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. Dysfunction or cell death of retinal pigment epithelium (RPE) is associated with the pathogenesis of both RP and AMD.1 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 (hPSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), with different protocols.9–11 While most protocols have used two-dimensional (2D) adherent conditions to induce hPSCs into RPE cells by spontaneous or directed differentiation,12–14 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 hiPSC-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...
hiPSC-RPE concomitantly generated with the same retina
gonoid induction system reported15 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 episcopal 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
passed every 5 to 7 days. To differentiate hiPSCs into RPE cell
fates, we applied a published protocol for retinal gonoid
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 supple-
ment (Invitrogen Corp., Carlsbad, CA, USA), 1X 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), 1X NEAA and 1X 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 cm² 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 passed 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% CO₂ 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 accord-
ing 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 Oph-
thalmic 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% paraformal-
dehyde (PFA) for 30 minutes on ice. The tissues were prepared
for frozen sections with the methods published15 or embedded
in 4% to 5% agarose for vibratome sections and cut in 50- to 70-
μm thickness.27 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 micro-
scope (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 protocol28 and labeled with
CM-Dil (C7001; Invitrogen) following manufacturer instruc-
tions. The phagocytosis assay was performed as previously
described.18,29,30 See supplementary materials for details.

**Transeptalional 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 voltohm-
eter (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·cm² 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·cm² were used to conduct this experiment.
Media cultured for 24 hours were collected separately from the
apical and basolateral chambers. The VEGF level was measured with
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 × 10⁶ 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

![Schematic view of RPE differentiation and spheroids formation from hiPSCs.](image)

**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 (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.
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). 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 $\times$ $10^6$ 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. 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). 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.
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. 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.

**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. 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). Immunohistochemistry showed that RPE specific transcrip-
tion 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
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
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-RPE 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. 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, 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. This procedure has been described in detail in a study reported by Vugler and colleagues, 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 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. Hence, this time-consuming and labor-intensive method requires skillful, experienced researchers. Slightly modified from the above approach, Julien and colleagues picked and dissociated deeply pigmented hiPSC-RPE cells from different passages (P1, P3, and P5). The expression level of these genes were not significantly different among P3 and P5. However, the expression level of MITF, OTX2, PAX6, PEDF and RPE65 in hiPSC-RPE 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) P3, and (C) hRPE. Mean ± SEM, *$P < 0.05$, **$P < 0.005$.

350 Ω·cm$^2$ (similar to those of hRPE; Fig. 7A) and significantly above the TER of 150 Ω·cm$^2$ in naive RPE. 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. 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-RPE 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.

![Figure 6](https://example.com/f6.png)

**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 increased, 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) P3, and (C) hRPE. Mean ± SEM, *$P < 0.05$, **$P < 0.005$.

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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). 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. 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 \( \times 10^4/cm^2 \)) 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 \( 3 \times 10^{11} \) 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. 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. 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 vivo32; further, more RPE cells are ideal for large-scale RPE cell banking. In this study, we demonstrated that non-pigmented, passages RPE cells (up to 8 times 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 markers33,39 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 RPE spheroids can self-form pigmented spheroids in suspension condition, generated with NRs on retinal organoid induction platform replacing degenerated RPE cells in AMD.

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