Generation of retinal pigment epithelium from human induced pluripotent stem cells showed polarized secretion of VEGF and PEDF.

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ABSTRACT   Age-related macular degeneration (AMD) is one of the major diseases that cause severe visual impairment in developed countries and is related to dysfunction of the retinal pigment epithelium (RPE). Human induced pluripotent stem cells (hiPSCs) have been drawing attention as a valuable source of RPE for basic research on or treatment of AMD. Here we investigated whether RPE could be generated from hiPSCs in Kawasaki Medical School. We maintained hiPSCs retaining pluripotent markers after a series of regular culture steps involving passaging, freezing, and thawing. hiPSCs were then cultured in RPE differentiation medium, and pigmented colonies were manually isolated for further differentiation into RPE. Differentiated cells formed pigmented cells with a typical RPE cobblestone appearance, abundant apical microvilli, adherens junctions, and tight junctions. Furthermore, generated pigmented cells expressed typical RPE marker genes, exhibited a barrier function, and secreted growth factors in a polarity-dependent manner similar to native RPE. These results indicate that RPE derived from hiPSCs in our facility can be used for in vitro and in vivo research.

INTRODUCTION

Age-related macular degeneration (AMD)[1] is caused by dysfunction of the retinal pigment epithelium (RPE), which plays an essential role in the maintenance of photoreceptors[2] and a healthy ocular environment. The recent introduction of treatments such as laser therapy[3], photodynamic therapy[4], and anti-vascular endothelial growth factor therapy[5] has resulted in significant overall maintenance or improvement of vision in AMD patients; however, a large number of AMD patients still have severe visual impairment with the limited effects of these therapies, such as non-responders, dry AMD, or advanced AMD. RPE transplantation with the aim of replacing pathological RPE with healthy RPE was introduced more than two decades ago, and RPE transplantation for AMD has been reported using allologous fetal RPE[6] or autologous peripheral RPE[7]. In summary, the former exhibited complications associated with immune rejection, while the latter required invasive surgery to harvest peripheral RPE and place them in the submacular

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area. Therefore, RPE derived from autologous human induced pluripotent stem cells (hiPSCs)[8] emerged as an ideal alternative cell source to overcome these disadvantages.

hiPSCs are typically reprogramed by transfecting stem cell-associated genes into adult somatic cells. Since hiPSCs share major characteristics with embryonic stem cells such as pluripotency, self-renewal capacity, gene/protein expression, and epigenetic status, hiPSCs have been generated from various resources for research on and treatment of various diseases including retinitis pigmentosa[9], Parkinson's disease[10], and cardiovascular disease[11]. The hiPSC culture is completely different from other types of cell lines and requires specialized methods. Here we investigated whether a widely used hiPSC line could be cultured that retained pluripotency markers after a series of culture steps involving passaging, freezing, and thawing, and also tested if we could differentiate hiPSC-derived RPE (hiPSC-RPE) from them in our facility.

MATERIALS AND METHODS

Culture of hiPSCs and hiPSC-RPE

The utilization of hiPSCs was approved by the Research committee of Kawasaki Medical School. The hiPS cell line, 253G1 produced from healthy human dermal fibroblast cells using 3 transcription factors (Oct3/4, Sox2, and Klf4) was purchased from RIKEN BioResource Center. These hiPSCs were seeded on Mitomycin C-treated mouse feeder cells (SNL cells) after frozen hiPSCs were immediately thawed by Primate ES Cell Medium (ReproCELL). hiPSCs were maintained in Primate ES Cell Medium supplemented with 5 ng/ml bFGF (Wako) and passaged by pipetting into small cell aggregates when hiPSC colonies became large. hiPSCs were put into freeze stock after three months culture using the vitrification method. To differentiate into RPE, hiPSCs were cultured on gelatin-coated dishes in the differentiation medium (GMEM; GMEM supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 0.1 mM 2-mercaptoethanol) and 20% KnockOut Serum Replacement (KSR) for 4 days, GMEM and 15% KSR for 6 days, and GMEM and 10% KSR for 20 days. The Rho-associated kinase inhibitor Y-27632 (10 μM, Wako), the ALK4 inhibitor SB431542 (5 μM, Sigma), and the casein kinase I inhibitor CKI7 (3 μM, Sigma) was added to the differentiation medium for 18 days. After the pigmented cells focally appeared, the differentiation medium was switched to SFRM [DMEM/F12 (7:3) supplemented with B27 (Invitrogen), 2 mM L-glutamine (Sigma)] for 7 days. Pigmented colonies were then transferred to laminin-coated dishes in SFRM supplemented with 10 ng/ml bFGF (Wako) and grown until confluence was reached.

Immunocytochemistry

hiPSCs and hiPSC-RPE were fixed with 4% paraformaldehyde (PFA) and, after permeabilization with 0.3% Triton™ X-100 and blocking with blocking one (NACALAI), samples were incubated with the primary antibody overnight at 4℃ followed by secondary antibody reactions for 1 hr at room temperature. Antigens detected with primary antibodies (spices, company, and dilution) were; Oct3/4 (mouse, Santa Cruz, 1/500), Nanog (rabbit, ReproCELL, 1/500), SSEA-4 (mouse, Millipore, 1/300), Tra-1-60 (mouse, Millipore, 1/300), Pax6 (rabbit, Covance, 1/600), MITF (mouse, Abcam, 1/1000), Bestrophin (mouse, Abcam, 1/500), RPE65 (rabbit, 1/1000), and ZO-1 (rabbit, Zymed, 1/100). The bound primary antibodies were detected with secondary antibodies labeled with Alexa Fluor 546; goat anti-rabbit IgG (1/1000; Molecular Probes), or goat anti-mouse IgG (1/1000; Molecular Probes), and nuclei were stained with 4'6-diamindino-2-phenylindole (DAPI) (Molecular Probes, 1 μg/ml). Specimens were imaged using a laser-scanning confocal microscope (FV1000-D, OLYMPUS).

Transmission electron microscopy (TEM)

hiPSC-RPE were fixed in 2.5% glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated with ascending concentrations of ethanol, and infiltrated by epoxy resin. Two days after drying under the hood, ultrathin sections, about 60 nm thick, were obtained with a microtome. hiPSC-RPE were visualized with a transmission electron microscope (JEM-1400, JEOL).

Reverse transcription-polymerase chain reaction

Total RNA was extracted from confluent hiPSCs (253G1) and hiPSC-RPE (253G1) using RNeasy Plus Mini Kit® (QIAGEN) according to the manufacturer's instructions. The RNA concentration and quality were assessed with a NanoDrop 1000 spectrophotometer (NanoDrop Technoligies). The RNA was reversetranscribed using 20 μl reaction consisted of: 11 μl RNA (1,000 ng) in DNase RNase Free water (QIAGEN), 1 μl 50 μM Oligo(dT)$_{20}$ (Invitrogen), 1 μl 10 mM dNTP Mix (Invitrogen), 1 μl Rnase OUT (20 U/μl; Invitrogen), SuperScript^{TM} III Reverse Transcriptase (4 μl 5 × First-Standard Buffer, 1 μl 0.1M DTT, 1 μl SuperScript III 200 U/μl; Invitrogen) and the cDNA synthesis was performed as follows: 15 min at 25℃, 60 min at 50℃, and 15 min at 70℃. PCR reactions were performed using Ex Taq® DNA polymerase (Takara Bio). Thermal cycling conditions were performed as follows: one cycle at 94℃ for 120 sec; 30 cycles at 94℃ for 30 sec, 57℃ for 30 sec, and 72℃ for 60 s; one cycle at 72℃ for 60 sec. Primer sequences used to amplify the three RPE-specific genes are listed in Table 1.

Measurement of transepithelial electrical resistance (TER)

TER was performed according to the manufacturer's instructions of the electrical resistance system (Millicell®, Millipore) at 37℃. The TER value ($\Omega\,cm^2$) was calculated by subtracting

the value of medium alone in the insert as a blank from the experimental value and multiplying it by the area of the insert membrane.

Enzyme linked immunosorbent assay (ELISA) for vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF)

The culture medium from confluent hiPSC-RPE was collected 24 hr after the medium was changed. Secretion levels were measured by the human VEGF-ELISA Kit (eBioscience) for VEGF and human PEDF-ELISA Kit (BioVender) for PEDF, according to the manufacturers' instructions.

RESULTS

Maintenance culture and characterization of hiPSCs

To determine that we could stably culture hiPSCs in Kawasaki Medical School, we investigated if we could maintain hiPSCs that retained pluripotency markers after performing a series of culture steps involving passaging, freezing, and thawing. Firstly, provided hiPSCs (253G1) were thawed and seeded on Mitomycin C-treated SNL feeder cells (SNL). A medium change was made every day and hiPSC colonies are transferred onto new dishes with SNL feeder cells every 4 to 5 days. Three months after initiating the culture, hiPSCs were frozen and stocked in liquid nitrogen (the passage number was 20). One week after being kept as a frozen stock, thawed hiPSCs were fed again for three months (the passage number was 38). The performed procedure

Table 1. Primer sequences used to amplify the four RPE-specific genes

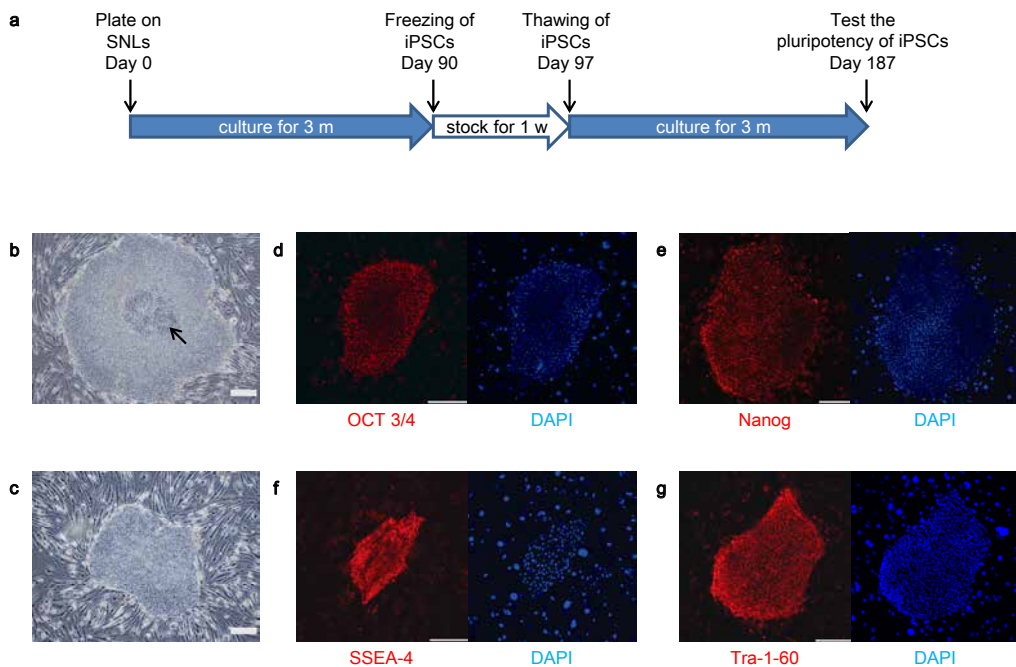| primers		Sequences
Bestrophin 1	Forward	TAGAACCATCAGCGCCGTC
	Reverse	TGAGTGTAGTGTGTATGTTGG
RPE65	Forward	TCCCCAATACAACTGCCACT
	Reverse	CCTTGGCATTCAGAATCAGG
MERTK	Forward	TCCTTGGCCATCAGAAAAAG
	Reverse	CATTTGGGTGGCTGAAGTCT
GAPDH	Forward	ACCACAGTCCATGCCATCAC
	Reverse	TCCACCACCCTGTTGCTGTA

Fig. 1. Maintenance culture and characterization of hiPSCs
(a) Culture steps involved in thawing, feeding, and freezing of hiPSCs (b) Phase image of a hiPSC colony with differentiated cells (arrow). Scale bar, 100 μm. (c) Phase image of hiPSCs. Scale bar, 100 μm (d-e) hiPSCs expressed the pluripotency markers OCT3/4, Nanog, SSEA4, and Tra-1-60. Scale bar, 200 μm.

was summarized in Figure 1a. When differentiated cells exhibiting a polygonal morphology appeared, we manually picked up the colonies including these differentiated cells (Fig. 1b). hiPSCs that went through the series of culture steps (Fig. 1c) retained the expression of pluripotency markers (OCT3/4, Nanog, SSEA-4, and Tra-1-60) in immunocytochemistry (Fig. 1d-g).

Generation of RPE from hiPSCs

hiPSCs were then differentiated to RPE using a modified protocol established previously[12]. Three weeks after initiating differentiation, pigmented colonies presented a typical RPE cobblestone appearance (Fig. 2a). To purify these cells, we manually picked up, plated them onto a laminin coat dish, and cultured them until confluency was reached (Fig. 2b). To become fully mature cells, purified pigmented cells were continuously

cultured at a confluent state (Fig. 2c, d). These pigmented cells had the structural characteristics of RPE including abundant apical microvilli, adherens junctions, and tight junctions under transmission electron microscopy (TEM) (Fig. 2e-g). Furthermore, generated pigmented cells expressed typical RPE markers related to chloride channels (BEST1; Bestrophin 1)[13], the retinoid cycle (RPE65)[14,15], and phagocytosis (MERTK)[16] in reverse transcriptase-polymerase chain reaction (RT-PCR) (Fig. 3a) and eye field specification markers (PAX6 and MITF), BEST1, RPE65, and tight junction marker (ZO-1) in immunocytochemistry (Fig. 3b-f).

Functional assessment of hiPSC-RPE in vitro

We investigated whether generated pigmented cells exhibited RPE functions typically observed in native RPE. Since RPE are known to contribute
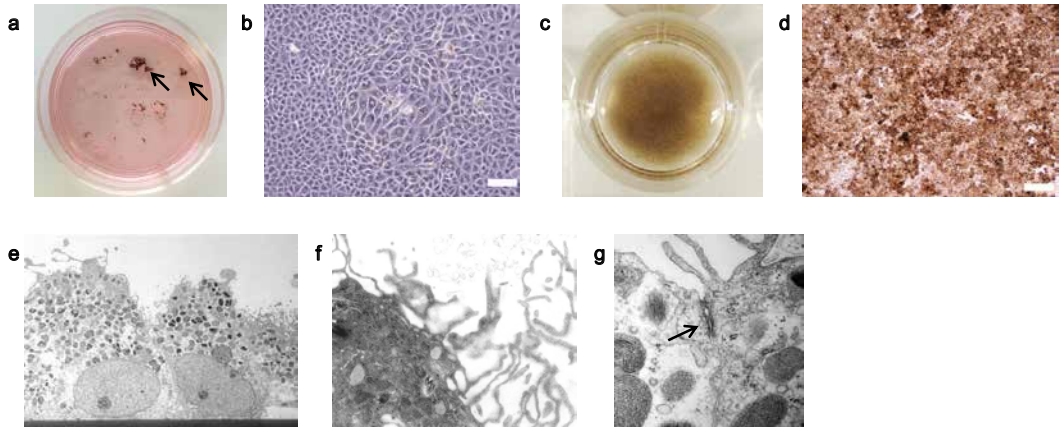
Fig. 2.   Generation of RPE from hiPSCs
(a) Approximately 3 weeks after the initiation of differentiation, some cells formed pigmented colonies with a typical RPE cobblestone appearance (arrow). (b) Phase image of transferred pigmented cells cultured until confluency. Scale bar, 100 μm. (c) Purified pigmented cells became confluent. (d) Phase image of purified pigmented cells forming a typical RPE cobblestone appearance. Scale bar, 100 μm. (e) Transmission electron microscopy image of pigmented cells, (f) apical microvilli, and (g) adherens junctions and tight junctions.
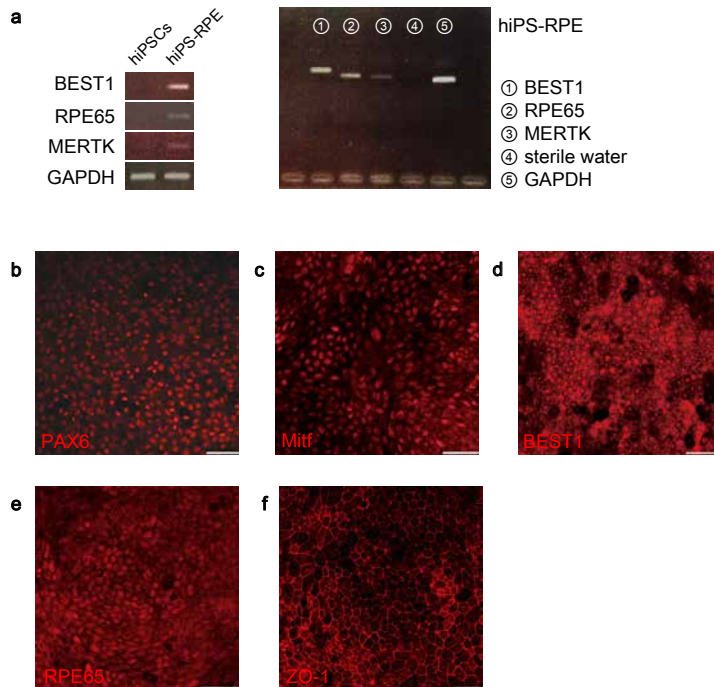

Fig. 3.   Gene expression of RPE from hiPSCs
(a) Purified pigmented cells expressed typical RPE markers (BEST1, RPE65, and MERTK) in RT-PCR. (b-f) Purified pigmented cells derived from hiPSC expressed early (MITF, PAX6) and mature (BEST1, RPE65, ZO-1) RPE markers in immunocytochemistry. Scale bar, 50 μm.

to the barrier function and secrete growth factors including vascular endothelial growth factor (VEGF)[17] and pigment epithelium-derived factor (PEDF)[18], we evaluated the functional tight junction, or a barrier function by measuring transepithelial electrical resistance (TER)[19], and measured the polarity-dependent concentration of VEGF and PEDF in the culture medium using ELISA. We seeded pigmented cells on the Transwell^{TM} for these experiments. Four weeks after they became confluent, we measured the TER and concentration of PEDF and VEGF in the apical and basal media of the Transwell^{TM}. The mean TER was 410 Ω cm^2 ($n = 6$) and concentration of VEGF was higher in the basal medium (5.9 $\pm$ 0.2 ng/well/24hr $n = 3$) than in the apical medium (1.2 $\pm$ 0.1 ng/well/24hr $n = 3$), whereas that of PEDF was higher in the apical medium (600 $\pm$ 99 ng/well/24hr $n = 3$) than in the basal medium (380 $\pm$ 27 ng/well/24hr $n = 3$) (Fig. 4). All data are expressed as mean $\pm$ SEM (standard error of the mean), with $P < 0.05$ being considered statistically significant. Statistical analyses were performed using StatMate4 (ATMS). Secretion of PEDF and VEGF was evaluated using paired t-tests.

DISCUSSION

In this study, we demonstrated that we could successfully maintain hiPSCs and induce hiPSC-RPE to be used for research in Kawasaki Medical School.

hiPSCs are currently a useful cell source for regenerative medicine, disease modeling, and drug testing, even though the hiPSC culture requires specialized methods. We had to demonstrate the pluripotency of hiPSCs and the function of generated differentiation cells. Therefore, we firstly confirmed that hiPSCs retained pluripotency after the series of regular culture steps involving passaging, freezing, and thawing. As shown Figure 1, our hiPSCs retained the expression of OCT3/4, Nanog, SSEA-4, and Tra-1-60, which indicated that we could maintain these cells without losing their pluripotency.

The limitation of existing clinical treatments for AMD has resulted in attention being given to replacing pathological RPE with healthy RPE. Research using hiPSC-RPE as an ideal cell source for transplantation is currently underway and RPE derived from embryonic stem cells is being examined in a clinical trial[20]. The differentiation methods of RPE from hiPSCs and generated hiPSC-
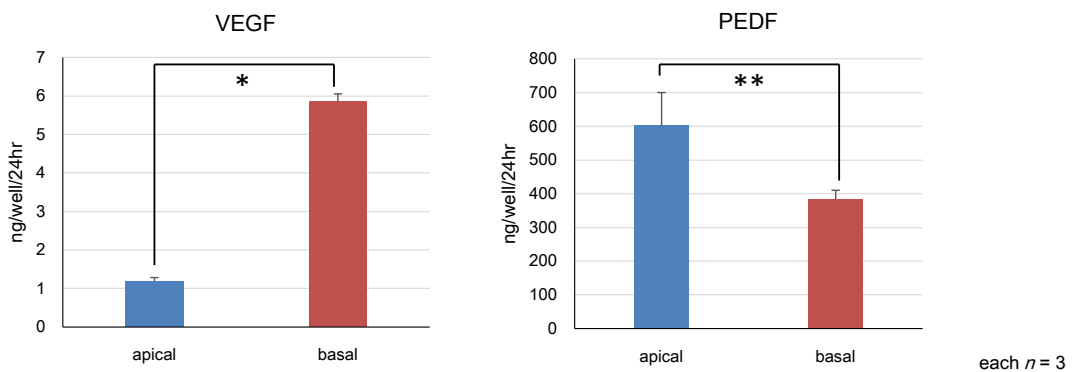

Fig. 4.   Functional assessment of hiPSC-RPE in vitro
The concentration of VEGF was higher in the basal medium (5.9 $\pm$ 0.2 ng/well/24hr $n = 3$) than in the apical medium (1.2 $\pm$ 0.1 ng/well/24hr $n = 3$), whereas that of PEDF was higher in the apical medium (600 $\pm$ 99 ng/well/24hr $n = 3$) than in the basal medium (380 $\pm$ 27 ng/well/24hr $n = 3$). All data are expressed as means $\pm$ SEM. *$P < 0.01$, **$P < 0.05$.

RPE have displayed appropriate physiological properties in vitro and in vivo in previous reports[21,22]. As was shown in the results of the present study, our differentiated RPE also exhibited morphological and genetic properties consistent with typical RPE characteristics, which indicates that they can be used as tools for further RPE research.

Another important observation in this study was that our hiPSC-RPE secreted VEGF and PEDF, as seen in Figure 4. Considering the roles of these factors in physiology and pathology, these results indicate that hiPSC-RPE transplantation could also be applied to various clinical purposes such as the protection of degenerating photoreceptors or regeneration of choroid vasculature. Therefore, we can utilize our hiPSC-RPE for clinically oriented research in vitro and in vivo in Kawasaki Medical School.

ACKNOWLEDGEMENTS

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