Supplemental Information

Supplemental Materials and Methods

Cryosection

For cryosectioning, retinal organoids were fixed for 15 min in 4% paraformaldehyde at 4°C and washed in PBS. Structures were incubated at 4°C in PBS / 30% Sucrose (Sigma-Aldrich) solution during at least 2 hrs. Structures were embedded in a solution of PBS, 7.5% gelatin (Sigma-Aldrich), 10% sucrose and frozen in isopentane at -50°C. 10 µm-thick cryosections were collected in two perpendicular planes.

Cell dissociation

Floating retinal organoids were collected at D84 or D100. Distal pigmented RPE was discarded from the structures under a stereomicroscope and retinal structures were washed 3 times in Ringer solution (NaCl 155 mM; KCl 5 mM; $CaCl_2$ 2 mM; $MgCl_2$ 1 mM; NaH_2PO_4 2 mM; HEPES 10 mM and Glucose 10 mM). RPE-free retinal organoids were dissociated with two units of pre-activated papain at 28.7 u/mg (Worthington) in Ringer solution during 25 min at 37°C. When cells were homogeneously resuspended with up and down pipetting, then papain was deactivated by adding 1 ml of ProB27 medium. Cells were centrifuged and resuspended in pre-warmed ProB27 medium. Retinal cells were plated in 24 well-plates previously coated with Poly-D-Lysin at 2 µg/cm² and Laminin at 1 µg/cm² (Sigma-Aldrich). Dissociated cells were incubated at 37°C in a standard 5% CO_2 / 95% air incubator and medium was changed every 2 days for the next 5 days, before immunostaining.

Calcium dye loading and two-photon calcium imaging.

Fura 2-AM (Thermo Fisher Scientific) was dissolved in dimethyl sulfoxide mixed with 20% Pluronic F-127 (Thermo Fisher Scientific) and then diluted to reach a final concentration of

10 µM. Dissociated retinal cells seeded on Poly-D-Lysin/Laminin-coated coverslips were dark adapted prior to every recording, and loaded with Fura2-AM (10 µM) for 45 min at 37°C in a standard incubator. Cells were then placed in the recording chamber of a 2-photon microscope and perfused (2 ml/min) with oxygenated (95% O_2/5% CO_2) AMES Ringer's solution (Sigma-Aldrich) for at least 1 hr, in the dark. Cells were later exposed to either 1 mM of 8-Br-cGMP (Sigma-Aldrich), diluted in AMES or AMES for control recordings. Glass pipettes (borosilicate glass capillaries) with a resistance of 4-5 MΩ, filled with the solutions and connected to a Picospritzer II pressure system (General Valve Corporation, pressure 5 psi) were used for applying 10 seconds puffs on small groups of recorded cells. Temperature was kept at 37°C for the duration of the experiment.

Imaging of cells stained by the Fura 2-AM dye was performed using a custom-made two-photon microscope equipped with a 25x water immersion objective (XLPlanN-25x-W-MP/NA1.05, Olympus) and a pulsed femto-second laser (InSightTM DeepSeeTM, Newport Corporation, Irvine, CA). Images were acquired using the excitation laser at a wavelength of 800 nm. Square regions (minimum 50x50 µm) of neighbor cells positioned in the puffing area were imaged and analyzed. For each experiment we started by recording 20 seconds of baseline spontaneous activity before puffing. The interval between two consecutive puff stimulations was at least two minutes. An increase in calcium concentration in a cell was detected as a decrease in Fura-2 fluorescence intensity, which was calculated using the formula % ΔF/F0, in which ΔF=F-F0, and where F0 was average baseline cell intensity during the 20 sec prestimulus period.

Immunostaining and imaging on retinal sections and dissociated retinal cells

Sections, retinal dissociated cells or hiRPE cells were fixed with 4% PAF in PBS for 5 min before immunostaining. After washes with PBS, nonspecific binding sites were blocked for 1 hr at room temperature with a PBS solution containing 0.2% gelatin and 0.25% Triton X-100 (blocking buffer) and then overnight at 4°C with the primary antibody (Table S1) [19, 45] diluted in blocking buffer. Slides were washed three times in PBS with 0.1% Tween and then

incubated for 1 hour at room temperature with appropriate secondary antibodies conjugated with either AlexaFluor 488, 594 or 647 (Interchim) diluted at 1:600 in blocking buffer with 4',6-diamidino-2-phenylindole (DAPI) diluted at 1:1000 to counterstain nuclei. TUNEL assays were performed using the in situ cell death detection kit, TMR red (Roche, Sigma-Aldrich) according to the manufacturer's recommendations. Fluorescent staining signals were captured with a DM6000 microscope (Leica Microsystems) equipped with a CCD CoolSNAP-HQ camera (Roper Scientific) or using an Olympus FV1000 confocal microscope equipped with 405, 488, 543 and 633 nm lasers. Confocal images were acquired using a 1.55 or 0.46 µm step size and corresponded to the projection of 20 to 40 optical sections.

Karyotype analysis

Samples for conventional cytogenetic analysis were prepared as previously described [46] with slight modifications. For obtention of mitotic preparations, cells were cultured 2 hours in E8 medium supplemented with 100 ng/ml Colcemid (ThermoFisher Scientific) before osmotic shock in 0.075 M KCl (ThermoFisher Scientific) and fixation with methanol/acetic acid solution (3:1). For each cell line, a minimum of 10 metaphasis were analyzed.

RNA extraction and Taqman Assay

Total RNAs were extracted using Nucleospin RNA II kit (Macherey-Nagel) according to the manufacturer's protocol, and RNA yields and quality were checked with a NanoDrop spectrophotometer (Thermo Fischer Scientific). cDNA were synthesized from 250 ng of mRNA using the QuantiTect reverse transcription kit (Qiagen) following manufacturer's recommendations. Synthesized cDNA were then diluted at 1/20 in DN^{ase}-free water before performing quantitative PCR. qPCR analysis was performed on a Applied Biosystems real-time PCR machine (7500 Fast System) with custom TaqMan® Array 96-Well Fast plates and TaqMan® Gene expression Master Mix (Thermo Fischer Scientific) following manufacturer's instructions. All primers and MGB probes labeled with FAM for amplification (Table S2) were purchased from Thermo Fischer Scientific. Results were normalized against 18S and

quantification of gene expression was based on the DeltaCt Method in three minimum independent biological experiments.

Transmission electron microscopy (TEM)

Retinal organoids were fixed with 2% glutaraldehyde and 1% PAF in 0.1 M PBS (pH 7.4), for 1 hr at room temperature and washed in PBS. Samples were postfixed in 1% osmium-bidistilled water for 1 hr at room temperature. After washes in bidistilled water, the samples were dehydrated in increasing concentrations of ethanol. Samples were then infiltrated in 1:1 ethanol: epon resin for 1 hr and finally 100% epon resin for 48 hrs at 60°C for polymerization. 70 nm-thick sections were cut with an ultracut UCT microtome (Leica Microsystems) and picked up on copper rhodium-coated grids. Grids were stained for 2 min in Uranyless (DELTA Microscopies) and for 5 min in 0,2% lead citrate and further analyzed on an electron microscope (EM 912 OMEGA, ZEISS) at 80 kV. Images were captured with digital camera (Side-Mounted TEM CCD, Veleta 2kx2k).

VEGF and PEDF ELISA

HiRPEp3 (hiPSC-2) were plated at 50 000 cells/cm² and cultured in ProN2 medium. The cultured medium from confluent hiRPE p3 cells was collected 48hrs after the medium change and stored at -80°C until the assay was performed. VEGFA and PEDF secretion levels were measured by respectively the Human VEGF Quantikine ELISA kit and the Human SerpinF1/PEDF DuoSet ELISA (R&D Systems), according to the manufacturer's instructions.

Phagocytosis assay.

Banked hiRPE cells at passage1 were thawed, cultured and confluent hiRPE cells were plated onto 96-well CellStart®-coated plates at $5x10^4$ cells/cm² and cultured in ProN2 medium for at least 4 weeks before the phagocytosis assay. HiRPEp3 cells were challenged for 3 hrs with $1×10^6$ FITC-labeled photoreceptor outer segments (POS) before detection of surface-bound and internalized FITC-POS particles according to established procedures

[37]. Each well was rinsed three times with PBS containing 1 mM $MgCl_2$ and 0.2 mM $CaCl_2$ (PBS-CM). For exclusive detection of internalized particles, fluorescence of surface-bound FITC-POS was selectively quenched by incubation in 0.2% trypan blue in PBS-CM for 10 min before cell fixation. Cells were fixed by incubation in ice cold methanol for 5 min followed by rehydration and incubation for 10 min at room temperature with DAPI for nuclei counterstaining. Fluorescent signals were quantified with the Infinite M1000 Pro (Tecan) and cells were imaged with a DM6000 microscope equipped with a CCD CoolSNAP-HQ camera.. To assess the specificity of the POS phagocytosis machinery used, cells were pre-incubated 30 min in the presence of 50 µg/ml of the anti-integrin $\alpha v\beta 5$ (function blocking antibody, P1F6 Millipore) and subsequently during POS phagocytosis as described above. The human ARPE-19 cell line was used as a positive control for phagocytic activity, and fibroblasts were used as a negative control for the phagocytic machinery used for POS uptake.

Statistical analysis

Statistical analysis was performed using Prism 6 (GraphPad software) with unpaired parametric Student's *t* test with Welch's correction for unequal variances when required (one-to-one comparisons). Values of $P< 0.05$ were considered statistically significant.

Supplemental References

45. Li A, Zhu X, Brown B, et al. Gene expression networks underlying retinoic acid-induced differentiation of human retinoblastoma cells. Invest. Ophthalmol. Vis. Sci. 2003;44(3):996–1007.
46. Tosca L, Feraud O, Magniez A, et al. Genomic instability of human embryonic stem cell lines using different passaging culture methods. Mol. Cytogenet. 2015;8:30.

Supplemental figure legends

Figure S1: Characterization of integration-free hiPSCs (hiPSC-2) adapted to Xeno Free and Feeder Free conditions.

(A) RT-qPCR analysis of pluripotency and self-renewal markers in hESCs, hiPSC-2 and adult human dermal fibroblasts (AHDF). Data are normalized to hESCs. (B-G) Immunohistochemistry of pluripotency markers (SSEA4, OCT4, TRA1-60, and SOX2) in hiPSC-2. Scale bars = 100 µm (H) Karyotype analysis of hiPSC-2.

Figure S2: Reproducibility of the retinal organoid formation with different hiPSC lines. Morphology of the self-forming neuroretina-like structures at D28 (A, E, G) and derived retinal organoids at D28 (B, F), D35 (C, H) and D42 (D), generated from three different integration-free hiPSCs: hiPSC-2 derived from adult dermal fibroblasts reprogrammed by an episomal approach (A-D); hiPSC-3 derived from neonatal foreskin fibroblasts reprogrammed by an episomal approach (E, F) and hiPSC-4 derived from adult dermal fibroblasts reprogrammed by the Sendai virus method (G, H). Scale bars = 100 µm. Efficiency of retinal organoid formation was determined by calculating the number of structures per cm^2 at D28 for the three different hiPSC lines (I). The number of independent differentiation experiments is noted for each hiPSC lines.

Figure S3: Expression of surface antigen CD73 in hiPSC-derived photoreceptors. Immunofluorescence staining of cryosectioned retinal organoids at different times of differentiation for CD73 (A-D) and either PAX6 (A') or different photoreceptor markers, RECOVERIN (B'), BLUE OPSIN (C') and R/G OPSIN (D'). A" to D" correspond to merge image of panels A-A' to D-D' without DAPI staining, demonstrating the co-localization of CD73 exclusively with different photoreceptor markers. Scale bars = 25 µm.

Figure S4: Efficient generation of RGCs and photoreceptors in retinal organoids derived from different hiPSC lines.
Immunofluorescence staining of cryosectioned retinal organoids derived from hiPSC-3 and hiPSC-4 at different times of differentiation using markers for RGCs (BRN3A) and photoreceptors (OTX2, RECOVERIN, CONE ARRESTIN, R/G OPSIN, RHODOPSIN, BLUE OPSIN). Scale bars = 100 µm (A-I), 50 µm (J).

Figure S5: Absence of apoptosis in the rosettes containing hiPSC-derived photoreceptors. Immunohistological staining of D195-old retinal organoid sections for apoptotic cells (TUNEL) and photoreceptor markers, RECOVERIN (A', A'"), RHODOPSIN (A", A'") and BLUE OPSIN (B'). Merged images showed no TUNEL-positive photoreceptors in the rosettes. Scale bars = 50 µm.

Supplemental movie legends

Movie S1: 3D Movie of a D195 retinal organoid labelled with anti-Recoverin (green) and anti-Rhodopsin (red).

Movie S2: 3D Movie of a D195 retinal organoid labelled with anti-Cone Arrestin (green) and anti-Rhodopsin (red).

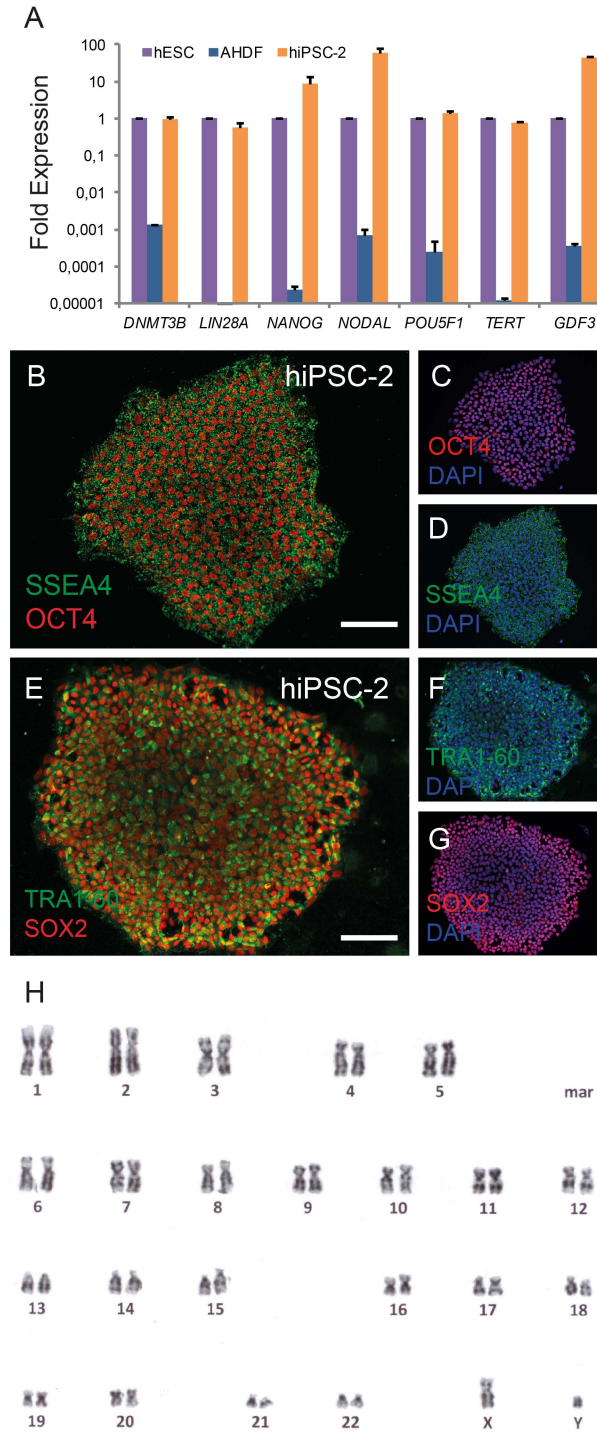
Table S1. List of antibodies used for immunohistochemistry analysis.

Antigen	Species	Dilution	Source
Acetylated TUBULIN	Mouse monoclonal	1:1000	Sigma
AP2	Mouse monoclonal	1:100	DSHB
BESTROPHIN	Mouse monoclonal	1:1000	Novus
BLUE OPSIN	Rabbit polyclonal	1:500	Millipore
BRN3A	Mouse monoclonal	1:250	Millipore
CD73	Mouse monoclonal	1:100	BioLegend
CONE ARRESTIN	Rabbit polyclonal	1:2000	Millipore
	Rabbit polyclonal	1:10 000	gift from Dr C. Craft
CRX	Mouse monoclonal	1:5000	Abnova
EZRIN	Mouse monoclonal	1:250	Sigma
GLUTAMIN SYNTHASE	Mouse monoclonal	1:500	Millipore
KI67	Mouse monoclonal	1:200	BD Pharmagen
LIM1 (LHX1)	Mouse monoclonal	1:20	DSHB
LHX2	Goat polyclonal	1:100	Santa Cruz
MITF	Mouse monoclonal	1:200	DAKO
OCT4	Rabbit monoclonal	1:100	Cell Signaling
R/G OPSIN	Rabbit polyclonal	1:500	Millipore
OTX2	Rabbit polyclonal	1:5000	Millipore
PAX6	Rabbit polyclonal	1:1000	Millipore
PKCα	Rabbit polyclonal	1:5000	Santa Cruz
RAX/RX	Rabbit polyclonal	1:10 000	Abcam
RHODOPSIN	Mouse monoclonal	1:250	Millipore
RECOVERIN	Rabbit polyclonal	1:2000	Millipore
SSEA4	Mouse monoclonal	1:200	Cell Signaling
SOX2	Rabbit monoclonal	1:400	Cell Signaling
SOX9	Rabbit polyclonal	1:1000	Millipore
TRA1-60	Mouse monoclonal	1:100	Cell Signaling
VSX2 (CHX10)	Goat polyclonal	1:2000	Santa Cruz
ZO1	Rabbit polyclonal	1:250	ThermoFischer Sci

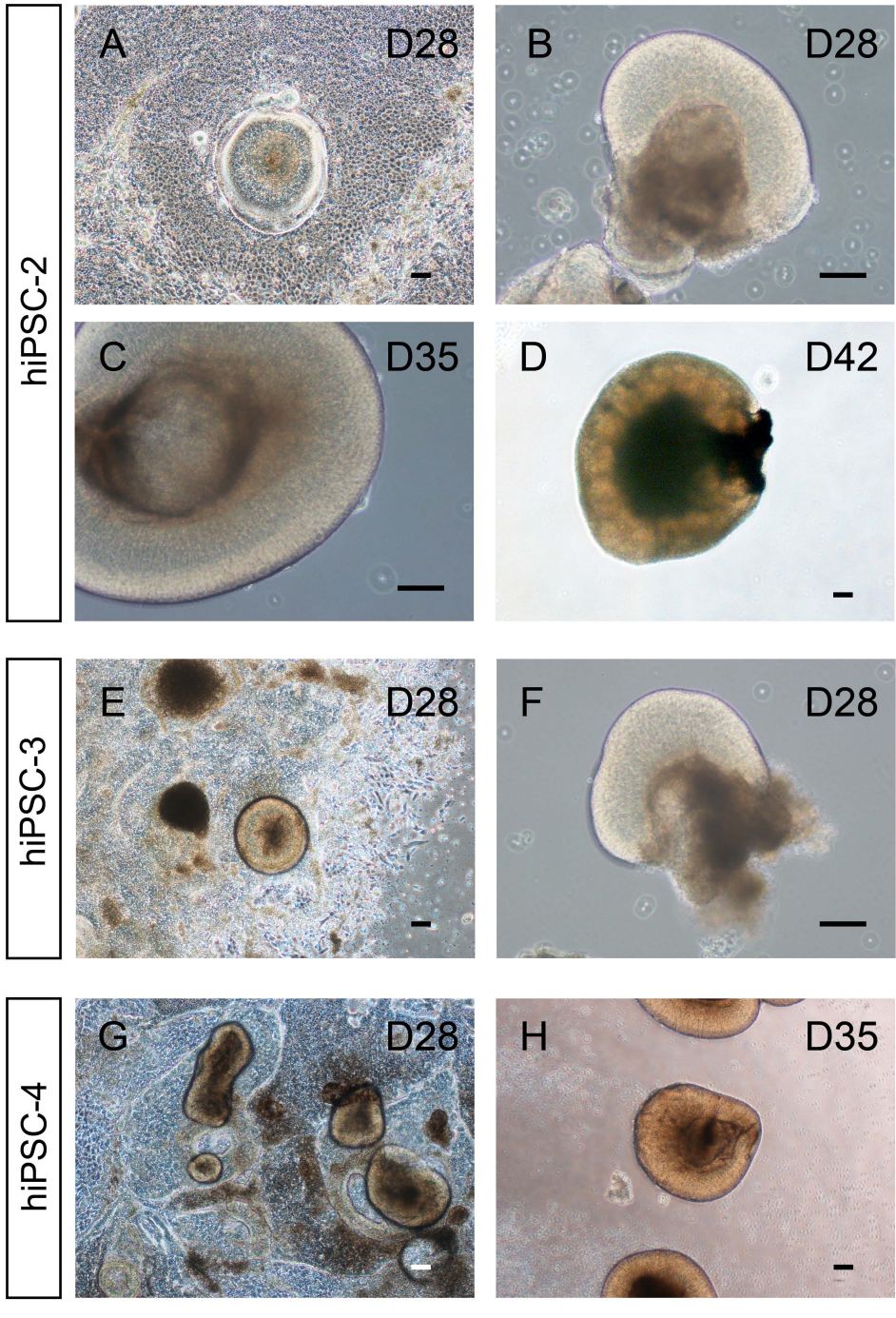
Table S2. List of TaqMan® Gene Expression ID Assays used for qRT-PCR:

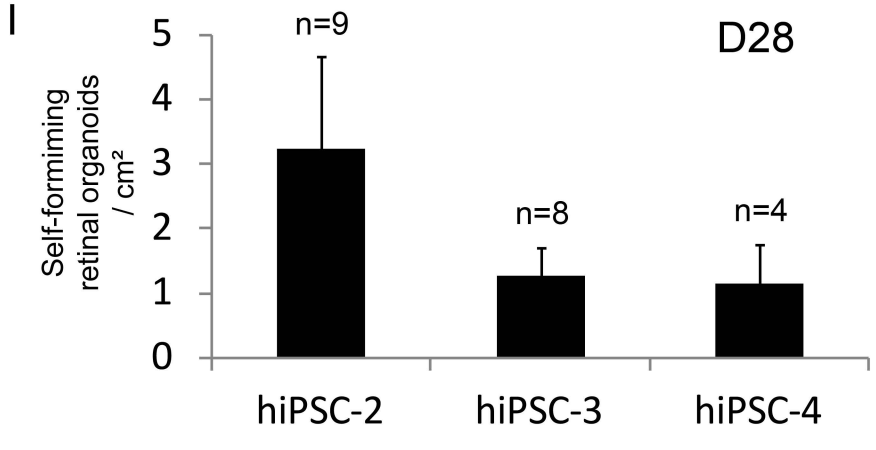
Gene Symbols	Assays IDs
18S	18S-Hs99999901_s1
BEST1	BEST1-Hs00188249_m1
BLUE OPSIN	OPN1SW-Hs00181790_m1
BRN3A	POU4F1-Hs00366711_m1
BRN3B	POU4F2-Hs00231820_m1
CONE ARRESTIN	ARR3-Hs00182888_m1
CRX	CRX-Hs00230899_m1
DKK1	DKK1-Hs00183740_m1
DNMT3B	DNMT3B-Hs00171876_m1
GAD2	GAD2-Hs00609534_m1
GDF3	GDF3-Hs00220998_m1
GLAST1	SLC1A3-Hs00188193_m1
LHX2	LHX2-Hs00180351_m1
LIM1	LHX1-Hs00232144_m1
LIN28A	LIN28A-Hs00702808_s1
MERTK	MERTK-Hs01031973_m1
MITF	MITF-Hs01117294_m1
NANOG	NANOG-Hs02387400_g1
NEUROD1	NEUROD1-Hs00159598_m1
NODAL	NODAL-Hs00415443_m1
NOGGIN	NOG-Hs00271352_s1
NRL	NRL-Hs00172997_m1
OCT4 (POU5F1)	POU5F1-Hs00999632_g1
PAX6	PAX6-Hs00240871_m1
PDEF	SERPINF1-Hs01106934_m1
PKCα	PRKCA-Hs00925195_m1
R/G OPSIN	OPN1MW-Hs00241039_m1
RAX	RAX-Hs00429459_m1
RECOVERIN	RCVRN-Hs00610056_m1
RHODOPSIN	RHO-Hs00892431_m1
*RLBP**1***	RLBP1-Hs00165632_m1
RPE65	RPE65-Hs01071462_m1
SIX3	SIX3-Hs00193667_m1
TERT	TERT-Hs00972656_m1
VEGF	VEGFA-Hs00900055_m1
VSX2	VSX2-Hs00766959_s1

Goureau, Supplemental figure 1, top

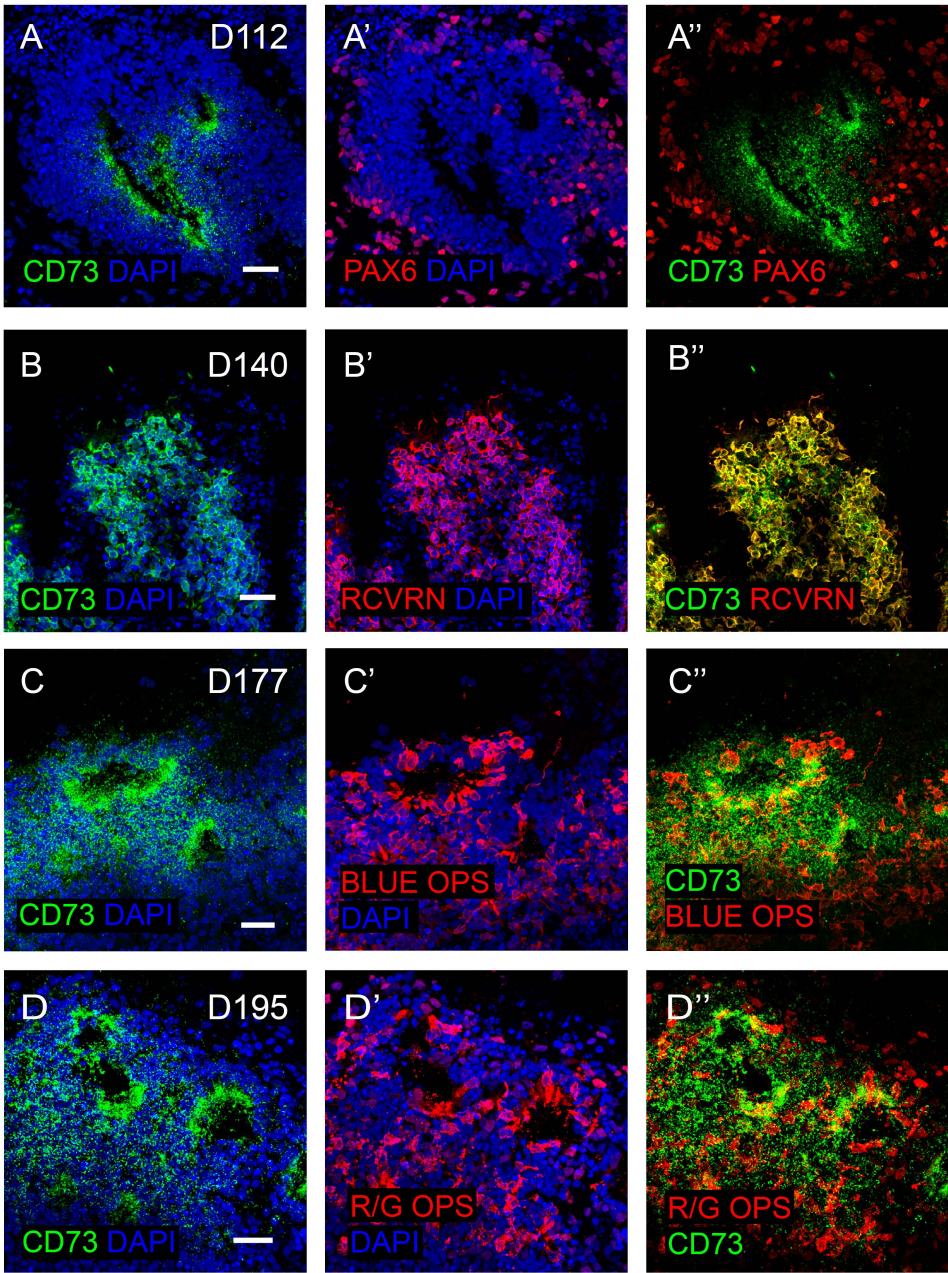


Goureau, Supplemental figure 2, top

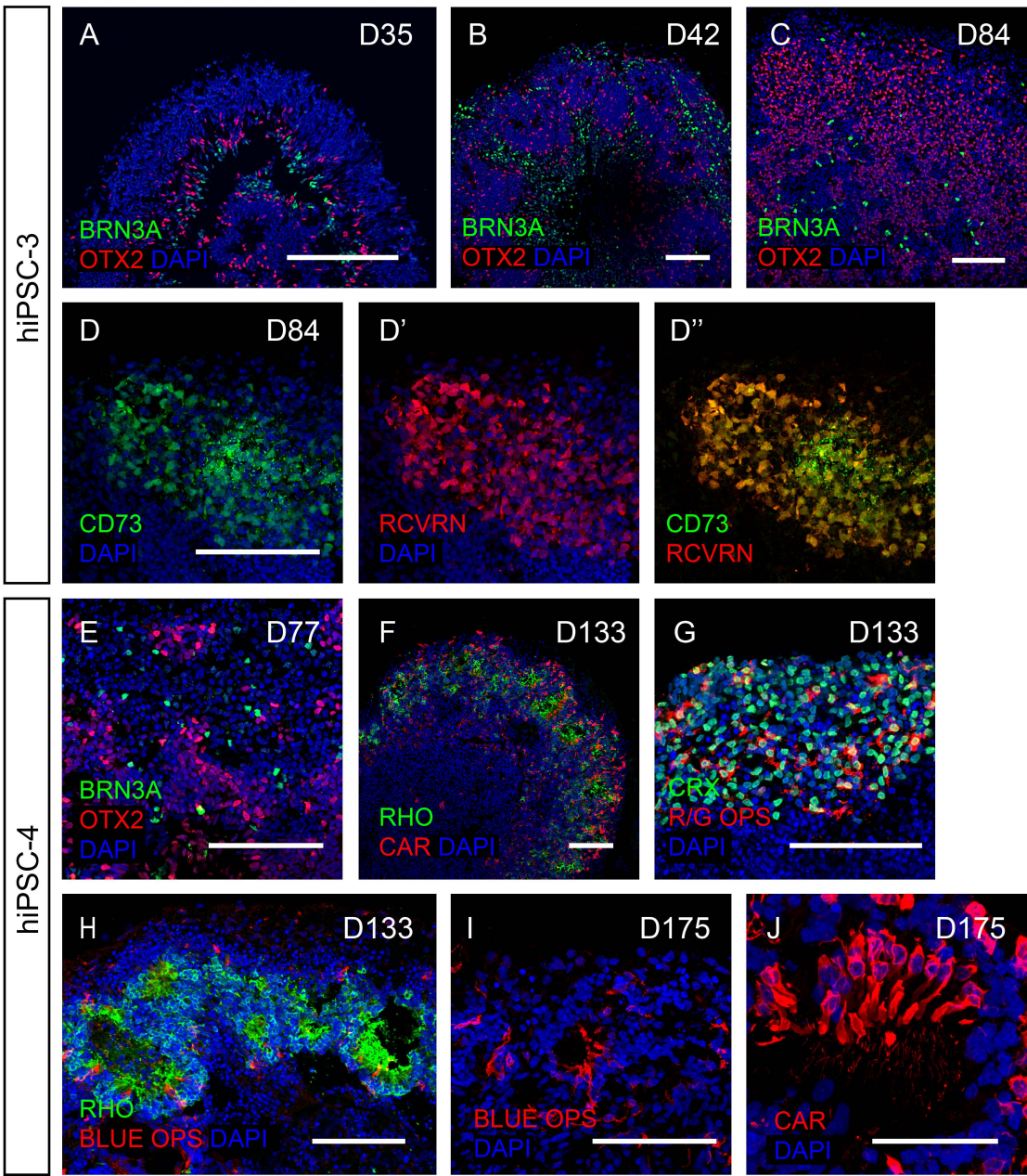




Goureau, Supplemental figure 3, top



Goureau, Supplemental figure 4, top



Goureau, Supplemental figure 5, top