRNA was detected after 7 weeks in culture, but RPE65 protein was not detected.[32] In studies of primary RPE, isolated from both adult and fetal tissues, the RPE

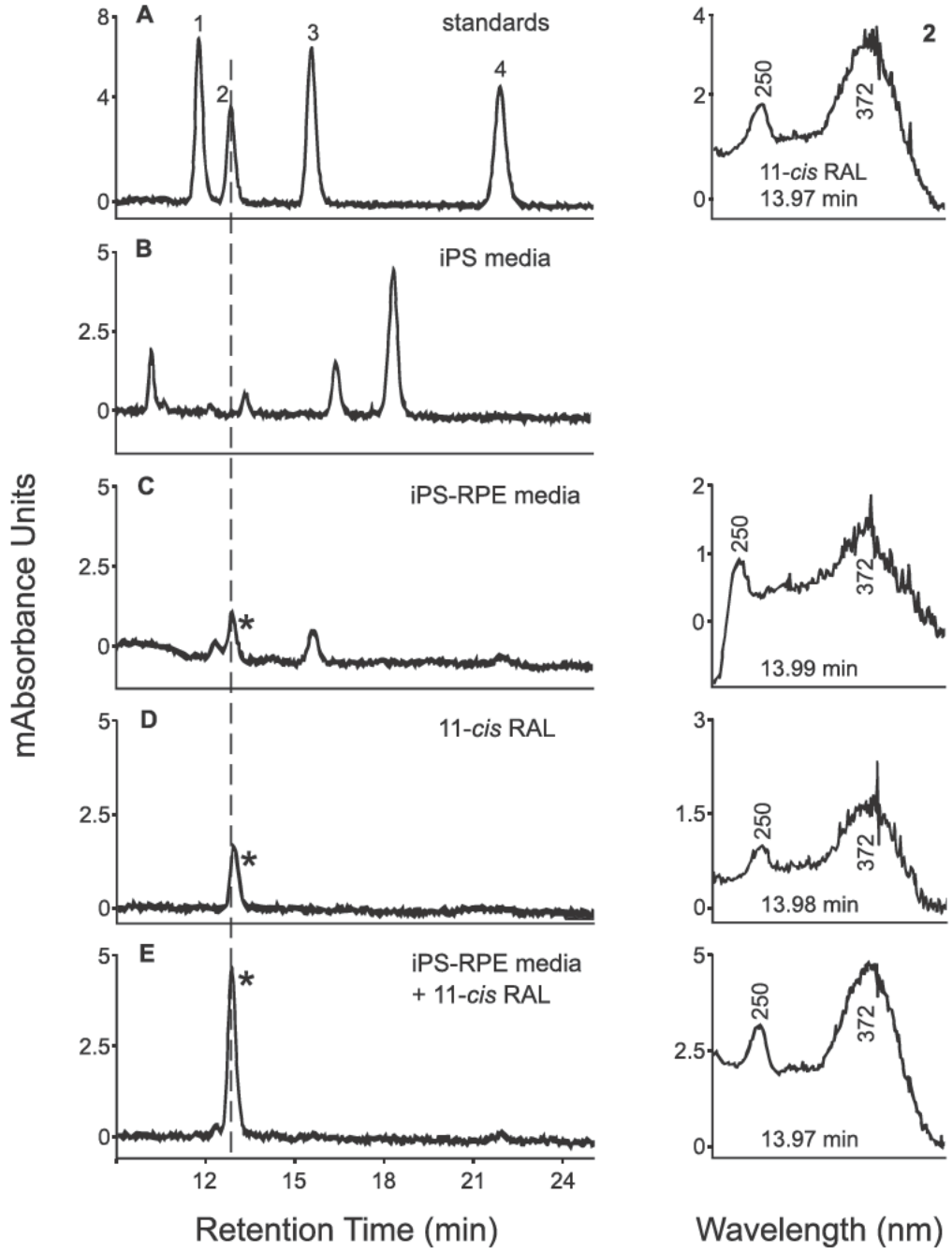

Figure 7. iPS RPE synthesized and released 11 *cis* RAL from exogenous all *trans* retinol. iPS RPE cultured for 5 months and iPS cells were incubated with all *trans* retinol for 24 hours. Retinoids were then extracted from the culture media and analyzed by isocratic HPLC. (**A**) Chromatogram of authentic retinaldehyde standards. *Peak 1*, 13 *cis* RAL; *Peak 2*, 11 *cis* RAL; *Peak 3*, 9 *cis* RAL; *Peak 4*, all *trans* RAL. (**B**) Chromatogram of media extract form iPS cells incubated in the presence of all *trans* retinol. (**C**) Chromatogram of media extract from iPS RPE incubated with all *trans* retinol. (**D**) Chromatogram of authentic 11 *cis* RAL standard. (**E**) Chromatogram of retinoid extract from media of iPS RPE incubated with all *trans* retinol combined with authentic 11 *cis* RAL. The (*) marks peaks corresponding to 11 *cis* RAL. *Insets* are the absorbance spectra for the labeled peaks in the chromatograms. Monitoring λ 365. **B** does not include an absorbance spectrum because a peak corresponding to 11 *cis* RAL was not detected in the iPS cell media.

rapidly lost function and protein expression of the visual cycle isomerase.[53–55] The basis for the loss of isomerase in vitro has yet to be determined. Possible reasons include separation from the retina and loss of microenvironmental cues such as photoreceptor contact and a sudden decrease in the demand for 11 *cis* retinoids.[56]

Incubation of iPS RPE cultures with all *trans* retinol resulted in esterification of all *trans* retinol, intracellular accumulation of all *trans* retinyl esters, as well as the synthesis and release of 11 *cis* retinaldehyde into the culture media. We detected the production and accumulation of all *trans* retinyl palmitate in iPS and iPS RPE cells exposed to all *trans* retinol (Figs. 4B, 4C; respectively). This study found that iPS cell line iMR 90 1 cells do not express LRAT (Fig. 3A, 3N), one of the two retinyl ester synthase activities described. Thus, the formation of retinyl esters in iPS cells is likely owing to a separate retinyl ester

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synthase activity, possibly ARAT, which has been found in multiple tissues.[48,57–60] The formation of a retinyl ester pool in RPE is an important characteristic owing to the toxicity of vitamin A alcohols and aldehydes. Retinyl esters are the substrate for isomerization in the visual cycle; in vivo, all *trans* retinyl esters are the main storage form of retinoids.[5,61,62] As mentioned above, the production of all *trans* retinyl palmitate can be facilitated by either LRAT or ARAT synthase activities. However, LRAT is the major enzyme responsible for retinyl ester synthesis in RPE. It is a 25 kDa integral membrane protein that catalyzes the transfer of the fatty acid from the sn 1 position of phosphatidylcholine to retinol, resulting in retinyl ester.[5,6,63–65] Yet, ARAT activity uses fatty acyl coenzyme A as the acyl donor for esterification and in the RPE; ARAT has been suggested to complement LRAT.[47,66] To confirm LRAT expression and activity in iPS RPE, whole cell homogenate was incubated with all *trans* retinol and NEM, a potent inhibitor of LRAT.[43] Our results show that NEM reduced the production of all *trans* retinyl palmitate by 90% (Fig. 5) and clearly implicates LRAT as the enzyme responsible for most, if not all, all *trans* retinyl palmitate synthesis in the iPS RPEThe residual 10% of retinyl esters synthesis is in agreement with reported ARAT activity in RPE cells.[47]

Following the synthesis of all *trans* retinyl esters by LRAT, the next step in the visual cycle is the isomerization of the all *trans* retinyl esters into 11 *cis* retinoids, which is facilitated by RPE65.[10–12] In agreement with previous studies in which cultured human fetal RPE were observed to uptake and complete the visual cycle under specific culture conditions,[67,68] we demonstrated that human iPS RPE incubated with all *trans* retinol accumulated all *trans* retinyl esters (Fig. 4) and released 11 *cis* retinaldehyde into the culture media (Figs. 6, 7). Synthesis of 11 *cis* retinaldehyde from all *trans* retinol in iPS RPE completes retinoid isomerization and further demonstrates the presence of an active 11 *cis* retinol dehydrogenase (RDH), presumably RDH5. In vivo and in culture, the amount of 11 *cis* retinoids detected in the RPE is relatively small by comparison to all *trans* retinyl esters.[62,68] The lack of detectable 11 *cis* retinoids in iPS RPE agrees with the physiologically low levels of intracellular 11 *cis* retinoids in RPE. In vivo 11 *cis* retinoids are released to the IPM as 11 *cis* retinaldehyde. The exact mechanism of release or secretion of 11 *cis* retinaldehyde from the RPE remains unclear. However, retinoid binding proteins such as interphotoreceptor retinoid binding protein (IRBP) and BSA have been shown to enhance the release of 11 *cis* retinaldehyde into the culture media.[67,68] Here we demonstrate that iPS RPE is able to synthesize and secrete 11 *cis* retinaldehyde (Figs. 6, 7) presumably to support visual pigment regeneration. Minute amounts of 9 *cis* and all *trans* retinaldehyde were detected by HPLC analysis of the media from iPS RPE after incubation with all *trans* retinol. We attribute the presence of these isomers to isomerization of the 11 *cis* retinaldehyde during sample handling. The 9 *cis* retinaldehyde is not a physiological retinoid, and the amounts of both 9 *cis* and all *trans* retinaldehyde are much lower than that of 11 *cis* retinaldehyde, suggesting a gradient of isomerization toward a more stable isomer. HPLC analysis of media from iPS cells after incubation with all *trans* retinol revealed peaks that may correspond to 9 *cis* and *trans* retinaldehydes by retention time (Fig. 6C). However, the absorbance spectra of these peaks did not match the absorbance spectra of authentic retinaldehyde standards. Further analysis by isocratic elution revealed that these peaks did not match the retention times of the authentic retinaldehyde standards; therefore they could not be identified as retinaldehydes by this system (Fig. 7B). This result, in combination with the lack of retinoid processing proteins in iPS cells, clearly demonstrates that the iPS cells do not possess the ability to metabolize vitamin A, via the classic visual cycle, to produce 11 *cis* retinaldehyde, indicating that this ability is acquired as the cells differentiate into RPE. Our success at developing and maintaining expression and function of the visual cycle proteins in iPS RPE may be attributed to several factors, including a high cell density (10^5 cells/cm^2) at the time of seeding, a high concentration of FBS during culture, and culture of cells for up to 6 months prior to the experiment, which may have allowed the cells to gain visual cycle competence.

In summary, we have successfully derived RPE from iPS cells. The iPS RPE exhibits classic hexagonal morphology and pigmentation and expresses RPE specific visual cycle proteins RPE65, LRAT, and CRALBP. The iPS RPE maintain the expression of RPE65, LRAT, and CRALBP in culture and produce 11 *cis* retinaldehyde. The uptake and processing of retinoids to produce 11 *cis* retinaldehyde from all *trans* retinol confirms that iPS derived RPE are functionally competent and able to process retinoids to support visual pigment regeneration.

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