Self-Formation of RPE Spheroids Facilitates Enrichment and Expansion of hiPSC-Derived RPE Generated on Retinal Organoid Induction Platform

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PURPOSE. Retinal pigment epithelium (RPE) and neural retina could be generated concurrently through retinal organoid induction approaches using human induced pluripotent stem cells (hiPSCs), providing valuable sources for cell therapy of retinal degenerations. This study aims to enrich and expand hiPSC-RPE acquired with this platform and explore characteristics of serially passaged RPE cells.

METHODS. RPE has been differentiated from hiPSCs with a published retinal organoid induction method. After detachment of neural retina on the 4th week, the remaining mixture was scraped from the dish and subjected to suspension culture for the formation of RPE spheroids. RPE sheets were isolated and digested for expansion. The cellular, molecular, and functional features of expanded RPE cells were evaluated by different assays.

RESULTS. Under suspension culture, hiPSC-RPE spheroids with pigmentation self-formed were readily enriched by removing the non-retinal tissues. RPE sheets were further dissected and purified from the spheroids. The individualized RPE cells could be passaged every week for at least 5 times in serum medium, yielding large numbers of cells with high quality in a short period. In addition, when switched to a serum-free medium, the passaged RPE cells could mature in cellular, molecular, and physiological levels, including repigmentation, markers expression, and phagocytosis.

CONCLUSIONS. We developed a simple and novel RPE spheroids formation approach to enrich and expand hiPSC-RPE cells generated along with retinal neurons on a universal retinal organoid induction platform. This achievement will reduce the cost and time in producing retinal cells for basic and translational researches, in particular for retinal cell therapy.

Keywords: human induced pluripotent stem cells, retinal pigment epithelium, differentiation, organoids, retinal degenerations

Degenerative retinal disorders such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD) are leading causes of untreatable blindness, affecting millions of people throughout the world.[1,2] Dysfunction or cell death of retinal pigment epithelium (RPE) is associated with the pathogenesis of both RP and AMD.[3] With the advancement of stem cell technology and regenerative medicine, RPE cell replacement therapy has been considered as a promising therapeutic for these diseases, and several clinical trials in AMD/RP patients are underway worldwide.[4–8] Therefore, establishing methods for producing large amounts of RPE seed cells with high quality and at a low cost would be key to bringing stem cell therapy into effect.

It has been proven that RPE cells can be differentiated from human pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), with different protocols.[9–17] While most protocols have used two-dimensional (2D) adherent conditions to induce hPSCs into RPE cells by spontaneous or directed differentiation,[9–14] the latest retinal organoid induction platforms with hPSCs on 3D suspension or 2D/3D combined conditions could generate 3D retinal organoids and RPE simultaneously; both are needed as cell sources in treating advanced stages of retinal degenerations such as AMD.[15–18] With a simple retinal organoid induction approach, Zhong and colleagues reported that hiPSCs-derived 3D neural retina tissues could recapitulate the development of human retina in vivo with photoreceptors achieving an advanced degree of maturation, including outer segment disc formation, a functional structure.[15] However, the characteristics and expansion ability of hiPSC-RPE acquired with this approach remain unknown. In addition, RPE differentiation from hPSCs often takes place with the generation of other unwanted tissue phenotypes due to their pluripotency no matter what methods are used. Hence, it is necessary to optimize the protocols to enrich and expand the hPSC-derived RPE from the mixture for downstream applications.

In this study, we established a simple, novel RPE spheroid formation approach for efficient enrichment and expansion of

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hiPSC-RPE concomitantly generated with the same retinal organoid induction system reported[15] and with fully identified biological features of these RPE cells.

MATERIALS AND METHODS

hiPSCs Culture and RPE Differentiation

Three hiPSC lines were used in this study. The BC1 and BC1-GFP lines were gifts from Linzhao Cheng (Johns Hopkins University School of Medicine).[19,20] The Gibco episomal hiPSC line (also called CB-iPSC6.2)[15,21] was purchased from Thermo Fisher Scientific (A18945; Life Technologies, Carlsbad, CA, USA). All hiPSCs were maintained in mTeSR1 medium (05851; STEMCELL Technologies, Cambridge, MA, USA) on Matrigel coated plates (354277; Corning, Inc., New York, NY, USA) and passaged every 5 to 7 days. To differentiate hiPSCs into RPE cell fates, we applied a published protocol for retinal organoid induction with slight modifications (Fig. 1A).[15,22] In brief, hiPSCs were dissociated and cultured in low adherent dishes for embryoid bodies (EBs) formation. By day 7, the EBs were replated onto Matrigel-coated dishes with neural induction medium (NIM) containing DMEM/F12 (1:1), 1% N2 supplement (Invitrogen Corp., Carlsbad, CA, USA), 1× minimum essential media non-essential amino acids (NEAA), 2 μg/ml heparin (Sigma-Aldrich Corp., St. Louis, MO, USA). From D16, the culture medium was changed with retinal differentiation medium (RDM) containing DMEM/F12 (3:1), 2% B27 (with or without vitamin A; Invitrogen), 1× NEAA and 1× penicillin-streptomycin. By weeks 4 to 5, both RPE and neural retina (NR) domains had formed and were ready to lift up. After selection of NRs with surrounding RPE in a parallel experiment for other projects, all remaining mixtures containing scattered, presumptive RPE patches with or without pigmentation were scraped and cultured in suspension for RPE spheroids formation and pigmentation with RDM. Unless otherwise stated, the results in the figures were obtained from BC1-GFP hiPSC line.

Enrichment and Culture of hiPSC-RPE Spheroids

After detachment, cell masses or non-retinal tissues without RPE were manually removed from the mixtures each week to enrich RPE and reduce medium consumption (Supplementary Fig. S1). By week 8, the enriched RPE spheroids were used for RPE cell dissociation and expansion, or they were switched to RDM supplemented with 10% FBS and 100 μM taurine (serum medium [SM]) for long-term culture. The medium was changed twice a week. In a parallel NR experiments, RPE spheroids detached from NRs were collected for this study.

Dissociation and Expansion of hiPSC-RPE

After week 8, the RPE spheroids at different timepoints after differentiation were collected and treated with 2% Dispase II (D4693; Sigma-Aldrich Corp.) at 37°C for 5 to 8 minutes, washed, and dissected with tungsten needles under a dissection microscope. The isolated RPE sheets were then digested into single cells with TrypLE Express (12605-010; Life Technologies) for 5 to 10 minutes in a 37°C incubator. The individualized cells were plated at 5×10^4 cells per cm^2 on Matrigel-coated plates and cultured with serum medium, as previously described, for cell expansion. These cells were defined as primary culture, passage (P) 0, and passaged at 100% confluence. To evaluate maturation features, expanded hiPSC-RPE cells reaching 100% confluence on 5 to 7 days after passage were switched to RDM again (also called "serum-free medium" in this study [SFM]) unless otherwise stated.[15,23] Regardless of the medium used, all cultures were maintained in a 37°C, 5% CO$_2$ incubator. The medium was exchanged every 2 to 3 days. Passaged hiPSC-RPE cells at P5 from BC1-GFP hiPSC line were selected to test the expansion ability, following a published method.[23]

Culture of Human Adult RPE and ARPE-19

Donor adult eyes were acquired from Eye Bank of Guangdong Province (Guangzhou, China), and RPEs were isolated according to the published protocols.[24,25] The human adult RPE (hRPE) cells were expanded, passaged, and matured in a similar manner as previously described. The experiment was approved by Ethical Review Committee of Zhongshan Ophthalmic Center, Sun Yat-sen University. ARPE-19, a widely used human adult RPE cell line,[26] was a gift from David Wan-Cheng Li (Zhongshan Ophthalmic Center, Sun Yat-sen University) and expanded in serum medium as described.

Immunohistochemistry

RPE spheroids were collected and fixed with 4% paraformaldehyde (PFA) for 30 minutes on ice. The tissues were prepared for frozen sections with the methods published,[15] or embedded in 4% to 5% agarose for vibratome sections and cut in 50- to 70-μm thickness.[27] Cells on coverslips or in chambers were fixed with 4% PFA for 5 minutes. Immunostaining procedure was similar to the method published.[15] The primary antibodies used are listed in Supplementary Table S1 (see Supplementary Materials for details).

Transmission Electron Microscopy

Samples were fixed in EM fixative (2.5% glutaraldehyde/2% PFA) at 4°C, sent to Electron Microscopy Core Facility at Sun Yat-sen College of Medical Science, Sun Yat-sen University (Guangzhou, China) for dehydration, embedding, sectioning and staining, and observed by transmission electron microscope (Tecnai G2 Spirit; FEI, Inc., Carlsbad, CA, USA).

Phagocytosis Assay

Swine retinas were dissected from freshly slaughtered porcine eyes in a dark room. Photoreceptor outer segments (POS) were prepared according to a published protocol[28] and labeled with CM-Dil (C7001; Invitrogen) following manufacturer instructions. The phagocytosis assay was performed as previously described.[18,29,30] See supplementary materials for details.

Transepithelial Resistance (TER) Measurement

hiPSC-RPE and hRPE cells were plated on Transwell filters (Sigma-Aldrich Corp.) and cultured in serum medium, then switched to serum-free medium when 100% confluence was reached. TER was measured using an epithelial tissue voltohmmeter (EVOM2; World Precision Instruments, Sarasota, FL, USA) following manufacturer's instructions in Qiaobing Huang's Lab at Southern Medical University, Guangzhou, China. The net TERs were calculated by subtracting the value of a blank Matrigel-coated Transwell filter without cells from the experimental value. The final TER values in Ω*cm^2 were obtained by multiplying net TER values with the surface area of Transwell filters.

Measurement of VEGF Secretion by ELISA

Mature hiPSC-RPE cells grown on Matrigel-coated Transwells with TER more than 250 Ω*cm^2 were used to conduct this experiment. Media cultured for 24 hours were collected separately from the apical and basal chambers. The VEGF level was measured with
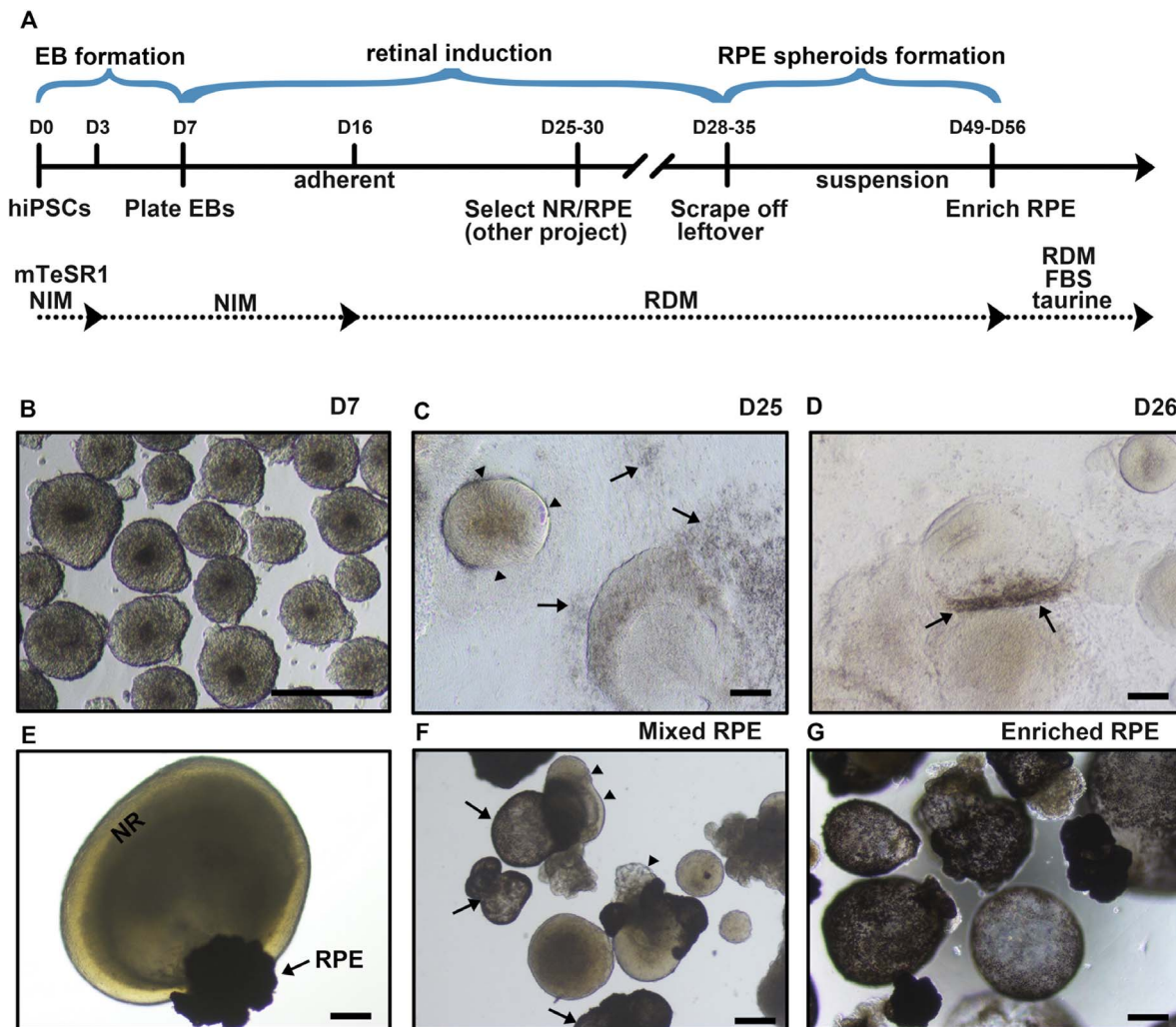
Figure 1. Self-formation and enrichment of hiPSC-RPE spheroids. (**A**) Schematic view of RPE differentiation and spheroids formation from hiPSCs. (**B**) EBs formed in suspension culture on day 7. (**C**, **D**) On 4th week after differentiation, a few pigmented RPE domains (*arrow*) appeared surrounding the neural retina (NR) domains (*arrowhead*) or scattering over the dish. (**E**) In suspension culture, detached NR domains formed 3D retinal organoids with a thick, transparent NR ring attached with a small RPE spheroid (*arrow*). (**F**) In detached remaining cultures, many pigmented RPE spheroids also self-formed, free-floating (*arrow*) or adherent to cell masses (*arrowhead*). (**G**) RPE spheroids separated from retinal organoids or enriched from the remaining cultures since 8th week after differentiation. *Scale bars*: 100 μm.

VEGF ELISA kit (CSB-E11718h; Abbkine Scientific, Wuhan, China) following the manufacturer's instructions.

RT-PCR and Quantitative RT-PCR

Samples from hiPSCs and hiPSC-derived RPE cells were collected at various timepoints after passage. RNA extraction was done with TRIzol Reagent (15596026; Invitrogen) following the manufacturer's instructions. The primers used are listed in Supplementary Table S2. (See Supplementary Materials for details.)

Flow Cytometry

ARPE-19, BC1-GFP hiPSCs, and expanded P5 hiPSC-RPE cells (BC1-GFP) on D5 after passage were dissociated into single cells with TrypLE Express (Thermo Fisher Scientific), fixed in 1% PFA for 15 minutes, washed, blocked, and permeabilized at RT for 30 minutes and then incubated with primary antibody MITF (2 μg/ 1×10^6 cells) at RT for 1 hour. After a PBS wash, cells were then incubated with secondary antibody donkey anti-mouse Alexa 647 (Thermo Fisher Scientific) for 30 minutes, washed and analyzed using LSRFortessa Flow Cytometer (BD, Franklin Lakes, NJ, USA). Cells without 1st antibody incubation were used as negative control. Data analysis was performed using FlowJo 7.6.1 software (https://www.flowjo.com/). Three biological replicates were performed.

Statistical Analysis

The values are expressed as mean ± SEM (standard error of mean) or mean ± SD (standard deviation). $P < 0.05$ was considered statistically significant. All experiments were performed at least in triplicate. GraphPad Prism Software 6.01 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis.

RESULTS

hiPSC-RPE Spheroids Formation and Enrichment

With our modified retinal organoid induction and RPE enrichment approach, hiPSCs concurrently produced retinal
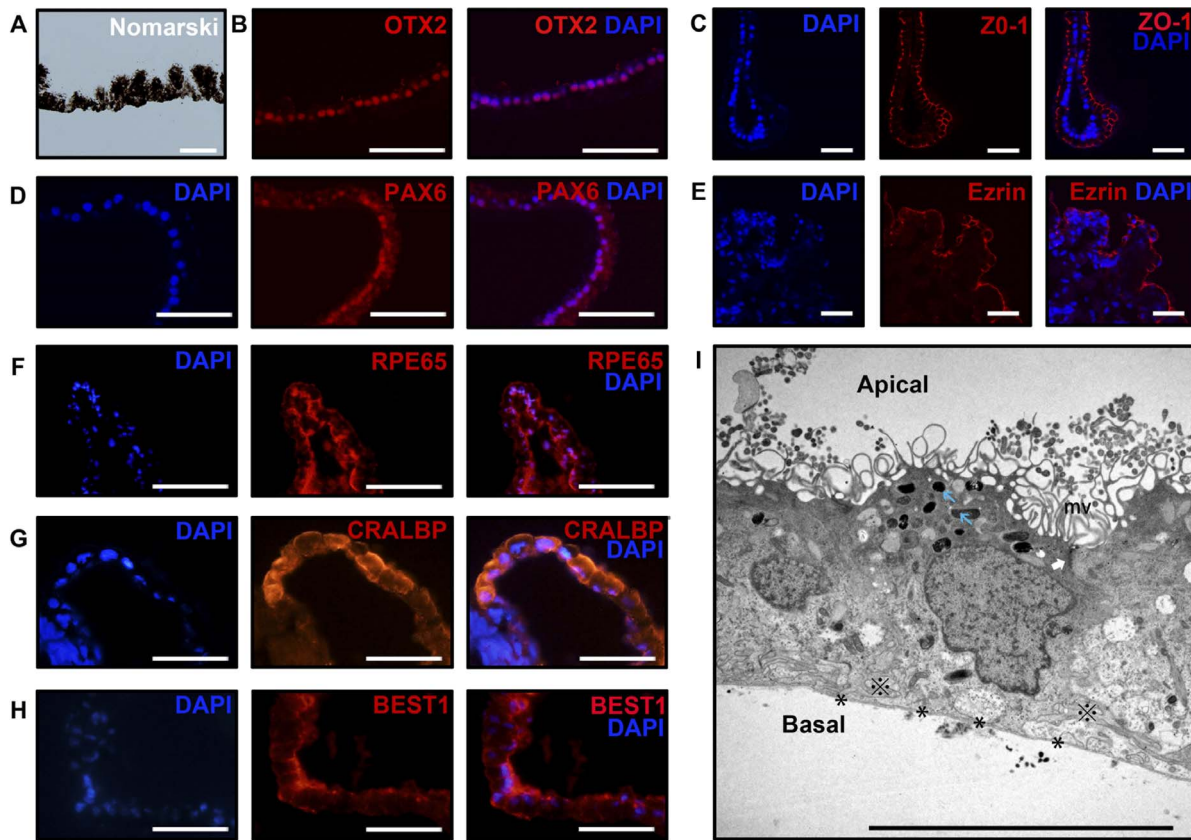
FIGURE 2. Characterization of RPE spheroids derived from hiPSCs. (**A**) Nomarski image of pigmented hiPSC-RPE. (**B–I**) Pigmented hiPSC-RPE spheroids expressed RPE specific protein markers (**B**) OTX2, (**C**) ZO-1, (**D**) PAX6, (**E**) Ezrin, (**F**) RPE65, (**G**) CRALBP, and (**H**) BEST1, indicating RPE achieved very high degree of maturation. (**I**) TEM showed RPE cells in spheroids developed apical microvilli (mv), melanin granules (*blue arrow*) in apical cytoplasm, apical tight junctions (*white arrow*), basement membrane (*) and basal infoldings (⁕), resembling ultrastructure features of mature RPE. *Scale bars*: 50 μm.

organoids as well as large amounts of RPE spheroids that were readily enriched from the mixture (Fig. 1, Supplementary Fig. S1). All hiPSCs (BC1, BC1-GFP, and 6.2) presented typical morphological features and expressed pluripotency markers OCT4, TRA-1-60, and TRA-1-81 (Supplementary Fig. S2). After EBs reattachment on adherent dishes at D7, pigmented foci gradually appeared surrounding NR domains or scattering over the dish (Fig. 1B–D). By week 4, NRs were often lifted up and cultured in suspension for retinal organoids formation in a parallel experiment (Fig. 1E). Afterwards, the leftover was scraped from the dish and cultured in suspension with RDM. Under this condition, RPE self-formed spheroids or aggregates with pigmentation, free floating, or adherent to cell masses or neural retinal tissues in the mixture over 2 to 4 weeks (Fig. 1F). During this period, non-retinal tissues were routinely cleared away from the culture, leading to the enrichment of pigmented RPE spheroids by week 8. The enriched RPE spheroids could grow and survive in serum medium for a long time (up to 150 days after differentiation), with some forming big RPE cysts (Fig. 1G).

Moreover, the pigmented RPE within spheroids expressed not only RPE pan markers OTX2, PAX6, Ezrin (an apical marker), and ZO-1 (a tight junction marker) but also mature RPE associated markers RPE-specific 65 kDa protein (RPE65), CRALBP, and bestrophin 1 (BEST1) (Fig. 2A–H). In addition, TEM showed typical ultrastructure features of mature RPE including cell polarity with apical microvilli, melanin granules in apical cytoplasm, apical tight junctions, basement membrane, and basal infoldings (Fig. 2I). These data indicated that

RPE cells enriched through spheroid formation achieved quite high levels of maturation in both protein and morphological levels. In general, one 6-well plate of hiPSCs could produce more than 600 RPE spheroids, releasing about 3×10^6 cells in week 8 to week 10. All three hiPSCs lines could reproducibly generate pigmented RPE spheroids. However, variations of RPE spheroid yield were also observed among the lines and experimental batches.

Efficient Expansion of RPE Cells from RPE Spheroids

Previous studies showed that human RPE could be isolated from the underlying choroid.[23,25] Here, we applied this method to successfully dissect RPE sheets from RPE spheroids at various time windows (<day 60, day 60–90, >day 90) after differentiation (Fig. 3A, 3B). Individualized hiPSC-RPE cells were attached to the dish within 30 minutes, forming a monolayer that reached 100% confluence in 1 week with their typical polygonal appearance containing more or fewer pigments (Fig. 3C–3E). All RPE cells from three hiPSC lines and hRPE succeeded in primary culture and could be routinely passaged in about 1 week for at least 5 times in serum medium. The expanded RPE cells started to lose pigment from P1 (Fig. 3F). The morphological change of hiPSC-RPE cells was similar to that of freshly isolated hRPE cells that were parallelly cultured in this study and reported before (Supplementary Fig. S3).[23,25] Even more, passaged RPE cells (P5–P8) from the tested BC1-GFP hiPSCs still showed strong proliferation ability
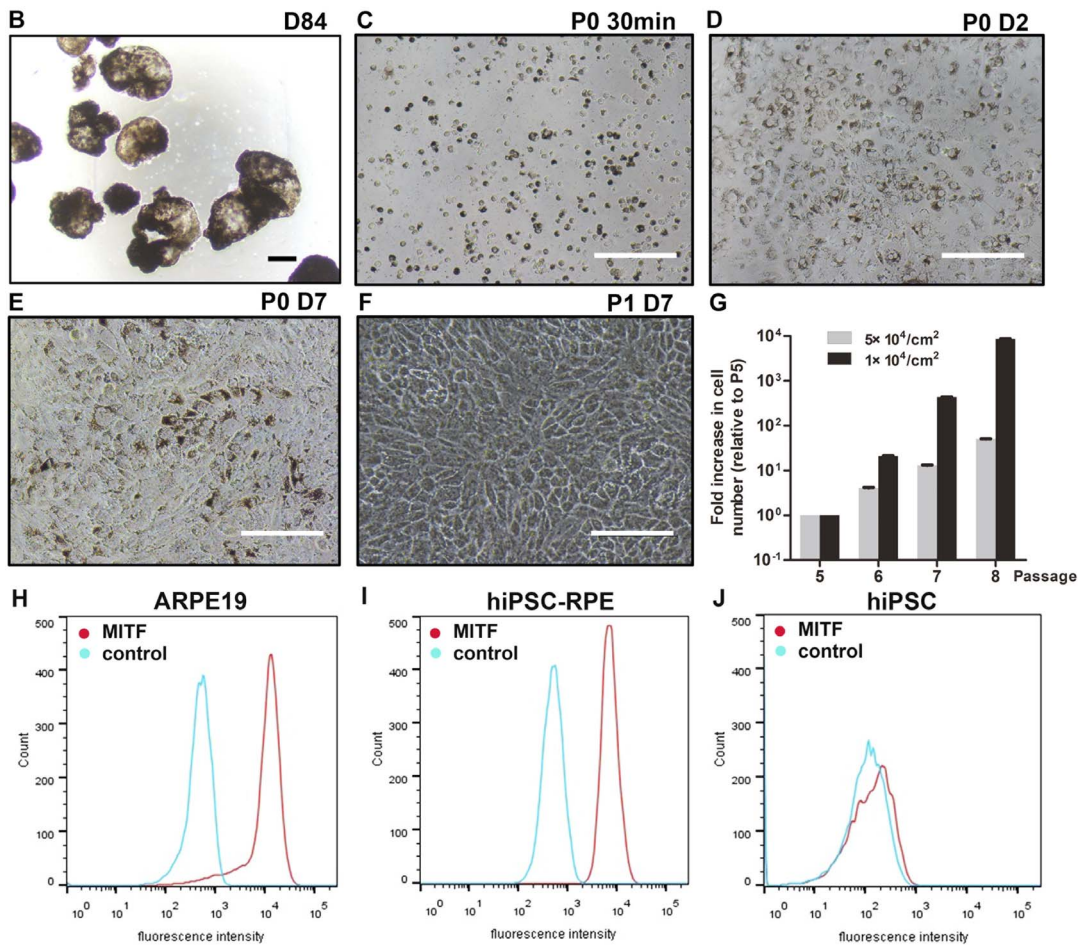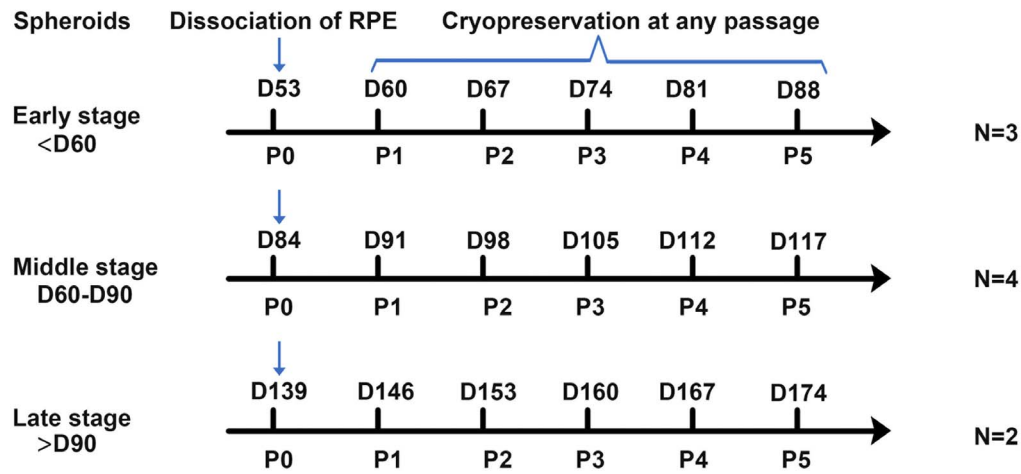
FIGURE 3. Primary and passaged culture of hiPSC-RPE cells isolated from RPE spheroids. (A) Diagram of expansion and passage of hiPSC-RPE cells from spheroids at various timepoints after differentiation. *N*, number of independent experiments. D, days after differentiation. (B) Purified RPE sheets from D84 spheroids by dispase treatment followed by dissection. (C–E) Primary culture of single RPE cells with pigmentation and epithelium-like morphology at 30 minutes, 2 days, and 7 days after dissociation. (F) Expanded P1 RPE cells cultured in serum medium lost pigments. (G) Fold changes of passaged BC1-GFP-RPE cells from P5. Quantification of fold changes in the total number of live hiPSC-RPE cells at P5, P6, P7, and P8 (all relative to P5). (H–J) Flow cytometry analysis showed approximately 98.8% of expanded hiPSC-RPE cells, 95.4% of ARPE-19 cells, and none of hiPSCs expressed MITF, a RPE specific marker. B–G, *scale bars*: 100 µm.
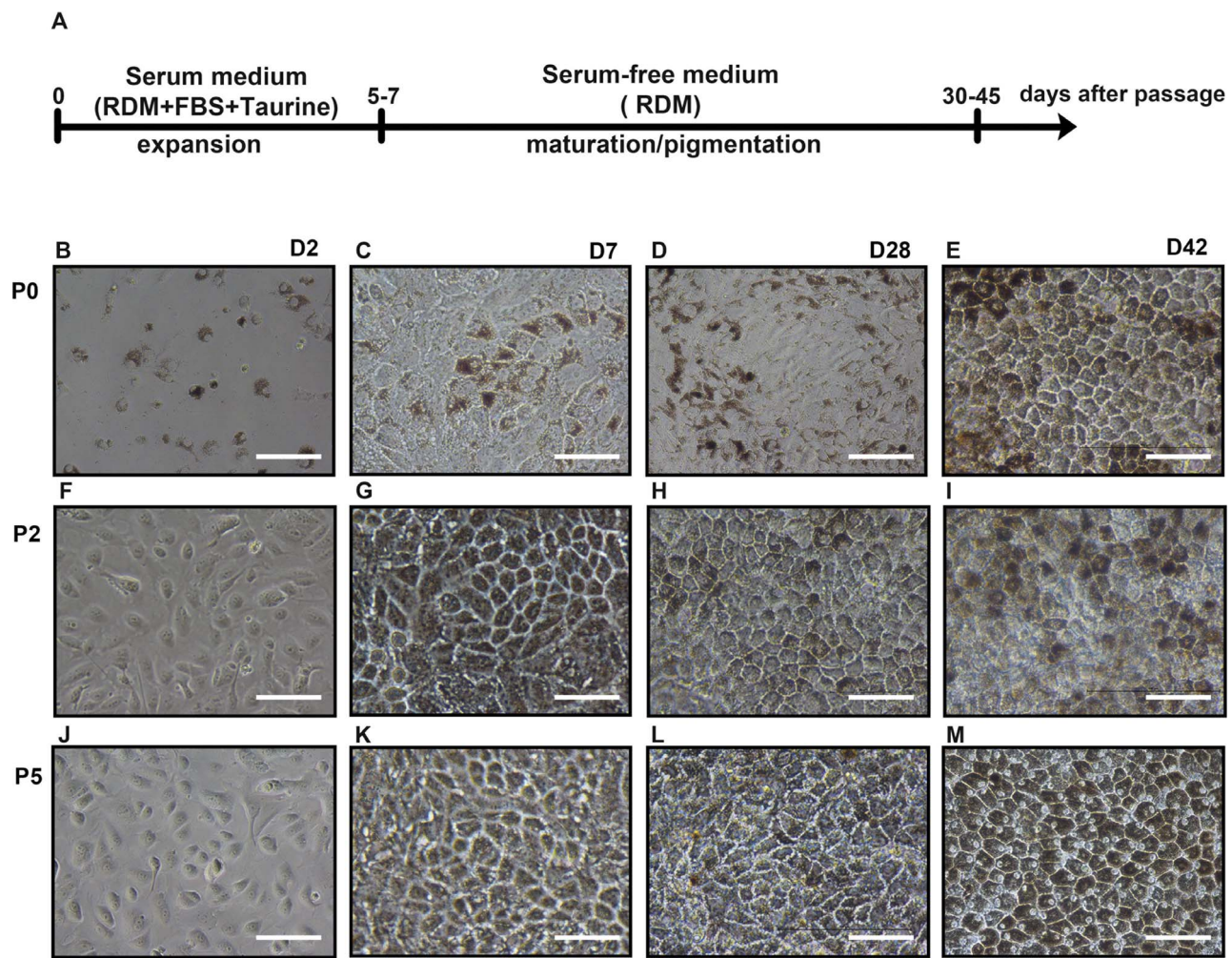
FIGURE 4. Serially passaged hiPSC-RPE cells had capacity of proper pigmentation. (**A**) Schematics of maturation culture of hiPSC-RPE cells switched to serum-free medium on day 5 to 7 after passage. (**F–M**) Under this two-step culture conditions, nonpigmented P2 and P5 hiPSC-RPE cells on D2 and D7 progressively regained melanin pigments on D28 and D42 when cells appeared typical hexagonal morphology, similar to the morphological features of P0 cells (**B–M**), *scale bars*: 50 μm.

with the cell growth of approximate 4 or 20 folds per generation when plating density at $5 \times 10^4/cm^2$ or at $1 \times 10^4/cm^2$, respectively (Fig. 3G). Therefore, a serial passage with SM could produce large numbers of RPE cells in a short period, which were routinely frozen and revived when needed. In addition, RT-PCR showed that pluripotency markers OCT4 and SOX2 were negative for both P1 and P5 hiPSC-RPE cells, but the retinal progenitor markers VSX2 and RX were weakly positive for P1 but negative for P5 (Supplementary Fig. S4), implicating serial passage reduced cell contamination risk. Flow cytometry analysis showed approximately 98.8% ($\pm 0.02\%$, $n = 3$) of hiPSC-RPE cells expressed MITF, a RPE progenitor marker.[31] The purity of these hiPSC-RPE cells (95.4% $\pm$ 2.05, $n = 3$) was comparable to that of ARPE-19. No hiPSCs expressed MITF (Fig. 3H–J). These data indicated that serial passage resulted in a high purity of RPE cells without contamination of undifferentiated hiPSCs or non-RPE cells, which is consistent with a previous report.[32]

Pigmentation and Markers Expression of Passaged hiPSC-RPE Cells

Considerable evidence indicated that the pigmentation of RPE cells was coincident with the functional maturation and used to assess the maturation status of RPE cells in culture.[33] To verify that the expanded RPE cells kept the proper maturation ability, they were switched to SFM on day 5 to 7 after passage for long-term culture unless otherwise stated (Fig. 4A). Under this two-step culture conditions, dynamic pigmentation changes of passaged hiPSC-RPE cells at P2 and P5 occurred within 6 weeks after passage, from non-pigmented cells on day 2 and day 7 to progressively pigmented cells on day 28 and day 42 when cells appeared typical hexagonal morphology, similar to the morphological features of P0 cells (Fig. 4B–M). On day 42 after passage, the pigmentation level of serially passaged hiPSC-RPE cells (Fig. 4I, 4M) was similar to that of the primary cells derived from the day 84 RPE-spheroids (Fig. 4E).

Immunohistochemistry showed that RPE specific transcription markers MITF and OTX2 were strongly positive in proliferative hiPSC-RPE cells (day 3 and day 7) cultured in SM (Fig. 5A, 5B) but gradually became negative or weak in the cells (day 14, day 28, and day 42) grown in SFM after day 7. The decreasing trend of these protein expression levels was similar to that of cell proliferative marker Ki67 in the corresponding timepoints (Fig. 5C) but opposite to the increasing trend of expression level of mature RPE associated markers ZO-1, BEST1, CRALBP, and RPE65 (Fig. 5D–G). It indicated the cells
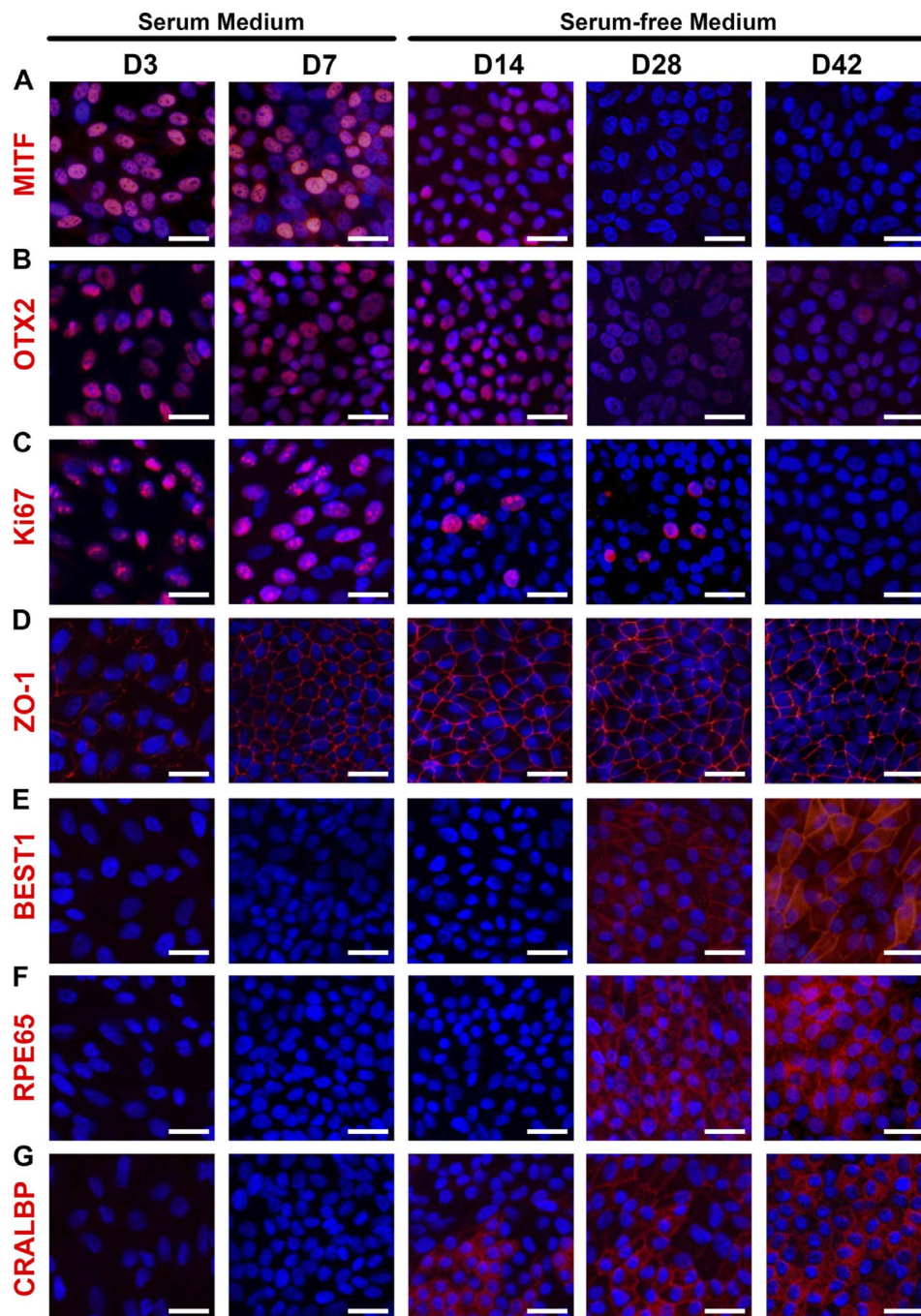
FIGURE 5. Protein expression profiles of expanded hiPSC-RPE cells cultured in SM and SFM evaluated by immunohistochemistry. (**A–C**) The expression level of RPE progenitor markers MITF and OTX2, and cell proliferative marker Ki67 were high in cells P3–P5, BC1-GFP cultured in serum medium (SM; D3, D7), gradually decreased to weak or negative in cells cultured in serum-free medium (SFM; D14, D28, and D42). (**D**) ZO-1, a tight junction marker, appeared as early as D3 and gradually increased its expression level in cells over time. (**E–G**) In contrary to the RPE progenitor markers MITF and OTX2, mature RPE associated markers BEST1, CRALBP, and RPE65 were negative in D3 and D7 cells but gradually increased its expression level in cells over time (D14, D28, and D42), indicating the cells progressively matured in SFM. *Scale bars*: 50 μm.

progressively matured in SFM, which were further confirmed by qRT-PCR (Fig. 6A). However, the expression level of these RPE-related markers was not significantly changed among day 28 hiPSC-RPE cells from different passages (Fig. 6B). Furthermore, the transcriptional expression level of RPE-related markers in hiPSC-RPE cells was not significantly different compared to that in hRPE except CRALBP, shown by qRT-PCR (Fig. 6C). Altogether, these results demonstrated that expanded RPE monolayers from different passages had the capacity to

mature in both morphological and molecular levels equivalent to the expanded hRPE cells.

Functional Features of Passaged hiPSC-RPE Cells

Next, we questioned whether expanded hiPSC-RPE cells were able to function after maturation like native RPE cells. TER measurements showed that TER in all cultures gradually increased in first 3 weeks, reached and maintained more than
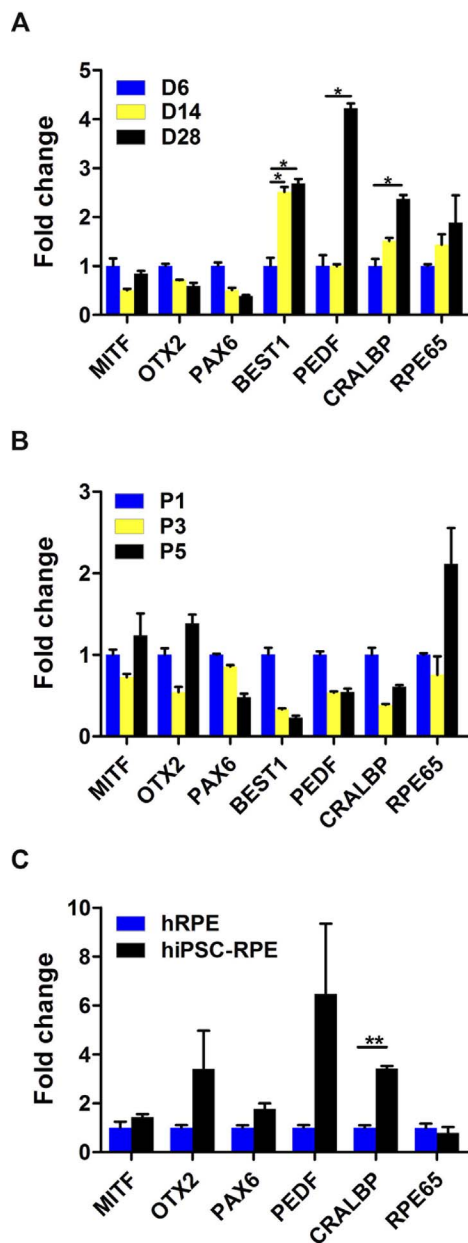
A

B

C

FIGURE 6. Gene expression profiles of serially passaged hiPSC-RPE cells evaluated by qRT-PCR. (**A**) qRT-PCR analysis showed the expression level of RPE transcription factors MITF, OTX2, and PAX6 gradually decreased, while maturation markers BEST1, CRALBP, PEDF, and RPE65 increased in P3 hiPSC-RPE cells on D6, D14, and D28 after passage. (**B**) The expression level of these genes were not significantly changed among D28 hiPSC-RPE cells from different passages (P1, P3, and P5). (**C**) The expression level of MITF, OTX2, PAX6, PEDF, and RPE65 in hiPSC-RPE cells (P3, D28) had no significant difference with hRPE cells (P3, D28) except PEDF. Ct values of each sample were normalized with that of hiPSCs, and results were presented as fold changes compared to (**A**) D6, (**B**) P1, and (**C**) hRPE. Mean ± SEM, *$P < 0.05$, **$P < 0.005$.

350 $\Omega*cm^2$ (similar to those of hRPE; Fig. 7A) and significantly above the TER of 150 $\Omega*cm^2$ in naïve RPE.[25] Following the increase of TER, the pigmented hiPSC-RPE cells from P5 presented polarized secretion of VEGF with the level being higher in the basal side than in the apical side (Fig. 7B). In addition, an essential function of RPE cells in vivo is phagocytosis of outer segments shed by photoreceptors every

day.[25,28] Phagocytosis of POS was evaluated in pigmented hiPSC-RPE fed with CM-Dil labeled POS for 3 and 12 hours at 37°C and 4°C, respectively. Compared to the 4°C negative control (physiological function decreased at 4°C), the POS number phagocytosed by hiPSC-PRE cells in the 37°C group was significantly higher in either 3-hour or 12-hour exposure period, with the 12-hour group significantly higher than the 3-hour group (Fig. 7C). Confocal imaging further showed that the POS was phagocytosed and appeared in the cytoplasm, which was confirmed by ZO-1 staining (an RPE apical side marker) with 3D z-stack images (Fig. 7D, 7E). Therefore, our results demonstrated that serial passage and expansion did not change the capacity of hiPSC-RPE cells to form a functional, polarized monolayer with normal phagocytosis and polarized secretion.

DISCUSSION

RPE has been concomitantly generated from hPSCs through retinal organoid induction platforms.[15–18,34–37] Here, we developed a simple RPE spheroid formation approach to enrich hiPSC-RPE produced along with NRs on the same retinal induction platform reported before,[15] and verified that the individualized RPE cells were able to serially passage every week for at least 5 passages in serum medium, yielding a large population of RPE cells in a short period, which could further mature in both molecular and morphological levels with serum-free culture conditions. The successful enrichment and expansion of hiPSC-RPE cells with the same induction system, including substrate and culture medium, would greatly decrease the cost in producing clinical grade retinal cells containing RPE and all retinal neurons, consequently reducing patient expenses for retinal cell therapy, especially in personalized medicine.

Several approaches have been used to enrich, purify, and expand hPSC-RPE from the mixture for further applications. Manually picking out pigmented clusters and reattaching them to the adherent surface for expansion and passage in serum-free medium is commonly used when pigmented foci become abundant in culture in about 8 to 13 weeks after differentiation.[14,32,38,39] This procedure has been described in detail in a study reported by Vugler and colleagues,[39] indicating that it was feasible only when the sizes of the pigmented foci were over 1 mm in diameter with every effort made to dissect the surrounding and non-pigmented material for acquiring high purity of RPE cells under inverted microscope. Further, many research groups applied the same conditions—serum-free or low serum media, high plating density (approximately $1 \times 10^5/cm^2$), and 30-day passage (SH30)—for both expanding and maturing hiPSC-RPE cells, taking a couple of months to produce a large population of RPE cells. For instance, Singh and colleagues[32] picked and dissociated deeply pigmented hiPSC-RPE sheets in day 60 to day 90 after differentiation, concomitantly generated with NR on an optic vesicle induction platform. By passaging the pigmented mature hiPSC-RPE cells roughly every month, these cells were subcultured only 3 times, which took about 90 days after RPE selection, implying the timeframe of producing large population of hiPSC-RPE cells would be 150 to 180 days after differentiation, similar to the timeline reported by Leach et al.[38] Hence, this time-consuming and labor-intensive method requires skillful, experienced researchers. Slightly modified from the above approach, Julien et al.[9] enriched hPSC-RPE cells by direct passage from week 8 after differentiation. The culture conditions (serum-free medium, high plating density, and 3-week passage) were similar to the earlier-described studies. However, with this method, the RPE cells were passaged twice as reported, so it is unclear how
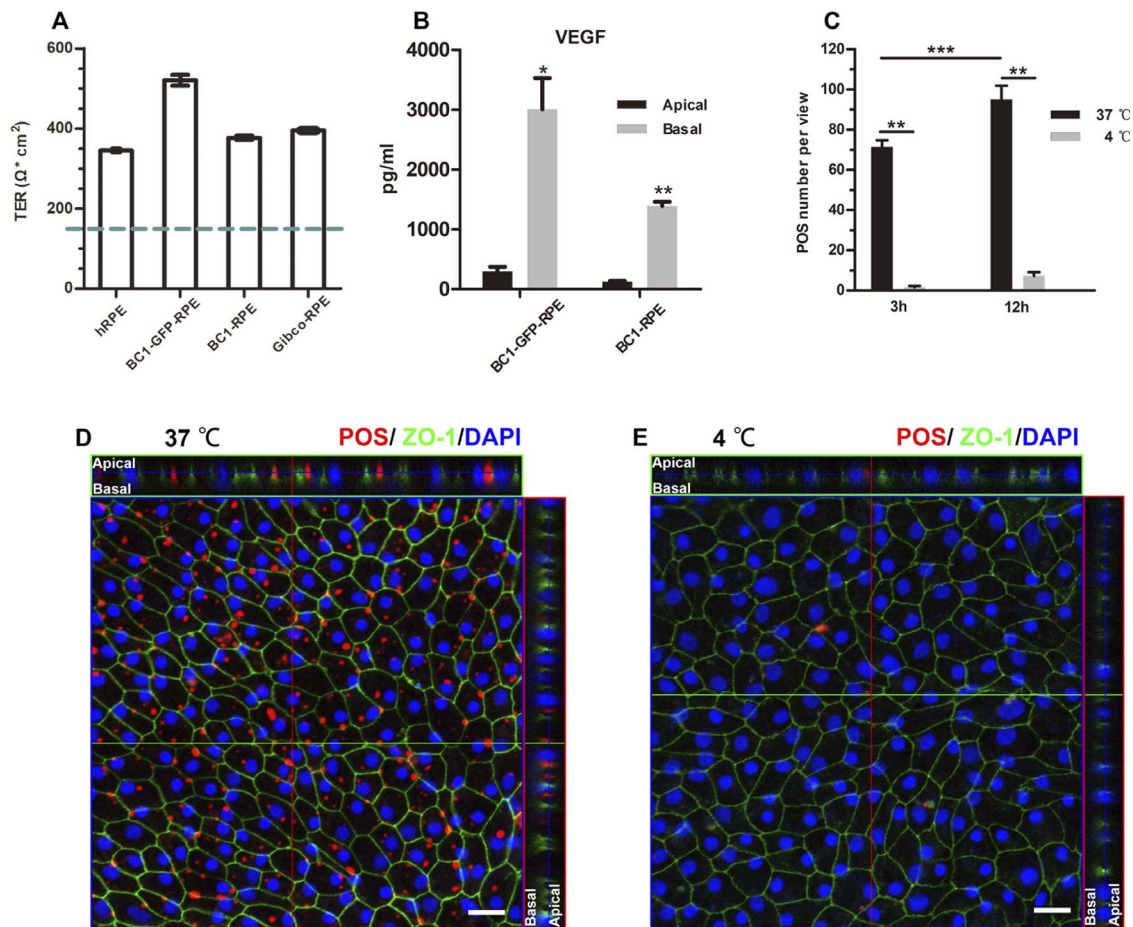
FIGURE 7. Functional tests of passaged hiPSC-RPE monolayers. (A) TER measurements of expanded P5 RPE cells from different hiPSC lines cultured in SFM for 6 weeks. Mean ± SD. (B) Polarized VEGF secretion of passaged hiPSC-RPE (P5). Mean ± SD, $*P < 0.05$, $**P < 0.005$. (C) Phagocytosis activity of hiPSC-RPE cells exposed to CM-Dil-labeled POS for 3 and 12 hours at $4°C$ and $37°C$, respectively, Mean ± SD, $n = 3$, $**P < 0.01$, $***P < 0.001$. (D, E) Z-stack confocal micrograph showing passaged hiPSC-RPE cells phagocytosed CM-Dil-labeled POS (_red_) located in the cytoplasm verified by an apical side marker ZO-1 (_green_). _Scale bars_: 20 μm.

far they could go. In our study, a novel procedure was developed to enrich and purify hiPSC-RPE from the mixture by formation and dissection of RPE spheroids, similar to isolating hRPE from human eyeballs (see "Methods" and "Results" sections).[23–25] Since the definite non-retinal tissues or cell masses could be routinely cleared from the mixture after detachment, our method readily enriched RPE spheroids and significantly reduced the medium consumption and cost. In addition, RPE spheroids could grow for a long time and achieve high degrees of maturation with proper pigmentation, specific markers expression, and cell polarity. RPE spheroids or aggregates were also observed in previous studies, which were often reattached to the adherent surface to allow RPE cells to migrate out and expand for 3 to 5 weeks.[23,37,40,41] However, in our study, RPE sheets were directly dissected from the spheroids and digested into single cells for quick expansion in our optimized culture conditions with serum medium, low seeding density (2.0 to 5.0×10^4/cm²) and 7-day passage (SL7). Under these conditions, singularized RPE cells from RPE spheroids at varying timepoints after differentiation (day 53 to day 139) were all successfully expanded and routinely passaged at least 5 times. For BC1 and BC1-GFP hiPSC-RPE cells, 8 to 10 passages were achieved, yielding large quantities of RPE cells in a relatively short period. For example, one 6-well plate of hiPSCs could produce at least 3×10^{11} hiPSC-RPE cells over 5 passages in about 1 month after cell dissociation, or

in about 80 to 90 days after differentiation if day 53 RPE-spheroids were used in the dissociation. Therefore, our SL7 expansion method significantly reduced the time for cell preparation compared to those SH30, which might take about 140 to 150 days after differentiation to provide a similar output if RPE clusters were selected at the same timepoint.[32,38] In a subset of parallel experiments, we expanded RPE cells with the SH30. The cell number after 1-month culture was roughly equivalent to that in 1-week culture with our SL7 expansion method, which implies that the prolonged culture did not increase cell yield (data not shown). The cell contact inhibition and decreased proliferative activity in SFM might be related to the low yield of a 1-month culture. In summary, this optimized method for RPE enrichment and expansion is simple, practical and highly efficient, which could be applied in retinal organoid induction platform for producing RPE and retinal neural cells together, facilitating the basic and translational research of retinal diseases.

There has been much controversy about whether serially passaged RPE cells can be used as cell source for RPE replacement therapy in the field. The major issues are that serially passaged RPE cells (more than 3 passages) might not function well after transplantation since they lose pigment and epithelioid morphology, undergo epithelial-mesenchymal transition, and do not (or weakly) express specific functional markers such as RPE65 and BEST1.[24,39,42,43] However, the

more times RPE cells are passaged, the less chance the cells will be contaminated with unwanted cells or undifferentiated stem cells, which might cause tumor in vivo[32]; further, more RPE cells are ideal for large-scale RPE cell banking. In this study, we demonstrated that non-pigmented, passaged RPE cells (up to P8 tested) cultured on SM could mature properly over time with progressive pigmentation when switched to SFM. Pigmentation has been regarded as an effective and simple indicator of RPE maturation status, coincident with the expression of mature RPE specific markers.[33,39] Similarly, the expanded RPE cells within 5 passages in this study not only regained pigment and polygonal morphology but also acquired features of mature RPE, including expression profile of mature RPE makers, high TER, polarized secretion of VEGF, and phagocytosis of POS, similar to human RPE. Taken together, hiPSC-RPE cells after serial passage with our methods are able to mature at molecular, morphological, and physiological levels, with the potential of serving as the cell source for replacing degenerated RPE cells in AMD.

In conclusion, hiPSC-derived RPE cells concomitantly generated with NRs on retinal organoid induction platform can self-form pigmented spheroids in suspension condition, facilitating their enrichment and expansion. The RPE spheroids culture system may also serve as a novel research model. The success of producing expandable RPE cells will definitely reduce the cost of retinal cell preparation for retinal cell therapy, in particular for personalized medicine. However, further studies will be needed to optimize the protocol for preparation of clinical grade retinal cells and to explore their efficacy and safety as sources in treating retinal diseases.

Acknowledgments

References

1. Milam AH, Li ZY, Fariss RN. Histopathology of the human retina in retinitis pigmentosa. *Prog Retin Eye Res*. 1998;17:175–205.

2. Gehrs KM, Anderson DH, Johnson LV, Hageman GS. Age-related macular degeneration – emerging pathogenetic and therapeutic concepts. *Ann Med*. 2006;38:450–471.

3. Nguyen HV, Li Y, Tsang SH. Patient-specific iPSC-derived RPE for modeling of retinal diseases. *J Clin Med*. 2015;4:567–578.

4. Nazari H, Zhang L, Zhu D, et al. Stem cell based therapies for age-related macular degeneration: the promises and the challenges. *Prog Retin Eye Res*. 2015;48:1–39.

5. Schwartz SD, Regillo CD, Lam BL, et al. Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. *Lancet*. 2015;385:509–516.

6. Lu B, Malcuit C, Wang S, et al. Long-term safety and function of RPE from human embryonic stem cells in preclinical

7. Kashani AH. Stem cell therapy in nonneovascular age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2016;57:ORSFm1–ORSFm9.

8. Canto-Soler V, Flores-Bellver M, Vergara MN. Stem cell sources and their potential for the treatment of retinal degenerations. *Invest Ophthalmol Vis Sci*. 2016;57:ORSFd1–ORSFd9.

9. Maruotti J, Wahlin K, Gorrell D, Bhutto I, Lutty G, Zack DJ. A simple and scalable process for the differentiation of retinal pigment epithelium from human pluripotent stem cells. *Stem Cells Transl Med*. 2013;2:341–354.

10. Miyagishima KJ, Wan Q, Corneo B, et al. In pursuit of authenticity: induced pluripotent stem cell-derived retinal pigment epithelium for clinical applications. *Stem Cells Transl Med*. 2016;5:1562–1574.

11. Maruotti J, Sripathi SR, Bharti K, et al. Small-molecule-directed, efficient generation of retinal pigment epithelium from human pluripotent stem cells. *Proc Natl Acad Sci U S A*. 2015;112:10950–10955.

12. Carr AJ, Vugler AA, Hikita ST, et al. Protective effects of human iPS-derived retinal pigment epithelium cell transplantation in the retinal dystrophic rat. *PLoS One*. 2009;4:e8152.

13. Hongisto H, Jylha A, Nattinen J, et al. Comparative proteomic analysis of human embryonic stem cell-derived and primary human retinal pigment epithelium. *Sci Rep*. 2017;7:6016.

14. Kokkinaki M, Sahibzada N, Golestaneh N. Human induced pluripotent stem-derived retinal pigment epithelium (RPE) cells exhibit ion transport, membrane potential, polarized vascular endothelial growth factor secretion, and gene expression pattern similar to native RPE. *Stem Cells*. 2011;29:825–835.

15. Zhong X, Gutierrez C, Xue T, et al. Generation of three-dimensional retinal tissue with functional photoreceptors from human iPSCs. *Nat Commun*. 2014;5:4047.

16. Nakano T, Ando S, Takata N, et al. Self-formation of optic cups and storable stratified neural retina from human ESCs. *Cell Stem Cell*. 2012;10:771–785.

17. Meyer JS, Shearer RL, Capowski EE, et al. Modeling early retinal development with human embryonic and induced pluripotent stem cells. *Proc Natl Acad Sci U S A*. 2009;106:16698–16703.

18. Reichman S, Slembrouck A, Gagliardi G, et al. Generation of storable retinal organoids and retinal pigmented epithelium from adherent human iPS cells in xeno-free and feeder-free conditions. *Stem Cells*. 2017;35:1176–1188.

19. Chou BK, Mali P, Huang X, et al. Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. *Cell Res*. 2011;21:518–529.

20. Zou C, Chou BK, Dowey SN, et al. Efficient derivation and genetic modifications of human pluripotent stem cells on engineered human feeder cell lines. *Stem Cells Dev*. 2012:2298–2311.

21. Burridge PW, Thompson S, Millrod MA, et al. A universal system for highly efficient cardiac differentiation of human induced pluripotent stem cells that eliminates interline variability. *PLoS One*. 2011;6:e18293.

22. Li G, Xie B, He L, et al. Generation of retinal organoids with mature rods and cones from urine-derived human induced pluripotent stem cells. *Stem Cells Int*. 2018;2018:4968658.

23. Gamm DM, Melvan JN, Shearer RL, et al. A novel serum-free method for culturing human prenatal retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 2008;49:788–799.

24. Maminishkis A, Chen S, Jalickee S, et al. Confluent monolayers of cultured human fetal retinal pigment epithelium exhibit morphology and physiology of native tissue. *Invest Ophthalmol Vis Sci*. 2006;47:3612–3624.

25. Sonoda S, Spee C, Barron E, Ryan SJ, Kannan R, Hinton DR. A protocol for the culture and differentiation of highly polarized human retinal pigment epithelial cells. *Nat Protoc*. 2009;4: 662–673.

26. Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Exp Eye Res*. 1996;62:155–169.

27. Iulianella A. Cutting thick sections using a vibratome. *Cold Spring Harb Protoc*. 2017;2017:pdb.prot094011.

28. Parinot C, Rieu Q, Chatagnon J, Finnemann SC, Nandrot EF. Large-scale purification of porcine or bovine photoreceptor outer segments for phagocytosis assays on retinal pigment epithelial cells. *J Vis Exp*. 2014;94:52100.

29. Croze RH, Buchholz DE, Radeke MJ, et al. rock inhibition extends passage of pluripotent stem cell-derived retinal pigmented epithelium. *Stem Cells Transl Med*. 2014;3: 1066–1078.

30. Ben M'Barek K, Habeler W, Plancheron A, et al. Human ESC-derived retinal epithelial cell sheets potentiate rescue of photoreceptor cell loss in rats with retinal degeneration. *Sci Transl Med*. 2017;9:eaai7471.

31. Capowski EE, Simonett JM, Clark EM, et al. Loss of MITF expression during human embryonic stem cell differentiation disrupts retinal pigment epithelium development and optic vesicle cell proliferation. *Hum Mol Genet*. 2014;23:6332–6344.

32. Singh R, Phillips MJ, Kuai D, et al. Functional analysis of serially expanded human iPS cell-derived RPE cultures. *Invest Ophthalmol Vis Sci*. 2013;54:6767–6778.

33. Kamao H, Mandai M, Wakamiya S, et al. Objective evaluation of the degree of pigmentation in human induced pluripotent stem cell-derived RPE. *Invest Ophthalmol Vis Sci*. 2014;55: 8309–8318.

34. Reichman S, Terray A, Slembrouck A, et al. From confluent human iPS cells to self-forming neural retina and retinal pigmented epithelium. *Proc Natl Acad Sci U S A*. 2014;111: 8518–8523.

35. DiStefano T, Chen HY, Panebianco C, et al. Accelerated and improved differentiation of retinal organoids from pluripotent stem cells in rotating-wall vessel bioreactors. *Stem Cell Reports*. 2018;10:300–313.

36. Volkner M, Zschatzsch M, Rostovskaya M, et al. Retinal organoids from pluripotent stem cells efficiently recapitulate retinogenesis. *Stem Cell Reports*. 2016;6:525–538.

37. Meyer JS, Howden SE, Wallace KA, et al. Optic vesicle-like structures derived from human pluripotent stem cells facilitate a customized approach to retinal disease treatment. *Stem Cells*. 2011;29:1206–1218.

38. Leach LL, Croze RH, Hu Q, et al. Induced pluripotent stem cell-derived retinal pigmented epithelium: a comparative study between cell lines and differentiation methods. *J Ocul Pharmacol Ther*. 2016;32:317–330.

39. Vugler A, Carr AJ, Lawrence J, et al. Elucidating the phenomenon of HESC-derived RPE: anatomy of cell genesis, expansion and retinal transplantation. *Exp Neurol*. 2008;214: 347–361.

40. Hazim RA, Karumbayaram S, Jiang M, et al. Differentiation of RPE cells from integration-free iPS cells and their cell biological characterization. *Stem Cell Res Ther*. 2017;8:217.

41. Idelson M, Alper R, Obolensky A, et al. Directed differentiation of human embryonic stem cells into functional retinal pigment epithelium cells. *Cell Stem Cell*. 2009;5:396–408.

42. Liu Y, Ye F, Li Q, et al. Zeb1 represses Mitf and regulates pigment synthesis, cell proliferation, and epithelial morphology. *Invest Ophthalmol Vis Sci*. 2009;50:5080–5088.

43. Alge CS, Suppmann S, Priglinger SG, et al. Comparative proteome analysis of native differentiated and cultured dedifferentiated human RPE cells. *Invest Ophthalmol Vis Sci*. 2003;44:3629–3641.