ADDITIONAL FILE

Multi-ocular organoids generated from human iPSC displayed retina, cornea, and RPE lineages

Helena Isla-Magrané, Anna Veiga, José García-Arumí, Anna Duarri*

Tables: 2

Figures: 9

METHODS

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) 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, 10 mM β-glycerolphosphate, 10 mM nicotinamide and recombinant human IGF1 (10 ng/ml). Electrical measurements were performed every 5 days for 3 weeks in biological triplicates using an epithelial voltohmmeter (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).

TABLES

Supplementary 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

Supplementary Table S2: List of primers used in 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

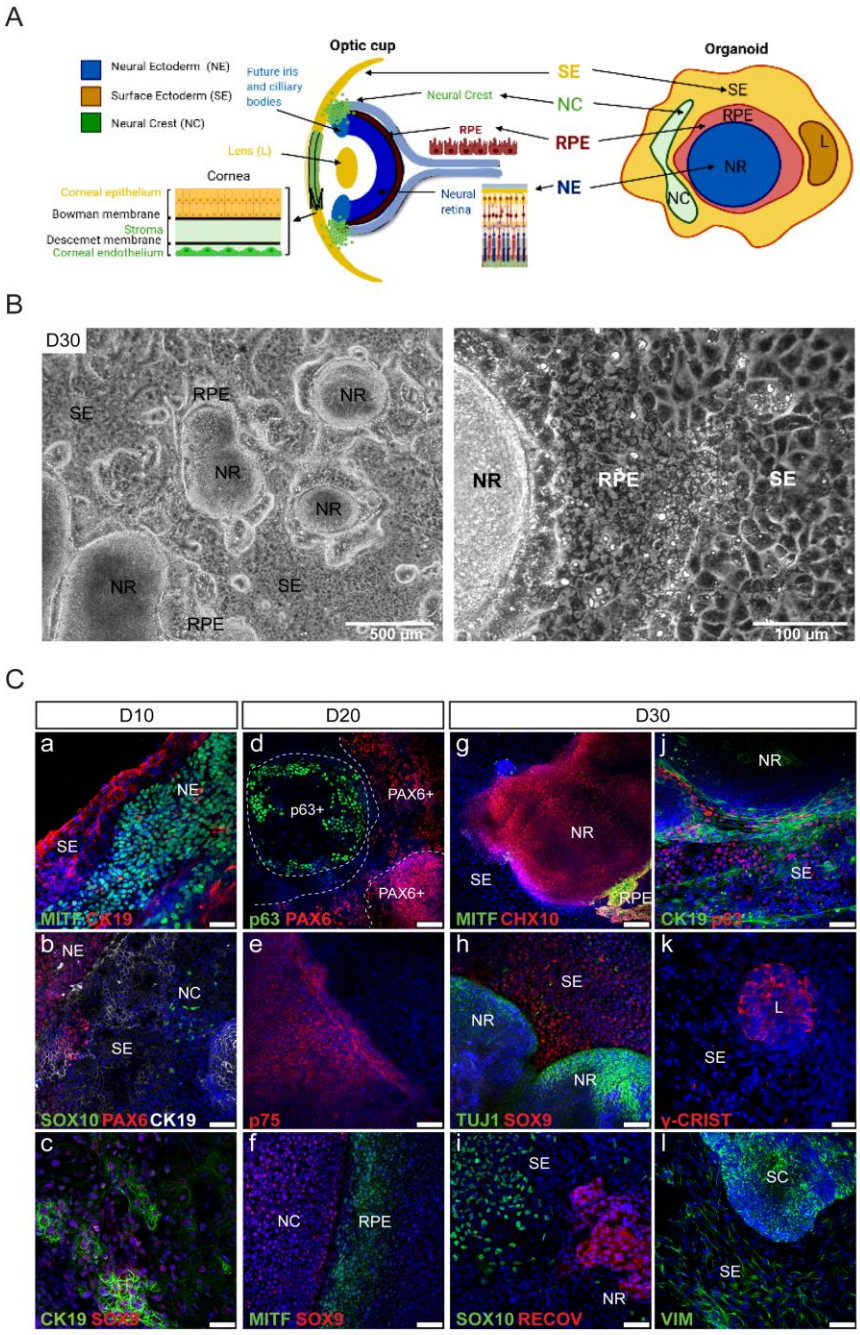

Figure S1: Generation of multi-zone ocular progenitor cells from CBiPS30-4F-5 hiPSC line. A) Schematic diagram of multi-zone ocular progenitor cells (mzOPC) differentiation showing different ectodermal lineages mimicking eye development. B) Representative phase-contrast image of mzOPC at day 30 of differentiation containing regions of surface ectoderm (SE), retinal pigment epithelium (RPE) and neural retina (NR). Scale bar: 500 µm. B) Immunofluorescence images of mzOPC 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 neuroectoderm marker PAX6; NR marker TUJ1 and NRL; RPE marker MITF; SE markers CK19 and p63; NC-markers SOX9, SOX10 and p75-NGFR; lens marker γ-crystallin; and stromal cell marker vimentin (VIM). Nuclei were stained in DAPI. Scale bars: 25 µm in a,c,l,k; 50 µm in b,e,f,h,j,l, 100 µm in d,g.

FIGURE S2

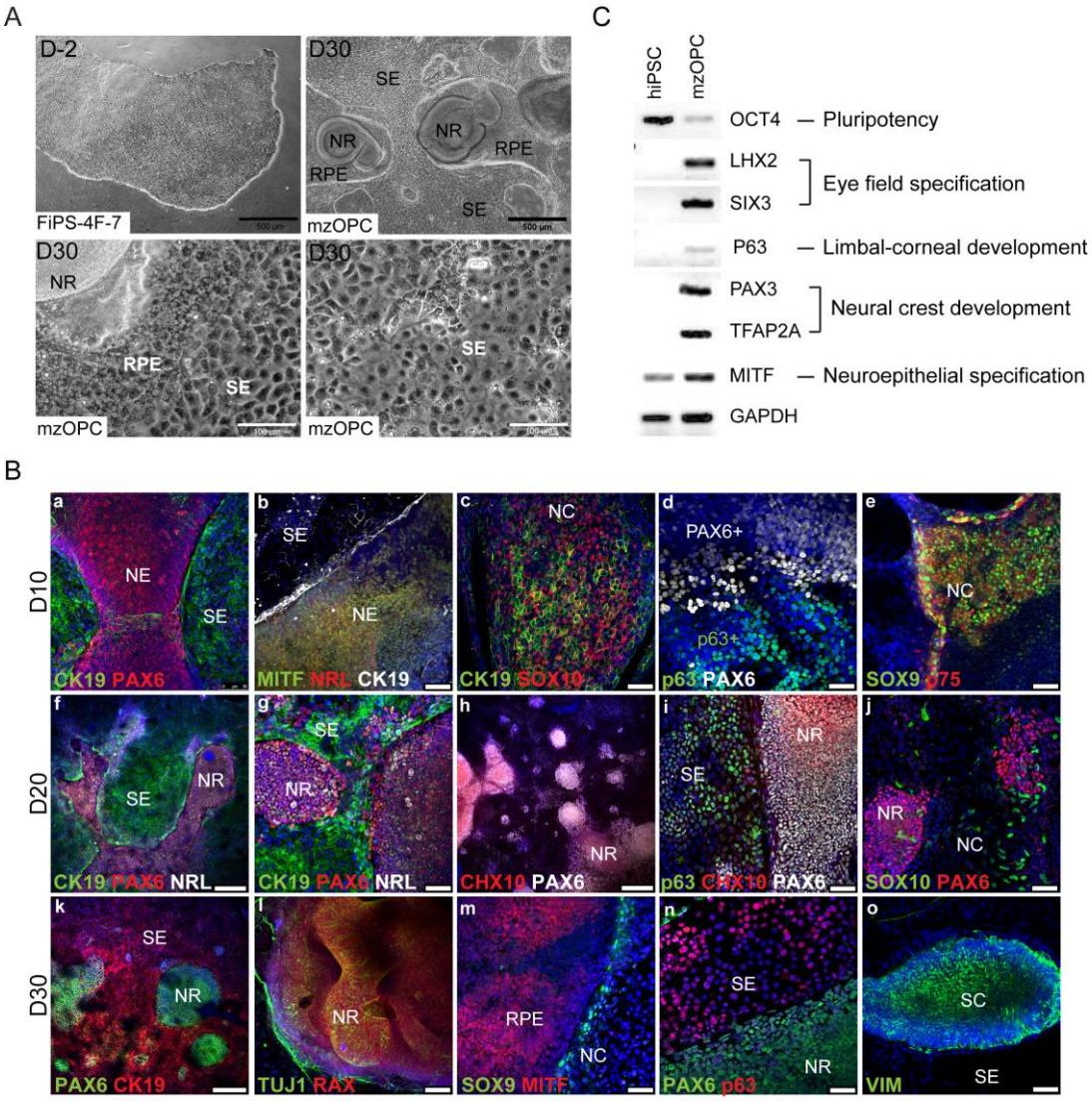

Figure S2: Generation of multi-zone ocular progenitor cells from FiPS-4F-7 hiPSC line. A) Phase contrast images of human induced pluripotent stem cells (hiPSC) at day -2 showing a compact stem cell colony, and multi-zone ocular progenitor cells (mzOPC) after 30 days of differentiation showing different ocular lineages: neuroretina (NR), retinal epithelium (RPE), and surface ectoderm (SE). Scale bars: 500 µm and 100 µm. **B)** Immunocytochemistry of mzOPC at days 10, 20 and 30 of differentiation showing neuroectoderm (NE), NR, RPE, SE, 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,l,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 mzOPC at day 30 of differentiation. Expression of *GAPDH* was used as internal control.

FIGURE S3

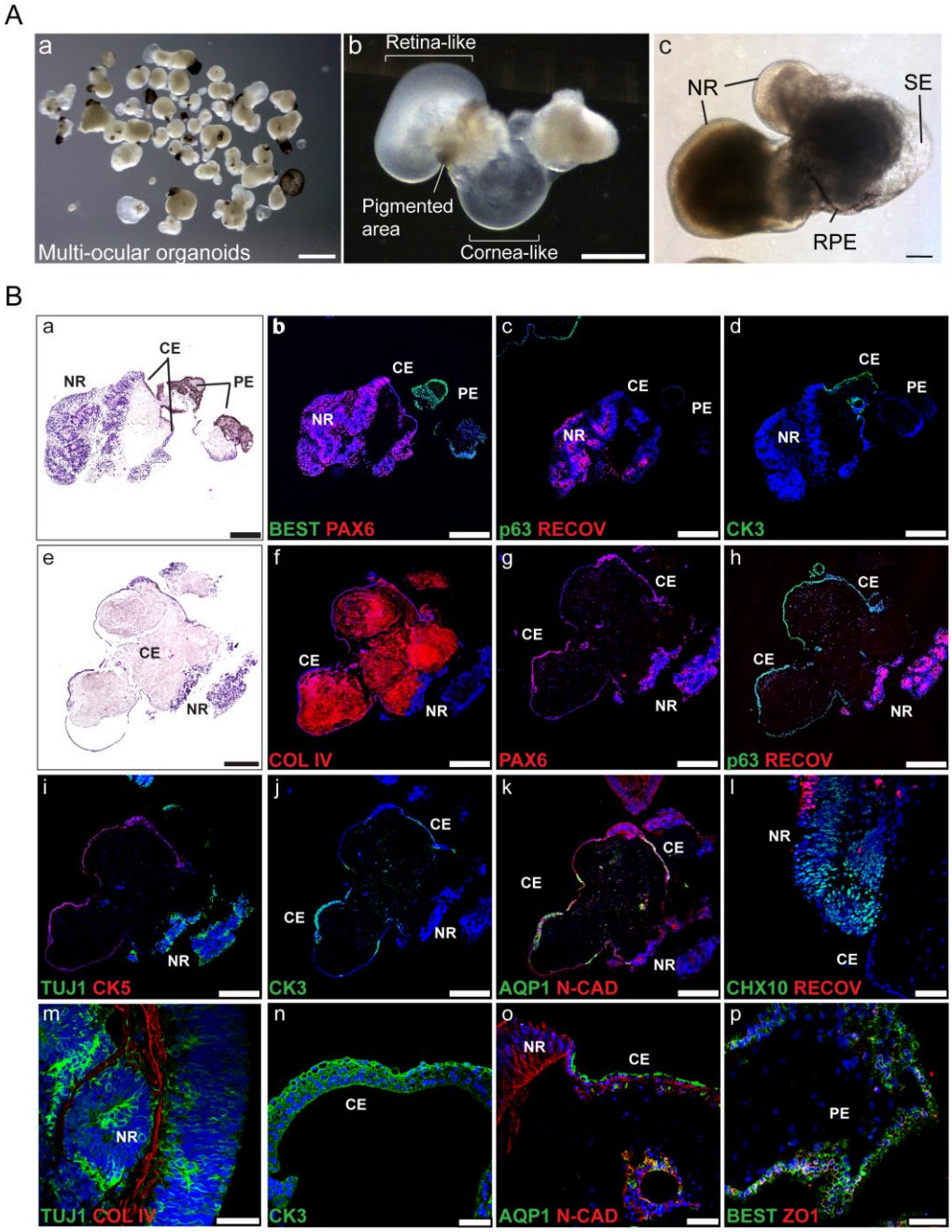

Figure S3: Generation of multi-ocular, retinal, corneal and RPE organoids from FiPS-4F-7 hiPSC line. **A)** (a) Microscopic image of a mixed ocular organoids culture generated from FiPS-4F-7 hiPSC line at day 90. (b,c) Bright field image of multi-ocular organoid showing different ocular regions: neuroretina (NR), pigmented epitheluim (PE) and surface ectoderm (SE). Scale bars: 2 mm (a); 500 µm (b); 150 µm (c). **B)** Hematoxylin and eosin (HE) staining (a,e) and immunofluorescence images of the multi-ocular organoid paraffin section with different ocular structures. Paraffin sections are labelled with specific antibodies against NR (PAX6, RECOV, TUJ1), corneal-conjuctival epithelium (CK3, p63, CK5, AQP1, N-CAD), and RPE (BEST and ZO1). Stroma is stained with collagen IV (COL IV). Scale bars: 250 µm (a-k); 50 µm (l-p). Nuclei are stained with DAPI.

FIGURE S4

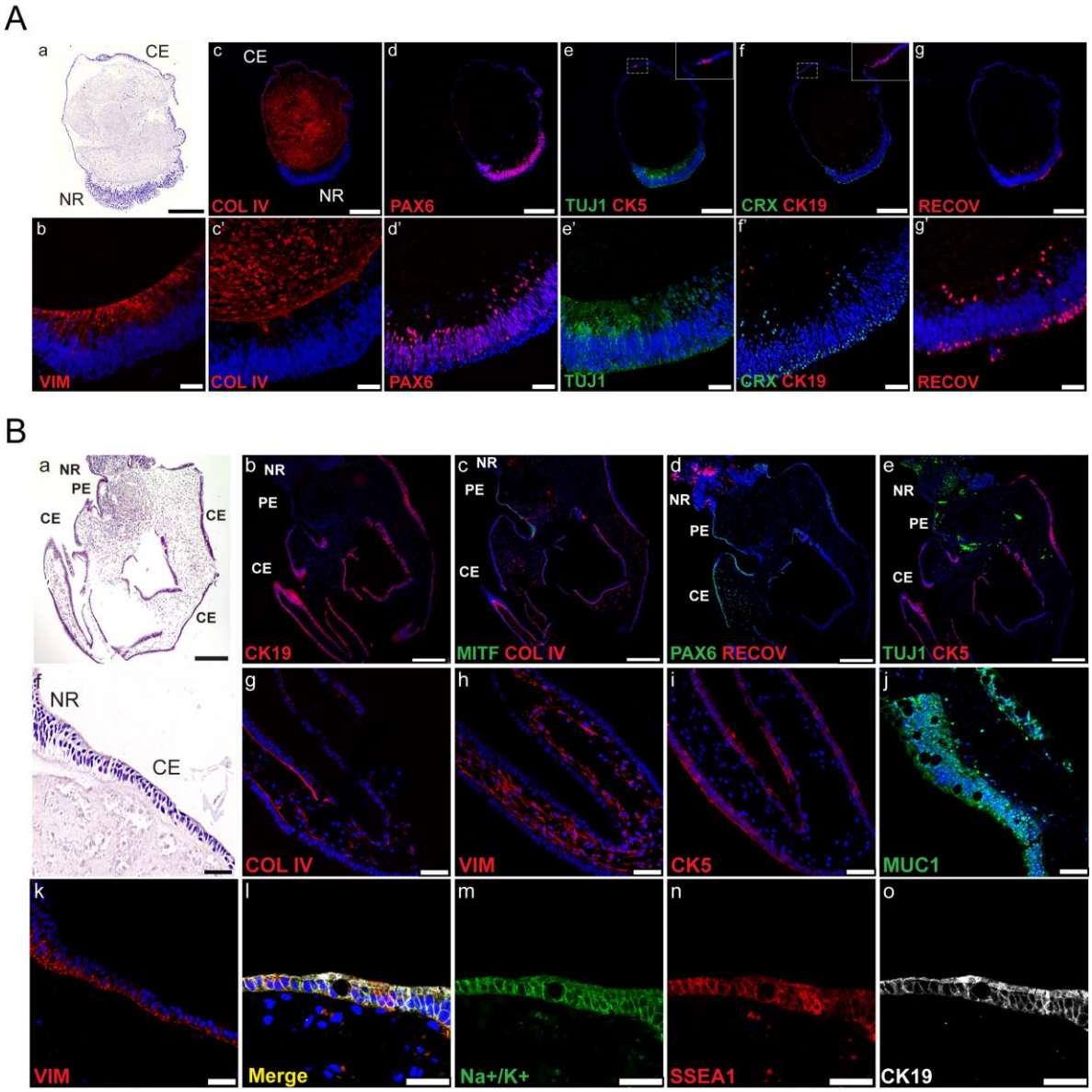

Figure S4: CBiPS30-4F-5-derived multi-ocular organoids composed by retinal, corneal and pigmented areas. **A)** Hematoxylin and eosin (HE) staining of multi-ocular organoid paraffin section formed by neuroretina (NR) and corneal epithelium (CE) (a). Immunofluorescent images showing expression of collagen 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, bright field and immunofluorescent images of multi-ocular organoid paraffin section formed by NR, CE and pigmented epithelium (PE). Stroma is stained with COL IV (b,g,n). PE is stained with bestrophin 1 (BEST; c,h), MITF (b,n) and PAX6 (c,h). Neuroretina is stained with CRX (d), RECOV (i), TUJ1 (e,j) and PAX6 (c,h). Scale bars: 250 µm in a-j; 100 µm in l; 50 µm in m,n; 25 µm in k. Nuclei are stained in DAPI. Na+/K+-ATPase (j') and SSEA1 (j''), and MUC1 (mucin-1, p)

FIGURE S5

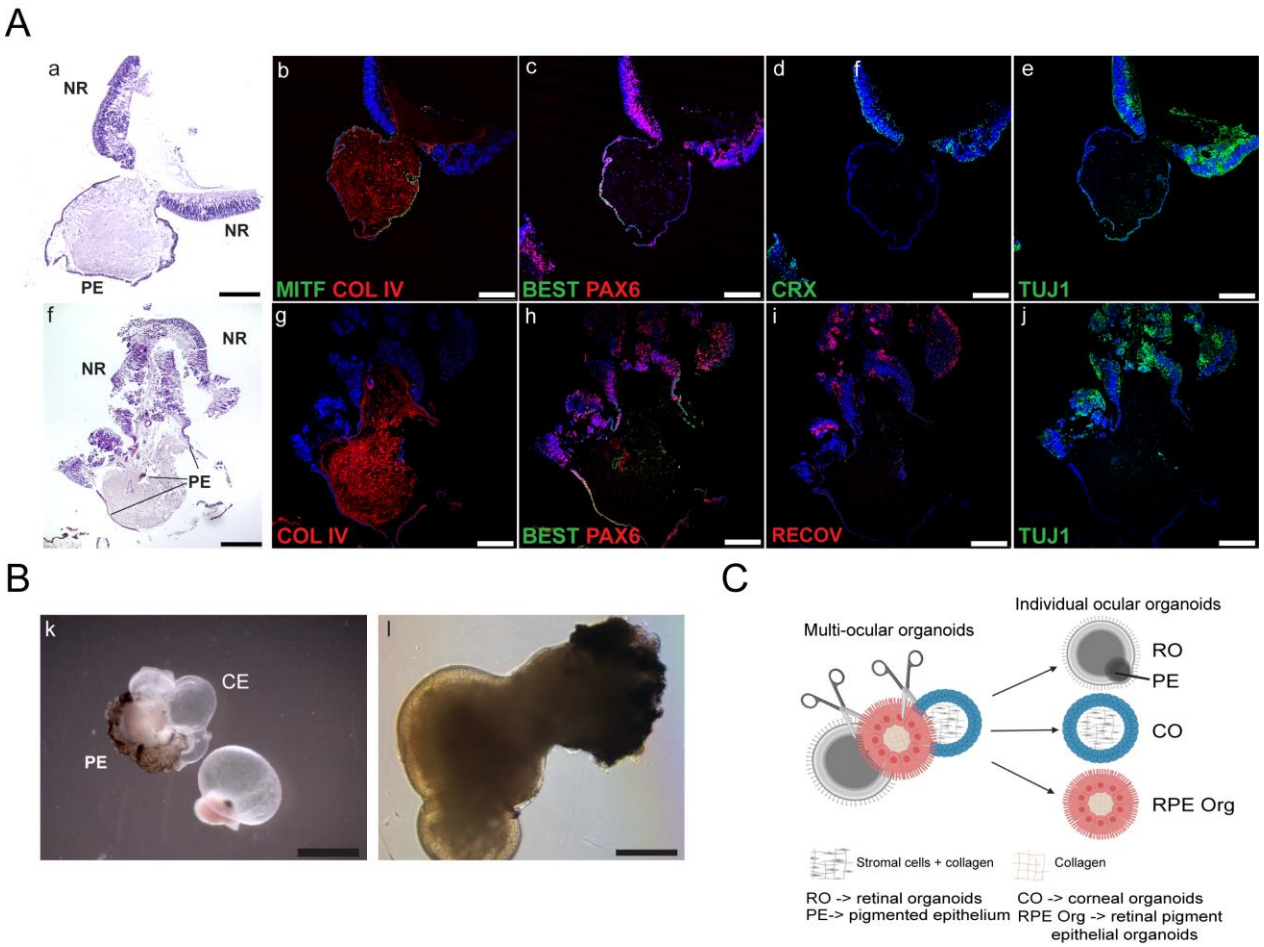

Figure S5: CBiPS30-4F-5-derived multi-ocular organoids composed by retinal and pigmented areas. **A)** Hematoxylin and eosin staining (a,f) and immunohistochemistry images of multi-ocular organois exhibiting retinal and RPE regions.

C) Schematic illustration of isolation of different ocular regions from multi-ocular organoids to generate retinal organoids (RO) and RO containing pigmented areas (RO+PE); corneal organoids (CO) and CO containing pigmented areas (CO+PE); and RPE organoids.

FIGURE S6

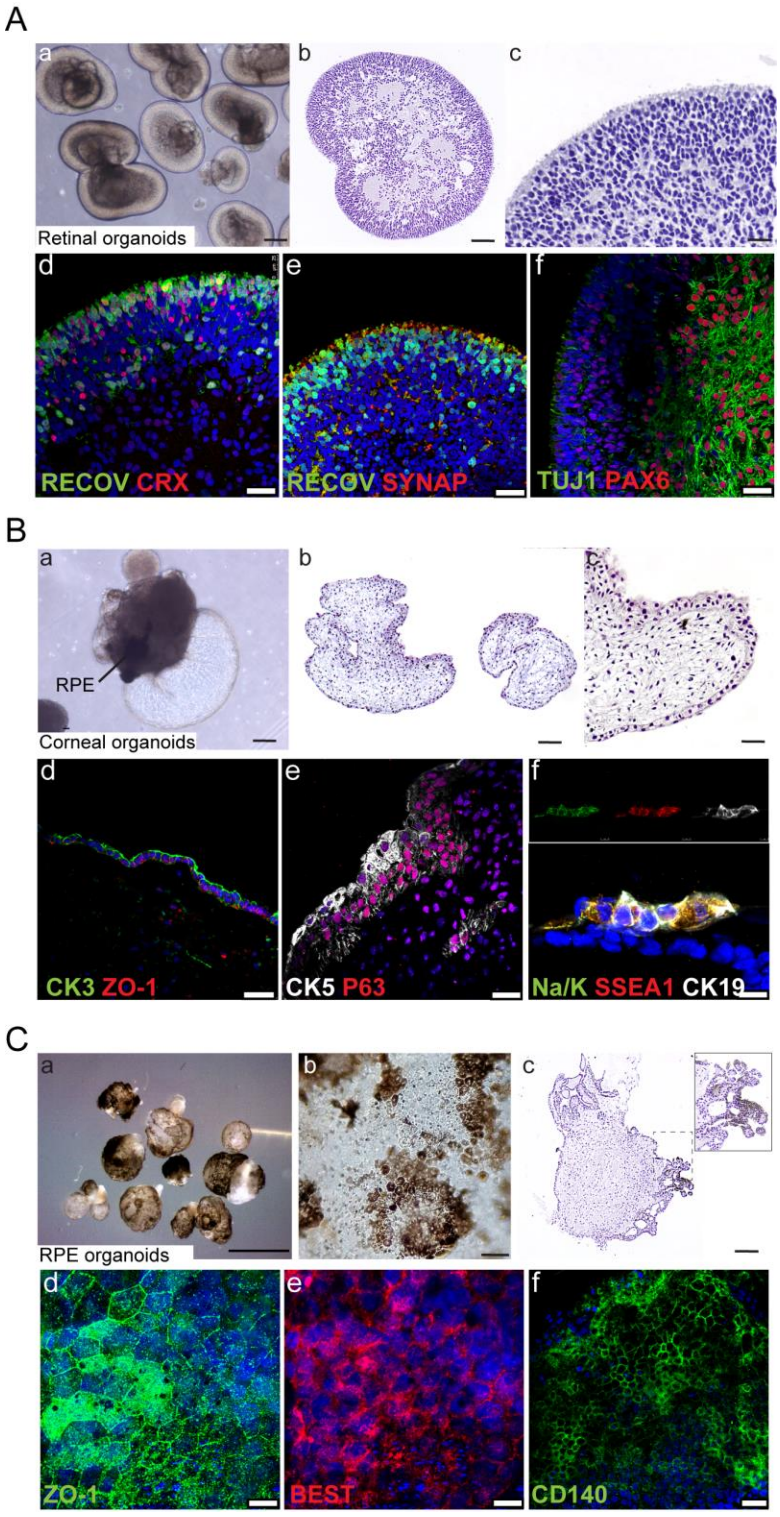

Figure S6: CBiPS30-4F-5-derived individual retinal, corneal and RPE organoids.

A) Bright filed image of retinal organoids in suspension culture at day 140 (a) showing laminated NR. HE staining images of retinal organoid paraffin sections (b,c) showing stratified NR at the apical site. (d-f) Immunofluorescence images of retinal organoid paraffin sections stained with specific antibodies against recoverin (RECOV) and CRX (photoreceptor markers), synaptophysin (SYNAP; presynapstic marker); PAX6 (bipolar and ganglion cell marker) and tubulun beta III (TUJ1; ganglion cell marker). Scale bars: 300

µm (a); 100 µm (b); 25 µm (c-f). **B)** Bright filed image of corneal-like organoids in suspension culture at day 140 in all-trans retinoic acid (a) showing spherical and transparent organoid corresponding to cornea/conjunctival epithelium. Some corneal organoids present a pigment epithelium (RPE) region in one side. HE staining images of corneal organoid paraffin sections (b,c) showing stratigied epithelium in the apical side fullfilled with stromal-like cells. (d-f) Immunofluorescence images of corneal-conjuctival 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 filed image of RPE organoids in suspension culture at day 140 in T3 (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. (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

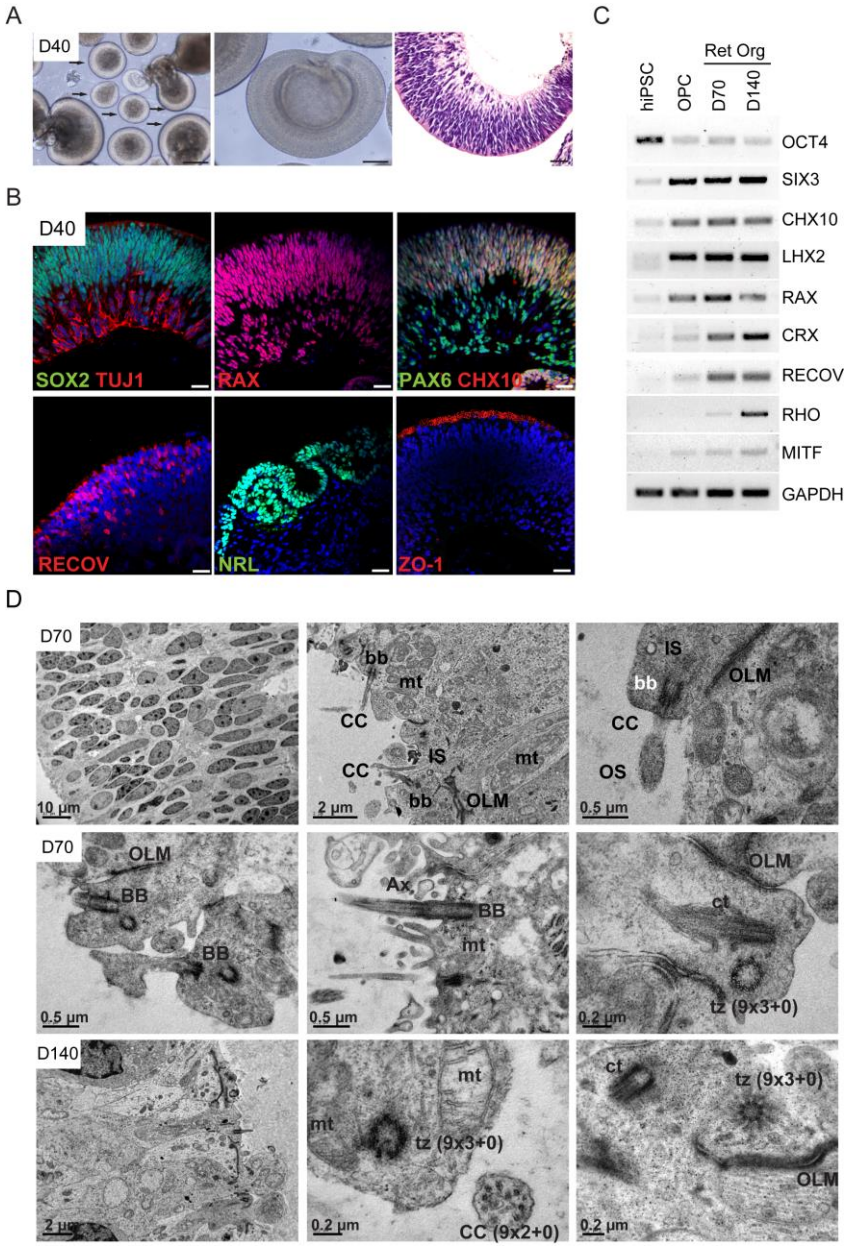

Figure S7: Characterization of retinal organoids generated from hiPSC-derived 2D multi-zone ocular progenitor cells. A) Bright-field images of retinal organoids derived from hiPSC at 40 DIV. Arrows indicate developing laminated retinal tissue. (Right) Hematoxylin and eosin staining of a paraffin section 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 40 DIV 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 internal control. **D)** Transmission electron microscopy analysis of retinal organoids at 70 and 140 DIV 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), classical array of nine triplet and doublet microtubles of bb and transition zone (tz) of the CC, respectively. Scale bars are indicated in the images.

FIGURE S8

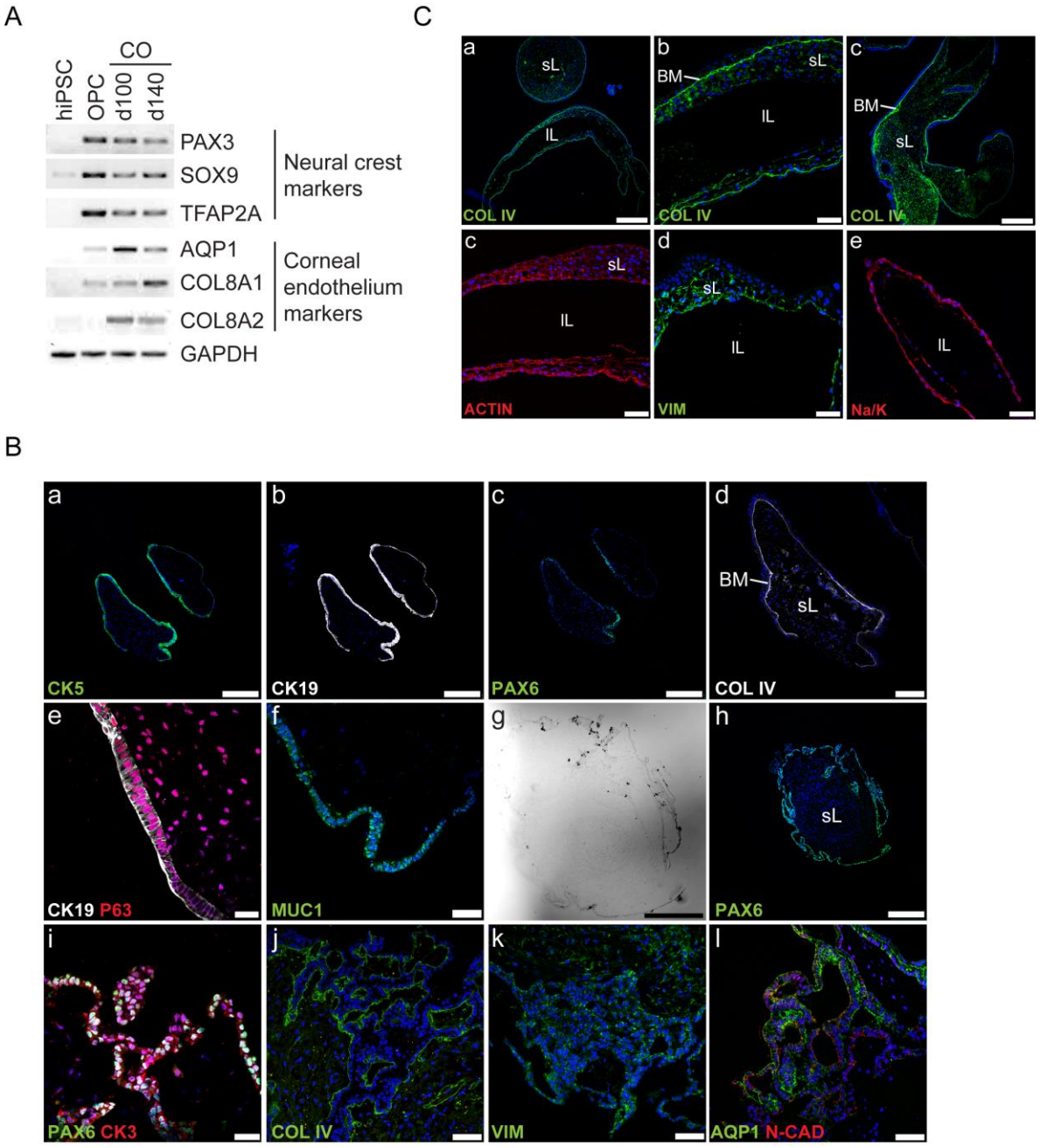

Figure S8. Characterization of neural crest cells and corneal endothelial-like organoids. A) PCR analysis of neural crest progenitors and corneal endothelium gene expression in samples from human induced pluripotent stem cells (hiPSC), ocular progenitor cells (OPC) at day 30 of differentiation and corneal organoids (CO) at days 100 and 140 in culture. Expression of GAPDH was used as internal control. B) Confocal images of corneal organoids at day 140 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). 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 stromal lumen (sL) and fluid-filled lumen (lL) stained with COL IV (collagen type IV) forming a basement membrane (BM) similar to Descemet's membrane, actin, VIM (vimentin) and Na+/K+-ATPase. Scale bars: 250 µm in a,c,; 50 µm in b,d-f. Nuclei are stained with DAPI.

FIGURE S9

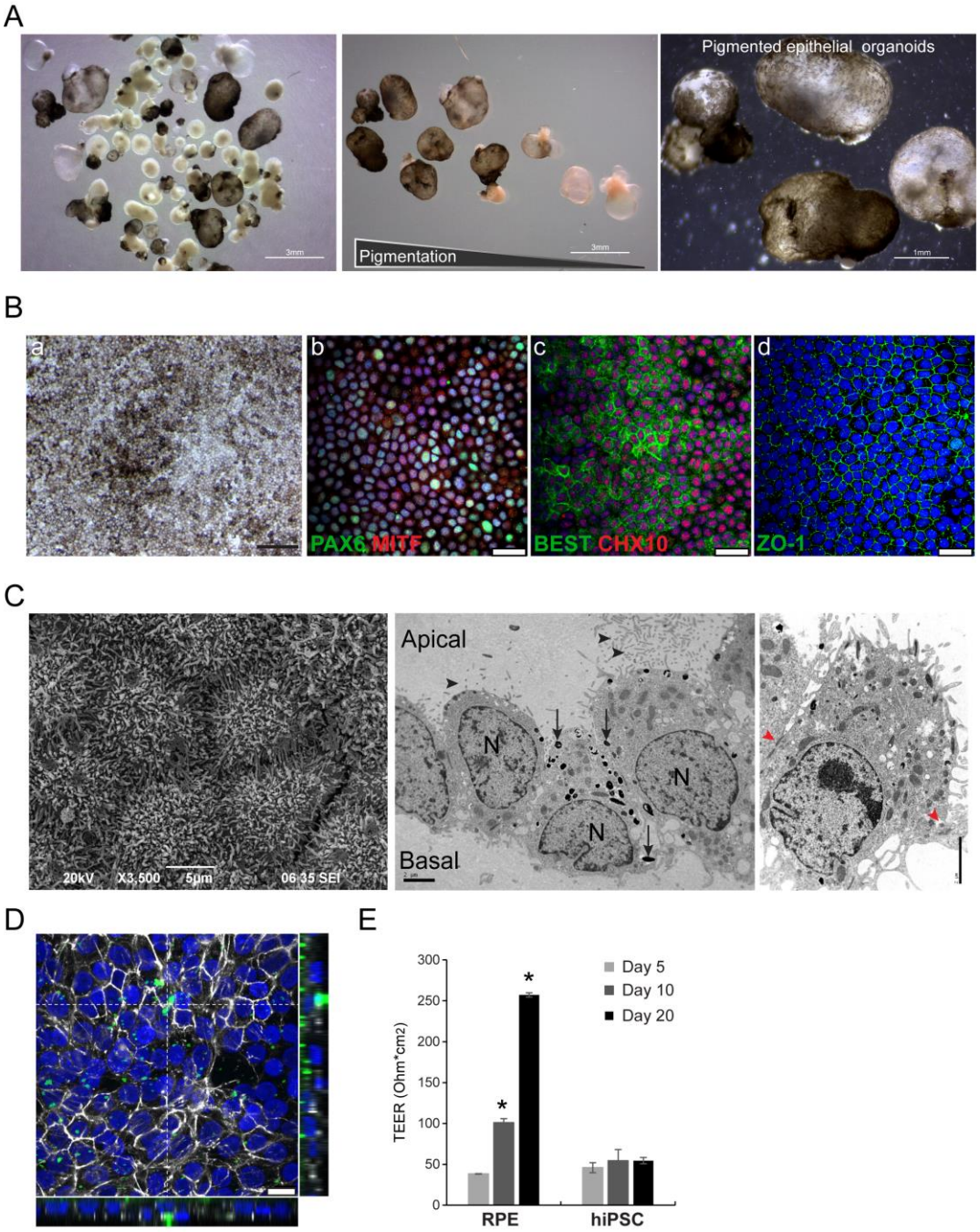

Figure S9. Pigmentation of retinal pigment epithelial (RPE) organoids and derivation of RPE cell culture. Bright field images of CBiPSC-30-5 hiPSC-derived RPE organoids in suspension at day 120, cultured in T3 from day 90. Scale bars: 3 mm in a-b; 1 mm in c. B) (a) Phase contrast image of RPE organoid-derived 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. E) (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. (Rigth) 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 cells cultured in transewells (n=3) at indicated days in culture. Data are expressed as mean ± SD, * p<0,01 using unpaired Student's t test.

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.