ADDITIONAL FILE

Multiocular organoids from human induced pluripotent stem cells displayed retinal, corneal, and retinal pigment epithelium lineages

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METHODS

Characterization of hiPSC lines

CBiPS30-4F-5 and FiPS-4F-7 cell lines were fully characterized and banked into the Spanish National Stem Cell Bank/ Banco Nacional de Líneas Celulares (BNLC). This information can be found at the website:

https://www.isciii.es/QueHacemos/Servicios/BIOBANCOS/BNLC/Paginas/LineasiPS.aspx

Transepithelial Electrical Resistance Measurements

Human iPSC and RPE cells were seeded on Matrigel-coated transwells filter inserts (Millipore, USA) and cultured until the complete formation of the cell monolayer. hiPSC were cultured in mTSeR1 (StemCell Technologies, Vancouver, BC, Canada) and RPE cells were cultured in IM medium consisting of Dulbecco's Modified Eagle´s Medium/Nutrient Mixture F-12 (DMEM/F12), 5% fetal bovine serum, 0.1 mM non-essential amino acids, 2 mM GlutaMax, 1% N2, 1% B27 (all the previous reagents were from Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10 mM β-glycerol phosphate (Sigma Aldrich, St. Louis, MO, USA), recombinant human IGF1 (10 ng/ml; R&D Systems, Minneapolis, MN, USA) and 10 mM nicotinamide (Sigma Aldrich, St. Louis, MO, USA) Electrical measurements were performed every 5 days for 3 weeks in biological triplicates using an epithelial Volt/Ohm Meter (EVOM3; World Precision Instruments Inc., USA). Transepithelial electrical resistance (TEER) values were corrected by subtracting values of a blank and multiplied by the cell growth area. Values were presented in Ω·cm2**.**

Photoreceptor Outer Segment Phagocytosis Assays

RPE phagocytosis activity was assessed by analyzing the binding and internalization of photoreceptor outer segments (POS) labeled with fluorescein isothiocyanate (FITC), as described previously [1]. RPE cells were incubated with POS-FITC for 12 hours at 37ºC with 5% (v/v) CO2 in IM medium, washed four times in phosphate-buffered saline with Ca2+ and Mg2+ and then fixed in 4% (w/v) paraformaldehyde for 20 minutes at room temperature. Immunocytochemistry was performed as described [2] using described in Table S1 and phalloidin-Alexa 647(1:200; Invitrogen). Confocal images were taken with a TCS SP5 confocal microscopy (Leica Microsystems, Wetzlar, Germany).

References

1 Nandrot EF, Anand M, Almeida D, et al. Essential role for MFG-E8 as ligand for αvβ5 integrin in diurnal retinal phagocytosis. Proc Natl Acad Sci U S A 2007;104:12005–12010.

2 Salas A, Duarri A, Fontrodona L, et al. Cell therapy with human induced pluripotent stem cell-derived retinal pigment epithelium and retinal precursor cells prevents visual function loss in a rat model of retinal degeneration. Molecular Therapy - Methods & Clinical Development 2021;0.

Table S1: List of primary antibodies

Antibody	Supplier and reference	Species	Dilution
Actin	MP Biomedicals, 691001	Mouse	1:1000
Aquaporin-1	Santa Cruz Biotechnology, sc-25287	Mouse	1:50
AP2	Santa Cruz, sc-184	Mouse	1:100
Bestrophin 1	Santa Cruz Biotechnology, sc-32792	Mouse	1:25
B- Opsin	Millipore, AB5407	Rabbit	1:100
Calbindin	Swant, 300	Mouse	1:50
CHX10	Abcam, ab16141	Sheep	1:100
Collagen I	Abcam, ab34710	Rabbit	1:100
Collagen IV	Abcam, ab6586	Rabbit	1:100
CRX	Abnova, H00001406-M02	Mouse	1:50
Cytokeratin 19	Genetex, GTX112666	Rabbit	1:100
Cytokeratin 19	Santa Cruz Biotechnology, sc-374192	Mouse	1:100
Cytokeratin 19	Santa Cruz Biotechnology, sc- 33119	Goat	1:100
Cytokeratin 3/2p	Santa Cruz Biotechnology, sc-80000	Mouse	1:100
Cytokeratin 5	Abcam, ab24647-50	Rabbit	1:100
GFAP	Dako, Z0334	Rabbit	1:1000
Ki67	Abcam, AB16667	Rabbit	1:100
MITF	Santa Cruz Biotechnology, sc-56725	Mouse	1:25
Na+/K+-ATPase	Millipore, 05-369	Mouse	1:50
NANOG	R&D Systems, AF1997	Goat	1:25
NRL	R&D Systems, AF2945	Goat	1:25
OCT4	Santa Cruz, sc-5279	Mouse	1:25
P63	Abcam, ab735	Mouse	1:50
PAX6	Covance, PRB278P	Rabbit	1:100
PKCα	Santa Cruz, sc-8393	Mouse	1:100
RAX	Abcam, ab23340	Rabbit	1:50
Recoverin	Millipore, AB5585	Rabbit	1:500
RG-Opsin	Millipore, AB5405	Rabbit	1:100
Rhodopsin	Sigma, O4886	Mouse	1:500
p75 NGFR	Santa Cruz Biotechnology, sc- 271708	Mouse	1:50
Pericentrin	Abcam, ab28144	Mouse	1:50
RPE65	Santa Cruz Biotechnology, sc-73616	Mouse	1:100
SOX2	Santa Cruz Biotechnology, sc-17320	Goat	1:25
SOX9	Invitrogen, 711048	Rabbit	1:200
SOX10	Santa Cruz Biotechnology, sc-365692	Mouse	1:50
SSEA1	Iowa, MC-480	Mouse	1:2
SSEA3	Iowa, MC-631	Rat	1:2
SSEA4	Iowa, MC-813-70	Mouse	1:2
Synaptophysin	Millipore, MAB329-C	Mouse	1:100
TRA-1-60	Chemicon, MAB4360	Mouse	1:100
TRA-1-81	Chemicon, MAB4381	Mouse	1:100
Tuj1	Covance, MMS-435P	Mouse	1:1000
vGUT1	Millipore, AB5905	Guinea pig	1:50
Vimentin	Cell signaling kit Arigobio, SQab1721	Rabbit	1:100
ZO-1	Millipore, ab2272	Rabbit	1:100
γ-cristallin	Santa Cruz Biotechnology, sc-22415	Goat	1:50

Table S2: List of primers used in PCR and RT-qPCR

Gene	Forward	Reverse
AQP1	ACCTCCTGGCTATTGACTACA	CCCTTCTATTTGGGCTTCATCT
CHX10	GGCGACACAGGACAATCTTTA	TTCCGGCAGCTCCGTTTTC
CK12	AGCAGAATCGGAAGGACGCTG	ACCTCGCTCTTGCTGGACTGAAA
CK19	ACAGCCACTACTACACGACC	CCTGTTCCGTCTCAAACTTGGT
CK3	ACGTGACTACCAGGAGCTGATG	ATGCTGACAGCACTCGGACACT
COL8A1	CCTGGGTCAGCAAGTACCTC	TTGTTCCCCTCGTAAACTGG
COL8A2	ATGCTGGGGACTCTGACAC	GGTAGAGGCATTTCCAGGTACT
CRX	TCCAGGGTTCAGGTTTGGTT	CATCTGTGGAGGGTCTTGGG
GAPDH	CCTGCACCACCAACTGCTTAG	TGGCATGGACTGTGGTCATG
LHX2	ATGCTGTTCCACAGTCTGTCG	GCATGGTCGTCTCGGTGTC
MITF	GTGCCAACTTCTTTCATCA	ACCTAAACCGTCCATTCA
Na+/K+- ATPase	CAGGGCAGTGTTTCAGGCTAA	TCGACGATTTTGGCGTATCTT
NR2E3	GGCTTCTTCAAGAGGAGCGT	CGGGACTCAGTGTTGGACTC
OCT3/4	GTTCTTCATTCACTAAGGAAGG	CAAGAGCATCATTGAACTTCAC
P63	GAAAACAATGCCCAGACTCAATTT	TCTGCGCGTGGTCTGTGTTAT
PAX3	CCACAAGATCGTGGAGATGG	ACCGCGTCCTTGAGTAATTT
PAX6	TCTAATCGAAGGGCCAAATG	TGTGAGGGCTGTGTCTGTTC
RAX	GCGAAGCGAAACTGTCAGAG	TTCTGGAACCACACCTGGACC
RECOVERIN	TCTACGACGTGGACGGTAACG	CGTCCTCGGGAGTGATCATT
RHODOPSIN	GGGAGAACCATGCCATCAT	TCGTCTCCGTCTTGGA
SIX3	CACTCCCACACAAGTAGGCA	GCTGGAGGTTACCGAGAGGA
SIX6	ACGGCGAACAGAAGACACAC	TGCTGGAGTCTGTTCTTGGCT
SOX9	TGAAGAAGGAGAGCGAGGA	CGCGGCTGGTACTTGTAAT
TFAP2	GTCTCCGCCATCCCTATTAAC	GGACAGCTTCTCCCTCTACTA

FIGURE S1

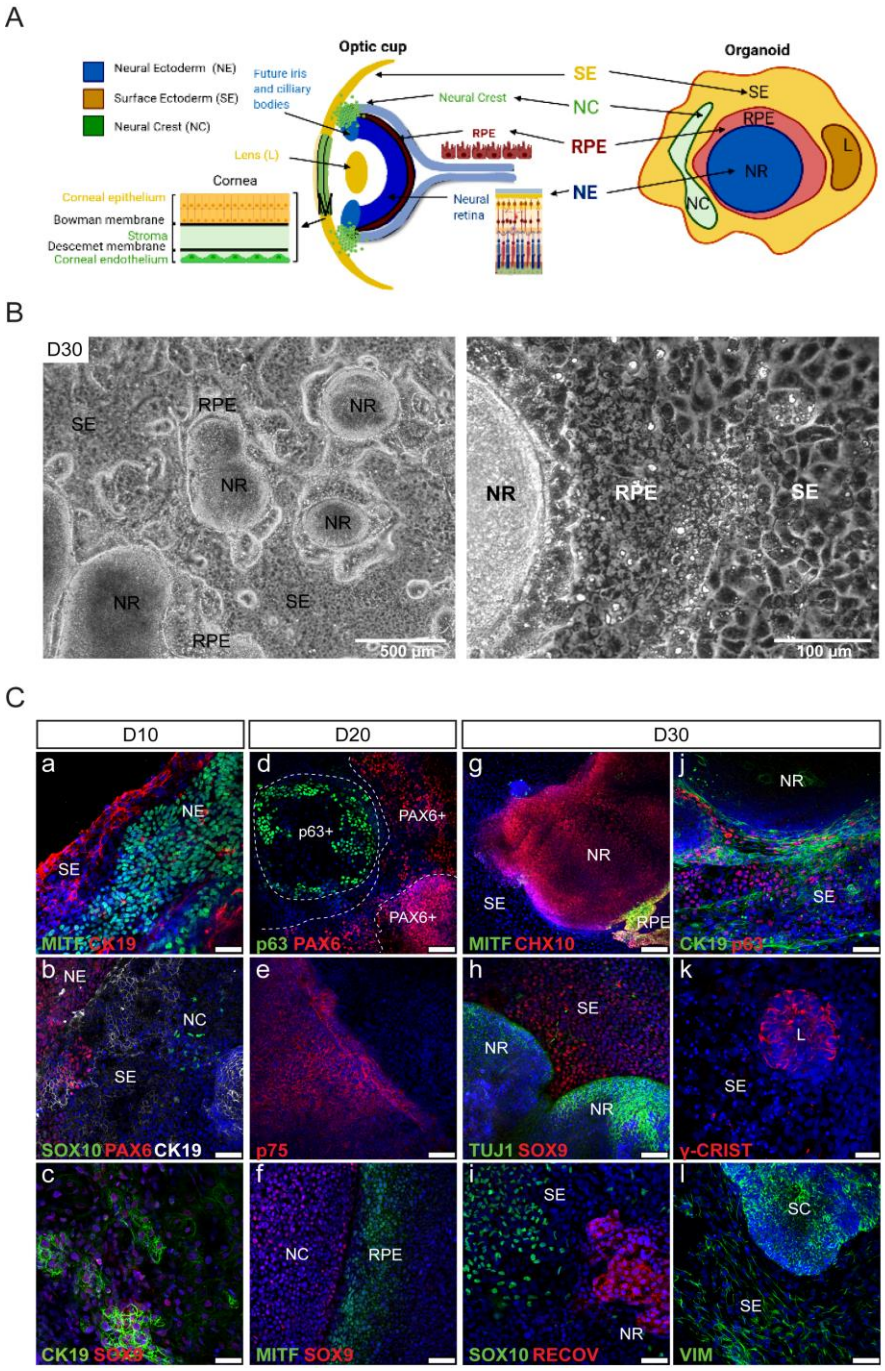

Figure S1: Generation of multizone ocular progenitor cells from CB30 hiPSC line. A) Schematic diagram of multizone ocular progenitor cells (mzOPCs) differentiation showing different ectodermal lineages mimicking eye development. B) Representative phase-contrast image of mzOPCs at day 30 of differentiation containing regions of surface ectoderm (SE), retinal pigment epithelium (RPE), and neural retina (NR). Scale bar: 500 µm; 100 µm. C) Immunofluorescence images of mzOPCs at days 10, 20, and 30 of differentiation presenting areas of neuroectoderm (NE), SE, RPE, NR, neural crest (NC), lentoid cell cluster (L), and stromal cells (SC), immunolabelled with PAX6 (neuroectoderm marker); TUJ1 and NRL (NR markers); MITF (RPE marker); CK19 and p63 (SE markers); SOX9, SOX10, and p75-NGFR (NC markers); γ-crystallin (lens marker); and vimentin (VIM) (SC marker). Nuclei were stained in DAPI. Scale bars: 100 µm in d, g; 50 µm in b, e, f, h, j, l;.25 µm in a, c, I, k.

FIGURE S2

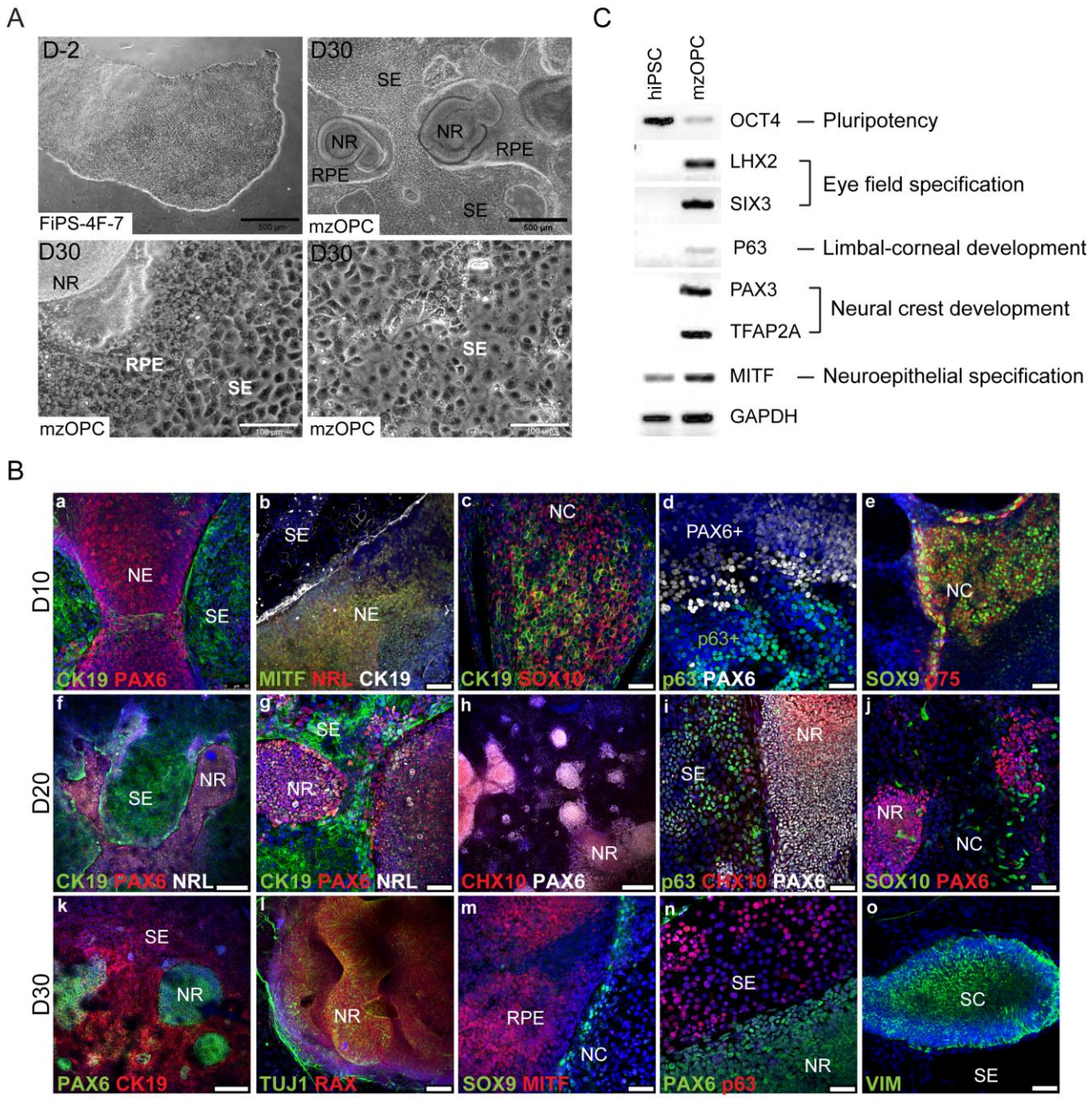

Figure S2: Generation of multizone ocular progenitor cells from FiPS hiPSC line. A) Phase-contrast images of human induced pluripotent stem cells (hiPSC) at day -2 showing a compact stem cell colony, and multizone ocular progenitor cells (mzOPCs) after 30 days of differentiation showing different ocular lineages: neuroretina (NR), retinal epithelium (RPE), and surface ectoderm (SE). Scale bars: 500 µm; 100 µm. **B)** Immunocytochemistry of mzOPCs at days 10, 20, and 30 of differentiation showing neuroectoderm (NE), NR, RPE, SE, neural crest cells (NC), and stromal cells (SC). Cells were stained with antibodies against PAX6 (NE marker), MITF (RPE marker), NRL, RAX, CHX10, and TUJ1 (NR markers), p63 and CK19 (SE markers), SOX9, SOX10, and p75-NGFR (NC markers), and VIM (stromal fibroblasts marker). Nuclei are stained with DAPI. Scale bars: 250 µm in f, h, k; 50 µm in b-d, g, I, j, l-o. **C)** PCR analysis of specific eye-field, NE, SE, RPE, and neural crest (NC) gene expression levels in samples from hiPSC and mzOPCs at day 30 of differentiation. Expression of *GAPDH* was used as an internal control.

FIGURE S3

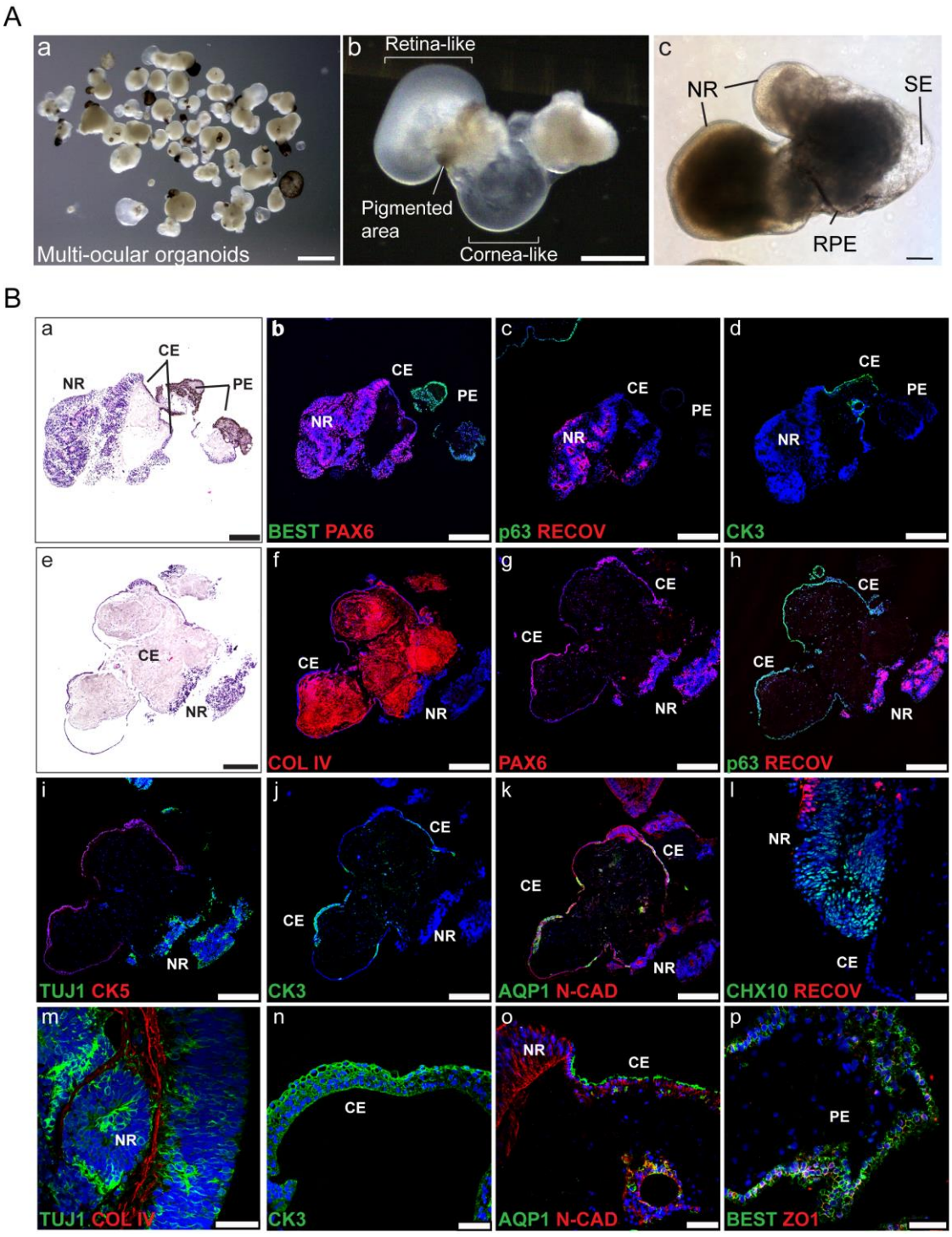

Figure S3: Generation of multiocular organoids from FiPS hiPSC line. A) (a) Microscopic image of a mixed multiocular organoids culture at day 90. (b, c) Bright-field image of multiocular organoid showing different ocular regions: neuroretina (NR), pigmented epithelium (PE), and surface ectoderm (SE). Scale bars: 2 mm in a; 500 µm in b; 150 µm in c. B) Hematoxylin and eosin (HE) staining (a, e) and immunofluorescence images of the multiocular organoid paraffin section with different ocular structures. Sections are labeled with specific antibodies PAX6, RECOV, and TUJ1 (NR markers); CK3, p63, CK5, AQP1, N-CAD (corneal-conjunctival epithelium markers); and BEST and ZO1 (RPE markers). The stroma is stained with collagen IV (COL IV). Nuclei are stained with DAPI. Scale bars: 250 µm in a-k; 50 µm in l-p.

FIGURE S4

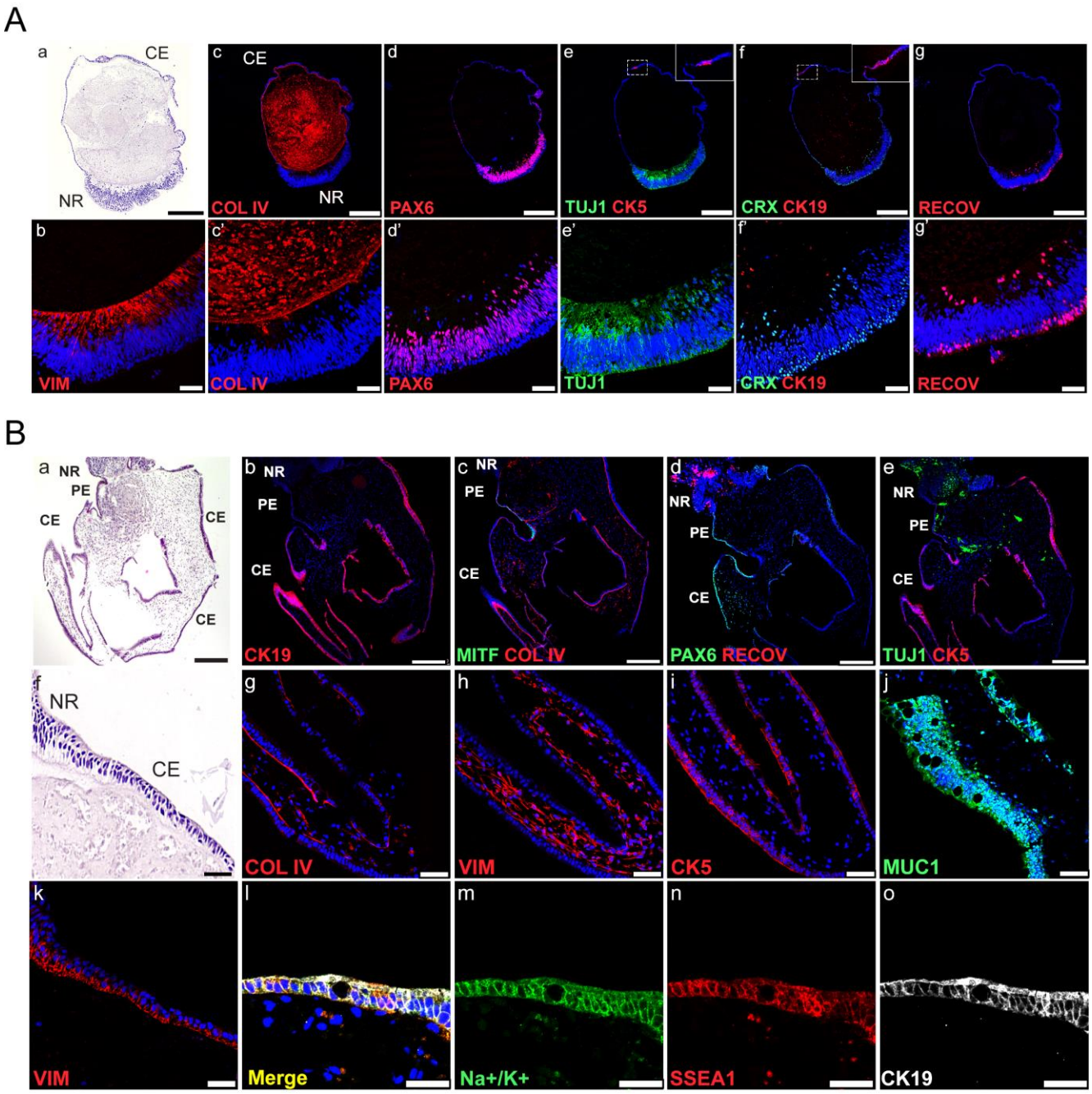

Figure S4: CB30-derived multiocular organoids composed of retinal, corneal, and pigmented areas. A) Hematoxylin and eosin (HE) staining of multiocular organoid paraffin section formed by neuroretina (NR) and corneal epithelium (CE) (a). Immunofluorescent images showing expression of collagen IV (COL IV) and vimentin (VIM; b, l, m, n) in the stroma of the organoid; PAX6 (d, d') and TUJ1 (e, e') in the inner retina, and CRX (f, f') and recoverin (RECOV; g, g') in the outer retina; CK5 (e) and CK19 (f) is detected in the corneal-conjunctival epithelium. Scale bars: 250 µm in a, c-g; 50 µm in b, c'-g'. B) HE (a, f) and immunofluorescent images of multiocular organoid paraffin section formed by NR, CE, and pigmented epithelium (PE). Corneal-conjunctival epithelium is stained with CK19 (b, o), CK5 (e, i), MUC1 (mucin-1, j), Na+/K+-ATPase (m) and SSEA1 (n). Stroma is stained with COL IV (c, g) and VIM (h, k). PE is stained with MITF (c) and PAX6 (d). Neuroretina is stained with RECOV (d), TUJ1 (e). Nuclei are stained in DAPI. Scale bars: 250 µm in a-e; 50 µm in f-k; 25 µm in l-o.

FIGURE S5

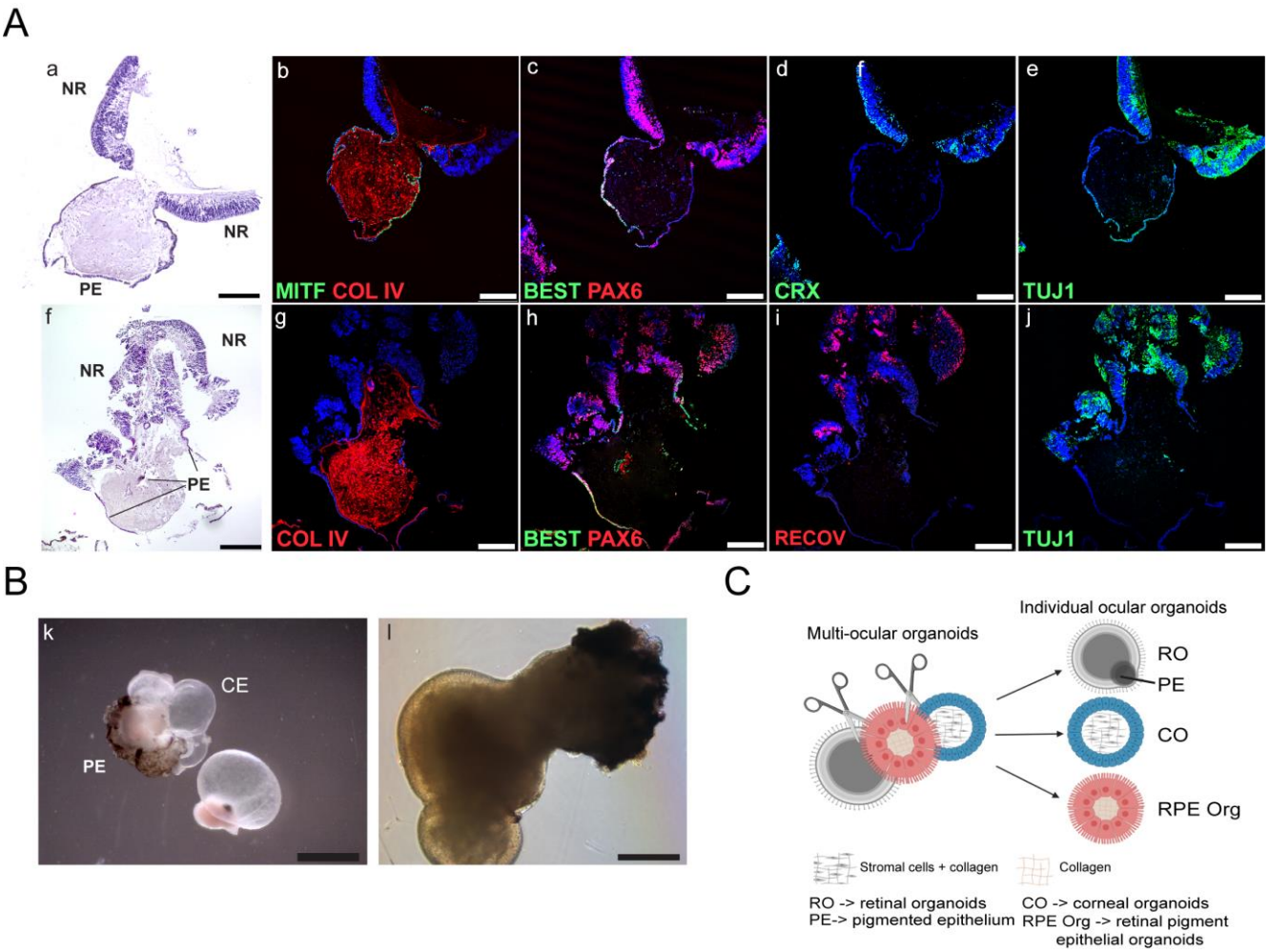

Figure S5: CB30-derived multiocular organoids composed by retinal and pigmented areas. A) Hematoxylin and eosin staining (a,f) and immunohistochemistry images of multiocular organoids exhibiting retinal (CRX, TUJ1, recoverin (RECOV), and PAX6), and RPE regions (MTIF, BEST, and PAX6) filled with collagen type IV (COL IV) (b-e; g-j). Scale bars: 250 µm. B) Bright-field images of multiocular organoids composed of the pigmented epithelium (PE) and the cornea (k), and the PE and the retina (l). Scale bars: 500 µm (k); 150 µm (l). C) Schematic illustration of isolation of different ocular regions from multiocular organoids to generate retinal organoids (RO) and RO containing pigmented areas (RO+PE); corneal organoids (CO) containing stromal cells and collage, and CO containing pigmented areas (CO+PE); and RPE organoids containing collagen.

FIGURE S6

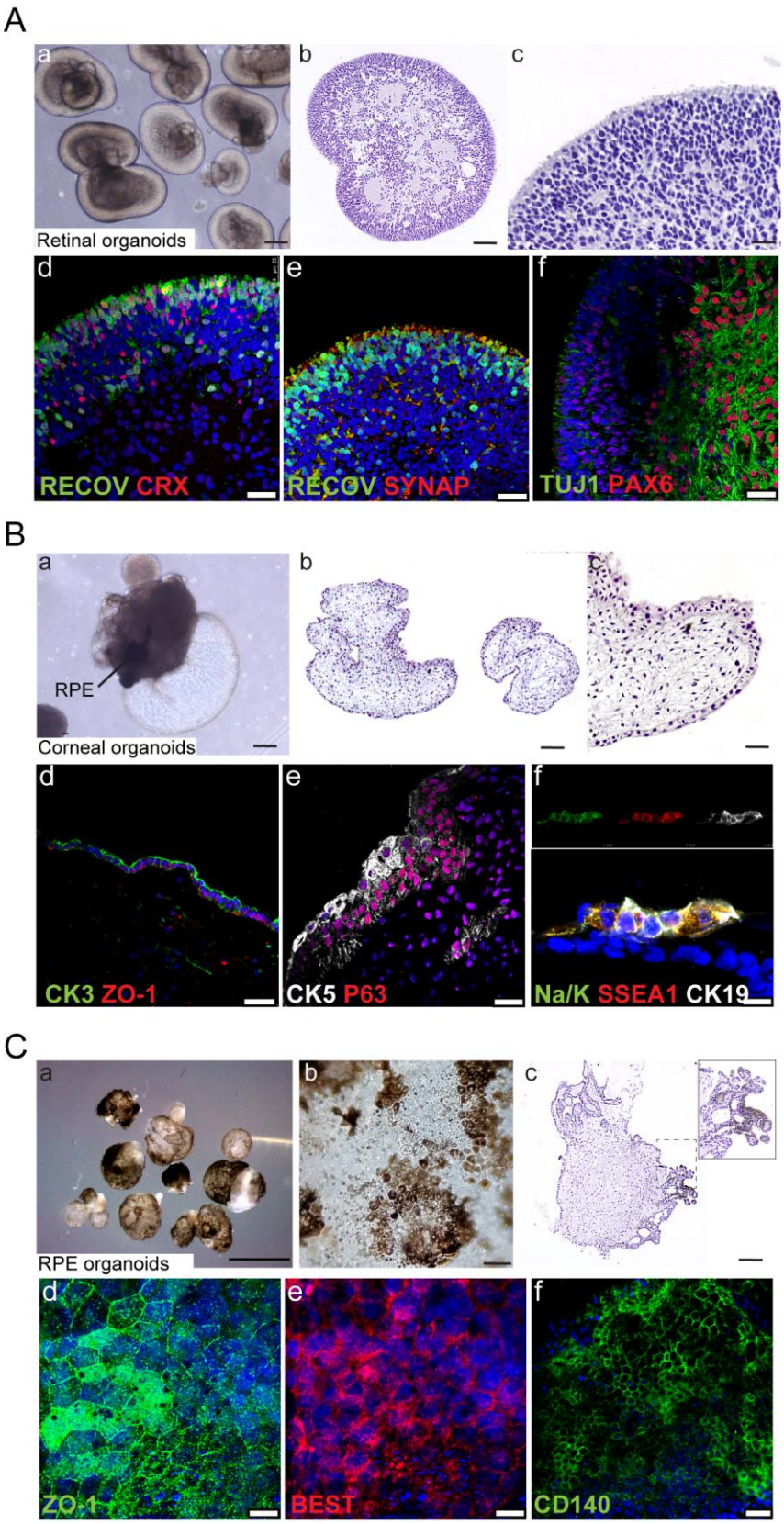

Figure S6: FiPS-derived individual retinal, corneal, and RPE organoids. A) Bright-field image of retinal organoids in suspension culture at day 140 (a) showing laminated neuroretinas (NR). Hematoxylin and eosin

staining (HE) staining images of retinal organoid paraffin sections (b, c) showing stratified NR at the apical site. Immunofluorescence images of retinal organoid paraffin sections (d-f) stained with specific antibodies against recoverin (RECOV) and CRX (photoreceptor markers), synaptophysin (SYNAP; presynaptic marker); PAX6 (bipolar and ganglion cell marker), and tubulin beta III (TUJ1; ganglion cell marker). Scale bars: 300 µm (a); 100 µm (b); 25 µm (c-f). B) Bright-field image of corneal-like organoids in suspension culture at day 140 in all-trans retinoic acid (a) showing spherical and transparent organoid corresponding to corneal-conjunctival epithelium. Some corneal organoids present a pigment epithelium (RPE) region on one side. HE staining images of corneal organoid paraffin sections (b, c) showing stratified epithelium in the apical side fulfilled with stromal-like cells. (d-f) Immunofluorescence images of corneal-conjunctival epithelium stained with cytokeratin 3 (CK3) and zonula occludens (ZO-1) (d), CK5 and p63 (e) and CK19, Na+/K+-ATPase (Na/K), and SSEA1 (f). Scale bars: 300 µm (a); 100 µm (b); 50 µm (c, d); 25 µm (e); 10 µm (f). C) Bright-field image of RPE organoids in suspension culture at day 140 (a) and magnification of the organoid apical surface (b) showing cuboidal, pigmented epithelial cells. HE staining image of retinal organoid paraffin sections (c) showing pigmented epithelium on the surface and lumen filled with stroma. Insets: magnification of the region of interest (dashed square). (d-f) Immunofluorescence images of RPE organoid surface stained with ZO-1, bestrophin1 (BEST), and CD140 antibodies. Scale bars: 2 mm (a); 100 µm (c); 25 µm (b, f); 10 µm (d, e). Nuclei are stained with DAPI.

FIGURE S7

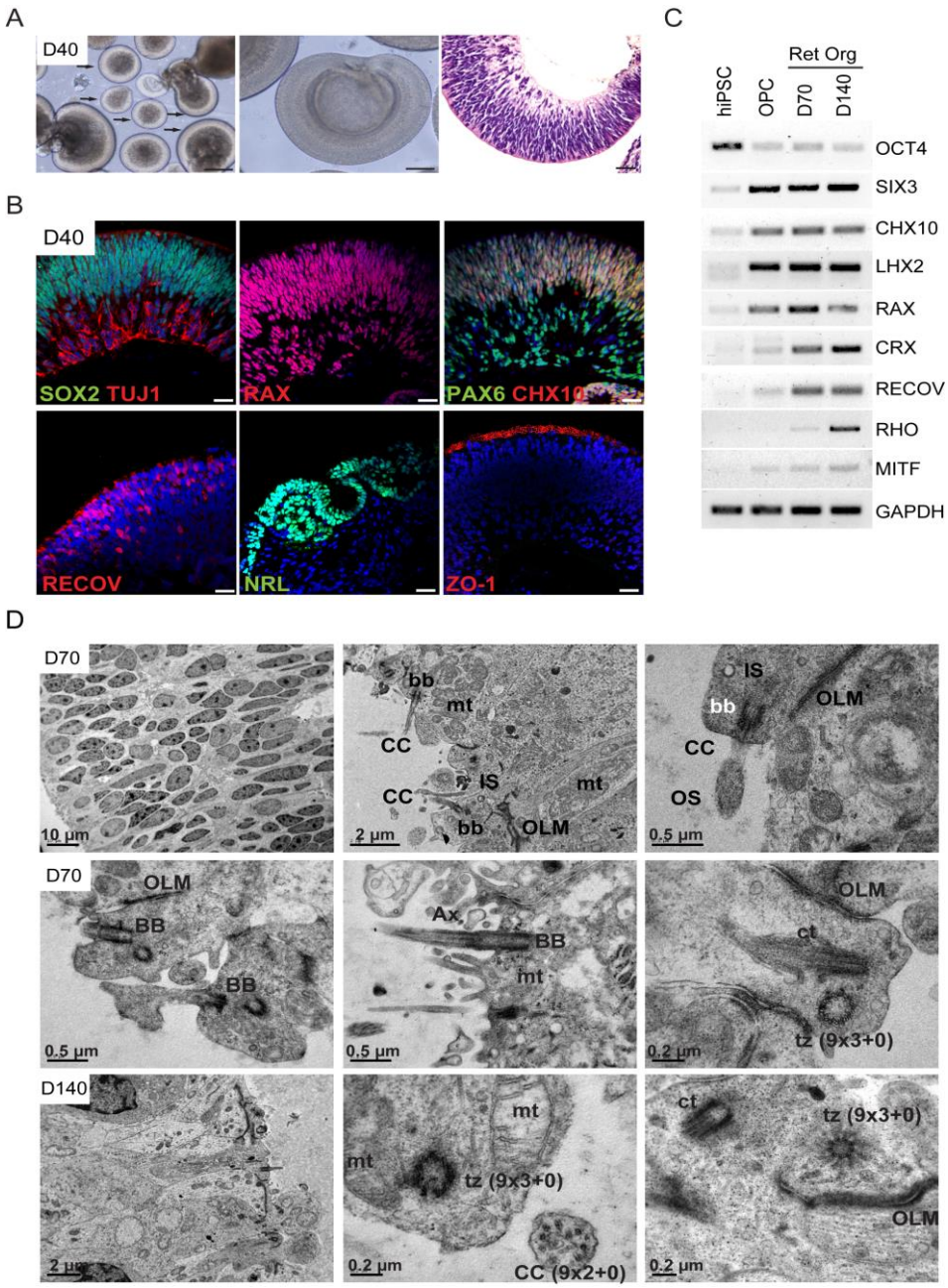

Figure S7: Characterization of retinal organoids generated from CB30 hiPSC line. A) Bright-field images of retinal organoids derived from hiPSC at day 40 of differentiation. Arrows indicate developing laminated retinal tissue. (Right) Hematoxylin and eosin staining of a paraffin section showing lamination of retinal tissue. Scale bars: 300 µm (left); 100 µm (middle); 50 µm (right). B) Confocal images of retinal organoid paraffin sections at day 40 immunostained with SOX2, TUJ1, PAX6, CHX10, RECOV, NRL, and ZO-1. Scale bars: 25 µm. C) PCR analysis of specific eye-field, retinal, and retinal pigment epithelium gene expression levels in samples from human induced pluripotent stem cells (hiPSC), ocular progenitor cells (OPC) at day 30 of differentiation, and retinal organoids (Ret Org) at days 70 and 140 in culture. Expression of *GAPDH* was used as an internal control. D) Transmission electron microscopy analysis of retinal organoids at days 70 and 140 show several retinal structures of connecting cilia (CC), basal bodies (bb), centriole (ct) inner segments (IS), mitochondria (mt), outer limiting membrane (OLM), outer segments (OS), the classical array of nine triplet and doublet microtubules of bb and transition zone (tz) of the CC, respectively. Scale bars are indicated in the images.

FIGURE S8

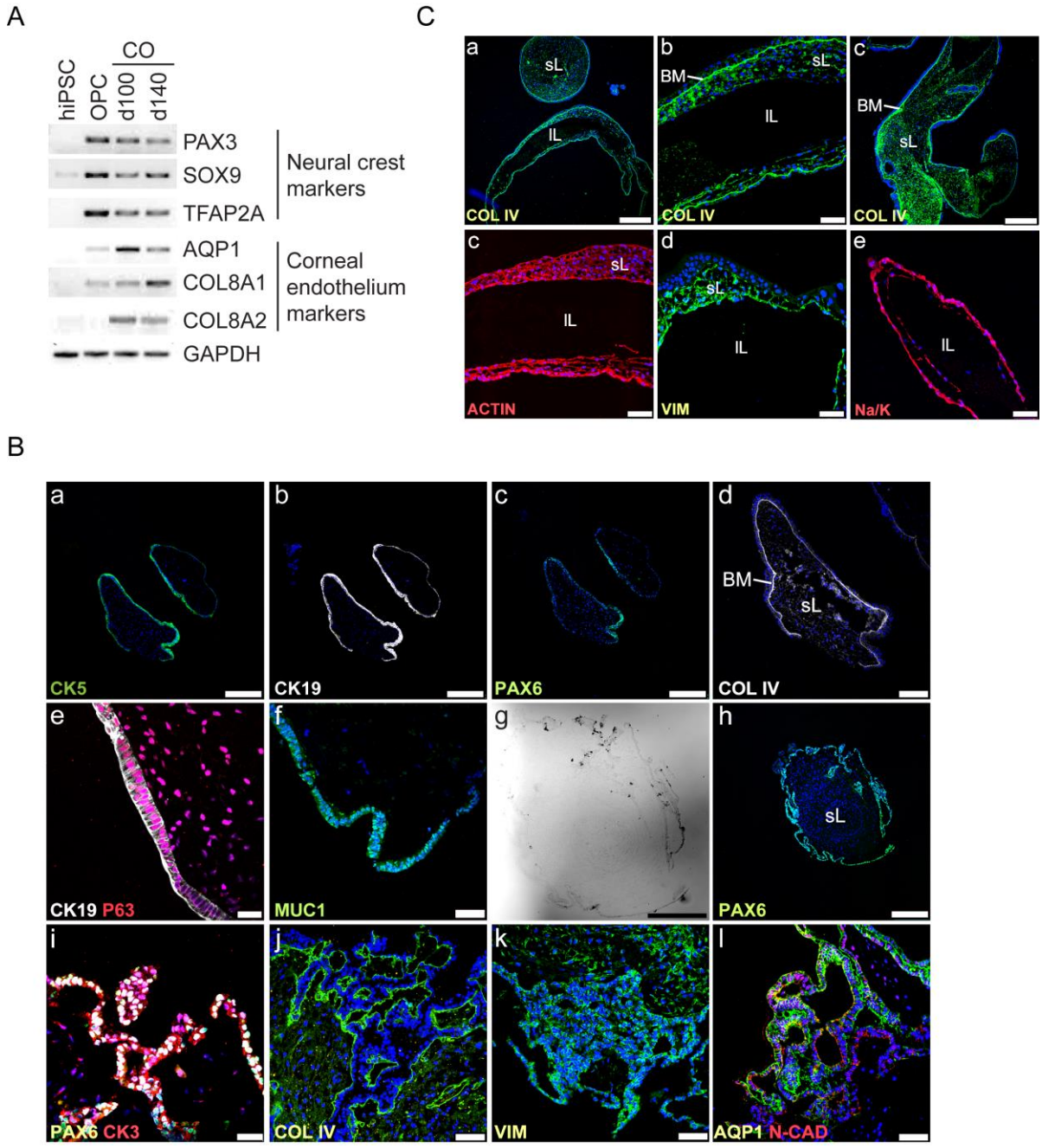

Figure S8. Characterization of corneal organoids derived from CB30 hiPSC line. A) PCR analysis of neural crest progenitors and corneal endothelium gene expression in samples from human induced pluripotent stem cells (hiPSC), ocular progenitor cells (OPC) at day 30 of differentiation, and corneal organoids (CO) at days 100 and 140 in culture. Expression of *GAPDH* was used as an internal control. B) Confocal images of corneal organoids at day 140 paraffin sections immunolabelled with cytokeratin 3, 5, and 19 (CK3, CK5, CK19), PAX6, p63, mucin 1 (MUC1), collagen type IV (COL IV), vimentin (VIM), aquaporin-1 (AQP1) and N-cadherin (N-CAD). Lumen was filled with stroma (sL). Pigmented cells are shown in the bright-field image (g). Scale bars: 250 µm in a-c, g, h; 100 µm in d; 50 µm in f, j-l; 25 µm in e, i, C) Immunofluorescence images of corneal endothelial-like organoids with a stromal lumen (sL) and fluid-filled lumen (lL) stained with COL IV (forming a basement membrane (BM) similar to Descemet's membrane), actin, VIM and Na+/K+-ATPase (Na/K). Scale bars: 250 µm in a, c; 50 µm in b, d-f. Nuclei are stained with DAPI.

FIGURE S9

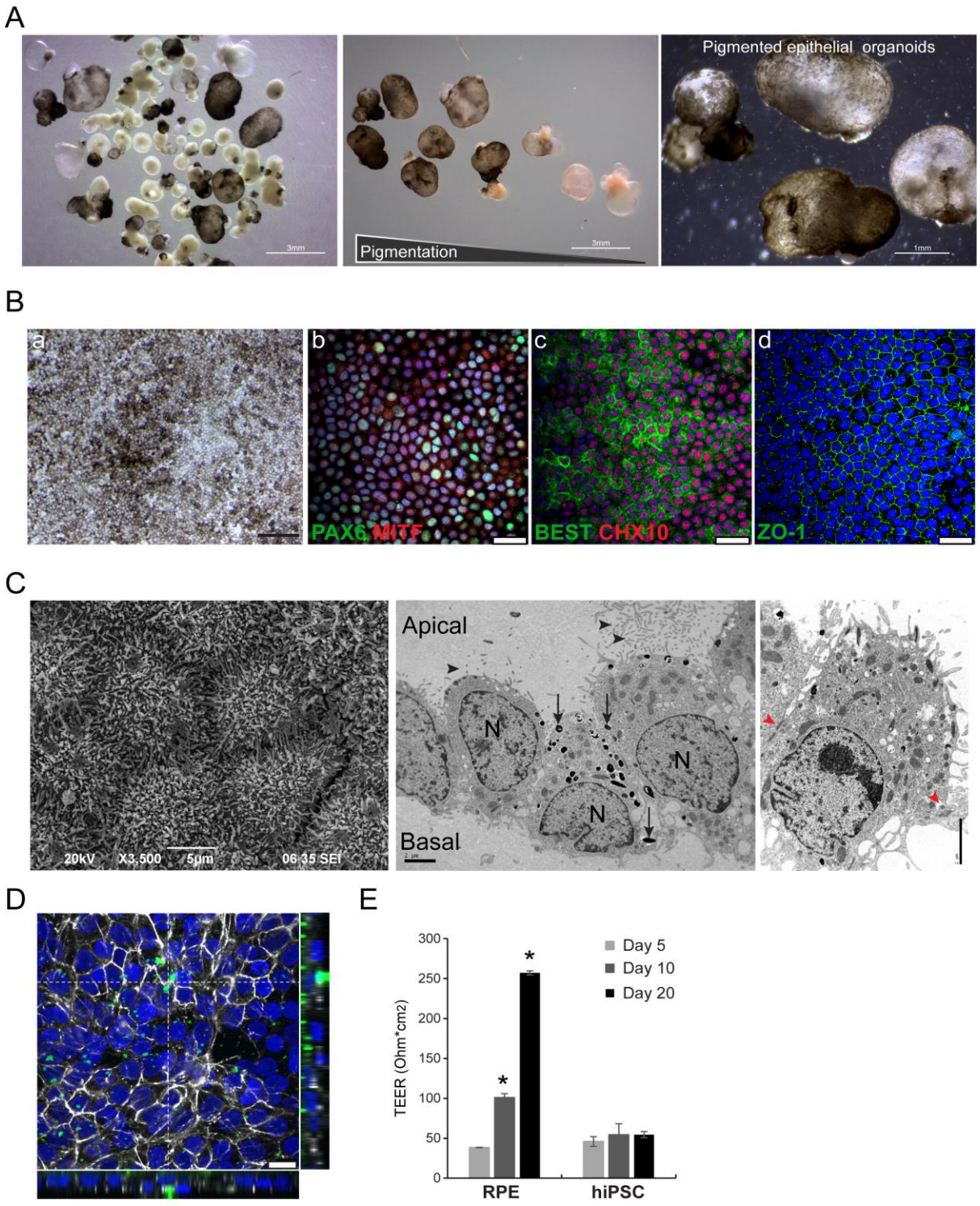

Figure S9. Pigmentation of retinal pigment epithelial (RPE) organoids and derivation of RPE cell culture. A) Bright-field images of CB30 hiPSC-derived RPE organoids in suspension at day 120 cultured without *all-trans* retinoic acid from day 90. Scale bars: 3 mm in a-b; 1 mm in c. B) (a) Phase-contrast image of RPE organoid-derived RPE cells cultured in monolayers after cell expansion. Scale bar: 100 µm. (b-c) Immunofluorescence staining of RPE cell culture with BEST, ZO-1, PAX6, MITF, RPE65, and CHX10. Scale bars: 25 µm. C) (Left) Scanning electron microscopy image of the RPE cell culture. RPE apical cell surface shows hexagonal cell

morphology and apical microvilli. Scale bar: 5 µm. (Right) Transmission electron microscopy image of an RPE cell monolayer containing melanosomes (arrows), basal location of nuclei (N), apical microvilli (arrowheads), and tight junctions (red arrowheads). Scale bar: 2 µm. D*) In vitro* phagocytosis assay with FITC-labelled photoreceptor outer segments (POS). Z-stack fluorescent images with orthogonal views showed the location of internalized FITC-POS in RPE cells. Tight junctions were stained with ZO-1 (white) to show the apical side. Scale bar: 10 µm. Nuclei are stained with DAPI. E) Graph showing transepithelial electrical resistance (TEER) for hiPSC and RPE cell cultured in transwells (n = 3) at indicated days in culture. Data are expressed as mean ± SD, * p<0,01 using unpaired Student's t-test.