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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