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Contacting co-culture of human retinal microvascular endothelial cells alters barrier function of human embryonic stem cell derived retinal pigment epithelial cells

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ABSTRACT

Here we evaluated the effects of human retinal microvascular endothelial cells (hREC) on mature human embryonic stem cell (hESC) derived retinal pigment epithelial (RPE) cells.

The hESC-RPE cells (Regea08/017, Regea08/023 or Regea11/013) and hREC (ACBRI 181) were co-cultured on opposite sides of transparent membranes for up to six weeks. Thereafter barrier function, small molecule permeability, localization of RPE and endothelial cell marker proteins, cellular fine structure, and growth factor secretion of were evaluated.

After co-culture, the RPE specific CRALBP and endothelial cell specific von Willebrand factor were appropriately localized. In addition, the general morphology, pigmentation, and fine structure of hESC-RPE cells were unaffected. Co-culture increased the barrier function of hESC-RPE cells, detected both with TEER measurements and cumulative permeability of FD4 – although the differences varied among the cell lines. Co-culturing significantly altered VEGF and PEDF secretion, but again the differences were cell line specific.

The results of this study showed that co-culture with hREC affects hESC-RPE functionality. In addition, co-culture revealed drastic cell line specific differences, most notably in growth factor secretion. This model has the potential to be used as an in vitro outer blood-retinal barrier model for drug permeability testing.

1. Introduction

Retina is a part of the central nervous system and thus protected by a blood brain barrier analogue, blood retinal barrier (BRB), consisting of inner (iBRB) and outer part (oBRB). oBRB consists of three layers: retinal pigment epithelial cells (RPE), Bruch's membrane and choroidal endothelial cells (CEC). RPE cells face the neural retina and are connected to each other by tight junctions, forming a selective barrier. RPE has several vitally important functions such as phagocytosis of shed photoreceptor outer segments, vitamin A metabolism, regulation of the transport of nutritive substances, absorption of stray light, and control of retinal ion balance [1,2]. On the other side, oBRB is in contact with the blood stream via the fenestrated CECs. Interactions between RPE and CEC are vitally important for transporting nutrients and water to the retina, and removing the metabolic waste to the blood stream [2,3]. Between these two cell types lies a layered extracellular matrix (ECM) structure - Bruch's membrane [4]. Besides offering support and separating RPE from the CEC, Bruch's membrane it is also a playing ground of growth factors secreted by the RPE, such as vascular endothelial growth factor (VEGF) and pigment epithelial derived growth factor (PEDF). An imbalance in this homeostasis might lead to dysregulation in angiostasis [5,6], for example to choroidal neovascularization and age-related macular degeneration (AMD) [7].

Due to its excellent protective properties oBRB also acts as a barrier to many drug molecules, making medical therapy of many retinal diseases demanding. This is the case in diabetic retinopathy (DR), AMD, posterior uveitis and retinitis pigmentosa [8]. For many decades ocular drug delivery to posterior segments of the eye has been assessed

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with in vivo animal studies, but recently due to their high costs and ethical considerations, alternative methods, such as *ex vivo* and in vitro techniques have gained popularity [9]. *Ex vivo* cultures of isolated tissues have been used to evaluate morphology, integrity and function of oBRB [10–12]. *In vitro* models can provide a valuable pre-clinical testing platform for disease modeling, testing nutrient/pharmaceutical trafficking, as well as drug delivery and targeting [13,14]. *In vitro* models based on primary RPE cell cultures of animal origin [14] have been widely used, although they suffer from species-related applicability problems. The functionality of oBRB has been extensively studied only in RPE cells alone (referred onwards as RPE solo-culture), generally using the immortalized RPE cell line ARPE-19 [14–17]. These RPE solo-cultures, however, do not develop cell-cell interactions typical to native human RPE, thus several oBRB co-culture models have been developed [7,18,19]. RPE cells have been co-cultured with endothelial cells in "contacting" cultures as mixtures permitting direct contact [20], sandwiched with ECM proteins [18,21], or cultured on opposite sides of amniotic membrane [22] or Transwell inserts [23,24]. In addition, "non-contacting" co-cultures have been performed where one cell type is cultured on top of a cell culture insert and the other cell type on the bottom of the well plate [23–26]. These various oBRB models have been generated either with primary RPE cells, which have a limited availability, or with immortalized RPE cell lines which have morphological and developmental abnormalities [9,27]. Thus, there is a need for new ophthalmic in vitro models with closer resemblance to the functionality and morphology of the native tissue [9,13]. RPE cells derived from human embryonic stem cells (hESC) provide an unlimited source of cells which closely resemble their native counterpart [28–34]. They have been shown to express RPE specific genes and proteins, become highly pigmented and polarized, display several RPE specific functions such as PEDF secretion and phagocytosis of photoreceptor outer segments, and express functional membrane proteins important for maintaining barrier properties [35,36]. When transplanted to rabbits, hESC-derived RPE cell sheets restore ERG and outer nuclear layer (ONL) thickness for a short period of time [37].

In this work we aimed to develop a hESC-RPE based contacting oBRB co-culture model by utilizing three different hESC lines. The effects of co-culture on hESC-RPE barrier properties were evaluated with trans-epithelial electrical resistance (TEER) measurements and permeability tests, the morphology with confocal and electron microscopy and cellular functionality with growth factor secretion assays. The results of this work could pave the way towards an in vitro model that could be useful for drug delivery research and retinal disease modeling.

2. Materials and methods

2.1. Cells

2.1.1. Endothelial cells

Human Umbilical Vein Endothelial Cells (HUVECs) were extracted at BioMediTech, University of Tampere from the umbilical cords acquired from scheduled Cesarean sections according to Hamilton et al. [38]. HUVECs were cultured in HUVECmem: Gibco M199 medium (Life Technologies, Carlsbad, CA, USA) with 1% Gibco GlutaMAX™ (Life Technologies), 2% Penicillin-Streptomycin (Lonza Group Ltd., Basel, Switzerland), 10% fetal bovine serum (FBS Gold, PAA, GE Healthcare Ltd., Little Chalfont, UK), 0.3% Endothelial Cell Growth Supplement (First Link Ltd., Wolverhampton, UK), 0.08% Fungizone amphoterizin B (Life Technologies) and 0.1% heparin sodium salt from porcine intestinal mucosa (Sigma-Aldrich, St. Louis, MO, USA). The HUVECs were routinely cultured on 5 μg/cm^2 collagen I (COLI, Sigma-Aldrich) coating, passaged at 80–90% confluency and used for experiments at passages 5–10.

Primary human retinal microvascular endothelial cells (hREC), ACBRI 181, are a commercially-available cell line (Cell Systems, Kirkland, WA, USA). The cells were grown on ECM Attachment Factor™ (AF, Cell Systems). For the culture of ACBRI 181 a special CSC medium was used, consisting of Complete Serum-Free Medium with RocketFuel and antibiotic Bac-Off (Cell Systems), supplemented with 10% fetal bovine serum (FBS Gold) and 1% penicillin-streptomycin (Cambrex Bio Science, Walkersville, MD, USA). ACBRI181 were used for experiments at passage 8.

Before the co-culture experiments the preference of cell culture substrata of HUVEC and ACBRI181 cells was assessed by plating the cells on COLI, collagen IV (COLIV, Sigma-Aldrich) and AF. Polystyrene cell culture plates were coated with 5 μg/cm^2 of ECM by incubating for 1–3 h at 37 °C. Thereafter the wells were briefly washed with phosphate buffered saline (DPBS, Lonza Group Ltd.). Cells were washed thrice with DPBS, dissociated with Trypsin-EDTA (Lonza Group Ltd.), counted and passaged on top of coatings at a density of 100,000 cells/cm^2. Cells were cultured for two days and fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) solution and subjected to the indirect immunofluorescence staining (below). These preliminary tests (Supplemental Fig. 1) demonstrated that there was no marked difference in the cell number on different substrata. Thus the COLIV which is routinely used for hESC-RPE cell culture [39], can also be used for endothelial cells.

2.1.2. Human embryonic stem cell derived retinal pigment epithelial cells

The hESC lines Regea11/013 (46, XY), Regea08/017 (46, XX) and Regea08/023 (46, XY) established previously by our group in University of Tampere, were used [40]. The undifferentiated hESCs were cultured similarly as previously described [41] at +37 °C in 5% CO_2 on human foreskin fibroblast feeder cells (hFFs; 36,500 cells/cm^2; CRL-2429™; ATCC, Manassas, VA, USA) which were mitotically inactivated either with γ-irradiation (40 Gy) or mitomycin C (10 μg/ml, Sigma-Aldrich), in serum-free conditions in Knock-Out Dulbecco's Modified Eagle Medium (KO-DMEM) containing 20% Knock-Out serum replacement (KO-SR), 2 mM Glutamax, 0.1 mM 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA, USA), 1% Minimum Essential Medium nonessential amino acids, 50 U/ml penicillin/streptomycin (both from Cambrex Bio Science), and 8 ng/ml human basic fibroblast growth factor (bFGF; R & D Systems Inc., Minneapolis, MN). The culture medium was replenished five times a week. Undifferentiated colonies were passaged onto new feeder cells either manually once a week or enzymatically at ten-day intervals with TrypLE Select (Invitrogen).

Spontaneous RPE differentiation was induced by reducing KO-SR concentration from 20% to 15% and removing bFGF from the hESC culture medium. This modified medium is called RPEbasic. The hESC colonies were manually dissected and cultured in suspension in low cell bind six-well plates (Nalgene, NUNC, Tokyo, Japan), as floating aggregates (embryoid bodies). The embryoid bodies were allowed to mature for 48–109 days until sufficient pigmentation appeared, changing the medium three times a week. The pigmented areas of floating aggregates were manually selected, cut and subsequently dissociated with Trypsin–EDTA. Acquired single cell suspensions were filtered through 100 μm BD Falcon cell strainer (BD Biosciences, San Jose, USA), and cells were plated onto well plates coated with human COLIV (5 g/cm^2) to expand cell numbers and purify the cell population. After expansion of this passage 2 for 65–349 days, the hESC-RPEs were dissociated with Trypsin–EDTA, filtered through a strainer and counted to be plated for experiments, in which the hESC-RPE cells were in passage 3.

Cells secrete several soluble factors to the culture medium. The human fetal RPE cell [42] and mature hESC-RPE cell [43] conditioned media have previously been shown to induce neuronal cell differentiation [42,43]. As in the native retinal tissue, the endothelial cells receive soluble queues from RPE. Therefore, prior the co-culture experiments we wished to test the ability of HUVEC (always between the passage 7

Fig. 1. The illustration how cells cultured for 4–6 weeks on PET filter insert clamped to Minusheet carrier was transferred to barrier function tests. After the 4–6 weeks of cell culture, the filter insert with cells was cut out from the Minusheet carrier (A). In order to have two technical replicates, the insert was cut to two pieces (see arrows), and each one was placed to one P2307 slider (B). The transepithelial electrical resistance was measured in a custom-made P2307 slider chamber (in the insert in C) with Millicell ERS-2 Voltohmmeter (C). The sample was then placed to EM-CYS-8 Ussing chamber without the electrodes (D) for the permeability assay. After the assay the same cell samples were processed for immunofluorescence or electron microscopy.

and passage 9), and ACBRI181 (always passage 8) cells to grow in fresh RPEbasic medium and RPEbasic conditioned by mature hESC-RPE cells. The effects were compared to the cultures where both endothelial cell types were grown in their own media. Cells were cultured for 7 days and new test media were changed every day. This preliminary test suggested that endothelial cells prefer conditioned RPEbasic (Supplemental Fig. 1) over the fresh RPEbasic medium. Therefore all co-cultures were maintained in co-culture medium – 1:1 mixture of hESC-RPE conditioned RPEbasic and fresh RPEbasic.

2.2. Treatment of polyethylene terephthalate substrata for the cell culture

In order to have equal culture conditions for both cell types the transparent polyethylene terephthalate (PET) membranes (Millipore 12 Well Millicell, 1.0 μm pore diameter; Merck Millipore, Billerica, MA, USA) were removed from the supporting hanging culture inserts with a scalpel and placed within Minusheet (part no. 1300, Minucells and Minutissue-Vertriebs GmbH, Bad Abbach, Germany) carriers, which were cleaned using EtOH (first 10 min in 70% EtOH, then 5 min in 99.5% EtOH followed with drying) before use. Minusheet constructs were coated with COLIV (5 μg/cm^2), for 1 h at +37 °C, with the membranes flipped after 30 min to ensure that the coating was equal on both sides. After coating, the membranes were washed once with 1xPBS. The PET membrane was always oriented similarly within the Minusheet carrier, to ensure that all samples were uniform. All work was carried out under the laminar flow hood. The pilot tests revealed that the endothelial cells had rather low adherence on the COLIV coated PET membrane. Therefore we increased the amount of reactive binding sites with plasma treatment. PET membranes with Minusheet carriers were placed onto a 12-well plate, with the untreated side facing up. The plasma treatment was carried out with O_2 gas in Pico, Diener Electronic (Ebhausen, Germany) using constant 0.4 mBar pressure and 50 W power for 1 min or with Reactive Ion Etcher (RIE Advanced Vacuum Scandinavia AB, Sweden) with parameters 30 scm, 30 W 30 mtorr, 1 min). Thereafter Minusheet constructs were coated similarly as without plasma treatment. The secondary pilot test exhibited that plasma treatment increased the number of endothelial cells on PET membrane after 9–10 days of culture (Supplemental Fig. 2).

2.3. Cell plating and maturation for co-culture

Human RECs and hESC-RPE cells were co-cultured with the "contacting" method [23]. Both cell types were plated immediately after coating of the Minusheet constructs with COLIV. First, the endothelial cells (100,000 cells/cm^2) were plated in 150 μl of CSC medium, and were allowed to adhere at 37 °C, 5% CO_2 for 4 h. Thereafter the Minusheet carriers were flipped, the CSC medium was removed and the hESC-RPE cell suspension (100,000 cells/cm^2) was applied onto the other side of the membranes in RPEbasic. Then, additional 500 μl of the co-culture medium was added to the constructs. After 4 h of incubation i.e. after hESC-RPE cells had attached to the membrane surface, an additional 1.5 ml of the co-culture medium was added.

The co-culture samples were allowed to mature for 4–6 weeks (Regea08/017 36d ± 7d; Regea08/023 32d ± 4d and Regea11/013 34d ± 4d), changing the medium three times a week. The co-culture medium was used during the first three weeks, and pure RPEbasic for the remaining time. The co-cultures were analyzed once the hESC-RPE cells regained their pigmentation.

2.4. Trans-epithelial electrical resistance

The barrier properties of the solo-cultured and co-cultured samples were evaluated with TEER measurements at the end of the maturation period: Each sample, cultured on the PET insert placed to the Minusheet carrier (Fig. 1**A**), was cut out (Fig. 1**A**). The culture insert was cut to two pieces (Fig. 1B), and clamped to P2307 slider (Physiologic Instruments, San Diego, CA, USA) (Fig. 1**AB**), placed into a custom-made teflon chamber [36] filled with 1xPBS, and TEER was measured with the Millicell ERS-2 Voltohmmeter (Merck Millipore) (Fig. 1**C**). TEER values (Ω cm^2) were calculated by subtracting the TEER of a similarly treated substrata-coated PET membrane without cells, and by multiplying the result by the surface area. TEER values were obtained from five individual differentiation experiments with all three different hESC lines with multiple parallel samples, and two technical replicates.

2.5. Permeability tests

After the TEER measurement, the samples in sliders P2307 were placed into the Ussing Chamber device (EM-CSYS-8 (Physiologic

instruments)) with P2300 EasyMount Diffusion Chambers to measure the barrier properties of the co-cultures (Fig. 1**D**). The permeability assessments were carried out in 2.25 ml of RPEbasic without KO-SR (thereafter referred to as Ussing medium) both on the donor and acceptor side. On the donor side 1 mg/ml of 4 kDa fluorescein isothiocyanate–dextran (FD4, Sigma-Aldrich) was used as the test molecule. Blank samples were collected prior to the test from both donor and acceptor media. The pH in chambers was kept constant with CO_2 gas (5% CO_2, 10% O_2, 85% N_2) bubbling. After 1 h, 2 h, 3 h and 4 h, two parallel 100 µl samples were taken from the acceptor side of the chamber, and 200 µl of fresh medium was added to balance the removed volume. Samples were analyzed with Wallac Victor2 1420 multilabel counter spectrophotometer (Turku, Finland).

The cumulative permeability i.e. the cumulative amount of fluorescence appearing in the acceptor solution versus time, was calculated from the average value of the two parallel absorbance measurement values of the acceptor (basal side) from which subtracting the value of pure Ussing medium (i.e. blank). This value was then divided by the total volume of acceptor side. The dilution effect of the added fresh medium after every hour. This was calculated according to Pitkanen et al. [44].

$$Cumulativepermeability = \frac{(Facceptor - Fpure\ medium)}{medium\ vol\,(ml)*)}$$

F is the emitted fluorescence value. Acceptor is in the beginning devoid of fluorescence, situated in chamber basal to hESC-RPE, apical to EC. Pure medium is fresh medium without fluorescence. (*) in equation is the dilution effect of fresh medium after every hour.

The cumulative amount permeated was calculated from cumulative permeability by dividing by the spectrophotometer value of the exposure medium and multiplied by 100%.

$$Cumulative\ permeability\% = \frac{Cumulative\ permeability}{F\ donor*100}$$

The diffusion of the solutes across cell cultures was characterized by calculating the apparent permeability coefficient (Papp, cm/sec) according to Pitkanen et al. [44], cumulative permeability of FD4 after every hour was plotted, and the slope (kx+b) of the linear portion, was divided with the surface area (0.031 cm^2) multiplied with the spectrophotometric value (i.e. maximum intensity) of the probe molecule in the donor solution.

$$Permeability\ coefficient = \frac{(kx + b)}{surface\ area\ (cm2)*F\ donor}$$

Donor in the equation is the maximum intensity from the Ussing medium that contains 1 mg/ml of FD4.

2.6. Indirect immunofluorescence

After the permeability test, indirect immunofluorescent staining was performed in order to evaluate cell morphology, structure, and polarity of the samples. All samples were first washed with 1×PBS, fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min at RT, washed four times with 1×PBS, and permeabilized with 0.1% TritonX-100 (Sigma-Aldrich) in 1×PBS for 10 min at RT. After that, the samples were again washed repeatedly with PBS, and 3% bovine serum albumin (BSA, Sigma-Aldrich) in PBS was added to the samples for 1–1.5 h at RT, or overnight at 4 °C, to block the non-specific binding sites. Then, the samples were incubated for 1–1.5 h at RT or overnight at 4 °C with the primary antibodies in 0.5% BSA-PBS. The endothelial cells were stained with rabbit-anti-von Willebrand factor (vWf, 1:500, Daco, Glostrup, Denmark); hESC-RPE cells were stained with mouse-anti-CRALBP (1:500, Abcam, Cambridge, UK). The tight junctions were stained with mouse-anti-ZO-1 (1:250 Invitrogen), and filamentous actin was stained with amanita phallotoxin Phalloidin 680 (1:200, Sigma-Aldrich). The primary antibody incubations were followed by 4× washes with 1×PBS. The secondary antibodies, donkey-anti-mouse and goat-anti-rabbit (both from Life Technologies) were both used at dilution of 1:1000 in 0.5% BSA-PBS. The secondary antibodies were incubated for 1–1.5 h at RT. Finally, the samples were washed ×4 with 1×PBS mounted between two cover glasses with Vectashield® mounting medium with 4′,5-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc.). The visualization and imaging of the stained samples was carried out with LSM 780 confocal microscope (Carl Zeiss, Jena, Germany) using magnification of 40× and resolution of 1200×1200.

2.7. Enzyme-linked immunosorbent assay

The concentrations of secreted PEDF and VEGF were determined using Chemikine PEDF Sandwich ELISA Kit (Millipore, Temecula, CA, USA) and Quantikine VEGF ELISA Kit (R & D Systems), respectively, according to the manufacturer's instructions.

2.8. Transmission electron microscopy

After 4–6 weeks of co-culture, the constructs were fixed for 2 h at RT with 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1 M phosphate buffer, and washed with 0.1 M phosphate buffer. The samples were post-fixed with 1% osmium tetroxide (Ladd Research, Williston, VT, USA) for 1 h at RT. The samples were then dehydrated through an ordered acetone series: 70% acetone, 94% acetone, and absolute acetone (J.T. Baker; Avantor Performance Materials, B.V. Deventer, Netherlands). Thereafter the samples were impregnated with 1:1 mixture of absolute acetone and epoxy resin (Ladd Research) for 1.5 h at RT. The embedding of pure epoxy resin was done overnight at RT, and polymerization of epoxy resin for 48 h at 60 °C. Thin sections were stained with 1% uranyl acetate for 30 min and with 0.4% lead citrate (Fluk, Steinheim, Switzerland) for 5 min. Samples were examined and imaged with JEM-2100F TEM (Jeol Ltd., Tokyo, Japan). The length of microvilli, cell thickness, and basal lamina thickness were measured with Image J Image Processing and Analysis Software from the TEM images.

2.9. Statistical analyses

The statistical significance of TEER, permeability assay and ELISA were analyzed with PASW Statistics, version18 with two tailed Mann-Whitney U. The image analysis from TEM images was calculated with R statistical computing and graphics program (version 3.3.1) (R Foundation for Statistical Computing, Vienna, Austria Foundation for R Foundation for Statistical Computing, Vienna, Austriatatistical Computing, Vienna, Austria) with two-sample Wilcoxon rank sum test. The number of replicates is indicated in the figure legends. Results were considered significant at $p < 0.05$.

2.10. Ethical issues

The extraction of HUVECs from the umbilical cords from full term scheduled Cesarean sections has the supportive statement from the Ethics Committee of Pirkanmaa Hospital District (Miettinen/R13019). The National Authority for Medicolegal Affairs Finland has given approval to study human embryos (Dnro 1426/32/300/05) and we have a supportive statement from the Ethics Committee of Pirkanmaa Hospital District to derive hESC lines from early stage embryos that are not used in infertility treatments (Skottman/R05116). No new hESC lines were generated in this study.

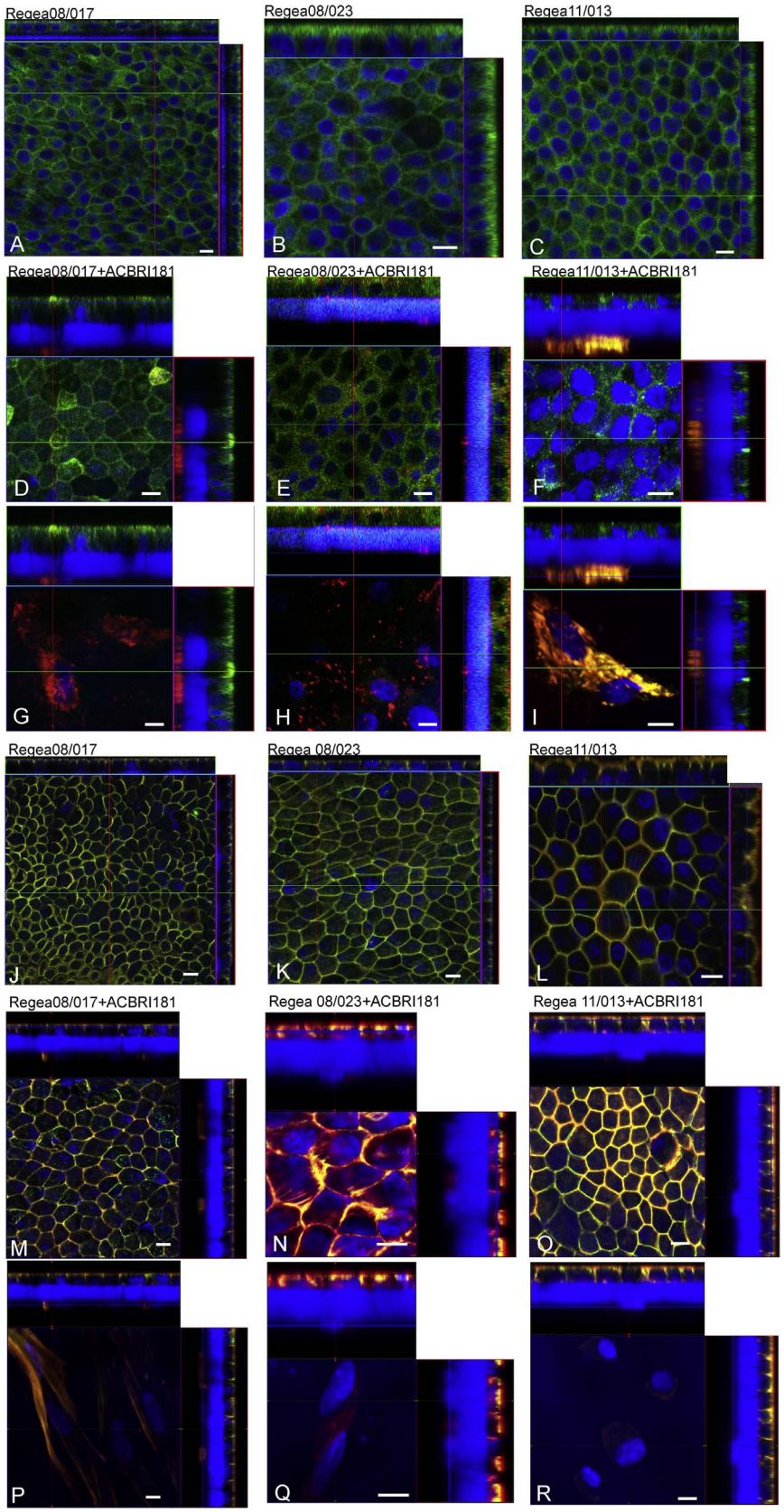

Fig. 2. Representative confocal ortho-images after 4–6 weeks of hESC-RPE solo-cultures (**A-C, J-L**) and hESC-RPE and ACBRI181 co-cultures (**D-I, M-R**) (n= 4, all with 2 biological and 2 technical replicates). Samples were immunostained with RPE specific marker CRALBP (green) and endothelial cell specific von Willebrand factor (vWF, red) (**A-I**) or tight junction marker ZO-1 (green) or filamentous actin binding phalloidin (red) (**J-R**). Nuclei were counterstained with DAPI (blue). Images **D**) Regea08/017, **E**) Regea08/023 and **F**) Regea11/0113 from the hESC-RPE level and **G,H, and I** from the endothelial level of the same co-cultures. Similarly images **M**) Regea08/017, **N**) Regea08/023 and **O**) Regea11/0113 from the hESC-RPE level and **P,Q** and **R** from the endothelial level of the same co-cultures. Scale bars 10 μm.

3. Results

3.1. Cell morphology

Expression and localization of cell type specific proteins was evaluated using indirect immunofluorescence after 4–6 weeks of co-culture of hESC-RPE cells and ACBRI181 hRECs. Localization of CRALBP, the RPE-specific protein, was similar in solo-cultured hESC-RPEs (Fig. 2**A, B, C**) and co-cultured hESC-RPEs (Fig. 2**D, E, F**). Also the endothelial cell specific vWF labeling localized to hRECs (Fig. 2**G, H, I**) in co-cultures. As hRECs were visualized with the confocal microscope through the pigmented hESC-RPE cells and through the 1 μm pores in the PET membrane the intensity of the label is slightly fainter on one side. The ZO-1 tight junction protein had a similar appearance both in solo-cultured (Fig. 2**J, K, L**) and co-cultured (Fig. 2**M, N, O**) hESC-RPEs.

The co-culture of hESC-RPE cells appeared to effect on epithelial culture: the endothelial cell soloculture had fewer islands than endothelial cells co-cultured with hESC-RPE cells (Supplemental Fig. 3). Also the endothelial cells cultured with Regea08/017 and Regea08/023 were more confluent than those cultured with Regea11/013 (Supplemental Fig. 4 and Supplemental Fig. 5). The endothelial cells and hESC-RPEs might interact with each other as seen in a confocal image where endothelial cell has sent a vWF positive process though the 1 μm pores of PET filter insert (Supplemental Fig. 6). In addition, endothelial cells interacted with each other by forming capillary-like structures which were visible in all cultures (white arrows in Supplemental Fig. 4) and (white arrowhead in Supplemental Fig. 5).

Cell morphology and pigmentation of hESC-RPE cells were unaffected by the co-culture, as seen from the low magnification electron microscopy images (Fig. 3**A**). During maturation and polarization the RPE cells are known to adopt a more columnar shape, thus the hESC-RPE cell height was quantified (Fig. 3**B**) for solo- and co-cultured hESC-RPEs from TEM photographs (Table 1). We found that co-culture significantly decreased the cell height of Regea08/017 hESC-RPE line (p=0.0014), but not Regea08/023 (p=0.331) or Regea11/013 (p=0.364) hESC-RPE cell lines.

During cell maturation and polarization the brush border is developed on the apical side of RPE cells. Microvilli length was quantified from TEM images of the solo- and co-cultured hESC-RPE (Fig. 4**A-C**), (Table 2). In Regea08/023 hESC-RPE cells the brush border was significantly shorter in solo-cultures than in co-cultures ($p < 0.001$). In Regea11/013 the microvilli were also slightly shorter in solo-cultures than in co-cultures but this difference did not reach statistical significance ($p = 0.058$). Finally, due to several outliers in Regea08/017 hESC-RPE, the differences were not statistically significant ($p = 0.896$). It should be noted that ultrathin sections (approx. 70 nm) do not capture the full length of the microvilli but rather a projection of them in specific layers of the cross-section. However, qualitatively, one can estimate that both the length and the number of microvilli are increased in co-cultures.

The cell-cell interaction can affect ECM organization and deposition. In all solo-cultures and co-cultures the deposited ECM had a highly organized structure (Fig. 4**D,E)**. Quantitation of the differences would not give reliable results. Therefore, only the ECM thickness was measured from TEM images of the solo- (Fig. 4**D**) and co-cultured hESC-RPEs (Fig. 4**E**), (Table 3). The co-culture significantly increased the ECM deposition underneath the Regea11/013 hESC-RPE (p=0.020). The differences were not statistically significant for Regea08/017 (p=0.443) or Regea08/023 (p=0.068) hESC-RPE cells.

3.2. Barrier properties

The integrity of mature cell cultures was first analyzed via TEER measurement immediately after cutting the insert from the Minusheet carrier and placing it to the P2307 slider. This also ensured that the cells were placed tightly on the slider. After the 4–6 weeks solo-culture, ACBRI181 had reached the TEER of 3.9 Ωcm^2 (Fig. 5**A**). Co-culture with ACBRI181 endothelial cells significantly increased TEER of Regea08/023 hESC-RPE (p=0.013). For the other two cell lines, although TEER was higher in co-culture, the differences were not significant due to high variation (p=0.059 for Regea08/017 and p=0.310 for Regea11/013). When all the solo-cultures of the three cell lines were compared to all the co-cultures, the overall difference in TEER was statistically significant ($p = 0.002$), (Fig. 5**B**). The TEER of pristine PET filter insert without cells (0.3 cm^2) analyzed with Millicell ERS-2 Voltohmmeter prior cutting, was 45 $\Omega\ cm^2$–54 $\Omega\ cm^2$, whilst the same clamped insert (0.031 cm^2) with the same Millicell ERS-2 Voltohmmeter was 25 $\Omega\ cm^2$–29 $\Omega\ cm^2$. These results indicate that the TEER was slightly compromised by cutting and clamping.

The tightness of the cultures was also evaluated by measuring fluorescent dye permeability in Ussing chamber for 4 h (Fig. 5**C**). The cumulative permeability of the empty PET membrane increased from 0.036% to 1.94% in this time. For the ACBRI181 endothelial cells in solo-culture the increase was from 0.26% to 1.47%. Co-culture with ACBRI181 cells significantly decreased the cumulative permeability of Regea08/023 hESC-RPE (p=0.013). The differences for the other two cell lines were not significant (p=0.221 for Regea08/017 and p=0.445 for Regea11/013). Similarly to TEER values, when all solo-cultures of the three cell lines were compared to all the co-cultures, the decrease in cumulative permeability was statistically significant ($p = 0.018$) (Fig. 5**D**). The co-culture with ACBRI181 cells also decreased the permeability coefficient (10 −6 cm/sec) but that was not statistically significant Regea08/017 hESC-RPE ($p = 0.17$), Regea08/023 ($p = 0.11$) or Regea11/013 (p=0.73) (Fig. 5**E**). But when all solo-cultures of the three cell lines were compared to all the co-cultures, the decrease in cumulative permeability was statistically significant ($p = 0.036$) (Fig. 5**F**).

3.3. Growth factor secretion

Finally, we assessed how the co-culture of hESC-RPE cells with ACBRI181 endothelial cells affects growth factor expression. ACBRI181 cells did not secrete detectable PEDF (Fig. 6**A**), as expected, and only secreted 0.56 ng/ml VEGF (Fig. 6B). PEDF secretion was significantly higher in co-cultured Regea08/017 ($p = 0.00004$) and Regea08/023 ($p = 0.000005$) hESC-RPE cells, but not in Regea11/013 hESC-RPE. VEGF expression, on the other hand, was significantly higher in co-cultured Regea08/017 and Regea11/13 hESC-RPE (p=0.032 and p=0.014, respectively), but lower for co-cultured Regea08/023 hESC-RPE (p=0.025).

4. Discussion

In vitro modeling of healthy functional RPE requires a polarized and mature monolayer of RPE cells characterized by strong barrier properties and low permeability [23]. In addition, these RPE cell cultures should function similarly to native cells. Cell culture methods should be consistent and efficient, yielding high quality cell populations with little variation between batches. Primary cells extracted from tissue tend to differ from isolate to isolate and the yield is always rather low [9]. Immortalized RPE cell lines provide a high yield, but they lack some of the characteristics, gene, and protein expression, critical to cell functions normally observed in RPE [45]. Human ESCs offer a solution to the limitations of primary and immortalized cell lines – they provide an unlimited source of RPE cells, which have many functionally important characteristics common to native RPE cells [28–33]. Previously, hESC-RPE cells have been used for in vitro modeling of oBRB in solo-cultures [28,36,46]. To our knowledge our study is the first to demonstrate the use of hESC-RPE co-cultured with hRECs as a potential in vitro model of oBRB.

In our earlier studies, we have shown that differentiated hESC-RPE

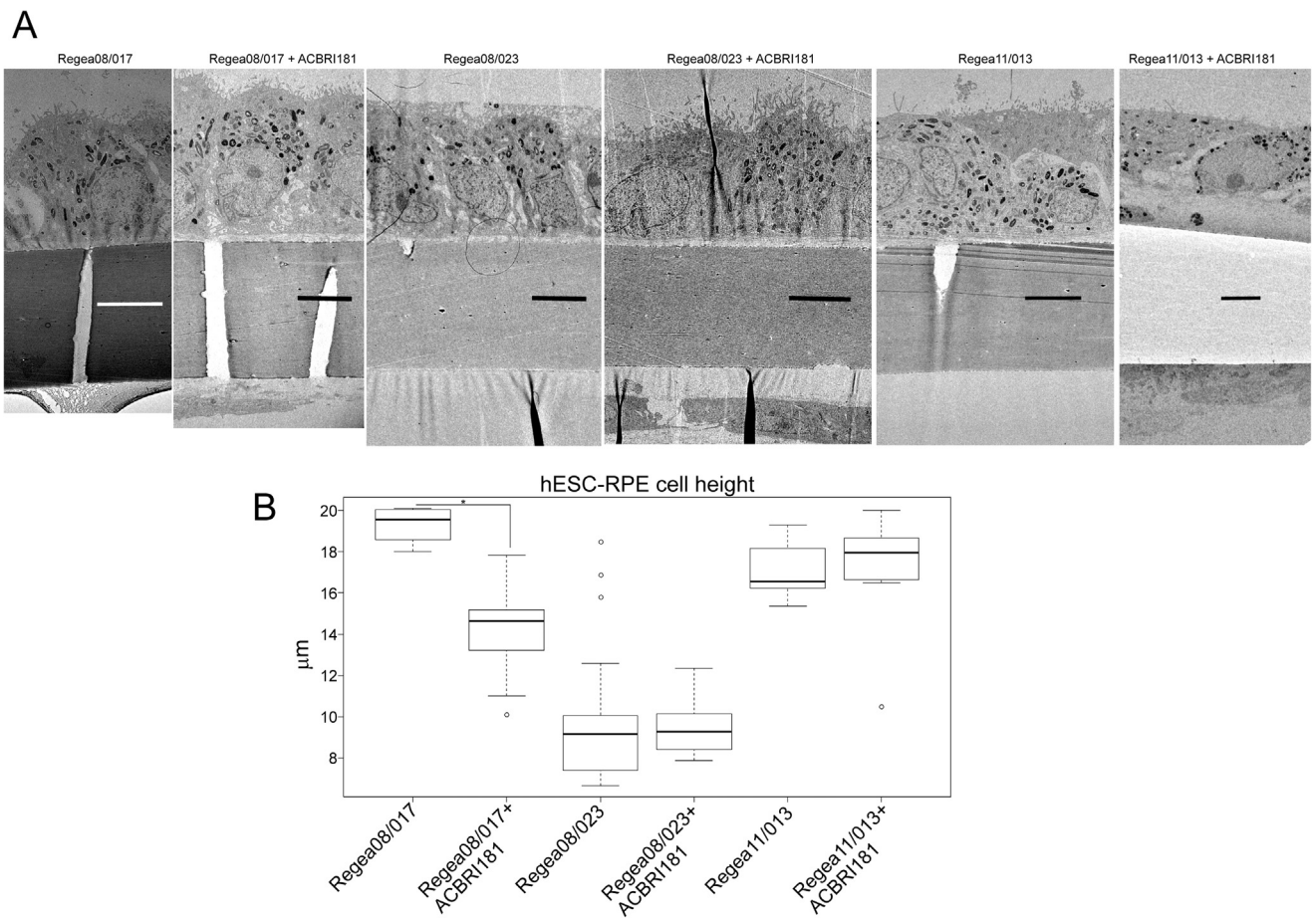

Fig. 3. A) The transmission electron micrographs after 4–6 weeks of hESC-RPE solo-cultures or hESC-RPE and ACBRI181 co-cultures (n= 3 with 2 biological replicates). Order from top to bottom: hESC-RPE cells, PET membrane, and in co-cultures hRECs. From left to right: Regea08/017 solo-culture; Regea08/017+ACBRI101 co-culture; Regea08/023 solo-culture; Regea08/023+ACBRI181 co-culture; Regea11/013 solo-culture; and Regea11/013+ACBRI181 co-culture. Scale bars 10 µm. **B)** The height of hESC-RPE cells was measured from the micrographs using Image J analysis software and presented as box plots. The thicker lines indicate medians. Statistical significance $p < 0.05$ is indicated with *.

cell cultures adopt cobblestone morphology, express RPE specific proteins and localize them correctly in solo-cultures [41,47]. The present study shows that co-culturing hESC-RPE cells with endothelial cells yield similar cobblestone morphology and localization of the RPE-specific protein CRALBP. Also, expression and localization of vWF in hRECs was unaltered in these co-cultures. There is no published corresponding data, but the results are in accordance with the data previously reported in ARPE-19 co-cultured with HUVECs [22,48]. ARPE-19 cell line, however, has important dissimilarities with human primary RPE cell cultures, most importantly low or lacking pigmentation and polarization [22,49]. In our study, co-culturing hESC-RPE cells with ACBRI181 did not affect either cell pigmentation or overall cell morphology. Co-cultured hESC-RPE had high amounts of pigmentation, visible tight junctions, thick brush border and highly organized ECM. Although there were differences in cell height, microvilli length and ECM thickness, these all were within the range of expected biological variation.

The queues from endothelial cells alter RPE cell functions [3], and biological solutes they secrete modify RPE barrier function in co-cultures [22,50]. Human CECs and RECs secrete different solutes [51], and for the purpose of an oBRB model hCECs would likely be the optimal choice. However, according to our current knowledge hRECs are commercially available, while hCECs are not. Primary bovine RPE have previously shown to have higher TEER in solo-culture than in contacting and non-contacting co-cultures with endothelial cells [50], but human fRPE and primary EC co-cultures had higher TEER than fRPE soloculture [52]. On the other hand, co-culturing ARPE-19 with HUVEC for 4 weeks on opposite sides of polyester membranes increased TEER [48]. In our study, co-culturing hESC-RPEs with hRECs clearly increased TEER, although the difference was only significant for one of the three cell lines. When the tightness of the cultures was evaluated by assessing the leakage or flux of small molecules from donor to acceptor side of the culture, all our cell lines demonstrated decreased permeability in co-cultures. Similar findings

Table 1
The hESC-RPE cell heights measured from the TEM photographs.

Culture	number of samples	total number of measurements	range	median	statistical significance solo- vs. co-culture
Regea 08/017	2	6	18.00 µm - 20.09 µm	19.54 µm	p=0.0014
Regea 08/017+ACBRI181	9	28	10.12 µm - 17.82 µm	14.65 µm	
Regea 08/023	10	23	6.67 µm - 18.49 µm	9.16 µm	p=0.3311
Regea 08/023+ACBRI181	9	32	7.88 µm - 12.33 µm	9.27 µm	
Regea 11/013	2	8	15.37 µm - 19.28 µm	16.57 µm	p=0.3638
Regea 11/013+ACBRI181	5	10	10.50 µm - 19.99 µm	17.95 µm	

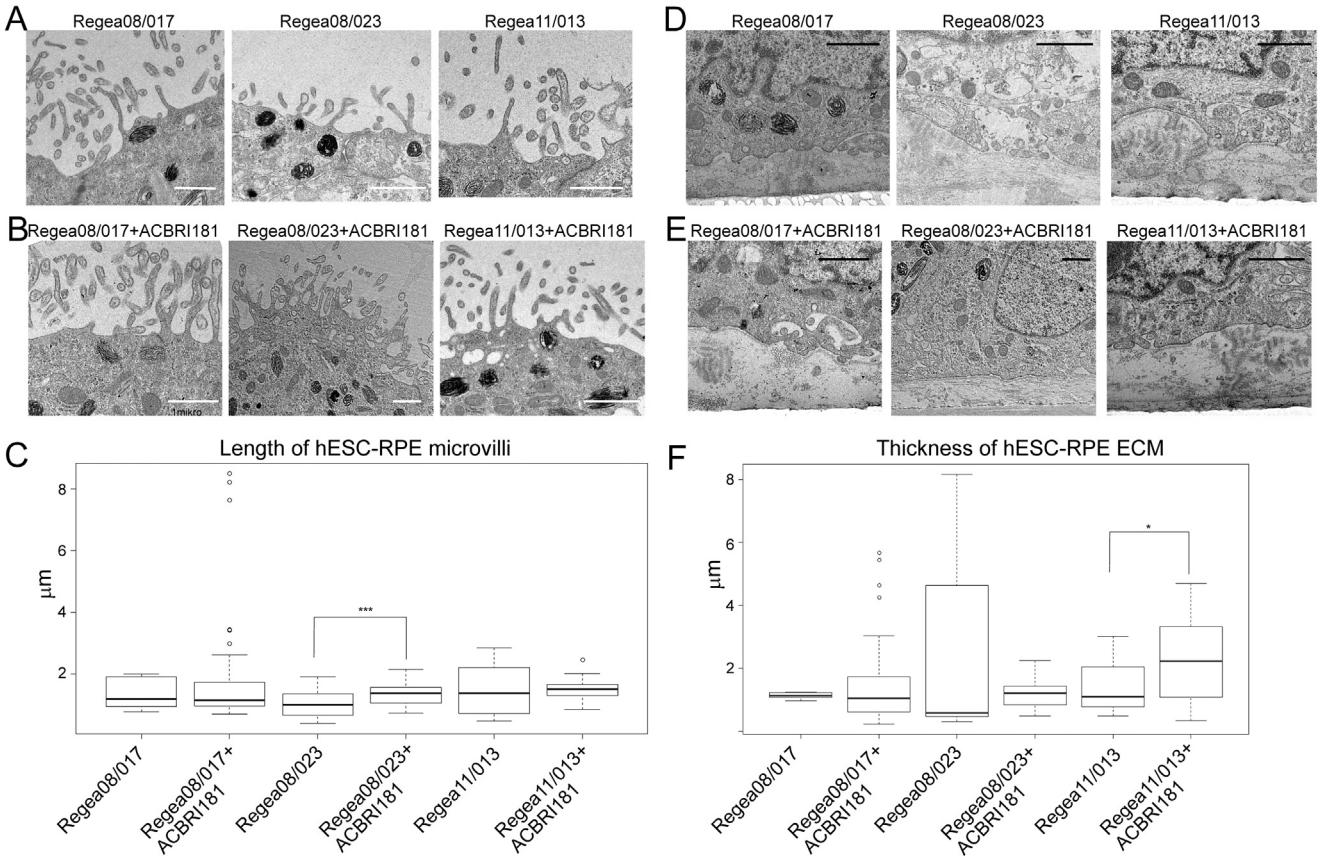

Fig. 4. The transmission electron micrographs after 4–6 weeks of hESC-RPE solo-cultures or hESC-RPE and ACBRI181 co-cultures (n= 3 with 2 biological replicates). A) Representative images of the apical brush border of solo-cultured hESC-RPE cells, and (B) hESC-RPE+ ACBRI181 co-cultures. Scale bar 1 µm. (C) The length of apical microvilli of hESC-RPE cells measured from the micrographs using Image J analysis software and presented as box plots (**C**). The thicker lines indicate medians. Statistical significance was analyzed but not found. D) Representative image of ECM underneath solo-cultured hESC-RPE cells, and (E) hESC-RPE+ ACBRI181 co-cultures. Scale bar 1 µm. (F) The height of hESC-RPE cells was measured from the micrographs using Image J analysis software and presented as box plots. The thicker lines indicate medians. Statistical significance $p < 0.05$ is indicated with *.

Table 2
The microvillus lengths in hESC-RPE cells measured from the TEM photographs.

Culture	number of samples	total number of measurements	range	median	statistical significance solo- vs. co-culture
Regea 08/017	2	7	0.78 µm - 2.02 µm	1.20 µm	p=0.8956
Regea 08/017+ACBRI181	13	51	0.71 µm - 8.49 µm	1.16 µm	
Regea 08/023	11	36	0.49 µm - 1.92 µm	1.03 µm	p=0.0002
Regea 08/023+ACBRI181	11	54	0.75 µm - 2.15 µm	1.39 µm	
Regea 11/013	5	14	0.50 µm - 2.86 µm	1.395 µm	p=0.0580
Regea 11/013+ACBRI181	5	14	0.86 µm - 2.47 µm	1.53 µm	

Table 3
The ECM thickness underneath hESC-RPE cells measured from the TEM photographs.

Culture	Number of samples	Total number of measurements	Range	Median	Statistical significance solo- vs. co-culture
Regea 08/017	2	6	0.97–1.25 µm	1.13 µm	p = 0.4434
Regea 08/017+ ACBRI181	11	40	0.23–5.67 µm	1.04 µm	
Regea 08/023	14	54	0.31–0.82 µm	0.58 µm	p = 0.0676
Regea 08/023+ ACBRI181	13	51	0.48–2.24 µm	1.21 µm	
Regea 11/013	7	30	0.48–3.02 µm	1.09 µm	p = 0.020
Regea 11/013+ACBRI181	5	20	0.33–4.86 µm	2.22 µm	

have been reported previously with ARPE-19 and HUVEC co-cultures [22] and primary bovine [50] RPE or primary human RPE [52] and EC co-cultures. The permeation coefficient (P_{app}) of the FD4 through co-cultures was 10 times higher ($2.93 + 10^{-6}$ cm/s) than though bovine RPE-choroid (2.36×10^{-7} cm/s) [44], but still the co-culture was 2 times tighter than hESC-RPE solocultures ($5.64 + 10^{-6}$ cm/s). The increase in TEER and decrease in permeability indicates that hRECs improve the barrier functions of RPE at least in vitro. This is also important to recognize when these in vitro assays are used for permeability and transport analysis of drugs or other molecules.

The balance of PEDF and VEGF levels is important for retinal homeostasis as it stimulates endothelial cell migration and RPE maturation [53,54]. In the developing retina PEDF is a differentiating factor for RPE cells. However, in mature retina PEDF is a trophic factor

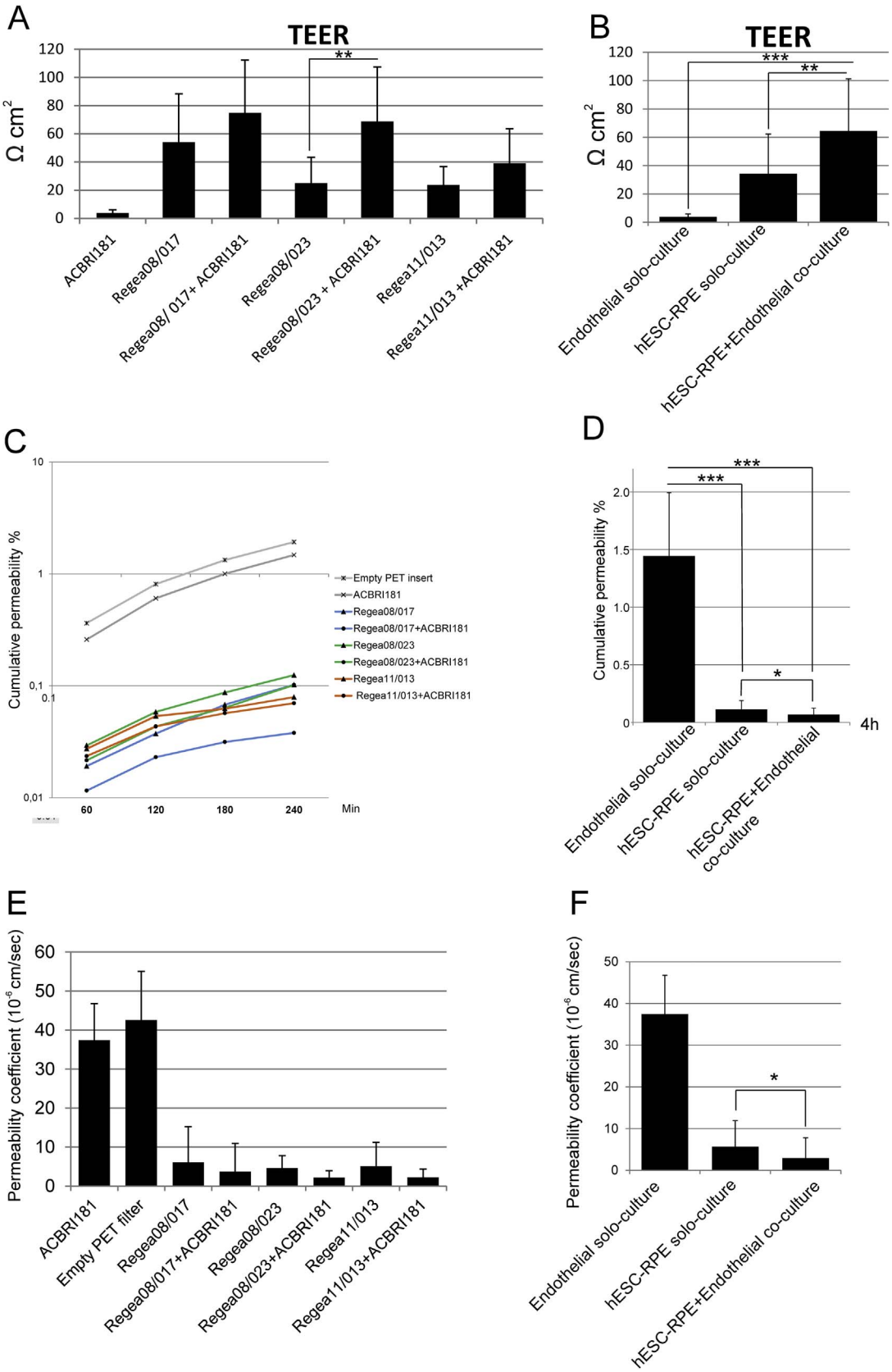

Fig. 5. A) The barrier properties of after 4–6 weeks of hESC-RPE solo-cultures of hESC-RPE and ACBRI181 co-cultures (n= 3, with 2 biological and 2 technical replicates. Data are expressed as mean +/-SD). (**A**) TEER values from individual cell lines as solo-cultures of endothelial cells, solo-cultures of hESC-RPEs, and hESC-RPE+ACBRI181 co-cultures, and (**B**) pooled cell lines, ** $p < 0.01$; *** $p < 0.001$. Cumulative permeability % of 4 kDa Fitc dextran (FD4, Sigma-Aldrich, Germany) of solo- and co-cultures after the 4–6 weeks culture (n= 3, with 2 biological and 2 technical replicates) in individual cell lines in solo- and co-cultures from 60 min to 240 min (**C**), and (D) in pooled cell lines at the 240 min time-point. Data are expressed as mean+/-SD, * $p < 0.05$; *** $p < 0.001$. Permeability of FD4 expressed as permeability coefficient (10^{-6} cm/s) (n= 3, with 2 biological and 2 technical replicates. Data are expressed as mean +/-SD) (E) from individual cell lines and in pooled cell lines, * $p < 0.05$.

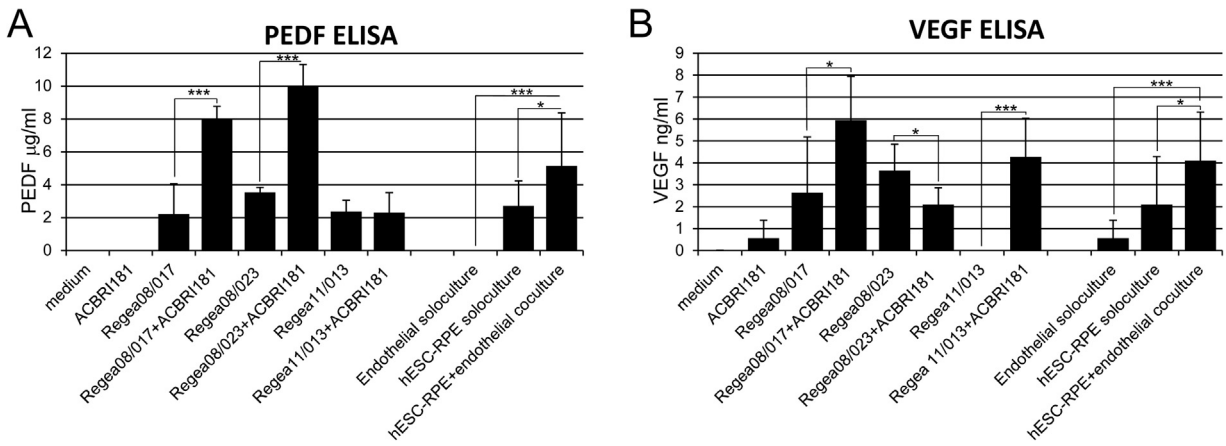

Fig. 6. The amount of growth factors ((A) PEDF and (B) VEGF) secreted during 48 h analyzed with ELISA after 4–6 weeks in culture (n=2 with 2 biological replicates). On the right side of the graphs the growth factor secretion between solo-cultures of hRECs, solo-cultures of pooled hESC-RPEs, and pooled hESC-RPE+endothelial co-cultures are compared. The data are expressed as mean +/-SD, * $p < p\ 0.05$, ***$p < 0.001$.

for RPE, which also keeps CECs quiescent [53]. PEDF secretion of RPE cells has been shown to be high in fetal RPE cells and hESC-RPE cells, but very low in ARPE-19 cells [41,55,56]. In a previous study, co-culture of dermal ECs with ARPE-19 induced a small, but statistically significant reduction in PEDF gene expression in ARPE-19 cells [20]. We have previously demonstrated that in hESC-RPE solo-cultures, PEDF secretion increases during maturation and polarization of RPE monolayer [41]. In this study, 48 h co-culturing of hESC-RPE cells with hRECs increased PEDF secretion in two of the three cell lines compered to solo-cultures. This reflects the inherent heterogeneity of hESC-RPE cell lines which has been shown previously [47,57].

VEGF is a chemotactic and angiogenic factor which is secreted by RPE cells in the retina [23,54]. VEGF is also an important pathologic factor in vaso-proliferative disorders, such as AMD [7]. In previous studies co-culture of RPE cells with ECs was shown to induce VEGF secretion [20,22,23,50]. The 48 h/72 h co-culture with CECs or 24-h co-culture with dermal ECs induced VEGF gene expression and VEGF secretion in ARPE-19 cells [58]. Similarly to PEDF, the effect of co-culturing of hESC-RPEs with hRECs seems to be cell line specific – for two cell lines, VEGF secretion increased as a result of co-culture, but decreased for the third cell line. This further illustrates the heterogeneity of hESC-RPE cell lines.

In this study which according to our knowledge is the first one in which hESC-RPE cells were co-cultured with with hRECs for up to six weeks, we show that does not alter RPE cell morphology or pigmentation, but increases the barrier properties. Co-culture also revealed cell line specific differences in induction of growth factor secretion and in hESC-RPE fine structure. Although this model is still quite preliminary, this data presented here propose that after critical selection of cell lines and careful validation process co-culture methods could be to be a valuable tool to model in vitro oBRB for drug permeability testing.

Authors contributions

Conceived and designed the experiments: KJU. Performed the experiments: JMu, HLa, EPa, KKa, AKo, KJU. Analyzed and interpreted the data: KJU, JMu, HLa, EPa, LKo. Contributed regents/ materials/ analysis tools: KMa, HUu. Drafted the manuscript: HSk, LKo, HSk, KKa, KJU. Participated in the critical revision of the manuscript and approval of the article: HSk, JMu, HLa, EPa, LKo, KMa HUu, KKa, KJU. Acquired the funding: KJU, HSk, HUu, KKa.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at. http://dx.doi.org/10.1016/j.yexcr.2017.08.004.

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