Development and characterization of an *in vitro* system of the human retina using cultured cell lines

Churm R. PhD[1], Dunseath GJ. PhD[1], Prior SL. PhD[1], Thomas RL. PhD[1], Banerjee S. PhD[2], Owens DR. MD[1]

[1] Diabetes Research Group, Grove Building, Swansea University, Swansea, UK

[2] Cardiff and Vale Health Board, University of Wales Hospital, Cardiff, UK

Running title

Novel triple-culture of the human retina

Conflicts of interest- None.

Finding Source- St David's Medical Foundation

Correspondence

Dr Rachel Churm

Tel: +44(0)1792 602310

Email: r.churm@swansea.ac.uk

Abstract

Background: Previously developed *in vitro* cultures of the human retina have been solo or dual cell cultures. We developed a triple-cell culture *in vitro* model utilizing a membrane system to produce a better representation of a functional and morphological human retina.

Methods: Retinal microvascular endothelial cells (HRMVEC/ACBRI181, Cell systems), retinal pigment epithelium cells (RPE/ARPE-19, ATCC) and Müller glial cells (MIO-M1, UCL) were grown in a triple-culture. Our optimized triple-culture media contained a mix of specific endothelial medium and high glucose Dulbecco's Modified Eagle's medium (DMEM), where all three layers were viable for up to 5 days. Co-culture effect on morphological changes (cell staining) and gene expression of functional genes (pigment epithelial derived factor (*PEDF*) and vascular endothelial growth factor (*VEGF*)) were measured from RNA via real time PCR. Expression of tight junction protein 1 (*TJP1*) was measured in RNA isolated from ARPE-19s, to assess barrier stability.

Results: The triple-culture promotes certain cell functionality through up-regulation of *TJP1*, increasing *PEDF* and decreasing *VEGF* expression highlighting its importance for the assessment of disease mechanisms distinct from a solo culture which would not allow the true effect of the native microenvironment to be elucidated.

Conclusion: This model's novelty and reliability allows for the assessment of singular cellular function within the retinal microenvironment and overall assessment of retinal health, whilst eliminating the requirement of animal-based models.

Key words; Human retina, Cell culture, Triple-culture, *In vitro* model

1. Introduction

The human eye is a complex organ that is comprised of three main areas; the cornea, lens, and the retina. The retina is a highly specialised organ of photoreception, involved in translating light energy into action potentials which are relayed to the brain, where the information is processed into vision. The integrity of the retina is dependent upon its immediate microenvironment, which is reliant on cell-cell interactions of the different human retinal cellular components. Disruption of which results in a variety of retinal diseases including diabetic retinopathy (DR)[1]. DR is associated with the breakdown of both the inner blood-retinal-barrier (BRB)[2, 3] and more recently linked to the outer BRB[4]. The inner BRB is composed of tight junctions between retinal capillary endothelial cells and the outer of tight junctions between the retinal pigment epithelial cells (RPE). The inter-relationship between vascular and endothelial cells that form the BRB is vital maintaining a specialized environment of the neural retina.

Current findings show that BRB breakdown is multifactorial, including; impaired endothelial cells, pericyte demise, thickening of capillary basement membrane and the alteration of tight junctions between RPE and endothelial cells[5]. Additionally RPE, endothelial and other cell types within the retina can be responsible for the release of neurotrophic factors that can alter the integrity of the BRB, such as vascular endothelial growth factor (VEGF) and pigment endothelial derived factor (PEDF)[6-8]. These factors can also be released from Müller cells[8-10], which are a subset of the retinal macroglia that are strongly linked to RPEs, establishing structural connections with the subretinal space and choroidal vasculature[8, 11]. It is evident that research into DR requires a

complex system that can encompass the interaction of all cells present within both the inner and outer BRB.

Currently research into retinal disease utilizes two main methodologies; *in vitro* analysis of solo or dual cell cultures, and animal models. The current literature and project designs relating to RPE cultures are often limited to them encompassing a dual-culture system with endothelial cells. The culture formations published comprise of cells either in direct contact[12], sandwiched with extracellular matrix proteins[13, 14], or cells cultured on either side of a membrane[15-17]. Alternatively, there are published models that encompass a dual system where these cells do not come into contact[18]. There are very few multi-culture systems within literature, with those published comprising of mixed species cell sources[19]. We aim to expand on current *in vitro* models to develop a triple-culture which utilizes both human cell lines and primary cells, to demonstrate a better representation of a functional and morphological human retina.

2. Methods

Cell line and isolated primary cell culture

Retinal pigment epithelial cells

RPE were authenticated and sourced as a commercially available cell line; ARPE-19 (ATCC® CRL-2302, USA). Upon receipt were grown in Dulbecco's Modified Eagle's medium (DMEM) containing glutamine supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen-Gibco, Rockville, MD). Cells were

incubated at 37°C at 5% CO_2, with media changed every 2-3 days and sub-cultured (1:3 split) at 80% confluency with 0.05% trypsin-EDTA (Invitrogen-Gibco, Rockville, MD).

Müller glial cells

Human Moorfield Institute of Ophthalmology-Müller 1 (MIO-M1) cell line were isolated, authenticated and purchased from Professor Limb (Institute of Ophthalmology and Moorfields Eye Hospital, UK)[20].Upon arrival, cells were cultured in the same media as ARPE-19, incubated at 37°C at 5% CO_2 and media changed every 2-3 days.

Retinal microvascular endothelial cells

Primary human retinal microvascular endothelial cells (HRMVEC) are commercially available, sourced from Applied Cell Biology Research Institute (ACBRI 181, Cell Systems, Kirkland, USA). All experimental procedures completed using primary cells were in compliance with the Human Tissue Act. Cells were grown in Cell Systems Culture (CS-C) medium, incubated at 37°C at 5% CO_2, with media changed every 2-3 days. Cells were passaged when confluent using Cell Systems Passage Reagent (all reagents sourced from Cell Systems, Kirkland, USA) and used in experiments at passage 8.

Co-culture formation

To assess cell viability in a microenvironment, cells were grown in solo, dual (MIO-M1 & ARPE-19, ARPE-19 & HRMVEC and MIO-M1 & HRMVEC) and triple formations (ARPE-19, MIO-M1 & HRMVEC), using the following protocol.

System set-up

During solo-, dual- and triple-culture formations all cell types were grown in the same location as seen within triple-culture schematic (Figure 1). Multi-culture system utilizes a 0.4 µm pore size polyester (PET) Transwell® membrane with a 10 µm membrane thickness (Corning, Thermo Scientific, UK). Cells were plated according to Figure 1, using an in-house optimized multi-culture media (MC media) consisting of 2:1 DMEM with glutamine: CS-C medium.

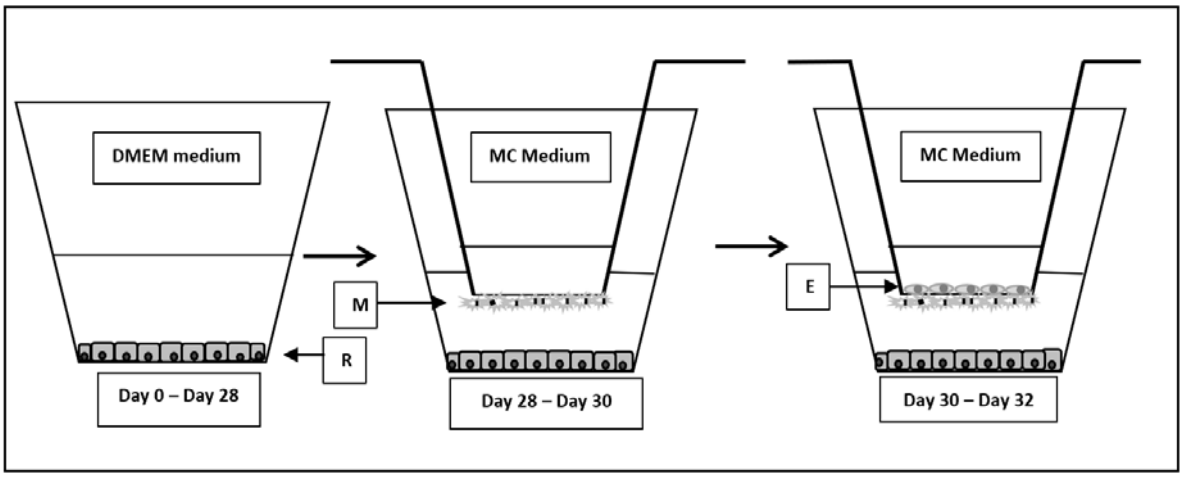


Figure 1. Triple-culture schematic for ARPE-19 (R), MIO-M1 (M) and HRMVEC (E). Annotated medium is the culturing medium present in both well insert and culture dish well as described in methodology, multi-culture. (MC).

Cell seeding and maturation

ARPE-19 was grown on the base of a 6-well culture dish, as shown (Figure 1). Cells were prepared for all experiments at passage 6, split ratio 1:3, requiring maturation and tight-junction formation for 28 days with growth media changed every 2-3 days (Supp. Figure 1A). At day 28, MIO-M1 cells were seeded at 100,000 cells per cm^2 in 150 µL (passage 34) onto the basal side of a Transwell® membrane by inverting the membrane structure and incubated for 3 hours at 37°C at 5% CO_2. Once adhered, excess medium was

removed, the membrane reversed and placed in the 6-well culture plate containing the ARPE-19 cells. At day 32, HRMVEC were seeded at 100,000 cells per cm^2 to the apical surface of the membrane insert washed with attachment factor, and grown in 1.5 mL MC medium, cells reached optimal confluency (70%) for experimentation after 48 hours (day 34). MC media of well and insert was replenished every 2-3 days, cell viability valid for up to 5 days of construction of complete model (i.e. day 37) due to cellular characteristics and phenotypes of specific cell lines being lost after prolonged culture (> 14 days)[20].

For permeability assay, an alternative layout was assembled to assess barrier properties as original triple-culture layout does not allow for this. Cells were cultured as described previously, but with cell seeding and maturation occurring in alternative positions i.e. ARPE-19 grown on apical surface of the membrane, MIO-M1 on basal surface and HRMVEC on bottom of the culture well (Figure 2).

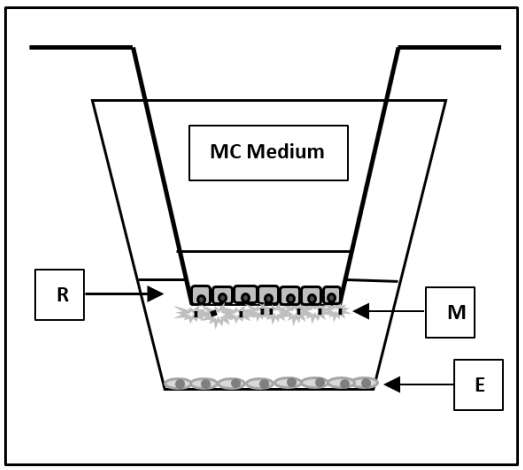


Figure 2. A- Alternative formation of multi-culture to assess Transwell® permeability in a multi-culture. R-ARPE-19, M-MIO-M1 and E-HRMVEC.

Morphological changes

Immunocytochemical analysis was conducted to ensure the presence of correct cell types within culture (See Supp. Work 1 and Supp. Figure 1). Basic cell morphologies and relative diameter were determined using Hematoxylin (Sigma) staining and digital imaging via light microscopy. Cell quantification, density and diameter were assessed using ImageJ software (NIH, Version 2). Limitations of a basic cell imaging only allows data to be used as a relative check for cell morphology instead of a quantification of absolute size. Each experimental well produced images in 5 fields, each field was pre-defined and set prior to cell seeding to remove experimental bias. A total of 20 cells per type at x50 magnification were analysed to determine cell diameter and morphologies (n=100 cells for data analysis, per biological repeat) (representation of images provided in Supp. Figure 2). Each cell type was analysed within 3 biological repeats.

Gene expression analysis

All expression data was collected from each culture layout from three biological repeats and three experimental repeats per plate (n=9 per culture layout). Individual cells types were harvested at day 32 of culture, homogenized in QIAzol lysis reagent and total RNA isolated using a RNeasy Mini Kit (Qiagen, UK). 1000ng/µl of total RNA was converted to cDNA using a reverse transcription kit (Invitrogen, Thermo Fisher, UK). 1:10 dilutions of cDNA transcripts were run on a CFX connect (BioRad, UK) using SYBR Green I (BioRad, UK) for each primer set (***VEGF***: Forward-ACT TCT GGG CTG TTC TC, Reverse-TCC TCT TCC TTC TCT TCT TCC; ***PEDF***: Forward-TGC AGG CCC AGA TGA AAG GG, Reverse-TGA ACT CAG

AGG TGA GGC TC. ***TJP1****:* sourced and optimized from BioRad, UK) at optimal cycling conditions of; 95°C for 3min, followed by 35 cycles of 95°C for 30s, 59.5°C for 30s and 72°C for 30s, followed by melt peak conditions. The average Ct value was taken from triplicate assays and normalised against the invariant expression of *β-actin* housekeeper gene. Result were analysed using the $2^{-\Delta\Delta Ct}$ method to produce relative fold change values in comparison between groups, standard error of the mean (SEM) was calculated from the average Ct value for each sample produced within the experiment cohort. Fold change range of -1.5 to 1.5 is indicative of no overall change in gene expression levels.

Permeability assay

Permeability assays were completed to assess barrier properties in a mixed cell environment, based on a horseradish peroxidase (HRP) (Sigma™) diffusion ELISA (n=9, per culture layout). For permeability assays, DMEM without FCS was used at all stages. An alternative layout of both dual- and triple-culture was used to assess Transwell® permeability due to the functional properties of each cell type. To assess the implication of culture formation on both ARPE-19 and HRMVEC, they were grown on the apical and basal side of the membrane, respectively. Medium within the well and insert compartments were replaced for assay media and the insert compartment dosed with HRP [1250 mU], incubated for 20 minutes and medium from the lower well compartment collected for analysis. Culture media was transferred to a 96-well clear microplate plate for colorimetric analysis via 3,3',5,5'-Tetramethylbenzidine (TMB) and stop solution (Invitron, UK) and absorbance recorded at a wavelength of 450 nm and

corrected at 620 nm. Membrane permeability was analysed in comparison to a control membrane with no cellular growth. Relative permeability was produced as a percentage;

$$Relative\ Permeability\ (\%) = \frac{Culture\ Experiment\ Value}{Control\ Value}\ x\ 100$$

Statistical analysis

Raw data was analysed using SPSS™ Version 23. Continuous data is summarized by mean and standard deviation (SD) when normally distributed, and by median and interquartile range (IQR) if not normally distributed. Normality of the data was verified by the Kolmogorov-Smirnov test and visualised on q-q plots. A Student t-test was used to compare the mean of two groups and analysis of variance (ANOVA) to compare the mean of more than two groups for normally distributed data. Alternatively, not normally distributed data was analysed using Mann Whitney U and Kruskal Wallis tests. For gene expression data, statistical analysis was run on threshold cycle (Ct) data normalised against *β-actin* housekeeper, assessed using Student t-test. P-values less than 0.05 were deemed statistically significant.

3. Results

Cell morphology

Across all three cell types, no significant alterations in cell morphologies were noted in any culture formation (Table 1). Analysis of HRMVEC morphologies using cellular staining and microscopy was unable to produce sufficient images within the triple-culture to assess cell diameter. RNA spectroscopy did confirm cell growth on the apical

surface and we believe a lack of imaging reflect limitations within methodology. Gene expression of *TJP1* was measured in RNA isolated from ARPE-19 to assess barrier stability in a multi-culture system. Data indicates a significant increase in fold change (FC) for *TJP1* when RPE are grown in combination with either MIO-M1 (+11.4 FC, $p<0.001$), HRMVEC (+12.9 FC, $p<0.001$) or both in a triple-culture (+7.8 FC, $p<0.001$) (Figure 3). Barrier permeability was not assessed within the primary formation of the dual- and triple-culture as described in methodology.

Table 1. Mean and standard deviation of cell diameter (µM) for the three cell types grown in all culture formations. P-value determined using one-way ANOVA.

Culture Formation	Cell Type		
	ARPE-19 (n=300)	**MIO-M1** (n=300)	**HRMVEC** (n=300)
Solo	**17.5 (6.1)**	**62.7 (13.3)**	**98.9 (24.4)**
Dual		**61.9 (10.6)**	**97.8 (28.0)**
Dual	**18.1 (4.3)**		**96.0 (22.9)**
Dual	**17.9 (4.5)**	**63.8 (13.9)**	
Triple	**18.2 (5.3)**	**62.5 (14.3)**	-
P-value	**0.48**	**0.54**	**0.66**

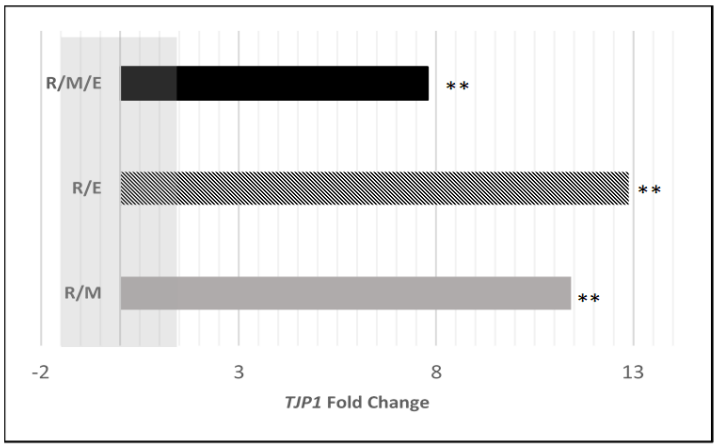


Figure 3. Relative fold change values for *TJP1* gene expression data in multi-culture formations in comparison to solo-culture. Fold change between -1.5 and 1.5 is classed as no relative gene expression change (grey shaded region). Statistical significance represented by asterisks (**p<0.01), determined using an independent t-test. R-ARPE-19, M-MIO-M1 and E-HRMVEC.

Implications of multi-culture on gene expression levels

PEDF expression (Figure 4A) demonstrated an increase in FC in both ARPE-19 (+5.2 FC, p<0.01) and MIO-M1 (+7.5 FC, p<0.05) in triple compared to solo-culture. Triple compared to solo-culture demonstrated no significant FC in *VEGF* expression in either MIO-M1 (+1.0 FC, p=0.49) or ARPE-19 (+0.6 FC, p=0.34) (Figure 4B). However, expression was significantly decreased within HRMVEC (-3.2 FC, p<0.05). Sub-analysis of dual-cultures implicates MIO-M1 for the observed change in *VEGF* expression (dual-culture of; MIO-M1+HRMVEC, -6.0 FC, p<0.05 and dual-culture; ARPE-19+HRMVEC, +1.80 FC, p=0.11).

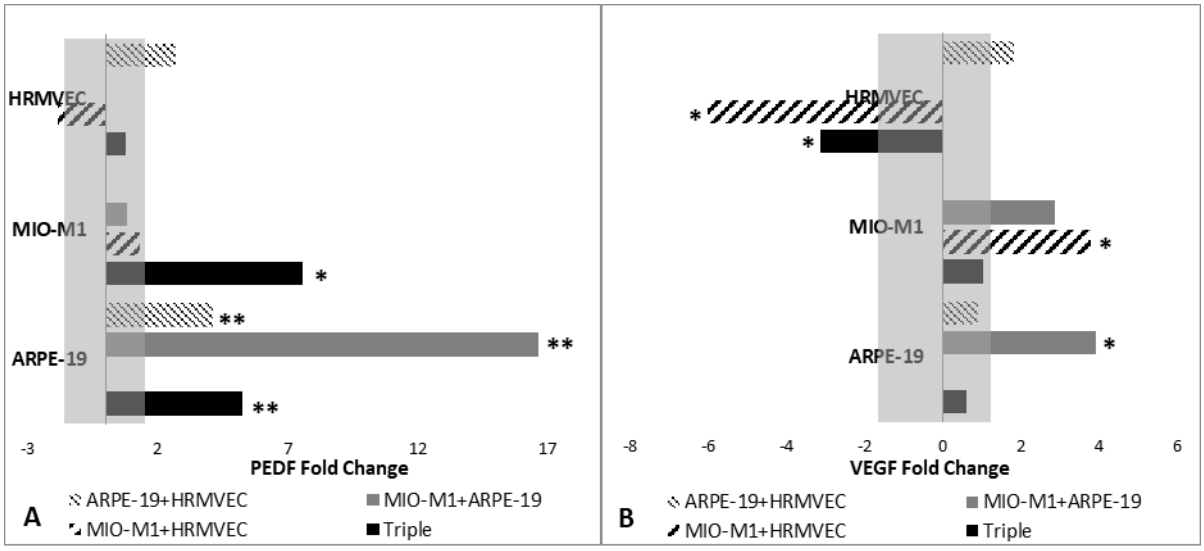


Figure 4A-B. Relative fold change values for gene expression data in multi-culture formations in comparison to solo-culture. Fold change between -1.5 and 1.5 is classed as no relative gene expression change (grey shaded region). (A) *PEDF*-fold change & (B) *VEGF*-fold change. Statistical significance represented by asterisks (*p<0.05, **p<0.01), determined using an independent t-test of the difference of ΔCt in multi-culture formation of cells versus solo-cultured cells.

Assessment of multi-culture on membrane properties

HRP diffusion across the Transwell® membrane altered significantly dependent on the cells grown on the surface. ARPE-19 monolayer formation resulted in the diminishing of HRP passing through the membrane, this barrier was significantly strengthened in the presence of MIO-M1 grown on the basal side of the membrane in both a dual- and triple-culture (ARPE-19 vs ARPE-19/MIO-M1; 34.1 (3.9)% v 25.7 (1.2)%: p<0.01; ARPE-19 vs ARPE-19/MIO-M1/HRMVEC; 34.1 (3.9)% vs 26.5 (0.5)%: p<0.01) (Figure 5). Diffusion of molecules through the Transwell® membrane is significantly reduced by cell growth on the surface, data demonstrated minimal relative permeability is 25.7% (±1.2) indicating cross membrane molecule diffusion is still occurring.

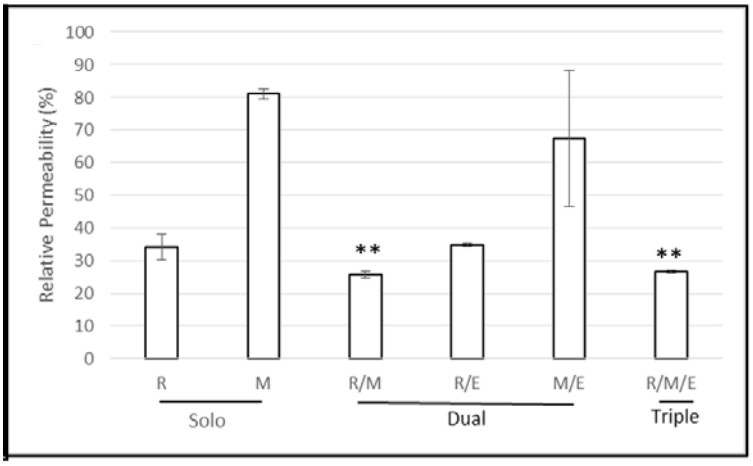


Figure 5. HRP diffusion assay, relative permeability (%) for all cell formations that require growth on the Transwell® membrane, error bars indicate standard deviation. Statistical significance represented by asterisks (**p<0.01), determined using an independent t-test. R-ARPE-19, M-MIO-M1 and E-HRMVEC.

4. Discussion

This study presents the evidence for a novel human *in vitro* model of the retina. This model is reproducible and provides flexibility in formation and structure to maximise the yield of experimental research that can be conducted, including mechanistic research into retinal disease, retina biology and pharmacology intervention. The culture model allows for the arrangement of cellular components that mimic the retinal microvasculature, neuronal retina and RPE *in vivo,* allowing us to explore the tissues cellular microenvironment. The triple-culture model represents the close contacting relationship between the Müller glial cells (MIO-M1) and the blood vessels (HRMVEC) within the retina and the distal location of the non-contacting RPE (ARPE-19) retinal layer (Figure 6)[21]. These cell types in particular play a pivotal role in the function of the BRB and investigation of their cross-communication could lead to great enhancements for understanding retinal disease that results in, or is the result of, BRB malfunction.

Our findings highlight the importance of cell to cell interaction in retinal biology, with multi-culture systems resulting in changes in both structural and genetic alterations when compared to solo-cultures. On the other hand, the assessment of cell morphology resulted in no significant changes when the cells were grown in solo or in formations with each other. However, the methodology used to assess morphology is a basic technique and is limited in quantification and should only be utilised as an indication of cell type presence. We propose that within future expansion of this model, investigation into an optimized confocal analysis of cell markers across the membrane will confirm and quantify our original findings.

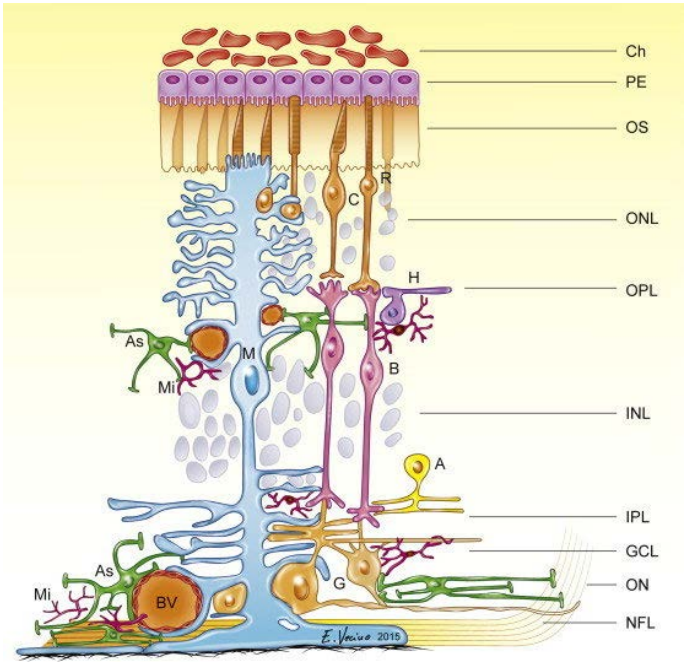


Figure 6. Schematic drawing of the cellular components of the retina taken from Vecino *et al,* 2016[21]. The different cell types are situated in a standard large mammalian retina, depicting the interactions between the Müller cells in blue (M) and blood vessels (BV) (represented in triple-culture model with HRMVEC). Different layers of the retina; optic nerve (ON), nerve fibre layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), outer segment layer (OS), retinal pigment epithelium (RPE) & choroid (Ch).

Structurally, these findings indicate a significant 11- and 7-fold increase in gene expression for *TJP1* or often referred to as zonula occludens (ZO1), when RPE are grown in either a solo-culture, or in combination with MIO-M1 as a dual-culture or in a triple-culture. This is also reflected within the barrier properties of the cultures, indicating a significantly strengthened barrier when MIO-M1 cells were grown on the basal side of the membrane. Previously published findings have associated Müller glial cell with tightened tight junctions and upregulated and polarized localization of specific blood-brain-barrier transporters[19, 22]. However, expansion of these findings would require protein analysis to determine the downstream impact.

Within DR and retinal disease, often researched is the alteration of key genes. Gerhardinger and colleagues report that in mice, 6 months post streptozotocin induction of diabetes, resulted in the alteration of 78 genes within Müller glial cells[23]. Over a third of these genes were reported to be associated with inflammation and inflammatory response including cytokines such as VEGF[5, 23]. These inflammatory cytokines can initiate a cascade of events within the retinal microenvironment and result in dysfunction of various cell types. The findings of this model indicate that within a triple-culture, expression of *VEGF* was significantly altered within HRMVEC; we hypothesise this is due to the presence of MIO-M1 cells. *PEDF* expression is often demonstrated in literature to promote the health of the BRB[24, 25]; within this model we show a marked increase in *PEDF* gene expression. We hypothesise that the triple-culture system promotes the functionality of the BRB, not only structurally through the promotion of *TJP1*/ZO1, but also by an increase in the expression of beneficial growth factors such as *PEDF* and decreasing the expression of detrimental growth factors such

as *VEGF*. This data highlights the significance of using a multi-culture system within the assessment of disease mechanism, as a solo-culture would not elucidate the effect of the native environment due to the implications of additional cell types. Let alone this model does not represent all cell types present within the retina, it does provide species specificity and three cell type interactions, therefore improving on the current retinal models available and published.

Often disputed in literature, is the use of cell lines in model design due to the presence of developmental abnormalities[17, 26]. The prolonged culture of both ARPE-19 and MIO-M1 can result in abnormalities in characteristic features of the cell such as the loss of pigmentation in ARPE-19 and MIO-M1 exhibiting progenitor characteristics[27]. However, within our study design we have allowed for maturation and low passage numbers to ensure manipulation of culture conditions will result in minimal difference from parent tissue[26]. The superior use of an alternative cell source i.e. primary cell sources will yield a truer representation of the native tissue. However, it can also produce poor efficiency of the model in practice allowing for restricted use of repeats and minimise use in pharmacological testing. Additionally, models do not allow for the manipulation of culture environments to exacerbate a disease effect, for example; chronic exposure to high glucose and varying glycaemic levels during cell seeding and growth, to induce phenotypes seen in DR. Due to difficulties in sourcing human primary cells for the modelling of the retina, to date only a rodent *in vitro* model[19] and no human multi-culture system have been published. We propose the compromise of two highly characterized and routinely used cell lines ARPE-19[28-30] and MIO-M1[20, 27]; and one primary sourced cell origin ACBRI-181 within this model, allowed for an *in vitro* multi-

346 culture model to be utilized in place of either an *in vitro* mixed species model or an *in*
347 *vivo/in vitro* animal model.

348 In conclusion, our methodology provides evidence for a novel multi-cell *in vitro*
349 culture model of the human retina, for the assessment of retinal biology and disease
350 mechanisms. The versatility of the model allows the assessment of both singular cellular
351 function within the retinal microenvironment in addition to a triple-cell assessment of
352 retinal structure and function in health and disease.

353

354

355 **Acknowledgement**

356 We would like to express a great gratitude to the St David's Medical Foundation for
357 funding this project.

358

References

[1] Simo R, Hernandez C: Novel approaches for treating diabetic retinopathy based on recent pathogenic evidence. Progress in Retinal and eye Research 2015, 48:160-80.

[2] Frey T, Antonetti DA: Alterations to the blood–retinal barrier in diabetes: cytokines and reactive oxygen species. Antioxidants & redox signaling 2011, 15:1271-84.

[3] Zhang X, Zeng H, Bao S, Wang N, Gillies MC: Diabetic macular edema: new concepts in patho-physiology and treatment. Cell & bioscience 2014, 4:27.

[4] Daruich A, Matet A, Moulin A, Kowalczuk L, Nicolas M, Sellam A, Rothschild P-R, Omri S, Gélizé E, Jonet L: Mechanisms of macular edema: beyond the surface. Progress in retinal and eye research 2017.

[5] Rübsam A, Parikh S, Fort P: Role of inflammation in diabetic retinopathy. International journal of molecular sciences 2018, 19:942.

[6] Miller JW, Adamis AP, Shima DT, D'Amore PA, Moulton RS, O'Reilly MS, Folkman J, Dvorak HF, Brown LF, Berse B: Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. The American journal of pathology 1994, 145:574.

[7] Ferrara N, Davis-Smyth T: The biology of vascular endothelial growth factor. Endocrine reviews 1997, 18:4-25.

[8] Sorrentino FS, Allkabes M, Salsini G, Bonifazzi C, Perri P: The importance of glial cells in the homeostasis of the retinal microenvironment and their pivotal role in the course of diabetic retinopathy. Life sciences 2016, 162:54-9.

[9] Fu S, Dong S, Zhu M, Sherry DM, Wang C, You Z, Haigh JJ, Le Y-Z: Müller glia are a major cellular source of survival signals for retinal neurons in diabetes. Diabetes 2015:db150180.

[10] Nishikiori N, Osanai M, Chiba H, Kojima T, Mitamura Y, Ohguro H, Sawada N: Glial Cell–Derived cytokines attenuate the breakdown of vascular integrity in diabetic retinopathy. Diabetes 2007, 56:1333-40.

[11] Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, Skatchkov SN, Osborne NN, Reichenbach A: Müller cells in the healthy and diseased retina. Progress in retinal and eye research 2006, 25:397-424.

[12] Dardik R, Livnat T, Halpert G, Jawad S, Nisgav Y, Azar-Avivi S, Liu B, Nussenblatt RB, Weinberger D, Sredni B: The small tellurium-based compound SAS suppresses inflammation in human retinal pigment epithelium. Molecular vision 2016, 22:548.

[13] Fan W, Zheng JJ, McLaughlin BJ: An in vitro model of the back of the eye for studying retinal pigment epithelial-choroidal endothelial interactions. In Vitro Cellular & Developmental Biology-Animal 2002, 38:228-34.

[14] Sakamoto T, Sakamoto H, Murphy TL, Spee C, Soriano D, Ishibashi T, Hinton DR, Ryan SJ: Vessel formation by choroidal endothelial cells in vitro is modulated by retinal pigment epithelial cells. Archives of Ophthalmology 1995, 113:512-20.

[15] Geisen P, McColm JR, Hartnett ME: Choroidal endothelial cells transmigrate across the retinal pigment epithelium but do not proliferate in response to soluble vascular endothelial growth factor. Experimental eye research 2006, 82:608-19.

[16] Wang H, Geisen P, Wittchen ES, King B, Burridge K, D'Amore PA, Hartnett ME: The role of RPE cell-associated VEGF189 in choroidal endothelial cell transmigration across the RPE. Investigative ophthalmology & visual science 2011, 52:570-8.

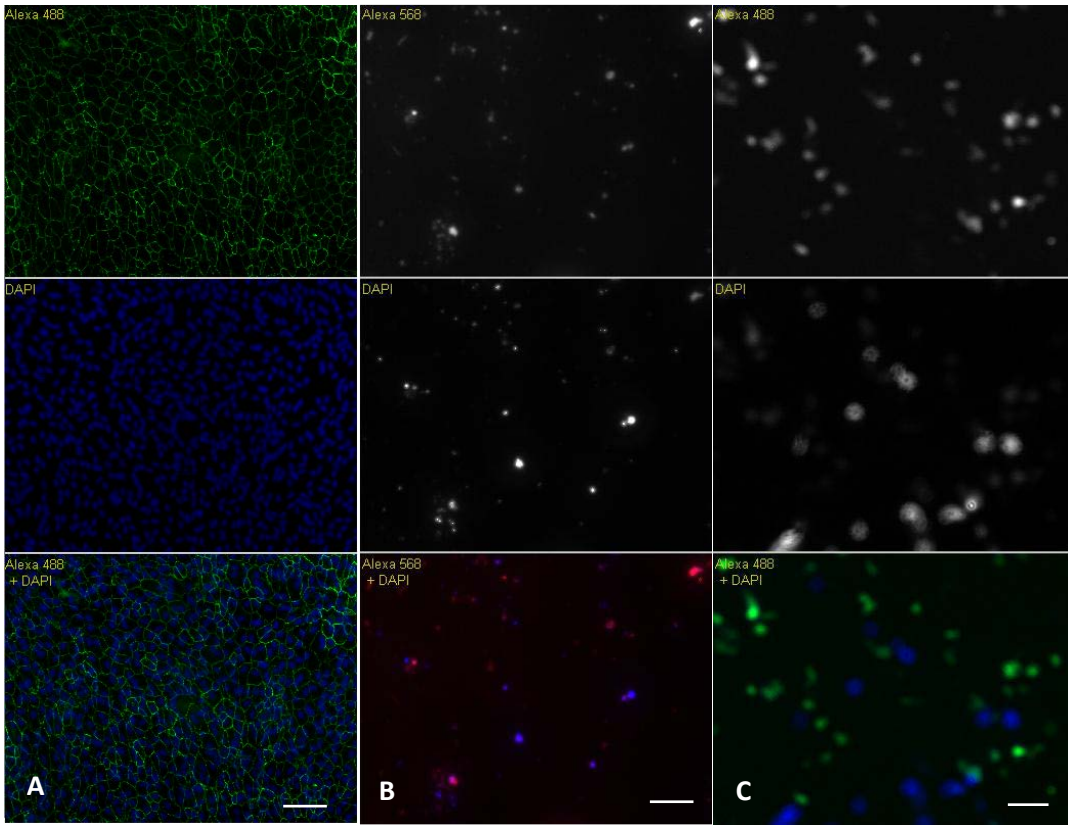
[17] Skottman H, Muranen J, Lähdekorpi H, Pajula E, Mäkelä K, Koivusalo L, Koistinen A, Uusitalo H, Kaarniranta K, Juuti-Uusitalo K: Contacting co-culture of human retinal microvascular endothelial cells alters barrier function of human embryonic stem cell derived retinal pigment epithelial cells. Experimental cell research 2017, 359:101-11.

[18] Liu X, Zhu M, Yang X, Wang Y, Qin B, Cui C, Chen H, Sang A: Inhibition of RACK1 ameliorates choroidal neovascularization formation in vitro and in vivo. Experimental and molecular pathology 2016, 100:451-9.
[19] Wisniewska-Kruk J, Hoeben KA, Vogels IM, Gaillard PJ, Van Noorden CJ, Schlingemann RO, Klaassen I: A novel co-culture model of the blood-retinal barrier based on primary retinal endothelial cells, pericytes and astrocytes. Experimental eye research 2012, 96:181-90.
[20] Limb GA, Salt TE, Munro PM, Moss SE, Khaw PT: In vitro characterization of a spontaneously immortalized human Muller cell line (MIO-M1). Investigative ophthalmology & visual science 2002, 43:864-9.
[21] Vecino E, Rodriguez FD, Ruzafa N, Pereiro X, Sharma SC: Glia–neuron interactions in the mammalian retina. Progress in retinal and eye research 2016, 51:1-40.
[22] Malina KC-K, Cooper I, Teichberg VI: Closing the gap between the in-vivo and in-vitro blood–brain barrier tightness. Brain research 2009, 1284:12-21.
[23] Gerhardinger C, Costa MB, Coulombe MC, Toth I, Hoehn T, Grosu P: Expression of acute-phase response proteins in retinal Muller cells in diabetes. Investigative ophthalmology & visual science 2005, 46:349-57.
[24] Zhang SX, Wang JJ, Gao G, Shao C, Mott R, Ma J-x: Pigment epithelium-derived factor (PEDF) is an endogenous antiinflammatory factor. The FASEB journal 2006, 20:323-5.
[25] Yoshida Y, Yamagishi SI, Matsui T, Jinnouchi Y, Fukami K, Imaizumi T, Yamakawa R: Protective role of pigment epithelium-derived factor (PEDF) in early phase of experimental diabetic retinopathy. Diabetes/metabolism research and reviews 2009, 25:678-86.
[26] Fronk AH, Vargis E: Methods for culturing retinal pigment epithelial cells: a review of current protocols and future recommendations. Journal of tissue engineering 2016, 7:2041731416650838.
[27] Hollborn M, Jahn K, Limb GA, Kohen L, Wiedemann P, Bringmann A: Characterization of the basic fibroblast growth factor-evoked proliferation of the human Müller cell line, MIO-M1. Graefe's Archive for Clinical and Experimental Ophthalmology 2004, 242:414-22.
[28] Weigel AL, Handa JT, Hjelmeland LM: Microarray analysis of H2O2-, HNE-, or tBH-treated ARPE-19 cells. Free Radical Biology and Medicine 2002, 33:1419-32.
[29] Dunn K, Aotaki-Keen A, Putkey F, Hjelmeland L: ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. Experimental eye research 1996, 62:155-70.
[30] Koirala D, Beranova-Giorgianni S, Giorgianni F: Data-independent proteome analysis of ARPE-19 cells. Data in brief 2018, 20:333-6.

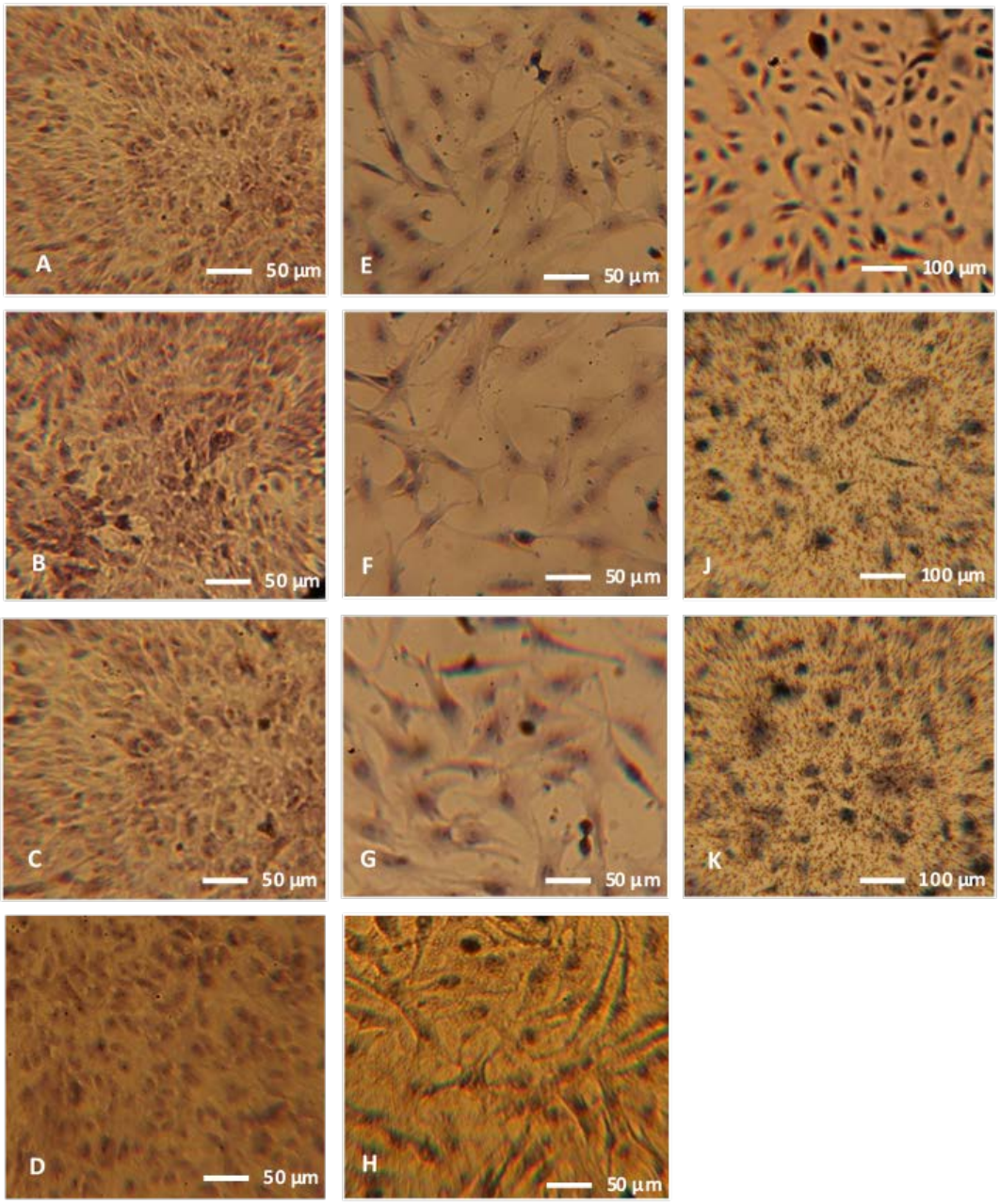
448 **Supplementary Work**

449 **Fluorescent immunocytochemical staining and imaging of cultures**

450 RPE were grown on 0.1% gelatin coated microscope cover slips and left to mature for 28
451 days. Both MIO-M1 and HRMVEC were grown as described on Transwell® membrane.
452 All culture formations (solo, dual and triple) followed the system set up as stated, they
453 were then rinsed in PBS and fixed in ice-cold PBS containing 4% formaldehyde for 30
454 minutes. Cultures were washed with wash buffer (1% BSA in PBS (Sigma Aldrich, UK)),
455 then blocking and permeabilization were achieved by incubation with 5% BSA in 1xPBS
456 with 0.3% Triton X-100 for 40 minutes prior to the addition of primary antibody
457 (prepared in blocking buffer) which was incubated overnight at 4°C. The following
458 conjugated primary antibodies were used: ZO-1 and AlexaFlour 488 (1:100, Thermo
459 Fisher Scientific, UK), Von Willebrand factor and AlexaFlour 594 (1:1500, Novus Bio, UK)
460 and Glutamine synthetase and AlexaFlour 488 (1:500, Novus Bio, UK). Post overnight
461 incubation, cells were washed in wash buffer. This was followed by two washes in PBS
462 and cultures were incubated with 1µg/ml 4', 6'-diamino-2-phenylindole (DAPI; D9542,
463 Sigma Aldrich, UK) for 5 minutes. All cultures were washed an additional two times in
464 PBS and once in deionized H_2O and mounted using PBS and 0.3% Tween-100 as
465 mounting medium (Sigma Aldrich, UK). RPE were mounted straight to slides by inverting
466 coverslips, MIO-M1 and HRMVEC grown on inserts were mounted between two glass
467 coverslips with prior to imaging with a Zeiss microscope with Axiocam MR3 and image
468 analysis with AxioVision 4.6.



Sup.Figure 1A-C. Immunocytochemical analysis of cells grown in triple-culture, A- ZO-1 expression in RPE after 28 day maturation marked with Alexa 488 (green), B- Von Willebrand factor expression in HRMVEC grown on the basal side of the membrane marked with Alexa 568 (red), C- Glutamine synthetase expression in MIO-M1 grown on the apical side of the membrane marked with Alexa 488 (green). A-C all cells counterstained with nuclear stain DAPI (blue). Scale 50 µM.



475

476 **Sup.Figure 2. Representation of Hematoxylin stained cells used in the assessment of cell morphology**
477 **including analysis of diameter (µM) by ImageJ software. A- RPE solo-culture, B- RPE dual-culture (+MIO-**
478 **M1), C- RPE dual-culture (+HRMVEC), D- RPE triple-culture, E- MIO-M1 solo-culture, F- MIO-M1 dual-**
479 **culture (+RPE), G- MIO-M1 dual-culture (+HRMVEC), H- MIO-M1 triple-culture, I- HRMVEC solo-culture,**
480 **J- HRMVEC dual-culture (+RPE) & K- HRMVEC dual-culture (+MIO-M1).**

481