CHARACTERIZATION OF hESC-RPE CELLS – APPENDIX A

Supplementary methods

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PURITY OF hESC-RPE CELLS

Immunohistochemistry and quantitative real-time polymerase chain reaction (Q RT-PCR) were used to confirm the purity of RPE cells culture (Figure A2). Undifferentiated hESC WA09 cell line was taken as positive control. For gene expression analysis, total RNA was extracted with miRNeasy Tissue/Cells Advanced Mini Kit (Qiagen) following manufacturer's instructions and RNA concentration was calculated with Qubit 2.0 Fluorometer (Thermo Fisher Scientific). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), according to the kit protocol. Q RT-PCR was performed with TaqMan Universal Matermix and predesigned TaqMan assays with FAM-labels (all from Thermo Fisher). The reactions were carried out on ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Relative gene quantification was measured by using the $2^{-\Delta\Delta Ct}$ method. GAPDH was the internal housekeeping gene reference for normalization. Stem cell markers OCT-3/4 and Nanog were not expressed in derived RPE cells, while high expression levels could be detected in hESC WA09 cell line. Immunofluorescence was carried out using the protocol described in the paragraph below.

IMMUNOFLUORESCENCE

hESC WA09 and hESC-RPE cells were washed with PBS and fixed in 4% paraformaldehyde (Santa Cruz Biotechnology) for 15 minutes at room temperature (RT). After several PBS washings, the cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 15 minutes at RT. Cells were washed with PBS and blocked with 3% BSA (Sigma-Aldrich) in PBS at RT for 1 hour to minimize unspecific binding sites. Samples were incubated at 4°C overnight with primary antibodies diluted in 3% BSA at the specified concentrations: Bestrophin 1:100 (PA589606), zonula occludens 1 (ZO-1) 1:200 (61-7300) (both from Thermo Fisher), Na+/K+ ATPase 1:200 (ab7671, Abcam), MERTK 1:50 (H00010461-M01, Abnova), OCT-3/4 1:200 (AF1759) and Nanog 1:150 (AF1997) (both from R&D Systems). The following day cells were washed with PBS and secondary antibodies were added at a dilution of 1:200 in 3% BSA for 1 hour at RT. Alexa Fluor 594-conjugated goat anti-mouse IgG (A11032), 594 donkey anti-rabbit IgG (A21207), 488-conjugated donkey anti-mouse IgG (A21202), A488 goat anti-rabbit IgG (A11034) and 488 donkey anti-goat IgG (A11055) were used as secondary antibodies (all from Thermo Fisher). Nuclei were counterstained with the nuclear dye Hoescht diluted 1:3000 in PBS at RT for 5 minutes. The samples were mounted in ProLong Gold Antifade Mountant without DAPI (Thermo Fisher). Images were captured with Nikon fluorescent microscope (Nikon) using 40x oil immersion objective (Figure A3).

TRANSEPITHELIAL RESISTANCE (TEER)

Transepithelial electrical resistance (TEER) was measured on hESC-RPE cells on TC insert with the Millicell® ERS-2 meter and electrode system (Merck Millipore) at days 28, 35,42,49 and 56 (Figure A4). To obtain true resistance value, resistance measured across the samples was subtracted from resistance read across the controls, respectively denuded hAM and TC insert without cells. Finally, TEER (Ω cm^2) measure was acquired by normalising the resistance value with the surface area of the nitinol ring or the TC insert.

ENZIME-LINKED IMMUNOSORBENT ASSAY

hESC-RPE cell culture supernatants from the upper and lower compartments of the TC inserts were collected after 48 hours of cell culture at 37°C. Harvested media were diluted 1:2 and apical and basal PEDF secretion levels were analysed with Human SERPIN F1 ELISA kit (Thermo Fisher). The remaining media were diluted 1:10 for VEGF quantification using VEGF Human ELISA kit (Thermo Fisher), following manufacturer's instructions (Figure A5). Media volume and insert growth area were used to calculate PEDF and VEGF levels.

FIGURES LEGEND – APPENDIX A

Figure A1: Schematic view with bright-field images of the differentiation process carried out to generate hESC-RPE cells. hESC, human embryonic stem cell; EB, embryoid bodies; RPE, retinal pigment epithelium; LN-521, recombinant laminin-521; CIV, collagen type IV; E8, Essential 8™ Flex Medium; Blebb, blebbistatin; KO-SR, Knock-out™ serum replacement.

Figure A2: Confirmation of a pure culture of RPE cells. (A) Immunofluorescence showing lack of staining for pluripotency associated factors such as OCT-3/4 and Nanog (all green) in hESC derived-RPE cells (upper panel). hESC WA09 cell line was used for comparison (lower panel). Cell nuclei were stained with DAPI (blue). Scale bars = 100µm. (B) Quantitative polymerase chain reaction revealed no expression of OCT-3/4 and Nanog in hESC-RPE. Data are normalized to hESC WA09 cell line. Data represent mean ± SD of N = 3 experiments for each sample. *** $p<0.0001$. hESC, human embryonic stem cell; RPE, retinal pigment epithelium.

Figure A3: Representative immunofluorescence staining indicating the correct expression and localization of typical RPE markers in hESC-RPE cells merged with Heoscht nuclear staining (blue). Scale bars = 100µm. Best1, Bestrophin 1; ZO1, zona occludens-1.

Figure A4: TEER was measured to monitor the barrier function of the hESC-RPE cells on TC inserts over time (days) using a Millicell volt/ohm meter. Data represent mean ± SD of N = 3 experiments for each sample. TEER, transepithelial electrical resistance.

Figure A5: Spent culture media were collected from the apical and the basal chambers of the TC inserts and quantified for PEDF (A) and VEGF (B) concentration using ELISA assays. Data represent mean ± SD of N = 3 experiments for each sample.