OMTM, Volume *20*

Supplemental information

Cell therapy with hiPSC-derived RPE cells and RPCs prevents visual function loss in a rat model of retinal degeneration

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SUPPLEMENTARY FIGURES

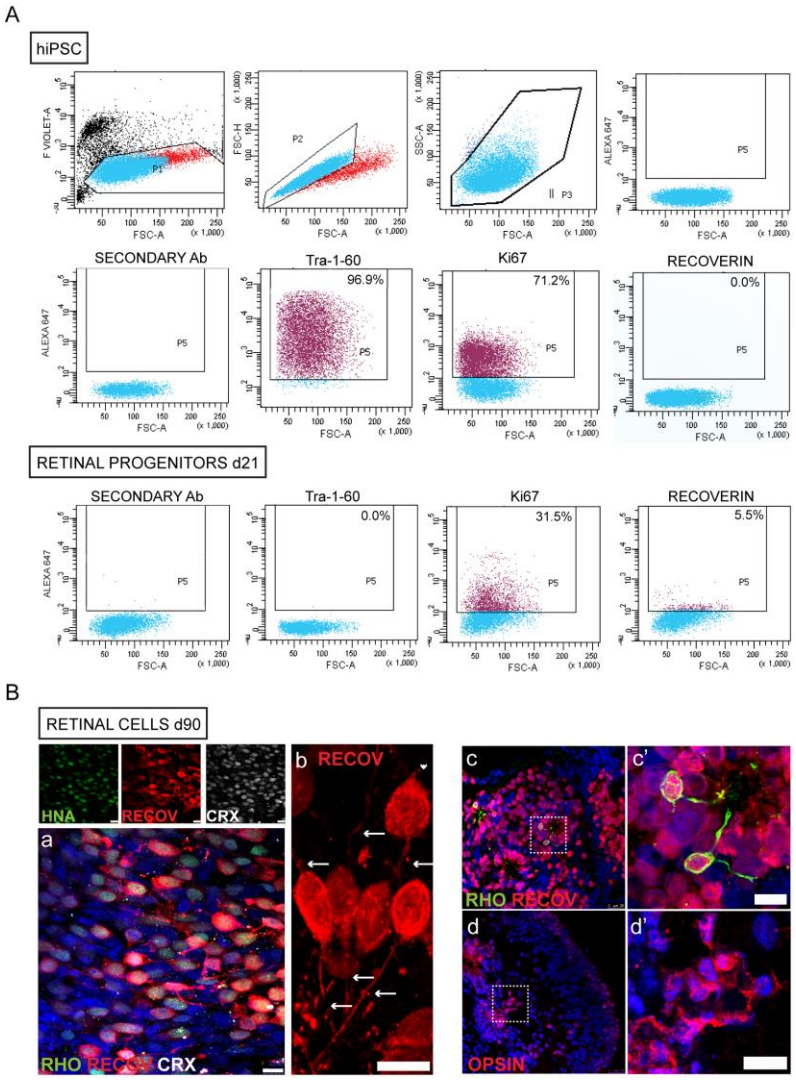

Figure S1: Analysis of hiPSC-derived retinal cells. A) Dot plot examples (upper panels) showing flow cytometry strategy indicating the live cells, dead cells, doublets and debris in hiPSC. Flow cytometric analyses of Tra-1-60, Ki67 and recoverin expression by hiPSC and retinal progenitors at day 21 of differentiation. Unstained cells and secondary antibodies were used as negative controls. Results are representative of 2 biological replicates. B) Confocal images of retinal cells at day 90 expressing human nuclear antigen (HNA), recoverin and CRX (a). Magnification shows recoverin+ cells with round cell bodies and axonal projections (b; white arrows). Retinal cells also expressed rhodopsin (RHO) (c) and red/green-opsin (OPSIN) (d). Magnifications show RHO+ (c') and OPSIN+ cells (d'). Nuclei are stained with DAPI. Scale bars: 25 μm in c,d and 10 μm in a,b,c',d'.

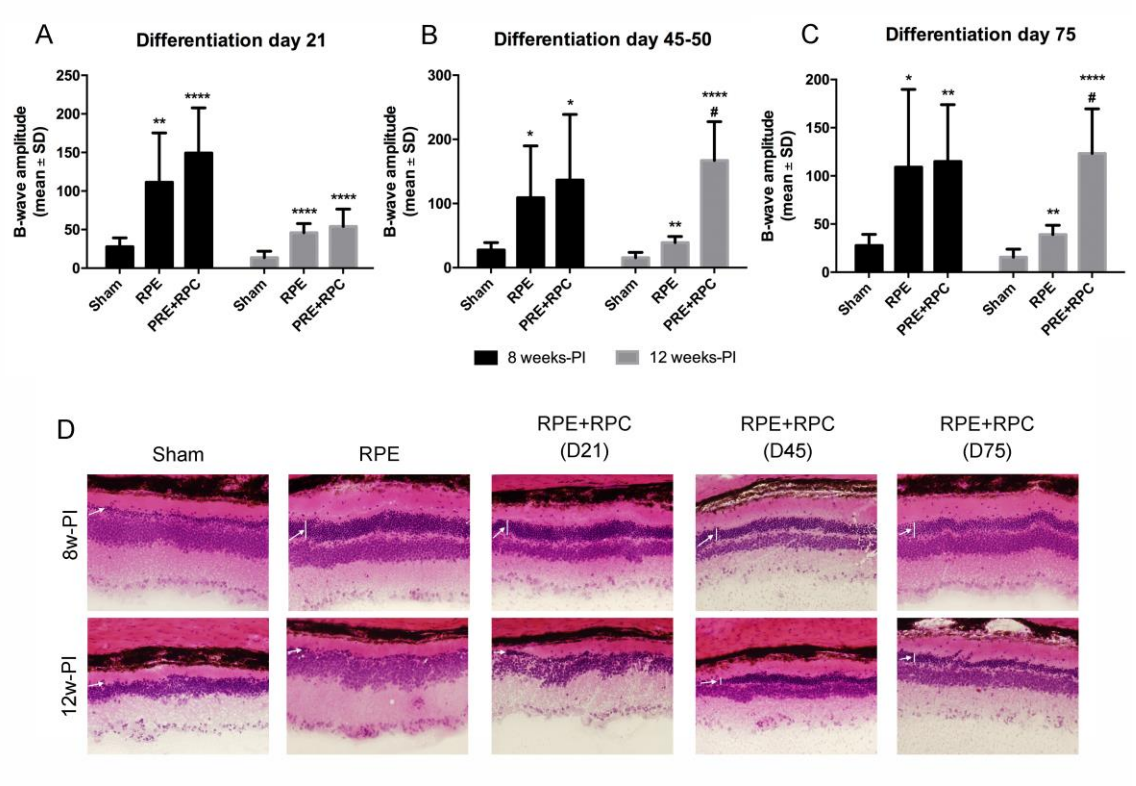

Figure S2: *In vivo* study of the best RPC differentiation time-point for transplantation. Post-natal day 21 RCS rats were subretinally injected with sham media (n=9), hiPSC-derived RPE cells (n=9) or RPE cells + retinal progenitors at 21 (n=9), RPC at 45 (n=7) and 75 days of differentiation (n=6). A-C) Visual function analyses at 8 weeks (black columns) and 12 weeks (grey columns) post-injection by electroretinogram. The mean ± SD b-wave amplitudes at 1 log cd·s·m^{-2} were compared between sham and RPE or RPE+RPC groups (*) and between RPE and RPE+RPE groups (#) at 21 days (A), 45 days (B) and 75 days of differentiation (C). D) Hematoxylin and eosin staining of representative retina sections of each treatment group in the grafted areas at 8 and 12 weeks post-injection. At 12 weeks post-injection, ONL (white arrow) disappeared in sham group and in RPE and RPE+RPC (D21) groups similarly. By contrast, ONL was maintained in RPE+RPC group at D45 and D75. Statistical significance was calculated by one-way ANOVA. * $p<0.05$; ** $p<0.005$; **** $p<0.0001$; # $p<0.05$. Abbreviations: RCS, Royal College of Surgeons; hiPSC, human induced pluripotent stem cells; PI, post injection; RPE, retinal pigment epithelium; GCL, ganglion cell layer; ONL, outer nuclear layer; INL, inner nuclear layer; RPC, retinal precursor cells; SD, standard deviation of mean.

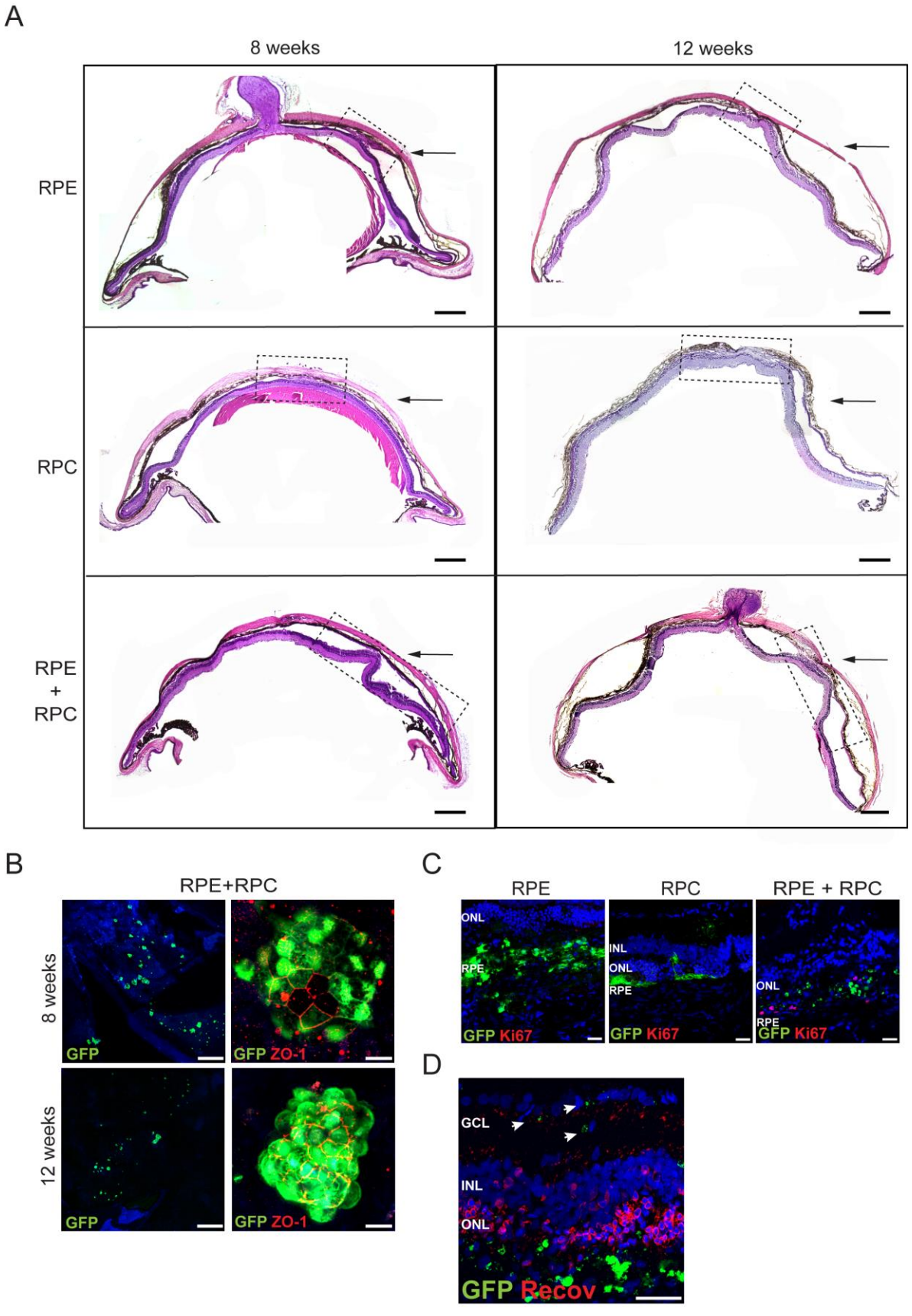
A
8 weeks
12 weeks
RPE
RPC
RPE
+
RPC
B
RPE+RPC
8 weeks
12 weeks
GFP
GFP ZO-1
GFP
GFP ZO-1
C
RPE
RPC
RPE + RPC
ONL
RPE
INL
ONL
RPE
ONL
RPE
GFP Ki67
GFP Ki67
GFP Ki67
D
GCL
INL
ONL
GFP Recov

Figure S3. Post-mortem analysis of retinal conservation after RPE and RPC transplantation in the RCS rat retina. A) Hematoxylin and eosin cross section of transplanted eyes with subretinal injection of RPE, RPC and RPE+RPC at 8 weeks and at 12 weeks post-injection. Arrows indicate the injection site and dashed square frames the area surrounding the injection site where ONL is conserved. Scale bars: 500 µm. B) Flat-mounted RPE layer of engrafted RPE cells (GFP in green) forming tight junctions (ZO-1 in red) at 8 and at 12 weeks post-injection with RPE+RPCs. Scale bars: 250 µm (left panels) and 10 µm (right panels). C) Immunostaining with Ki67 of RPE, RPC and RPE+RPC groups at 8 weeks post-injection showing few Ki67+/GFP- cells restricted in the subretinal space only in RPE+RPC group. Scale bars: 25 µm. D) Immunostaining with recoverin of RPE+RPC transplanted group at 8 weeks showing GFP+ material transfer to GCL (white arrowheads). Scale bar: 25 µm. Nuclei are stained with DAPI. Abbreviations: GCL, ganglion cell layer; GFP, green fluorescent protein; INL, inner nuclear layer; ONL, outer nuclear layer; RPC, retinal precursor cells; RPE, retinal pigment epithelium.

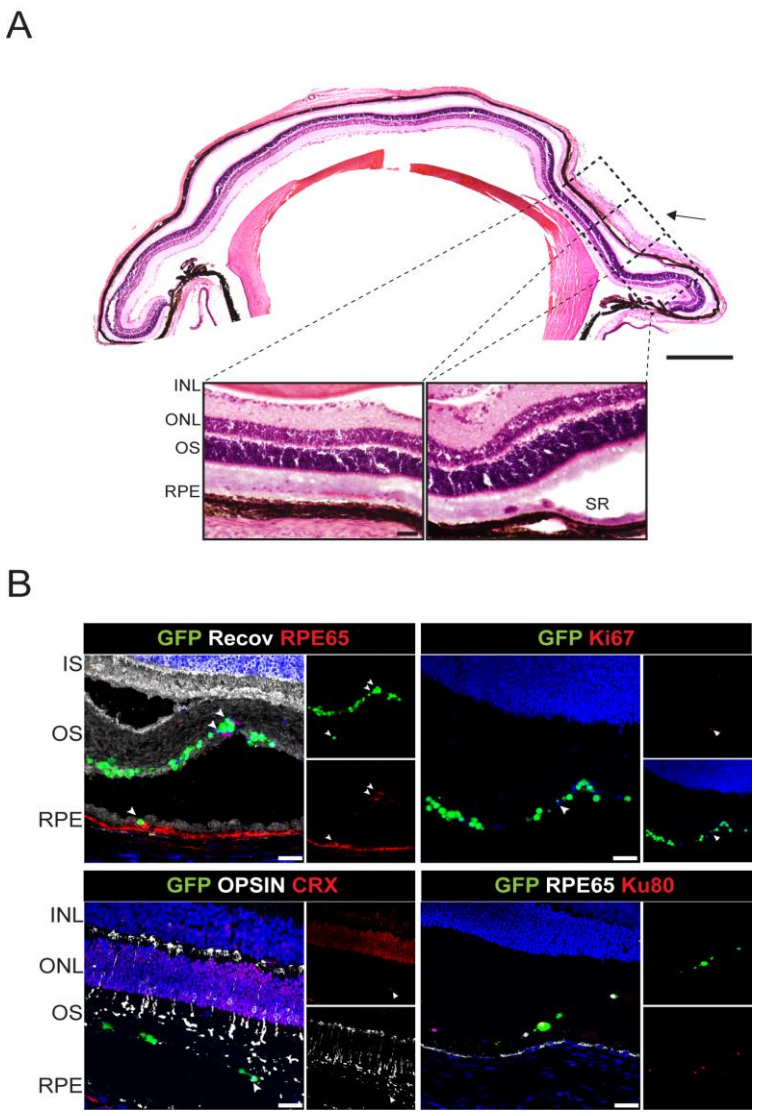

Figure S4: Analysis of transplanted mixture of hiPSC-derived RPE cells and RPC (D45) expressing GFP in the subretinal space of RCS rats at one week post-injection. A) Photomerged composition of hematoxylin and eosin (H-E) staining of the transplanted RCS rat retina section. Arrow indicates the injection site and dashed square frames the area surrounding the injection site. Scale bar: 500 µm. (Bottom panels) Magnifications of H-E staining of engrafted cells in the subretinal space, some of them showing pigmentation. Scale bar: 100 µm. B) Confocal images of transplanted area showing GFP-positive cells in the subretinal space stained with RPE marker RPE65, photoreceptor markers recoverin (Recov), opsin and CRX, and proliferation marker Ki67. White arrowheads indicate positive cells. Scale bars: 25 µm. Abbreviations: CRX, Otx-like homeodomain transcription factor; GFP, green fluorescent protein; IS, inner segments; INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segments; RPC, retinal progenitor cells; RPE, retinal pigment epithelium; RPE65, retinal pigment epithelium-specific 65 kDa protein; SR, subretinal space.

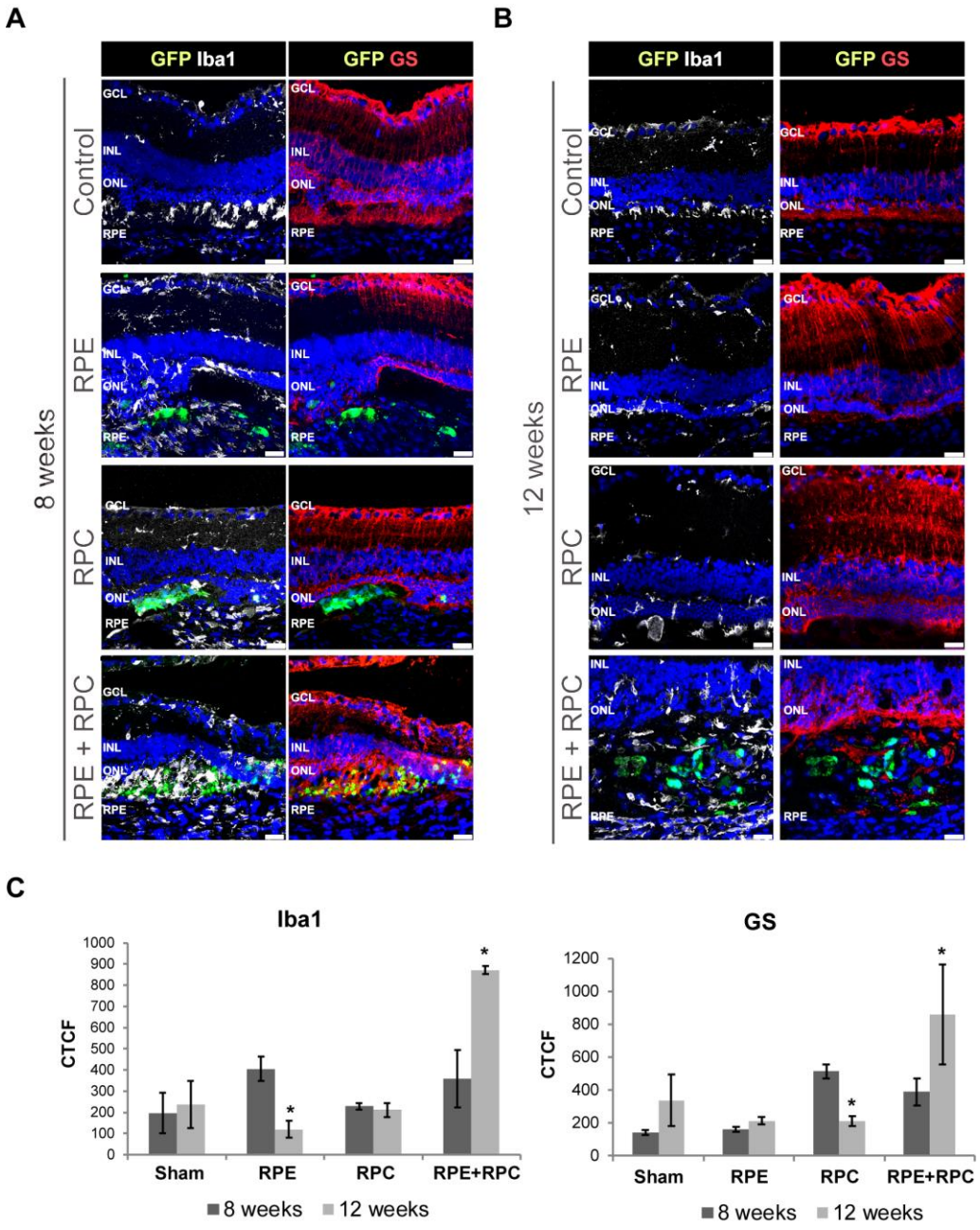

Figure S5: Analysis of microglia and Müller glia in the transplanted RCS rat retinas. A-B) Immunohistochemistry of paraffin sections of eyes transplanted with RPE cells, RPC or a combination of RPE+RPC (GFP+ cells in green) stained with Iba1 (microglia in white) and glutamine synthetase (GS; Müller glia in red) at 8 weeks post-injection (A) and 12 weeks post-injection (B). Sham injected eyes were used as controls. Microglia are mainly observed in the GCL and in the subretinal space in all conditions, however, they are more abundant surrounding RPE+RPC grafts. Similarly, Müller glia processes are more prevalent in the subretinal space of RPE+RPC grafts at 8 and 12 weeks. Scale bars, 25 µm. C) Quantification of fluorescence intensity in ONL and subretinal

spaces of Iba1 and GS staining, respectively. The fluorescence intensity was quantified using ImageJ and displayed in corrected total cell fluorescence (CTCF). Results shown represent the mean ± SD, *n* = 2-4 and $p < 0.05$ versus Sham calculated using Student's t test. Abbreviations: GCL, ganglion cell layer; GFP, green fluorescent protein; INL, inner nuclear layer; ONL, outer nuclear layer; RPC, retinal precursor cells; RPE, retinal pigment epithelium.

SUPPLEMENTARY TABLES

Table S1. Primer sequences used for qRT-PCR analysis

Gene	Forward	Reverse
CHX10	GGCGACACAGGACAATCTTTA	TTCCGGCAGCTCCGTTTTC
CRX	TCCAGGGTTCAGGTTTGGTT	CATCTGTGGAGGGTCTTGGG
GAPDH	GTCAGTGGTGGACCTGACCT	AGGGGAGATTCAGTGTGGTG
MERTK	TTGCAGCATTCAGGTCAAGGAAGC	GGCTTGCAGCTGCTTGATTTGGTA
NANOG	ACAACTGGCCGAAGAATAGCA	GGTTCCCAGTCGGGTTCAC
OCT3/4	GTTCTTCATTCACTAAGGAAGG	CAAGAGCATCATTGAACTTCAC
OPSINSW	TAGCAGGTCTGGTTACAGGATG	GAGACGCCAATACCAATGGTC
OTX2	GACCACTTCGGGTATGGACT	TGGACAAGGGATCTGACAGT
PAX6	TCTAATCGAAGGGCCAAATG	TGTGAGGGCTGTGTCTGTTC
PEDF	AGATCTCAGCTGCAAGATTGCCCA	ATGAATGAACTCGGAGGTGAGGCT
RAX	GCGAAGCGAAACTGTCAGAG	TTCTGGAACCACACCTGGACC
RECOVERIN	TCTACGACGTGGACGGTAACG	CGTCCTCGGGAGTGATCATT
RHODOPSIN	GCTGGTCCAGGTACATCCCC	TGAAGACGAGCTGCCCATAG
SIL	GTTGATGGCTGTGGTCCTTG	CAGTGACTGCTGCTATGTGG
TYR	ACTTACTCAGCCCAGCATC	GGTTTCCAGGATTACGCC

Table S2. Primary antibodies used for immunochemical and flow cytometry analyses

Name	Commercial house and reference	Specie
Alexa 647-conjugated Tra-1-60	BD Biosciences, 560850	
Bestrophin-1	Santa Cruz Biotechnology, sc-32792	Mouse
CHX10	Abcam, Ab16141	Sheep
CRX	R&D Systems, AF7085	Sheep
GS	BD Transduction Laboratories, 610517	Mouse
HNA	Chemicon, MAB1281	Mouse
Iba1	Wako, 019-19741	Rabbit
Ki67	Thermo Fisher Scientific, RM-9106-S	Rabbit
Ku80	Cell Signaling, 2180	Rabbit
MITF	Santa Cruz Biotechnology, sc-56725	Mouse
NRL	R&D Systems, AF2945	Goat
Opsin	Antibodies-online, ABIN350673	Rabbit
OTX2	Santa Cruz Biotechnology, sc-30659	Goat
PAX6	Covance, PRB278P	Rabbit
PKC-alpha (H-7)	Santa Cruz Biotechnology, sc-8393	Mouse
RAX	Abcam, ab23340	Rabbit
Recoverin	Millipore, AB5585	Rabbit
RG-opsin	Millipore, AB5405	Rabbit
Rhodopsin	Sigma, O4886	Mouse
RPE65	Novus Biologicals, NB100-355	Mouse
SOX1	R&D Systems, AF3369	Goat
TUJ1	Covance, MMS-435P	Mouse
ZO-1	Millipore, AB2272	Rabbit

MATERIALS AND METHODS

SparQ-GFP lentiviral production and transduction of hiPSC-derived retinal pigment epithelial cells and retinal precursor cells

Human embryonic kidney 293T (HEK293T) cells were cultured in DMEM supplemented with 10% fetal bovine serum (Hyclone), 1% GlutaMax™ (Gibco), 1% non-essential amino acids (Gibco), 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco), at 37 °C under humidified air containing 5% CO_2. Lentiviral particles were produced using a second-generation lentiviral system. HEK293T cells were transfected with SparQ-GFP (System Biosciences), psPAX2 and pMD2.G (Addgene) at a ratio of 1:0.75:0.25 µg DNA, respectively, using polyethylenimine (Sigma). Culture medium was replaced with fresh supplemented DMEM medium after 16 h. Virus-containing medium was collected after 24 h and 48 h and viral particles were concentrated by ultracentrifugation. Multiplicity of infection was determined by serial dilution counting GFP-positive HEK293 cells. RPC and purified RPE cells were transduced with a MOI=1 of SparQ-GFP viral particles in the presence of 10 µg/mL polybrene in the corresponding medium for 2 days. RPE cells were then sorted to obtain a homogeneous GFP+ cell culture. In contrast, RPC, which could not be sorted, only exhibited around 70% of GFP+ cells.

Determination of messenger RNA levels by quantitative real-time PCR

hiPSC, derived RPE cells or RPCs were used to isolate messenger RNA using the RNeasy RNA Isolation Kit (Qiagen). RNA was quantified on the NanoDrop platform and cDNA was synthesized from 1 µg total RNA per sample using the Cloned AMV First-Strand Synthesis Kit (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed in triplicate

samples, five independent biological replicates for RPE cells and three biological replicates for RPCs. using Platinum SYBR Green Quantitative PCR Super Mix (Invitrogen) on an Applied Biosystem 7900 HT Fast qRT-PCR System Thermocycler. Ct values were normalized to the *GAPDH* housekeeping gene and compared with those of undifferentiated hiPSC, expressed as 2^-ddCt (log scale). Primer sequences are listed in Supplemental Table S1.

Immunocytochemistry

Cells were fixed with 4% (w/v) paraformaldehyde (PFA) for 20 min at room temperature (RT), permeabilized and blocked in Tris-buffered saline (TBS) containing 0.5% Triton X-100 and 6% normal donkey serum (NDS; Millipore) for 1 hour at RT. Primary antibodies were incubated at 4ºC overnight in TBS, 0.1% Triton X-100 and 6% NDS in a humidified chamber, and secondary antibodies were incubated at 37ºC for 2 h (antibodies are listed in Supplemental Table S2). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), slides were mounted in mounting medium and images were obtained on an DMI6000 confocal microscope (Leica Microsystems).

Electron microscopy

For transmission electron microscopy (TEM), hiPSC-derived RPE cells were fixed in 2.5% (w/v) glutaraldehyde in 0.1M cacodylate buffer pH 7.2–7.4 for 2 h at 4°C. Cells were post-fixed in 1% (w/v) osmium tetroxide for 2 h at 4°C, rinsed in cacodylate buffer three times, dehydrated in progressive concentrations of ethanol and 100% propylene oxide, and embedded in epoxy resin. Semithin (250 nm) and ultrathin (70 nm) sections were cut on an ultramicrotome and ultrathin sections were stained with lead citrate. Ultrathin sections were examined on a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan). For

scanning electron microscopy (SEM), hiPSC-derived RPE cells were fixed as described above. Cells were then dehydrated in an ascending ethanol series, treated with the critical point dryer and metalized. Sections were examined using a Jeol JSM-6390LV scanning electron microscope.

Phagocytosis assays

In vitro phagocytosis function of hiPSC-derived RPE-like cells was assessed by their capacity to bind and internalize tetramethylrhodamine (TRITC)-labeled bovine photoreceptor outer segments (POS), as described [1]. RPE cells were incubated with TRITC-POS for 4 h at 37ºC with 5% (v/v) CO_2 in RPE medium, washed four times in phosphate buffered saline (PBS) and then fixed in 4% (w/v) PFA for 20 min at RT. Confocal images were obtained using a DMI6000 confocal microscope (Leica Microsystems).

Flow cytometry

hiPSC, RPE cells, retinal progenitors at day 21 and RPC at day 45 were dissociated into single cells with TrypLE Select for 15 min, neutralized and washed in PBS and filtered through a 40-µm strainer. Then, cells were stained with the Live/Dead Fixable Violet dead cell stain kit (Invitrogen) for 30 min., fixed in 2% PFA and washed three times in PBS. For cell surface marker, 1×10^5 cells/100 µl in PBS containing 2 % BSA were stained with Alexa 647-conjugated Tra-1-60 (0.5 µl) for 30 min. at RT protected from light, washed twice with PBS and resuspended in 350 µl PBS containing 2 % BSA. For intracellular staining, fixed cells were permeabilized and blocked in PBS containing 0.1% Triton X-100, 2 % BSA and 10 % normal donkey serum for 15 min. Then, primary antibodies were diluted in the same buffer and incubated for 30 min at RT (antibodies are listed in Supplemental Table S2). Following PBS washes, cells were incubated with donkey-anti-mouse or donkey-anti-rabbit

Alexa 647-conjugated secondary antibodies (1:500) for 30 min at RT, washed twice with PBS and resuspended in 350 µl PBS containing 2 % BSA. Cells were analyzed on the LSR Fortessa Cell analyzer (BD Biosciences). Flow cytometry analysis was performed by gating out death cells, doublets and the debris. Unstained cells and secondary antibodies were used as negative controls. Results are from the analysis of 2 biological replicates.

References

[1] C. Parinot, Q. Rieu, J. Chatagnon, S.C. Finnemann, E.F. Nandrot, Large-scale purification of porcine or bovine photoreceptor outer segments for phagocytosis assays on retinal pigment epithelial cells, J. Vis. Exp. (94). doi (2014) 10.3791/52100