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Induction of Human Umbilical Mesenchymal Stem Cell Differentiation Into Retinal Pigment Epithelial Cells Using a Transwell-Based Co-culture System

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Abstract

There is an increasing interest in generating retinal pigment epithelial (RPE) cells from stem cells for treating degenerative eye diseases. However, whether human umbilical cord mesenchymal stem cells (HUCMSCs) can differentiate into RPE-like cells in a co-culture system has not been fully understood. In this study, induction of HUCMSC differentiation into RPE-like cells was performed by co-culturing HUCMSCs and a human RPE-like cell line (ARPE19) in a transwell system and then analyzed for biomarkers using quantitative reverse transcription polymerase chain reaction (RT-PCR) and immunofluorescence staining technique. Moreover, the functional characterization of induced cells was carried out by examining their phagocytic and neurotrophic factor–secreting activities. Our results showed that mRNA expressions of RPE-specific markers—MITF, OTX2, RPE65, PEDF, PME17, and CRALBP—and protein markers—RPE65, CRALBP, and ZO-1—were significantly increased in HUCMSC-derived RPE-like cells. Functional characteristic studies showed that these induced cells were capable of engulfing photoreceptor outer segments and secreting brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF), which are typical functions of RPE-like cells. Overall, the study findings indicate that the morphology and proliferation of HUCMSCs can be maintained in a serum-free medium, and differentiation into RPE-like cells can be induced by simply co-culturing HUCMSCs with ARPE19 cells. Thus, the study provides fundamental information regarding the clinical-scale generation of RPE-like cells from HUCMSCs.

Keywords

umbilical cord, mesenchymal stem cells, retinal pigment epithelium, differentiation, transwell

Introduction

Age-related macular degeneration (AMD) is the most common type of eye disease characterized by the gradual degeneration of photoreceptors and retinal pigment epithelial (RPE) cells[1]. In the early phase of AMD, thickening of the vitreous lamina and choroidal blood vessels occurs, which is followed by atrophy of RPE cells and vision loss[2]. RPE cells

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have multiple functions, such as the formation of the blood–retinal barrier, protecting oxidative stress–induced retinal damage, supply of nutrients and removal of waste materials, production of growth factors, maintaining ocular immunity, and phagocytosis of photoreceptor outer segments[3,4]. Because RPE cells are not capable of self-regeneration, the majority of treatment options for retinal degeneration primarily focus on delaying the rate of degeneration. Recently, stem cell–based therapies have drawn significant attention as a promising approach for replacing degenerated RPE cells and correcting visual loss[5]. In such approaches, stem cells from a wide variety of retinal and non-retinal sources are used, including mesenchymal, neural, and embryonic stem cells (non-retinal origin) and ciliary epithelia–derived stem cells and RPE stem cells (retinal origin)[6–8]. Currently, several clinical trials are undergoing to investigate the safety and efficacy profiles of stem cell transplantation in retinal degeneration[9–11].

A growing pool of evidence has indicated the effectiveness of human umbilical cord mesenchymal stem cells (HUCMSCs) in treating retinal diseases[6,12–14]. For instance, transplantation of HUCMSCs into the vitreous cavity of adult rats has been found to repair acute optical nerve injury by inhibiting apoptosis and stimulating regeneration of retinal ganglion cells[15]. Similarly, transplantation of HUCMSCs in the transected optical tract of neonatal rats has been found to promote neural repair by rescuing and regenerating injured neurons[16]. In contrast, there is evidence suggesting that although HUCMSCs grafted inside the vitreous cavity of immune-suppressed rats can survive for a long period, they are incapable of differentiating into retinal ganglion cells[17]. Regarding other retinal cell types, there is a handful of evidence indicating the functional association between HUCMSCs and RPEs[18–20]. To explore the usefulness of HUCMSCs in retinal degeneration, the present study was designed to investigate whether HUCMSCs can differentiate into RPE-like cells in a co-culture system.

Materials and Methods

Isolation of HUCMSCs

This study protocol was approved by the Research Ethics Committee of Hualien Tzu Chi Hospital (IRB No. 104-95A). Informed consent was obtained from the mother before the delivery of a baby. Briefly, a human umbilical cord was collected and stored in phosphate-buffered saline (PBS) (Gibco, Grand Island, NY, USA) before transferring to the laboratory. After separating three vessels and amnion membranes, the Wharton jelly (WJ) part was cut into small pieces and cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM-LG, Gibco) containing 10% fetal bovine serum (FBS; Biological Ind., Kibbutz, Israel). The culture was maintained at 37°C inside an incubator (95% air / 5% CO_2) for 5–7 days. Next, the explants were washed and migrated cells were collected (HUCMSC passage 1). The cells were passaged every 7 weeks at a 1:3 ratio. After three to seven passages, HUCMSCs were used for the experiments.

Flow Cytometry of HUCMSCs

The surface markers of HUCMSCs were characterized using flow cytometry. Using PBS solution containing Accutase (Millipore, Billerica, MA, USA), the HUCMSCs were detached for 5 min, followed by washing with PBS containing 2% bovine serum albumin (Sigma) and 0.1% sodium azide (Sigma). Next, the cells were incubated with a panel of fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin-conjugated antibodies, namely, CD29, CD34, CD44, CD45, CD73, CD105, and HLA-ABC (BD, Franklin Lakes, NJ, USA). Finally, the cells were analyzed using a flow cytometer (BD, San Jose, CA, USA).

Differentiation of HUCMSCs

Adipogenesis. The HUCMSCs (5×10^4) were seeded onto a 12-well plate and cultured for 14 days in the adipogenic medium comprising DMEM, 10% FBS, 0.5 mmol/l isobutylmethylxanthine, 5 μg/ml insulin, 1 μmol/l dexamethasone, and 60 μmol/l indomethacin (Sigma). The medium was changed every 3 days. To examine adipogenic differentiation, the cells were stained with Oil Red O (Sigma), and the presence of intracellular oil drops was determined under a microscope (Nikon, Tokyo, Japan).

Osteogenesis. The HUCMSCs (1×10^4 cells) were seeded onto a 12-well plate and cultured for 14 days in the osteogenic medium comprising DMEM, 10% FBS, 10 mmol/l β-glycerol phosphate, 0.1 μmol/l dexamethasone, and 50 μmol/l ascorbic acid (Sigma). The medium was changed every 3 days. To examine osteogenic differentiation, the cells were stained with Alizarin Red S (Sigma), and the presence of intracellular minerals was investigated under a microscope (Nikon).

Chondrogenesis. The HUCMSCs (25×10^6/ml) were seeded on the bottom of a 15-ml conical tube containing 30 μl of chondrogenic medium [DMEM, 10% FBS, DMEM, 10% FBS, 10 ng/ml of transforming growth factor-β1 (Pepro Tech, Rocky Hill, NJ, USA), 6.25 μg/ml of insulin (Sigma-Aldrich), and 50 μg/ml of ascorbic acid-2-phosphate (Sigma-Aldrich)]. The cells were cultured for 21 days, with medium changing every 3 days. To examine chondrogenic differentiation, the cells were stained with Alcian blue (Sigma), and the presence of intracellular glycosaminoglycans was determined under a microscope (Nikon).

Human RPE Cell Line

The human RPE cell line ARPE19 was cultured in DMEM/F12 (1:1; Invitrogen, Grand Island, NY, USA) containing

Table 1. Primer Sequences.

Genes	Forward	Reverse	Product size (bp)
MITF	CCCAGGCCCAGCTACCTTCC	GGCACGATCCCCGATTCGGA	104
OTX2	ACCCGGTAGTGTGTCCCGCT	TCGCCGCTCTCTCCAGGGT	208
Tyrosinase	TGCCAACGATCCTATCTTCC	GACACAGCAAGCTCACAAGC	52
RPE65	GTCCTCGCGCTCACAGCTC	GCAGGAGGGCTTGCCATCAAA	137
Bestrophin	TCCCACCTGCCTAGTCGCCA	TTGTAGATGCTGCCCGCCA	186
PEDF	GGGAGCGGAGCAGGGAACAG	GGTCCAAGCGAGGGTTGCCC	248
PMEL17	TGCCTGTGCCTGGGATTCTTCTCA	GATGCGGGGTACACGCAGCC	194
CRALPB	CCCCGCCACACCTTGCAGAA	TTCCGTGCGCGGATGAAGGG	191
GAPDH	TCTCCTCTGACTTCAACAGCGAC	CCCTGTTGCTGTAGCCAAATTC	126

10% FBS and antibiotics (penicillin and streptomycin; ThermoFisher, Waltham, MA, USA). The experiments with ARPE19 cell line were conducted at 75% confluency.

Co-culture of HUCMSCs With ARPE19 Cells

The co-culture experiment was performed in a 12-well transwell dish. The HUCMSCs (1×10^5 cells/ml, 2×10^5 cells/ml, or 5×10^5 cells/ml) were seeded at the bottom well and the ARPE19 cells (1×10^5 cells/ml) were seeded in the upper inserts (8-μm pore size; BD) containing DMEM/F12 serum-free medium. The cells were co-cultured for 14 days, with medium changing every 3 days.

Quantitative RT-PCR

The total RNA from HUCMSC-RPE was extracted using RNeasy Protect Mini Kit (Qiagen, Hilden, Germany), and reverse transcription polymerase chain reaction (RT-PCR) was performed using SuperScript III One-Step RT-PCR kit (Invitrogen). The complementary DNA (cDNA) samples obtained from RT-PCR were used for quantitative PCR (qPCR) with a FastStart SYBR Green QPCR Master (Rox) (Roche, Indianapolis, IN, USA), using a quantitative real-time PCR detection system (ABI Step One Plus system; Applied Biosystems, Foster City, CA, USA). A melting stage was added at the end of the amplification procedure (94°C for 10 s, 60°C for 30 s for 40 cycles). The melting curve was analyzed to rule out the possibility of nonspecific amplification. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The specific primer sequences are listed in Table 1.

Immunocytochemistry

For immunohistochemistry, the cells were initially fixed in 4% paraformaldehyde (Sigma) for 20 min, followed by permeabilization with 0.3% Triton-X 100 for 5 min at room temperature. Next, the cells were incubated with 10% sheep serum for 30 min at 37°C for protein blocking. To characterize the differentiated RPE-like cells, the following primary antibodies were used: RPE65 (MAB5428, 1:100; Merck, Darmstadt, Germany), CRALBP (ab1501, 1:100; Abcam, Cambridge, UK), and zonula occludens-1 (ZO-1, LS-B9774, 1:100; LifeSpan BioSciences, Seattle, WA, USA). The incubation with primary antibodies was conducted at 4°C overnight, followed by incubation with FITC-conjugated secondary antibodies at room temperature for 1 h. The ARPE19 cells served as a positive control.

Phagocytosis Assay

The confluent HUCMSC-differentiated RPE-like cells were cultured with a concentration of 10^7 particles/ml of 1 μm microsphere (polystyrene) which contained fluorescence (FluoSphere; Invitrogen) for 6 h. In the meantime, an anti-MERTK antibody (Invitrogen) was added or not for the experiments[21]. Then the cells were washed with PBS thrice and fixed with 4% formaldehyde. Then we used fluorescence microscopy to observe and count the remaining microspheres.

Enzyme-Linked Immunosorbent Assay

The neurotrophic factors [brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF)] secreted by HUCMSCs, ARPE19, or HUCMSC-differentiated RPE-like cells were measured using quantitative sandwich ELISA kit (Sigma). These three cell types (5×10^4 cells) were seeded onto a 24-well plate for 3 days. Then, the conditioned medium was collected, centrifuged, and stored at −20°C until analyzed via enzyme-linked immunosorbent assay (ELISA).

Statistical Analysis

All data are expressed as mean ± standard deviation (SD). To compare between the two groups, the data were analyzed by Student's t test. For comparing between more than two groups, the data were analyzed by analysis of variance (ANOVA) together with the post hoc Bonferroni test. All statistical analyses were conducted via SPSS 22 software (IBM, New York, NY, USA). A p value of ≤ 0.05 was considered statistically significant.

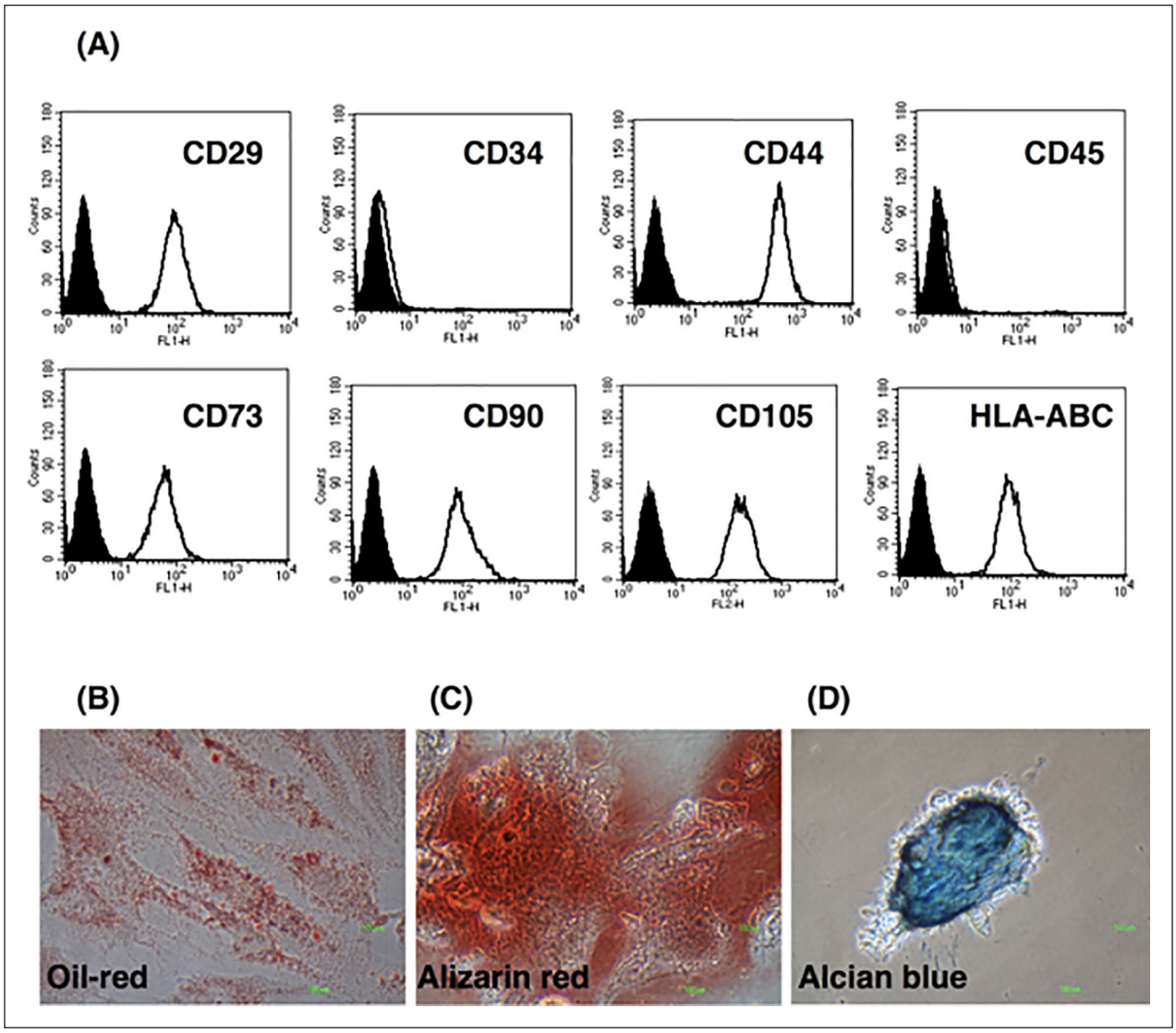

Figure 1. Characterization of HUCMSCs. (A) Representative flow cytometry histograms of HUCMSCs at passage 3 are negative for CD34 and CD45, but positive for CD29, CD44, CD73, CD90, CD105, and HLA-ABC. (B) Adipogenesis of HUCMSCs for 14 days shows a positive Oil Red staining. (C) Osteogenesis of HUCMSCs for 14 days shows a positive Alizarin Red staining. (D) HUCMSCs cultured under the chondrogenesis medium for 3 weeks show a positive Alcian blue staining. Scale bar=100 μm. HUCMSCs: human umbilical cord mesenchymal stem cells.

Results

Characterization of HUCMSCs

To investigate the mesenchymal characteristics of HUCMSCs, specific surface markers and mesoderm differentiation ability of HUCMSC were analyzed using flow cytometry and staining-based assays. The flow cytometric analysis of HUCMSCs at passage 3 showed that HUCMSCs were negative for CD34 and CD45 and positive for CD29, CD44, CD73, CD90, CD105, and HLA-ABC (Fig. 1A). These findings reveal that the characteristic features of HUCMSCs are similar to bone marrow–derived MSCs.

After culturing under adipogenic conditions for 14 days, the cytoplasm of differentiated HUCMSCs displayed large Oil Red O–positive lipid droplets (Fig. 1B). Similarly, after culturing under osteogenic conditions for 14 days, the differentiated HUCMSCs became positive for Alizarin Red staining (Fig. 1C). When cultured under chondrogenic conditions for 3 weeks, the HUCMSCs became positive for Alcian blue staining (Fig. 1D). Taken together, these findings clearly indicate that HUCMSCs are capable of fully differentiating into adipocytes, osteocytes, and chondrocytes.

Differentiation of HUCMSCs Into RPE-Like Cells

To induce RPE differentiation of HUCMSCs, both cell types were co-cultured in a transwell system. Three concentrations of HUCMSCs were tested (1×10^5 cell/ml, 2×10^5 cell/ml,

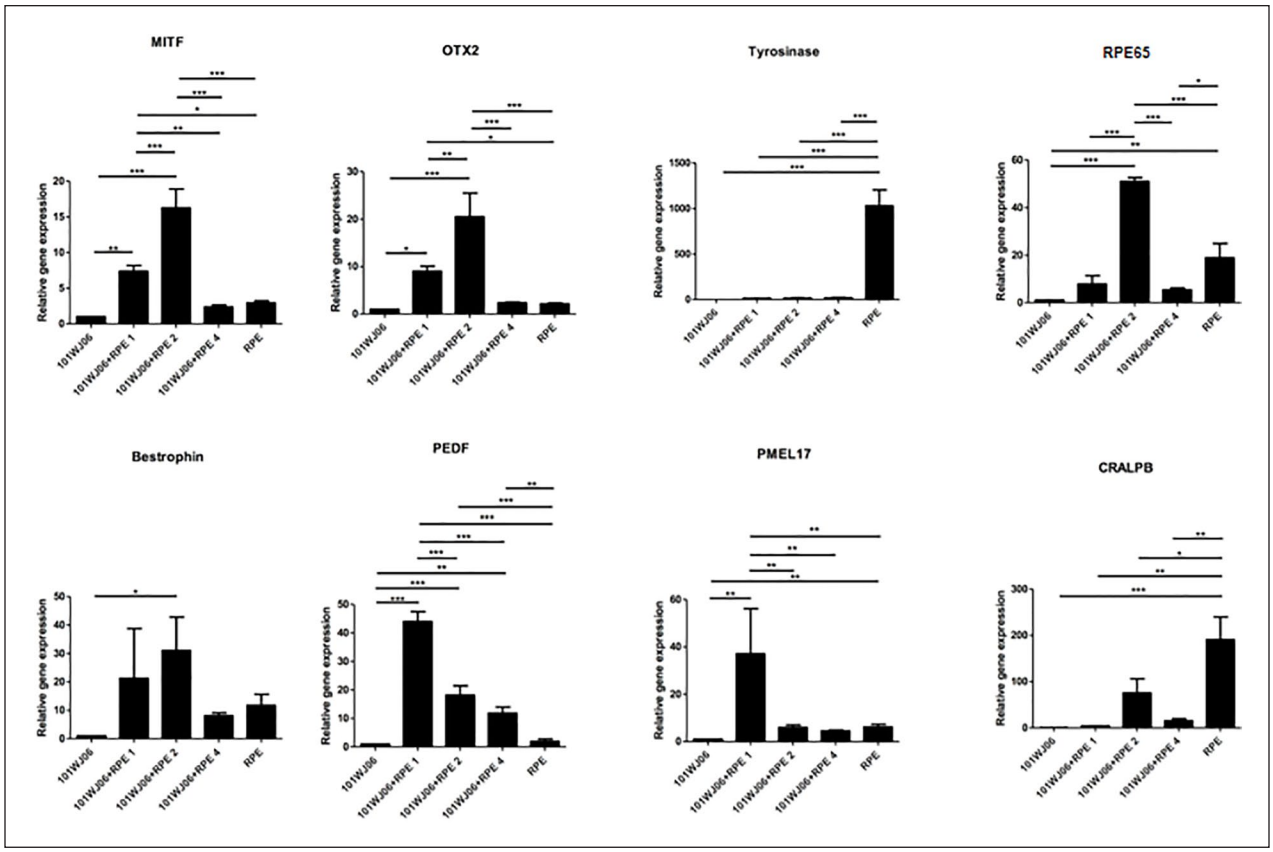

Figure 2. Determination of RPE-specific genes in HUCMSC-derived RPE-like cells following co-culture induction. The real-time PCR data demonstrate that HUCMSC-derived RPE-like cells express key RPE-specific transcription factors (mean ± SD, $n = 6$ independent experiments). RPE: retinal pigment epithelial; HUCMSCs: human umbilical cord mesenchymal stem cells; PCR: polymerase chain reaction. The ratio of HUCMSC:ARPE co-culture (1 = 1:1, 2 = 1:2, 4 = 1:4). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

4×10^5 cell/ml). After 14 days of co-culture, qRT-PCR was conducted to check the RPE-specific gene expression patterns in differentiated HUCMSCs (Fig. 2). The findings revealed that the expressions of two RPE-relevant transcription factors MITF and OTX2 increased by 15–20 folds in HUCMSC-derived RPE-like cells compared with that in control HUCMSCs without co-culture (Fig. 2). Similarly, a 40-fold increase in the expressions of other RPE-specific markers including RPE65, PEDF, and PME17 was observed in HUCMSC-derived RPE-like cells compared with that in control cells. Among all tested genes, the highest increase in gene expression was observed for CRALBP, which specifically showed a 100-fold higher expression in CRALBP than control cells. Importantly, the highest expression profile of most of the genes was observed in the 2×10^5 cells/ml HUCMSC group.

To validate RPE-specific characteristics at the protein level, immunohistochemistry was performed to evaluate the expression of RPE-associated proteins in HUCMSC-derived RPE cells (Fig. 3). A total of three RPE-specific proteins, namely, RPE65, CRALBP, and ZO-1, were included in the study. The findings revealed that both RPE65 and ZO-1 proteins were expressed in ARPE19 cells. Regarding HUCMSC-derived RPE-like cells, a weak expression of CRALBP was observed in the 1×10^5 cells/ml HUCMSC group, whereas a robust expression profile of all tested proteins was observed in the 2×10^5 cell/ml and 4×10^5 cell/ml HUCMSC groups.

The Phagocytic Activity of HUCMSC-Derived RPE-Like Cells

It is well established in the literature that the RPE cells are specialized phagocytes that play an indispensable role in clearing photo-oxidatively damaged photoreceptors and maintaining the turnover of the distal photoreceptor outer segments[22]. To examine the functionality of HUCMSC-derived RPE-like cells, polystyrene microbead–conjugated fluorescent dye was used to elucidate the phagocytic activity of differentiated HUCMSCs. The microbead was 1 μm in diameter, which is equivalent to the outer membrane of rod cells. After 6 h of co-culture, the cells with fluorescent microbeads were washed and counted under a microscope. The findings clearly revealed the intake of microspheres by HUCMSC-derived RPE-like cells (Fig. 4A, B). Because the MERTK protein is known to trigger RPE cell–mediated

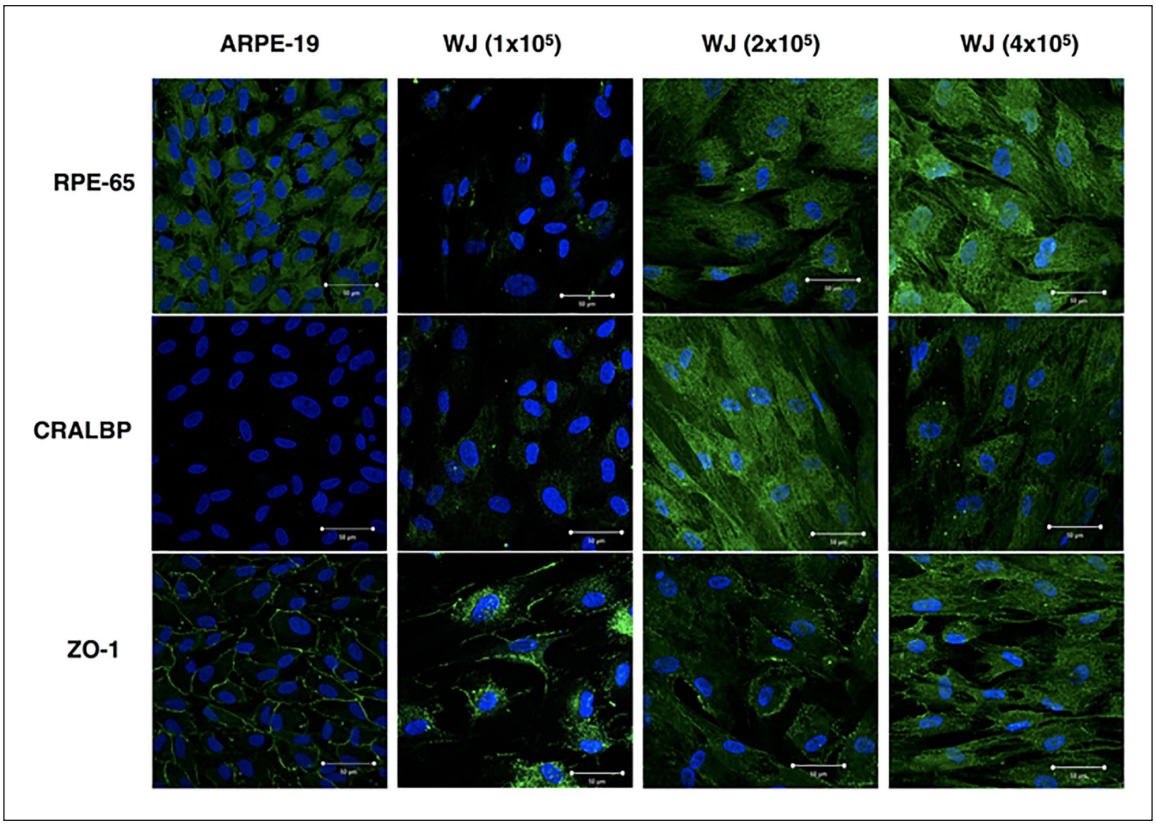

Figure 3. Determination of RPE-specific marker proteins in HUCMSC-derived RPE cells. The immunofluorescence staining data reveal that HUCMSC-derived RPE-like cells express RPE-specific makers including RPE65, CRALBP, and ZO-1. Cell nuclei were labeled with DAPI. Scale bar = 50 μm. RPE: retinal pigment epithelial; HUCMSCs: human umbilical cord mesenchymal stem cells; WJ: Wharton jelly.

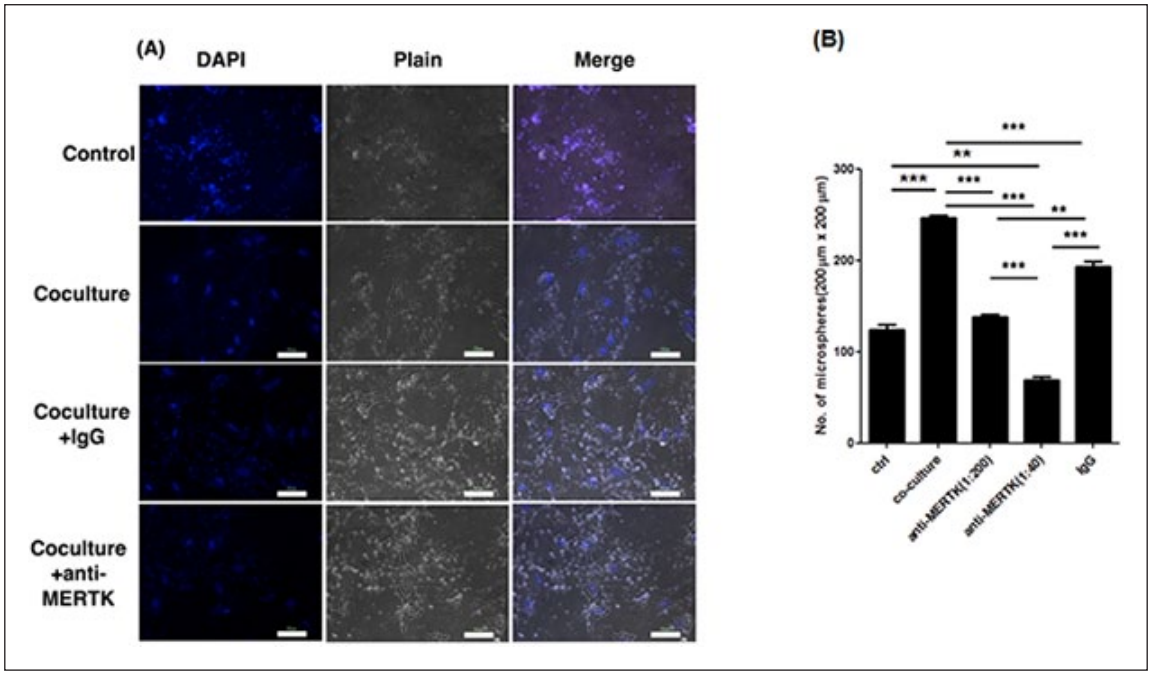

Figure 4. The phagocytotic activity of HUCMSC-derived RPE-like cells. (A) A clear intake of microspheres by HUCMSC-derived RPE-like cells indicates their phagocytic activity. The phagocytic activity of these is further validated by anti-MERTK antibodies. (B) Quantification of microspheres per 200 × 200 μm area. Error bars: SD; $n = 3$. HUCMSCs: human umbilical cord mesenchymal stem cells; RPE: retinal pigment epithelial. $^{**}P < 0.01$; $^{***}P < 0.001$.

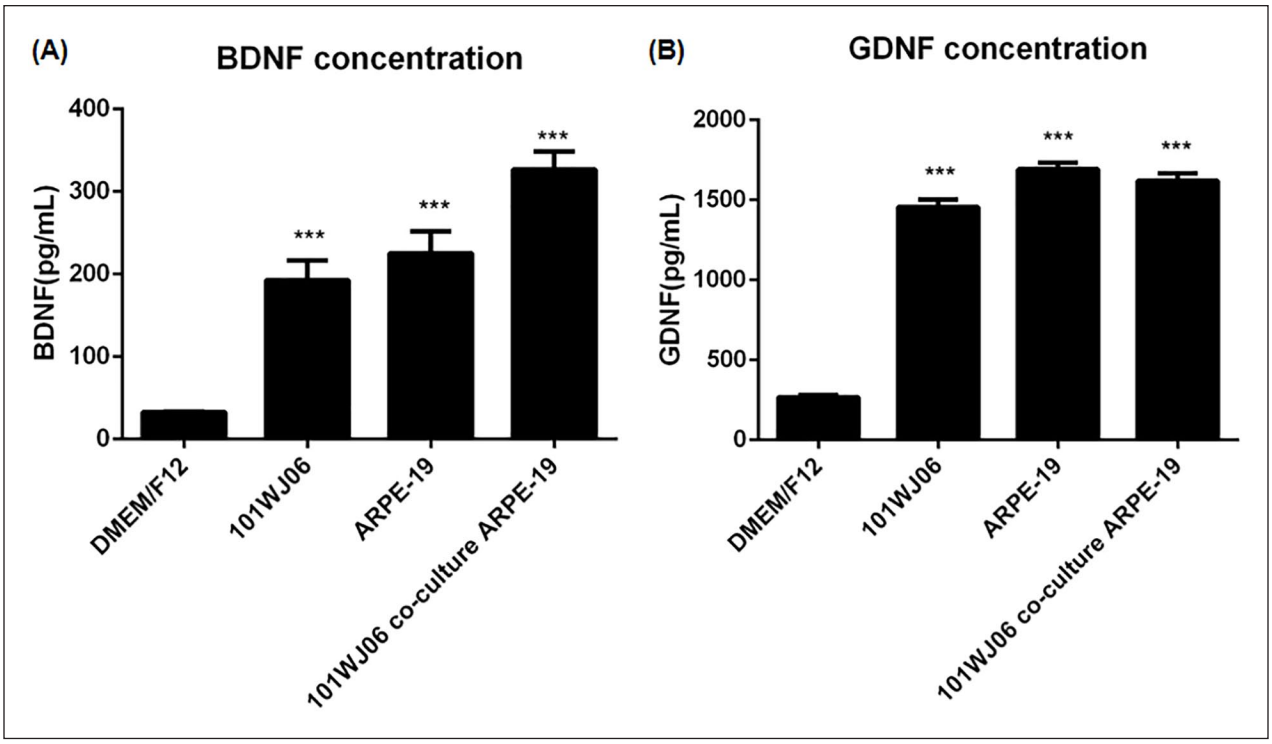

Figure 5. The neurotrophic factor–secreting ability of HUCMSC-derived RPE-like cells. The findings of the ELISA assay demonstrate the concentration of (A) BDNF and (B) GDNF secreted by HUCMSC-derived RPE-like cells. The bar diagrams represent mean ± SD from four independent experiments. HUCMSCs: human umbilical cord mesenchymal stem cells; RPE: retinal pigment epithelial; ELISA: enzyme-linked immunosorbent assay; BDNF: brain-derived neurotrophic factor; GDNF: glial-derived neurotrophic factor. ***$P < 0.001$ compared with medium control.

uptake of photoreceptors[23] the phagocytic activity of HUCMSC-derived RPE-like cells was further validated using anti-MERTK antibody. The findings revealed that in the presence of anti-MERTK antibodies, HUCMSC-derived RPE-like cells failed to engulf microspheres ($P < 0.001$). Taken together, these findings clearly indicate that HUCMSC-derived RPE-like cells act as phagocytes, which is a characteristic feature of RPE-like cells.

Secretion of Neurotrophic Factors by HUCMSC-Derived RPE-Like Cells

Previously, it has been observed that RPE-like cells secrete different types of neurotrophic factors that play several essential functions, such as nourishment of the neurosensory retina and prevention of phototoxic damage of photoreceptors[24,25]. In the present study, the neurotrophic factor–secreting ability of HUCMSC-derived RPE-like cells was assessed using ELISA. The findings revealed that compared with the control cells, the concentration of BDNF was significantly higher in HUCMSCs, ARPE19 cells, and HUCMSC-derived RPE-like cells (Fig. 5A). However, the highest secretion of BDNF was observed in HUCMSC-derived RPE-like cells. In contrast, all three types of cells showed a comparable secretion level for GDNF (Fig. 5B). Taken together, the findings reveal that although HUCMSCs are capable of secreting neurotrophic factors, the level of secretion is considerably higher in HUCMSC-derived RPE-like cells.

Discussion

The present study has developed and validated a co-culture model system to induce the differentiation of HUCMSCs into RPE-like cells. The observations made in the study reveal that by co-culturing HUCMSCs and ARPE19 cells (a human RPE cell line) in a transwell system for 14 days, it is possible to induce HUCMSC differentiation and produce RPE-like cells. This study is aimed to improve the RPE differentiation procedure for treating retinal diseases such as retinitis pigmentosa and macular degeneration through cell replacement therapy.

There have been many studies on the differentiation of stem cells to retinal cells[26] and culturing retinal cells alone[27,28]. Nevertheless, limited studies use the co-culture of retinal cells with RPE or choroid layer[29,30]. The current study showed RPE cells reserve important properties including tight junction, intracellular microfilaments, and morphology, which are fundamental for their function. Moreover, HUCMSCs can be differentiated into retinal progenitors by using the neural progenitor–conditioned medium[31]. One

previous study co-culturing human bone marrow stromal cells (HBMSCs) with pig RPE cells has shown that after 14 days of co-culture, the level of intracellular pigment granules in HBMSC-derived RPE cells becomes comparable to that in pig-derived primary RPE cells[32]. Another study has been shown three methods of co-culturing rabbit RPE and progenitors derived from human embryonic stem cells[33]. They found progenitor cells could express more early photoreceptor markers after 14 days (indirect co-culture, using an insert) and direct cell–cell contact co-culture than culture after 7 days[33]. Taken together, co-culture with RPE can facilitate stem cell–differentiated retinal-related cells.

Another advantage of the co-culture method is that it could mimic *in vivo* environments *in vitro*. In the human retina, the RPE cells nourish the neural retina. The microcapillaries between RPE and the neural retina supply oxygen and metabolites[34]. The co-culture technique can enable optimization of regenerative cell therapy for degenerative retinal diseases *in vitro*[34]. In our study, the co-cultures were allowed to survive up to 14 days and displayed RPE-like characteristics. Our previous study has shown that in a co-culture model of HUCMSCs and ARPE19, ARPE19-secreted PEDF could inhibit apoptosis in a serum-free medium and allowed HUCMSC proliferation[20]. This results are in evidence with the previous finding, a suitable time period of 14 days for the successful differentiation of RPE cells[33]. Another study also showed 14 days of co-culturing with RPE promoted differentiation of bone marrow stem cells into RPE-like cells[32]. However, the mechanisms of such interactions between these cells need further elucidation.

One of the interactions between RPE and photoreceptors is the secretome of RPE. In our previous study, the ARPE19 secretome analysis has identified a total of 77 extracellular proteins[20], including classical and non-classical secreted proteins and transmembrane proteins, which might play essential roles in inducing HUCMSC differentiation[35]. The microenvironment of the retina also contains many different kinds of cytokines which may contribute to the RPE differentiation of HUCMSCs[36].

For clinical application, the differentiated RPE can be transplanted in degenerative retinal diseases such as retinitis pigmentosa and macular degeneration. Stem cells derived from the patient himself are free for immune rejection. However, the differentiation potential of adult stem cells is less than fetal stem cells[37]. The fetal stem cells such HUCMSCs used in the current study showed promising differentiation and immunomodulation capabilities[37]. There are also studies using embryonic stem cell or pluripotent stem cell (iPSC)-differentiated RPE to treat eye diseases[38,39]. However, embryonic stem cells owned ethical concerns, and iPSCs owned tumorigenicity risk[40]. There are also studies using HUCMSCs for treating eye diseases[13,41]. Therefore, using HUCMSC-differentiated RPE could be a better option to treat degenerative retinal disease.

One potential limitation of the study is that the structural and functional characterization of HUCMSC-derived RPE-like cells has been conducted in *in vitro* conditions. No animal studies have been conducted to examine whether HUCMSC-derived RPE-like cells are capable of maintaining their functionality and inducing retinal regeneration after *in vivo* transplantation. Thus, future experiments with vision-impaired animal models are necessary for the clinical characterization of HUCMSC-derived RPE-like cells.

Conclusion

In conclusion, the study findings reveal that the differentiation of HUCMSCs into RPE-like cells can be induced by simply co-culturing HUCMSCs and ARPE-19 cells in a transwell system. Specifically, the study findings demonstrate that HUCMSC-derived RPE-like cells express RPE-specific markers at both the mRNA and protein levels. Moreover, these cells possess phagocytic activity and ability to secrete neurotrophic factors, which are the key characteristics of endogenous RPE-like cells. From a clinical perspective, this transwell-based co-culture system can serve as a promising approach in the field of stem cell replacement therapies.

Author Contributions

YHC was involved in manuscript preparation, data analysis, and manuscript preparation; VBK, CYH, YTW, and RKT were involved in study design and in providing the cell line; DCD was involved in study concepts, design, and manuscript preparation.

Availability of Data and Materials

All data generated or analyzed during this study are included in this article.

Ethical Approval

This study protocol was approved by the research ethics committee of Hualien Tzu Chi Hospital (IRB No. 104-95A).

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the Hualien Tzu Chi Hospital (IRB No. 104-95A) approved protocols.

Statement of Informed Consent

Written informed consent was obtained from the patient(s) for their anonymized information to be published in this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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