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Supplemental Information

Characterization of Human Induced Pluripotent Stem Cell-Derived Retinal Pigment Epithelium Cell Sheets Aiming for Clinical Application

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INVENTORY OF SUPPLEMENTARY INFORMATION

I. Supplementary Data

Figure S1, related Figure 4.

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Supplemental Figure Legends

II. Supplemental Experimental Procedures

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Figure S1. Immunogenicity of hiPSCs, hiPSC-RPE, and hRPE in vitro

Mean fluorescence intensity of MHC antigens on a second hiPSCs (59SV9), hiPSC-RPE (59SV9), and hRPE with and without IFN-γ. All cells behaved similarly to hiPSCs and hiPSC-RPE (253G1). Red line histograms represent the isotype control antibodies. Numbers in the histogram indicate the percentage of positive cells determined by isotype-specific control was less than 0.5%.

Figure S2. Immunogenicity of non-human primate iPSC-RPE cell sheets in vivo

(A-C) Image of the allogeneic miPSC-RPE cell sheet (red arrow) transplantation. Typical rejection signs such as formation of whitish fibrous tissues (red arrowhead), leakage of fluorescein (white arrow) on FA, and hyper-reflective material under the retina (white arrowhead) and retinal edema (blue arrowhead) with OCT were detected around the grafts until 1 month after surgery. Blue arrow is the injection site.

Table S1. The expression of RPE signature genes in hiPSC-RPE or ARPE19 compared with hRPE.

Group 1: under-expressed genes (fold change < −3); Group 2: similar expressed genes (fold change between −3 and +3), Group 3: over-expressed genes (fold change > +3).

Movie S1. A movie showing released hiPSC-RPE cell sheet without an artificial scaffold (pigmented circular sheet).

Movie S2. A movie showing released hiPSC-RPE cell sheet without an artificial scaffold being cut using the LMD system. The pigmented area is the hiPSC-RPE cell sheet and the transparent sphere formed after cutting by LMD system is the bubble.

Movie S3. A movie showing transplantation of miPSC-RPE cell sheet into subretinal space of monkey.

Figure S1. Immunogenicity of hiPSCs, hiPSC-RPE, and hRPE in vitro

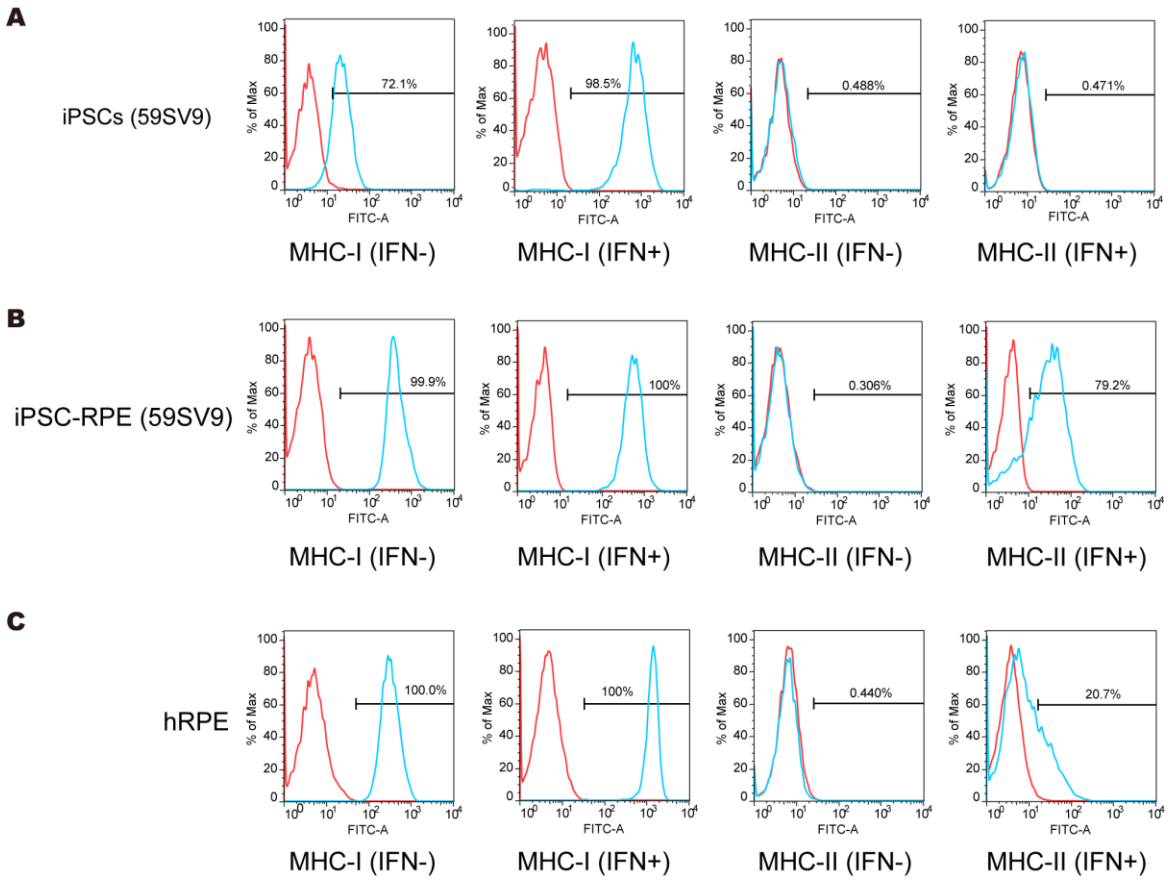
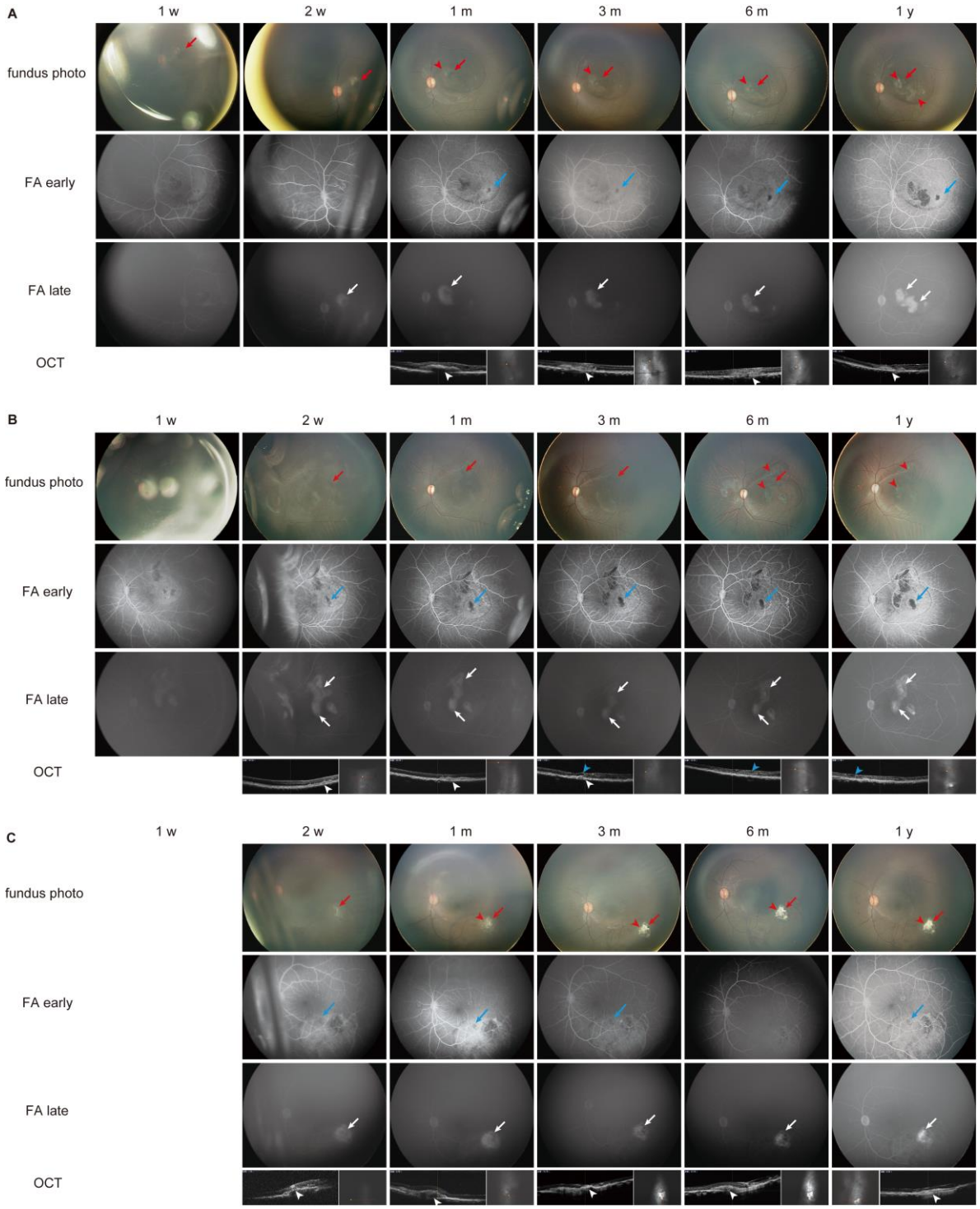


Figure S2. Immunogenicity of non-human primate iPSC-RPE cell sheets in vivo



Supplemental Experimental Procedures

Culture of human iPSCs and iPSC-RPE

The study was approved by the ethical committees of the Institute of Biomedical Research and Innovation Hospital and the RIKEN Center for Developmental Biology, Japan. Undifferentiated hiPSCs were maintained on Mitomycin C (Wako)-treated mouse embryo fibroblast feeder cells (MEFs) in Primate ES medium (ReproCELL) supplemented with 5 ng/ml basic fibroblast growth factor (bFGF, Wako) and the medium was changed every day. hiPSCs were normally passaged in small clumps after treatment with dissociation solution for iPSCs (0.25% trypsin [Invitrogen], 1mg/ml collagenase IV [GIBCO], 10% KSR [GIBCO], 1mM CaCl2, PBS [GIBCO]) and replaced onto MEFs every 4 to 6 days in average. To differentiate into RPE, hiPSCs were cultured on gelatin-coated dishes in the differentiation medium (GMEM; GMEM [Sigma] supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids [Sigma], and 0.1 mM 2-mercaptoethanol [Sigma]) and 20% KnockOut Serum Replacement (KSR, Invitrogen) for four days, GMEM and 15% KSR for six days, and GMEM and 10% KSR for 20 days. Y-27632 (10 μM, Wako), SB431542 (5 μM, Sigma), and CKI7 (3 μM, Sigma) were added to the differentiation medium for the initial 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 seven days. Pigmented colonies were then transferred to laminin (Sigma)-coated dishes in SFRM supplemented with 10 ng/ml bFGF and SB431542 (0.5 µM) and grown until confluence was reached. The differentiation media were changed every 2-3 days.

Culture of non-human primate iPSCs and iPSC-RPE

Undifferentiated miPSCs were maintained on Mitomycin C-treated STO feeder cells (STOs) in primate ES cell-maintenance medium (DMEM/F12 [Sigma] containing 20% KSR, 0.1 mM non-essential amino acidsand 0.1 mM 2-mercaptoethanol) supplemented with 5 ng/ml bFGF and the medium was changed every day. miPSCs were normally passaged in small clumps after treatment with dissociation solution for iPSCs and replaced onto STOs every 4 to 6 days in average. For induction of RPE cells, mESCs were cultured on gelatin-coated dish in PA6 stromal cells (standard SDIA method; Kawasaki et al., 2002) or cultured in the supernatant of PA6 stromal cells (modified SDIA method) in the differentiation medium (GMEM containing 10% KSR, 1mM sodium pyruvate, 0.1 mM non-essential amino acids and 0.1 mM 2-mercaptoethanol). Pigmented colonies were then transferred to laminin-coated dishes in SFRM supplemented with 10 ng/ml bFGF and SB431542 (0.5 µM) and grown until confluence was reached. The differentiation media were changed every 2-3 days.

Immunocytochemistry

hiPSCs and hiPSC-RPE were fixed with 4% PFA in 0.1 M phosphate buffer for 30 min at 4°C, permeabilized with 0.3% TritonTM X-100 for 15 min at room temperature, and blocked with blocking one (NACALAI) for 1 hr. at room temperature. hiPSCs and hiPSC-RPE were incubated with the primary antibody overnight at 4°C 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 (Molecular Probes, 1/1000), or goat anti-mouse IgG (Molecular Probes, 1/1000), and nuclei were stained with 4'6-diamindino-2-phenylindole (DAPI) (Molecular Probes, 1 μg/ml). AP staining was performed using the staining kit (Vector) per manufacturer's instructions. Samples were imaged using a laser-scanning confocal microscope (FV1000-D, OLYMPUS).

Immunohistochemistry

hiPSC-RPE cell sheets and RCS rat eyes were fixed in Superfix (Kurabo) for 10 min at 4°C, washed by PBS, and cryoprotected in 10% sucrose in PBS for 1hr and 30% sucrose in PBS

overnight at 4°C. Subsequently, they were embedded for 1 hr and rapidly frozen in OCT compound (Tissue Tec; Sakura Finetechnical Co), and cryostat sections (12 μm thickness) were cut onto charged glass slides (MATUNAMI) with a cryostat (HM560; Thermo). Specimens were permeabilized 0.3% Triton X-100 in PBS for 15 min at room temperature, blocked with blocking one for 1 hr at room temperature, and incubated with the primary antibody overnight at 4°C followed by secondary antibody reactions for 1 hr at room temperature. Antigens detected with primary antibodies (spices, company, and dilution) were; ZO-1 (rabbit, Zymed, 1/100), Laminin (rabbit, Abcam, 1/200), type I collagen (rabbit, Abcam, 1/40), type IV collagen (mouse, Calbiochem, 1/40), Rhodopsin (mouse, Sigma, 1/1000), and human EMMPRIN (mouse, R&D, 1/80). The bound primary antibodies were detected with secondary antibodies labeled with Alexa Fluor488; anti-rabbit IgG antibody with (Molecular Probes, 1/400), Alexa Fluor 546; goat anti-rabbit IgG (Molecular Probes, 1/1000), or goat anti-mouse IgG (Molecular Probes, 1/1000), 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

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 (RT-PCR)

Total RNA was extracted using TRIzol® reagent (Invitrogen)from hiPSCs (253G1), confluent hiPSC-RPE (253G1 and hRPE), and hiPSC-RPE cell sheet (253G1, 59SV9) 1 week and 4 weeks after they had reached confluence on collagen gel 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 (1000 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™ III Reverse Transcriptase (4 µl 5×First-Standard Buffer, 1 µl 0.1 M DTT, 1 µl SuperScript III 200 U/µl; Invitrogen) and the cDNA synthesis was performed as follows:15 min at 25°C, 60 min at 50°C,and 15 min at 70°C. PCR reactions were performed using Ex Taq® DNA polymerase (Takara Bio). Thermal cycling conditions were performed as follows: one cycle at 94°C for 120 sec; 30 cycles at 94°C for 30 sec, 57°C for 30 sec, and 72°C for 60 sec; one cycle at 72°C for 60 sec. Following forward and reverse (FP and RP) primer sequences used to amplify the four RPE specific genes as below.

RPE65: FP: TCCCCAATACAACTGCCACT; RP: CCTTGGCATTCAGAATCAGG

CRALBP: FP: GAGGGTGCAAGAGAAGGACA; RP: TGCAGAAGCCATTGATTTGA

MERTK: FP: TCCTTGGCCATCAGAAAAAG; RP: CATTTGGGTGGCTGAAGTCT

Bestrophin 1: FP: TAGAACCATCAGCGCCGTC; RP: TGAGTGTAGTGTGTATGTTGG

GAPDH: FP: ACCACAGTCCATGCCATCAC; RP: TCCACCACCCTGTTGCTGTA

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, following the manufacturers' instructions.

Fluorescence-activated cell sorting (FACS)

hiPSCs, hiPSC-RPE, and miPSC-RPE were dissociated with dissociation buffer for iPSCs (2.5% trypsin-EDTA [Invitrogen, 1/10], Collagenase IV [GIBCO, 1/10], KSR [1/5], 100 mM CaCl2, PBS [GIBCO, 3/5]) and 0.25% trypsin-EDTA (Invitrogen) respectively and washed and resuspended with 2% FBS in PBS. These cells incubated with FITC-conjugated anti-human SSEA4 (mouse, BD Biosciences 1/5), FITC-conjugated anti-human HLA-I (mouse, Abcam, 1/20), FITC-conjugated anti-human β2-Microglobulin (mouse, BD Biosciences, 1/5), FITC-conjugated anti-human HLA-DP,

DQ, DR (Dako, 1/10), FITC-conjugated anti-monkey HLA-I (Sigma-Aldrich), FITC-conjugated anti-monkey HLA-II (Dako Cytomation), and their respective isotype control antibodies (FITC-IgG1 κ isotype control, FITC-IgG2a κ isotype control, FITC-IgG3 κ isotype control, FITC-IgM κ isotype control; mouse, BD Biosciences, 1/5) for 30 min at 4°C (HLA-1 was at room temperature). After washing, 1 mg/ml propidium iodide was added (Dojindo, 1:1000), cells were passed through a cell strainer (BD), cell profiles were analyzed on FACSCanto™ II (BD Biosciences), and the data were analyzed with FlowJo software (Tree Star). Human IFN-γ (PeproTech) was added at 25 ng/ml 24 hr. before FACS analysis in some experiments.

PBMCs were isolated from two monkeys, Cyn46 autogenic and Cyn51 allogeneic monkey. Allogeneic immune responses were assessed for proliferation by carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) incorporation by the PBMCs. Labeling of PBMCs with CFSE was performed as described in a previous report (Sugita et al., 2004). CFSE-labeled PBMCs were cultured with iPSC-RPE from Cyn46 monkey in the presence or absence anti-monkey CD3 (Invitrogen). The culture medium was used RPMI1640 + 10% FBS containing human recombinant IL-2. The anti-monkey CD3 antibody was used in cultures for PBMCs plus iPS-RPE after 24 hr. After 96 hr., CFSE-labeled cells were washed and analyzed by flow cytometry.

Transepithelial electrical resistance (TER)

TER was performed according to the manufacturer's instructions of the electrical resistance system (Millicell®, Millipore). Briefly, we connected test electrode of electrical resistance system into input port on the meter and confirmed that the meter displayed 1000 Ω. The electrode was rinsed with 70% ethanol for 15 min and SFRM for 15 min. Measurement was within 3 min after taking out from the incubator to prevent reducing temperature. Net TER (Ωcm2) was calculated by subtracting the value of collagen gel only in the insert as a blank from the experimental value and multiplying by the area of the insert membrane.

Electroretinography

Conventional full-field ERGs were recorded in vivo using a PuREC system with two built-in white LED contact lens electrodes (Mayo, Aichi, Japan). RCS rats were dark-adapted overnight and all subsequent procedures were performed under dim red light. Before the ERG recordings, rats were anaesthetized and placed on a heating pad held at 37 °C throughout the experiments. The pupils were dilated with a cocktail of 0.05% tropicamide and 0.05% phenylephrine hydrochloride. 0.5% hydroxyethyl cellulose was applied to the eyes to maintain corneal hydration. Needle electrodes placed subcutaneously in the forehead and tail served as reference and ground electrode, respectively. Single-flash recordings were performed at a light intensity of 1.0 log cd sec/m2using a sampling frequency of 1,253 Hz and a flash duration of 1.0 msec. The band-pass filter was set

between 0.3 and 500 Hz. In addition, the obtained data were low-pass filtered at 300 Hz using a PuREC software (Mayo, Aichi, Japan).

Supplemental Reference

Sugita, S., Ng, T.F., Schwartzkopff, J., and Streilein, J.W (2004). CTLA-4+CD8+ T cells that encounter B7-2+ iris pigment epithelial cells express their own B7-2 to achieve global suppression of T cell activation. J Immunol. *172*, 4184-4194.