RESEARCH ARTICLE

Extracellular matrix nitration alters growth factor release and activates bioactive complement in human retinal pigment epithelial cells

Mark A. Fields[1]*, **Hannah E. Bowrey**[2,3]☯, **Jie Gong**[1]☯, **Ernesto F. Moreira**[4]☯, **Hui Cai**[1]☯, **Lucian V. Del Priore**[1]

1 Department of Ophthalmology and Visual Science, Yale School of Medicine, New Haven, CT, United States of America, **2** Brain Health Institute, Rutgers, The State University of New Jersey, Piscataway, NJ, United States of America, **3** Save Sight Institute, The University of Sydney, Sydney, NSW, Australia, **4** Department of Ophthalmology, Storm Eye Institute, Medical University of South Carolina, Charleston, SC, United States of America

☯ These authors contributed equally to this work.
* mark.fields@yale.edu

Abstract

Purpose

We have shown previously that non-enzymatic nitration (NEN) of the extracellular matrix (ECM), which serves as a model of Bruch's membrane (BM) aging, has a profound effect on the behavior of the overlying retinal pigment epithelial (RPE) cells, including altered phagocytic ability, reduced cell adhesion, and inhibition of proliferation. We know that transplanted RPE monolayers will encounter a hostile sub-RPE environment, including age-related alterations in BM that may compromise cell function and survival. Here we use our previous NEN model of BM aging to determine the effects of NEN of the ECM on growth factor release and complement activation in RPE cells.

Methods

Human induced-pluripotent stem cells (iPSCs) were differentiated into RPE cells, and confirmed by immunohistochemistry, confocal microscopy, and polymerase chain reaction. IPSC-derived RPE cells were plated onto RPE-derived ECM under untreated or nitrite-modified conditions. Cells were cultured for 7 days and barrier function measured by transepithelial resistance (TER). Vascular endothelial growth factor (VEGF), pigment epithelium-derived factor (PEDF), and complement component C3a were measured using enzyme-linked immunosorbent assay (ELISA).

Results

On average nitrite-modified ECM increased VEGF release both apically and basally by 0.15 ± 0.014 ng/mL ($p < 0.0001$) and 0.21 ± 0.022 ng/mL ($p < 0.0001$), respectively, in iPSC-derived RPE cells. Nitrite-modified ECM increased PEDF release in iPSC-derived RPE cells

apically by 0.16 ± 0.031 ng/mL ($p <0.0001$), but not basally (0.27 ± 0.015 vs. 0.32 ± 0.029 ng/mL, ($p >0.05$)). Nitrite-modified ECM increased production of C3a in iPSC-derived RPE cells by 0.52 ± 0.123 ng/mL ($p <0.05$).

Conclusion

Nitrite-modified ECM increased VEGF, PEDF release, and C3a production in human iPSC-derived RPE cells. This model demonstrates changes seen in the basement membrane can lead to alterations in the cell biology of the RPE cells that may be related to the development of age-related macular degeneration.

Introduction

Alterations in the basement membrane are antecedent events in the development of numerous human disorders, including age-related degeneration (AMD), dystrophic epidermolysis bullosa, and Alport Syndrome [1–4]. Within the eye, aging of Bruch's membrane (BM), whose inner-most layer is the basement membrane of the retinal pigment epithelial (RPE) cells is an important and early step in AMD. In the development of disease, these alterations precede RPE changes by 1–2 decades and exert a deleterious effect on RPE cell behavior [3, 4]. While the exact age-related molecular changes that develop within BM are still being elucidated, we know that structural changes within BM include diffuse membrane thickening, accumulation of drusen, basal laminar and basal linear deposits [5, 6], collagen cross-linking in the inner and outer collagen layer, calcification and fragmentation of the elastin layer [7], and BM lipidization [7, 8]. The contribution of RPE cells to Bruch's membrane health is significant. Large drusen that contribute to focal Bruch's membrane thickening, arise partially from the RPE cells and contribute to aspects of AMD pathology such as choroidal neovascularization and geographic atrophy (GA) via cracks or loss of inner layers due to inadequate basal membrane regeneration [9, 10].

In homeostasis, RPE cell release of vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF) in a polarized fashion is an important regulator of complement activation [11, 12]. Because of the importance of these factors, and the known deleterious effects of Bruch's membrane aging on RPE cell function [13], we attempt to investigate the effects of deleterious changes within the basement membrane on polarized release of VEGF, PEDF and production of the important complement component C3a [14]. To do this, we crosslink normal ECM using non-enzymatic nitration, which causes non-physiologic cross linking of proteins within the basement membrane and thereby mimics many of the effects of basement membrane aging that alter RPE cell behavior in vivo in elderly eyes with AMD.

We have shown that aging of human BM has a deleterious effect on critical cellular functions such as phagocytosis [13]. Collagen cross-linking of the extracellular matrix (ECM) derived from RPE cells serves as a relevant in vitro model of BM aging through non-enzymatic nitration of the basement membrane [13, 15]. This model has enabled the investigation of cellular behavior due to the age-related effects of BM substrate disease such as growth factor release and complement activation, and mimics the deleterious effects of substrate aging [16]. Here, we determine the effects of non-enzymatic nitration (NEN) of the ECM, which mimics the effects of BM aging on growth factor release as well as complement activation in an iPSC-derived RPE cell line.

VEGF is a critical survival factor for RPE cells, and its polarized release is required to stabilize the fenestrated structure of the endothelium of the choroid [17, 18]. The angiogenic effects of

VEGF in the pathogenesis of "wet" or exudative AMD have been well-documented [19, 20], and patients with "dry" or non-exudative AMD can progress to the exudative form in some cases, suggesting an overlap in pathogenesis [21, 22]. Genome-wide meta-analysis studies have shown that the VEGF-A locus is associated with both exudative and non-exudative AMD and the multi-factorial risk factors of the disease, such as oxidative stress and smoking, may contribute to both pathological forms [23–27]. Mice with increased VEGF-A levels exhibited both neovascular AMD and non-exudative AMD suggesting that VEGF dysfunction may play a role in both forms of the disease [28]. There is RPE cell and retinal dysfunction, as well as morphological abnormalities in mice, with increased VEGF-A at sites where no choroidal neovascularization (CNV) lesions appear to be present, thus supporting a role for this protein in the non-exudative form of AMD [21]. PEDF is an endogenous growth factor secreted by RPE cells that has neuro-protective, neurotrophic, and antiangiogenic activity [29, 30]. PEDF acts as a counterbalance to the effects of VEGF and is thought to help maintain the integrity of the RPE cell barrier [31–33].

Activation of the complement system has been shown to be a major contributor to the patho-genesis of AMD and is a reflection of the chronic inflammation seen at the site of disease [34–39]. Interestingly, bioactive complement components such as C5a and C3a have been identified in the drusen of patients with AMD, suggesting complement activation in the area of RPE cells and BM [14]. Both forms have been shown to induce VEGF production in RPE cells in vitro [14]. Activation of C3a has also been demonstrated in in vitro and in vivo models of smoke expo-sure and oxidative stress, and a key factor in the development of AMD [40–44]. Accumulation of these factors and local inflammation may contribute to the pathogenesis of this disease [45].

Materials and methods

Ethics statement

All experiments were conducted with the approval of the Institutional Review Board (IRB # Pro00023262) and after written informed consent by study participants. This work was per-formed in adherence to the tenets of the Declaration of Helsinki.

Human iPSC culture

Human iPSCs were derived from fibroblasts of a 71-year-old female donor (donor 1) with no his-tory of retinal disease and a 72-year-old female with GA secondary to AMD (donor 2). Skin spec-imens from punch biopsies were transplanted onto culture plates and incubated for 1 hour with 0.5% dispase (Invitrogen-Gibco, Life Technologies, Grand Island, NY) and phosphate-buffered saline (PBS) in a 37°C water bath, then separated as an intact sheet by gentle agitation or by using two forceps. Cells were then placed in Dulbecco's Modified Eagle's Medium (DMEM; Invitro-gen-Gibco, Life Technologies) and cultured in a humidified 37°C, 5% CO_2 incubator until fibro-blast outgrowth. Fibroblasts were grown to confluence and then treated with four Yamanaka factors, Oct3/4, Sox-2, Klf4, and c-Myc using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit according to the manufacturer's instructions (Invitrogen-Gibco, Life Technologies) [46–51]. Newly generated iPSC colonies were purified and expanded using MTeSR™1 media (Stem Cell Technologies, Vancouver, Canada). Cell numbers were expanded by passaging every 5–7 days using Accutase (Sigma-Aldrich) and cultured for use in downstream experiments.

Differentiation of human iPSCs into RPE cells

Human iPSCs were differentiated to iPSC-derived RPE cells using a modified protocol previ-ously described [52–54]. Human iPSC colonies were lifted with Accutase (1 mg/mL) and grown as embryoid bodies (EBs) by using AggreWell™ 400 plates (STEMCELL Technologies,

Vancouver, Canada) for 4 days in EB formation medium (STEMCELL Technologies). After 5 days, EB medium was replaced with neural induction medium (NIM) containing DMEM/F12 (1:1), 1% N-2 supplement (MEM non-essential amino acids and 2 μg/mL heparin). After 7 days, suspended EB aggregates were plated onto laminin-coated culture plates and allowed to reattach. Cells were grown for an additional 10 days in neural induction medium. After 16 days, neural induction medium was replaced with retinal differentiation medium (RDM) containing DMEM/F12 (3:1), 2% B-27 supplement (Invitrogen-Gibco, Life Technologies), MEM non-essential amino acids and penicillin/streptomycin. The adherent culture was maintained in RDM until the appearance of pigmented iPSC-derived RPE cells. Patches of pigmented iPSC-derived RPE cells were then micro-dissected, dissociated with trypsin-ethylenediamine-tetraacetic acid (EDTA, 0.05%) and plated onto laminin-coated Transwell® permeable inserts (Corning Inc., 3460-Clear, 0.4 mm pores, 12 mm inner diameter, polyester membranes). IPSC-derived RPE cells were cultured on Transwell® plates with RDM + 10% fetal bovine serum (FBS) for 2 days and then switched to RDM with 2% FBS until the cells were confluent. Human iPSC-derived RPE cells were then maintained in RDM and allowed to form monolayers and re-pigment within 60–90 days.

Preparation of ECM

Immortalized human RPE (ARPE-19) cells were obtained from the ATCC, cultured, and maintained in DMEM (Invitrogen-Gibco, Life Technologies) containing 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 100 μg/mL gentamicin (Invitrogen-Gibco, Life Technologies). These cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. RPE cell-derived ECM (RPE-ECM) nitrite-treated plates were prepared as previously described [13, 55]. ARPE-19 cells were grown on 24-well Transwell® permeable supports (Corning, Inc.) in 12-well plates or flat bottom 24-well plates for 6–8 weeks to allow the ECM to form. ARPE-19 cells were then removed by the addition of 20 mM ammonium hydroxide buffer for 20 min, and the ECM was washed with PBS. PBS was removed from the RPE-ECM plates and dried. These plates were used to create two experimental plating surfaces (untreated ECM and nitrite-modified ECM).

Nitrite modification of the ECM

Nitrite-modified ECM was prepared as previously described [13, 15, 55, 56]. Briefly, 100 mM sodium nitrite was added to the ECM and incubated at 37°C for 7 days. Plates were then washed with PBS and incubated with PBS for 4 h. Finally, plates were washed with PBS to completely remove the nitrite.

Immunohistochemistry

Cells were fixed with 4% paraformaldehyde at 4°C, then permeabilized with 0.1% Triton X-100 in PBS and incubated with 0.1% bovine serum albumin or 1% normal goat serum in PBS. Cells were incubated with various primary monoclonal or polyclonal antibodies for 2–3 h at room temperature, or overnight at 4°C. Cells were washed and incubated for 1 h at 37°C in the dark with rabbit anti-mouse or goat anti-rabbit IgG antibodies conjugated to either Alexa TM 594 (red fluorescence) or Alexa TM 488 (green fluorescence) (Invitrogen-Molecular Probes). Nuclei were stained with diamidinophenyl indole (DAPI, Sigma) for 5–10 minutes. A summary of antibodies used in this study is provided in Table 1. Immunologically-stained cell cultures were visualized by a Zeiss 510 NLO confocal laser scanning microscope using a Plan-Apochromatic 20x0.8 DIC objective.

Table 1. Antibodies used for staining target cells.

Antibody	Company	Dilution	Target Cells
OCT-4	EMD Millipore, Temecula CA	1:250	pluripotent stem cell
Sox-2	EMD Millipore, Temecula CA	1:100	pluripotent stem cell
Nanog	EMD Millipore, Temecula CA	1:100	pluripotent stem cell
TRA-1-60	EMD Millipore, Temecula CA	1:200	pluripotent stem cell
ZO-1	Invitrogen-Gibco, Life Technologies, Grand Island, NY	1:100	RPE cell (junction)
Na + /K + ATPase	EMD Millipore, Temecula CA	1:100	RPE cell
RPE65	Novus Biologicals, Littleton, CO	1:100	RPE cell
Bestrophin	EMD Millipore, Temecula CA	1:100	RPE cell

OCT-4, octamer-binding transcription factor-4; Sox-2, SRY (sex determining region Y)-box 2; Nanog, Nanog homeobox; TRA-1-60, podocalyxin; ZO-1, zonula occludens 1; RPE65, Retinal pigment epithelium-specific 65 kDa protein; Na + /K + ATPase, sodium-potassium adenosine triphosphatase; RPE cell, retinal pigment epithelial cell

https://doi.org/10.1371/journal.pone.0177763.t001

Transepithelial resistance

Human iPSC-derived RPE cells were plated onto one of two ECM conditions (untreated ECM and nitrite-modified ECM) in Transwell® permeable supports (Corning, Inc.) containing RPE-ECM as described above. Cells were grown to confluence for one week in RDM containing 2% FBS. To confirm confluence, barrier function of iPSC-derived RPE cell monolayers was assessed by monitoring transepithelial resistance (TER) using an epithelial volt/ohm meter and an electrode (STX2; World Precision Instruments, Sarasota, FL) on RPE cell-derived ECM. The resistance values ($\Omega^* cm^2$) were determined from the average of four independent measurements, and corrected for background resistance produced by the insert in the presence of RPE cell-ECM and media [33, 57].

VEGF/PEDF/C3a assays on nitrite-modified ECM

Human iPSC-derived RPE cells were either plated onto untreated ECM or nitrite-modified ECM as described above. Cells were grown to confluence in RDM containing 2% FBS. Media was removed and the cells were washed with PBS. Serum-free RDM was added to cultured human iPSC-derived RPE cell monolayers for 24 h before conducting experiments. The supernatants were then collected and assayed for VEGF and PEDF using a human VEGF Quantikine ELISA kit (R&D Systems, Minneapolis, MN) or human PEDF ELISA kit (BioProductsMD, Middletown, MD), respectively. Supernatants were also collected and assayed from flat bottom 24-well plates for C3a using a human C3a ELISA kit (BD Biosciences, San Jose, CA). The results were analyzed using a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices, Inc., Sunnyvale, CA) set at 450 nm. Aliquots were assayed in triplicate and values were compared with a $VEGF_{165}$, C3a-des Arg, or human PEDF antigen standard curve.

Data analysis

All data were analyzed with the average of two samples, and conducted in triplicate for each experiment. Independent, two-tailed t tests were performed using Prism (GraphPad Software, Inc., La Jolla, CA). A criterion of $\alpha = 0.05$ was adopted.

Results

Human iPSCs differentiate into RPE cells and express RPE markers

After Sendai virus transduction of octamer-binding transcription factor 3/4 (Oct3/4), (sex determining region Y)-box 2 (Sox-2), Kruppel-like factor 4 (Klf4), and regulator gene Myc

that codes for a transcription factor (c-Myc) the cells expressed four markers (Fig 1A), octamer-binding transcription factor 4 (OCT-4), Nanog homeobox (Nanog), SRY (sex determining region Y)-box 2 (Sox-2), and podocalyxin (TRA-1-60), indicating these cells were pluripotent. To obtain human iPSC-derived RPE cells, iPSCs (Fig 1B) were induced to generate embryoid bodies (Fig 1C) and differentiated using a modified protocol described previously toward an RPE cell fate displaying neural rosettes (Fig 1D) [52–54]. After 30 days of differentiation, RPE-like cells were visible, expressing a hexagonal monolayer of RPE cell phenotype,
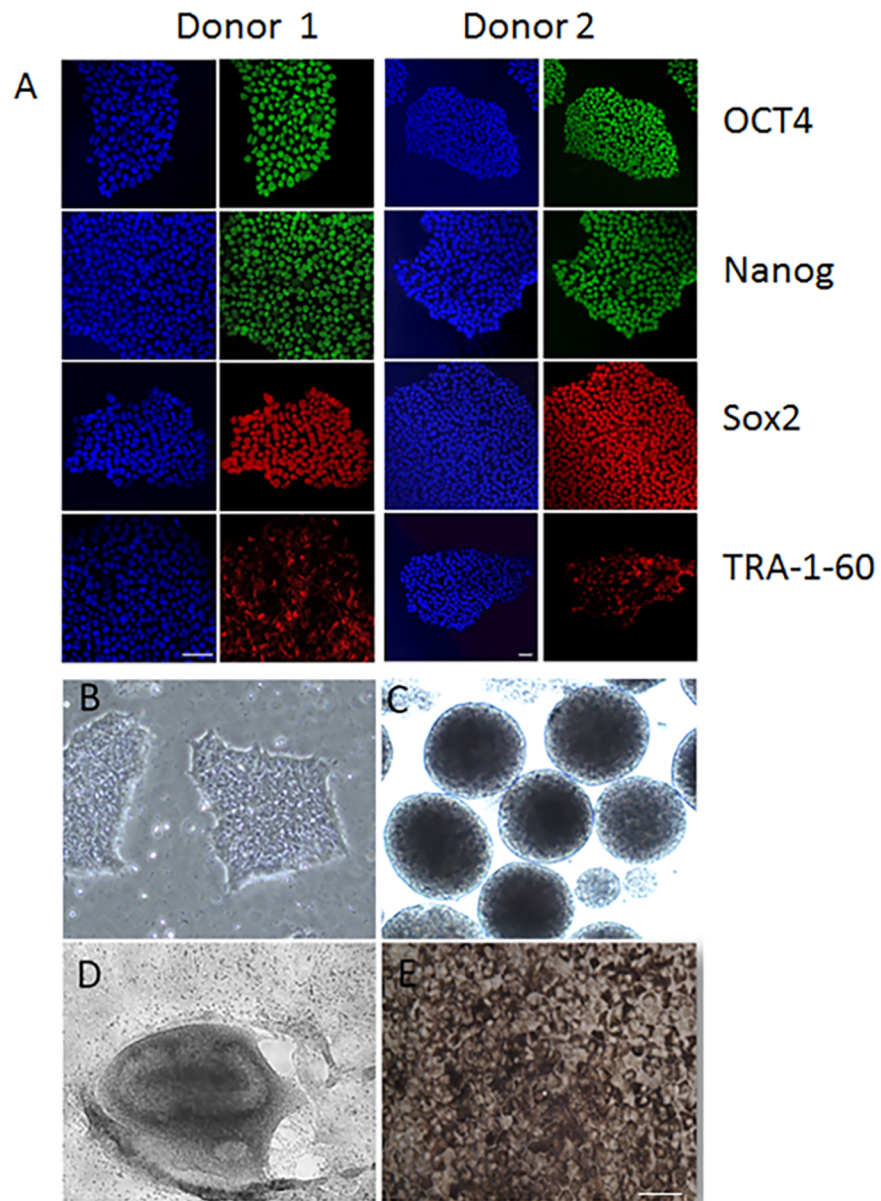
Fig 1. Differentiation of human induced-pluripotent stem cells (iPSCs) into iPSC-derived retinal pigment epithelial (RPE) cells from 2 different donors. Immunofluorescent staining was positive for Oct-4, Nanog, Sox-2, and TRA-1-60 (**A**). Nuclei stained with DAPI (blue). Undifferentiated human iPSC colony (**B**) and eventual formation into embryoid bodies (**C**). Formation of neural rosettes by day 14 post-differentiation (**D**), and a pigmented monolayer of iPSC-derived RPE cells forms by day 45 post-differentiation (**E**). Scale bar for **A** and **E** = 50 μm.

Fig 2. Expression and localization of retinal pigment epithelial (RPE) cell markers in human iPSC-derived RPE. Immunofluorescent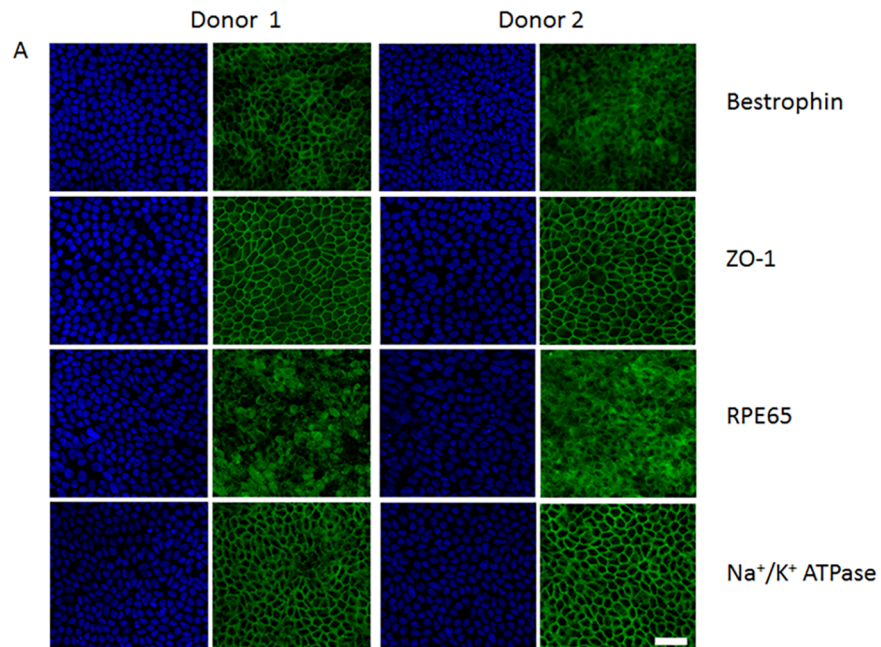 staining was positive for bestrophin, Na + /K + ATPase, RPE65, and ZO-1 (**A**). Nuclei stained with DAPI (blue). Scale bar for **A** = 50 μm.

https://doi.org/10.1371/journal.pone.0177763.g002

and were purified (Fig 1E). After 45 days, differentiated iPSC-derived RPE cells robustly expressed bestrophin, the tight junction marker ZO-1, RPE65, and Na + /K + ATPase (Fig 2).

ECM nitrite modification increased VEGF production in iPSC-derived RPE cells

In the following experiments we used a previously established approach of nitrite-modification of RPE cell-derived ECM to model the "aging" effect on BM [15]. The transepithelial resistance values for iPSC-derived RPE cells seeded on untreated ECM were 209.5 ± 8.4 and 209.3 ± 4.15 Ω^*cm^2, and nitrite-modified ECM were 225.8 ± 8.5 and 207.3 ± 7.0 Ω^*cm^2 from two donor samples, respectively ($p > 0.05$; Fig 3). The data were within reported experimental range based on TER studies of ex vivo RPE cell monolayers [58, 59]. We then investigated the release of VEGF on these surfaces. Nitrite modification increased the apical release of VEGF from iPSC-derived RPE cells (0.6679 ± 0.067 vs. 0.8017 ± 0.0151 ng/mL in donor one, and 0.7409 ± 0.0089 vs.0.9023 ± 0.0330 in donor two, respectively, $p < 0.0001$; Fig 4). Additionally, the basal release of VEGF was lower in iPSC-derived RPE cells seeded onto untreated ECM, relative to nitrite-modified ECM (0.7886 ± 0.063 vs. 1.061 ± 0.0091 ng/mL in donor one, and 0.9696 ± 0.0075 vs. 1.116 ± 0.0254 in donor two, respectively, $p < 0.001$; Fig 4). Thus relative to the untreated ECM, nitrite modification of the ECM surface led to increased levels of apical and basal VEGF in iPSC-derived RPE cells.

ECM nitrite modification increased PEDF release in iPSC-derived RPE cells

The apical release of PEDF was lower in iPSC-derived RPE cells seeded onto untreated ECM, relative to nitrite-modified ECM (0.3555 ± 0.0017 vs. 0.616 ± 0.0171 ng/mL in donor one, and
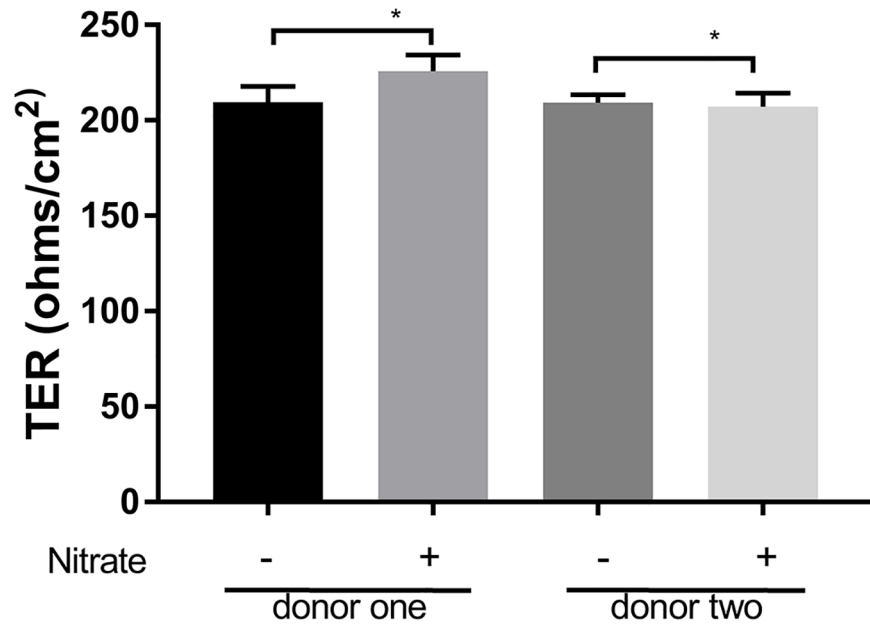
Fig 3. Transepithelial resistance (TER) of iPSC-derived RPE cells on RPE cell-derived ECM and nitrite-modified RPE cell-derived ECM. Monolayer permeability was assessed by TER in polarized iPSC-RPE cells. TER measurements were 209.5 and 209.3 $\Omega{*}cm^2$ for iPSC-derived RPE cells cultured on untreated extracellular matrix. iPSC-derived RPE cells cultured on nitrite-modified ECM were (225.8 and 207.3 $\Omega{*}cm^2$) from donor one and two samples, respectively.

https://doi.org/10.1371/journal.pone.0177763.g003

0.4255 ± 0.0013 vs. 0.488 ± 0.0021 in donor two, respectively, p <0.001; Fig 5). The basal release of PEDF from the overlying iPSC-derived RPE cells on untreated and nitrite-modified ECM samples was 0.2463 ± 0.0062 vs. 0.2413 ± 0.0070 in donor one (p >0.05), and 0.3023 ± 0.0223 vs. 0.3938 ± 0.0022 in donor two (p <0.001), respectively, (Fig 5). Thus, relative to the untreated ECM, nitrite modification of the ECM surface led to increased levels of PEDF in iPSC-derived RPE cells, apically, but not basally.

ECM nitrite modification increased C3a production in iPSC-derived RPE cells

The release of C3a was lower in iPSC-derived RPE cells seeded onto untreated ECM, relative to nitrite-modified ECM (1.096 ± 0.0063 vs. 1.29 ± 0.0102 ng/mL in donor one, and 1.453 ± 0.127 vs. 2.301 ± 0.0135 in donor two, respectively, p <0.001; Fig 6). Therefore, relative to untreated ECM, nitrite modification of the ECM surface led to increased production of C3a in iPSC-derived RPE cells.

Discussion

We used an in vitro model of BM "aging" to investigate cellular release of VEGF, PEDF, and production of C3a in iPSC-derived RPE cells. We demonstrated that the behavior of RPE cells differs when the ECM is nitrite-modified, a process that occurs during chronic inflammation and in the presence of risk factors associated with AMD such as cigarette smoking [15, 35–37, 60–64]. These findings are in line with previous studies in ARPE-19 and primary calf RPE cell
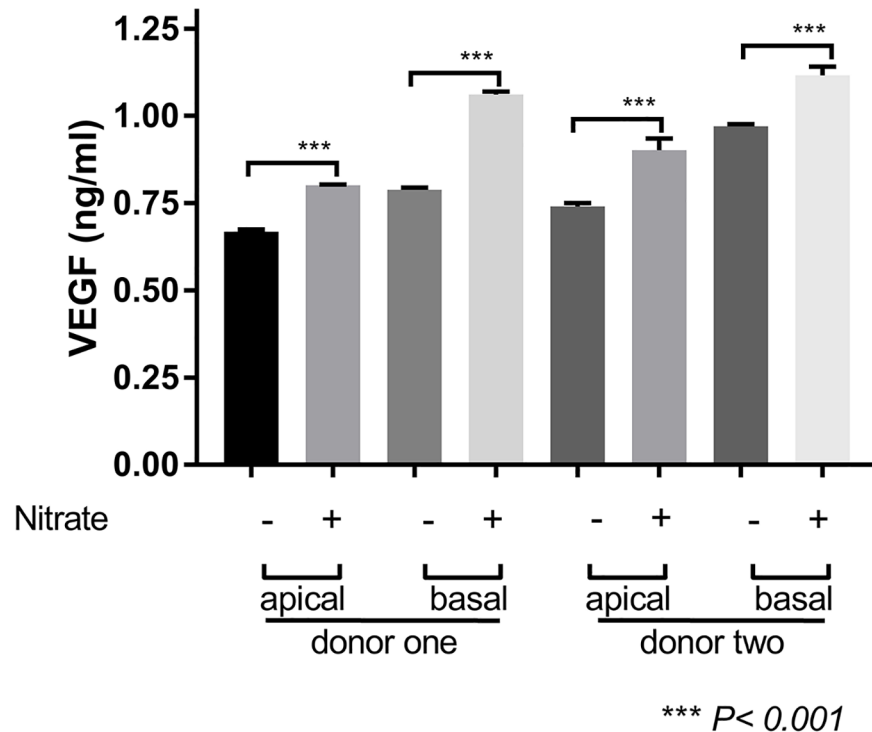
Fig 4. VEGF release in iPSC-derived RPE cell monolayers on RPE cell-derived ECM and nitrite-modified RPE cell-derived ECM. iPSC-derived RPE cells release of vascular endothelial growth factor (VEGF) on nitrite-modified ECM (nitrite) and untreated ECM (PBS) both apically and basally. ****p <0.0001.

https://doi.org/10.1371/journal.pone.0177763.g004

cultures that demonstrate nitrite modification of the ECM affects normal RPE cell behavior, such as attachment, proliferation, phagocytic ability and susceptibility to light-mediated damage by A2E [13, 15, 65].

Here we test similar parameters in therapeutically relevant cell lines generated from skin biopsies of older individuals with and without AMD [66, 67]. Using these iPS-derived RPE cells we demonstrated that secreted VEGF and PEDF levels were increased on nitrite-modified ECM (Figs 4 and 5). VEGF levels were increased both apically and basally in iPSC-derived RPE cells while PEDF levels were increased apically alone; there was no difference in the release pattern of PEDF basally on nitrite-modified ECM when compared to the untreated ECM when combining two donor samples together for statistical analysis. Consistent with past reports, the PEDF pattern of release is higher on the apical side in established monolayers of RPE cells [33]. Alternatively, VEGF production is typically higher on the basal side of established RPE cell monolayers, and amounts increase under stress such as hypoxic conditions in vitro [18]. While the release of PEDF increased apically, it did not exceed that of VEGF on the nitrite-modified ECM. Moreover, the basal release of PEDF did not change with nitrite modification of the surface, despite increased basal VEGF release. This suggests that the VEGF-PEDF ratio ultimately favored VEGF release after nitrite modification, which may favor progression to angiogenesis [68].

From a pathological standpoint, angiogenesis is a complex biological event that involves a balance between stimulators and inhibitors such as VEGF and PEDF. VEGF and PEDF have been shown to be expressed in the RPE cell-Bruch's membrane-choriocapillaris tissue complex in aged subjects [69]. It has been reported that VEGF increases the expression of PEDF by human RPE cells in a physiologic state [70]. The increased apical release of PEDF may suggest
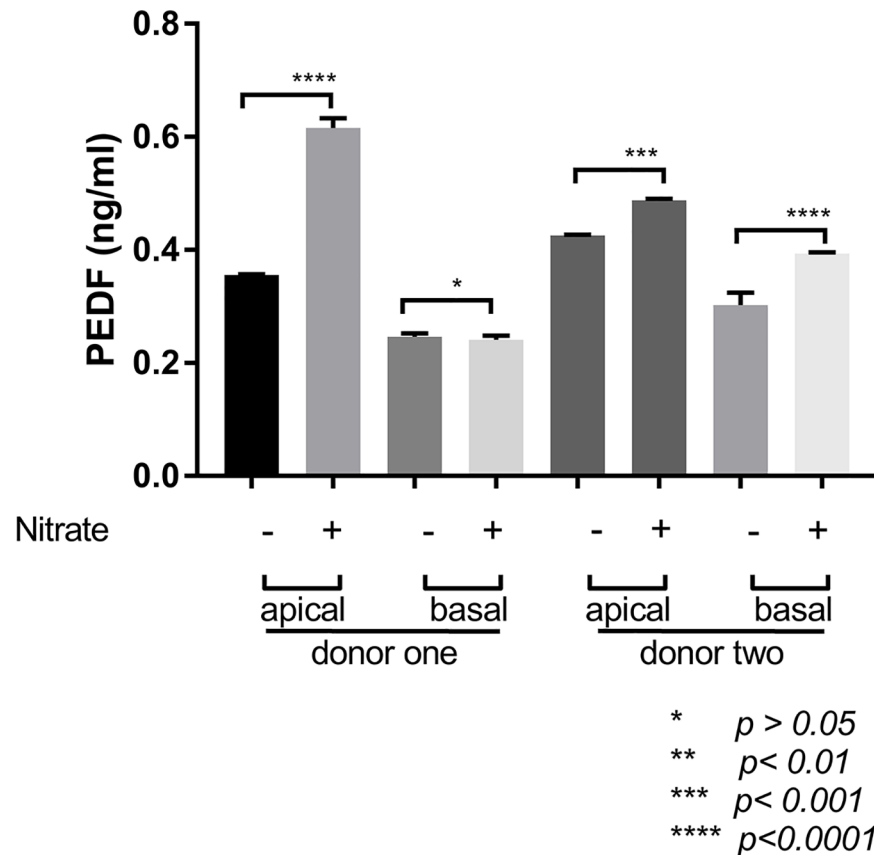
Fig 5. PEDF release in iPSC-derived RPE cell monolayers on RPE cell-derived ECM and nitrite-modified RPE cell-derived ECM. iPSC-derived RPE cell release of pigment epithelium-derived factor (PEDF) increased on nitrite-modified ECM (nitrite) apically. **** *p* <0.001.

https://doi.org/10.1371/journal.pone.0177763.g005

a regulatory role for the inhibitor in this model. There is evidence to suggest that endogenous antiangiogenic factors like PEDF maintain a vascular quiescence that is otherwise disturbed by factors such as VEGF [69]. Moreover, it has been demonstrated that PEDF is a major angiogenic inhibitor in the eye and may have a protective role against oxidative stress [30, 71, 72]. Our data suggest that in an environment of enhanced or altered VEGF release, PEDF may be acting as a counterbalance to this phenomenon. While these results are intriguing, more studies in therapeutically relevant cell lines remain to investigate the ratio of VEGF-PEDF release in this model and its relationship to RPE cell stress and BM aging.

We have also demonstrated that the production of C3a by iPSC-derived RPE cells is increased on the nitrite-modified ECM. Increased complement activation has been associated with disease progression in AMD [14, 73, 74]. This activation can lead to pro-inflammatory damage to the RPE cell-BM-choriocapillaris tissue complex, and continued damage that can potentially lead to neovascularization manifested as CNV or the atrophic damage manifested as geographic atrophy (GA) in late stage AMD [75, 76]. Moreover, complement components, such as C3a and C5a, have been identified in the drusen of AMD patients [14, 77]. Our observation that nitrite modification of the ECM increased C3a production, VEGF release, and apical PEDF release would suggest a relationship between the structural changes seen in aged or diseased BM and the cellular changes (atrophy and neovascularization) in RPE cells seen during the development of AMD.
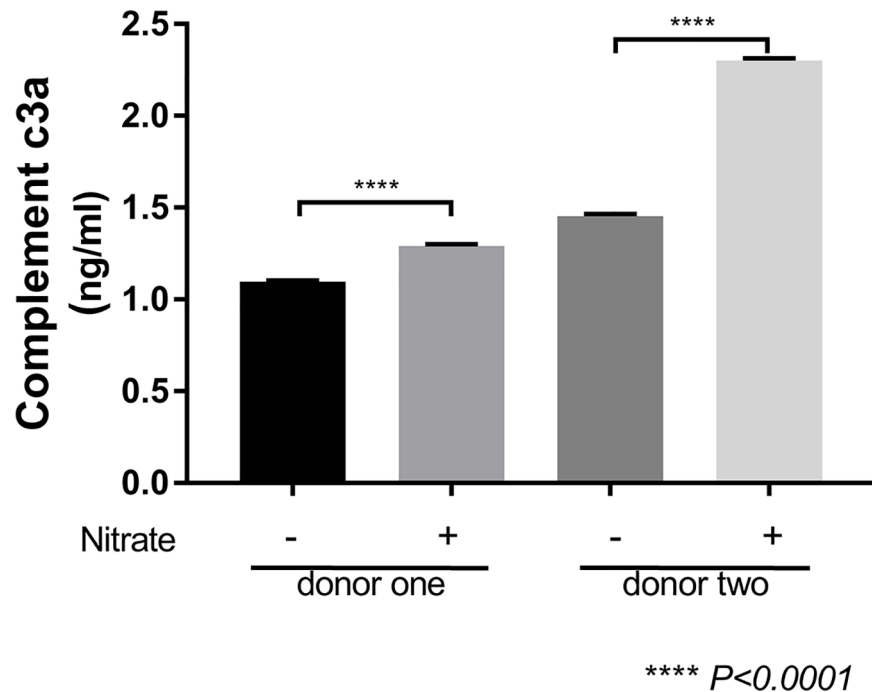
Fig 6. C3a production in iPSC-derived RPE cell monolayers on RPE cell-derived ECM and nitrite-modified RPE cell-derived ECM. iPSC-derived RPE cell C3a production increased on nitrite-modified ECM (nitrite). * p <0.0001.

C3a is the bioactive form of C3 and there is growing evidence of its role in AMD. Nozaki et al. observed the presence of C3a in drusen deposits of patients with AMD, suggesting that RPE cells are exposed to this inflammatory protein in this state [14]. Treatment with C3a protein both in vitro and in vivo has also been shown to induce VEGF in RPE cells and wild-type C57BL/6J mice, respectively [14]. Our data suggest that nitrite modification of the ECM plays a role in inducing C3a production in iPSC-derived RPE cells and may contribute to the release of VEGF in these cells. The relationship between complement activation and pro-inflammatory mediators in AMD continues to be investigated [75]. Models that mimic BM aging by treatment of the ECM with sodium nitrite, a nonenzymatic crosslinking agent, can help elucidate these disease mechanisms [13, 65]. While there appears to be a difference in the donor with GA in terms of VEGF and PEDF release as well as C3a production, we feel that the sample size is insufficient to decipher whether this is due to a disease state or other variables. In the future, we will address this by increasing the number of cell lines to draw valid conclusions between disease and non-disease states.

Our data add further support to the understanding that alterations in the basement membrane, an important portion of BM, can induce changes in the overlying RPE cells. Fig 7 summarizes the effects of basement membrane alterations on the overlying RPE cells as seen in this study. Aging is the most significant risk factor for the development of AMD, and BM undergoes major changes during this process [42]. As mentioned, these changes include drusen formation, development of basal laminar and basal linear deposits [5, 6], collagen crosslinking of the collagen layers, calcification and fragmentation of the elastin layer [7], and membrane lipidization [7, 78, 79]. Other key elements contributing to the structural changes in BM are exposure to nitrite during chronic inflammation from aging and smoking [15, 80]. Our
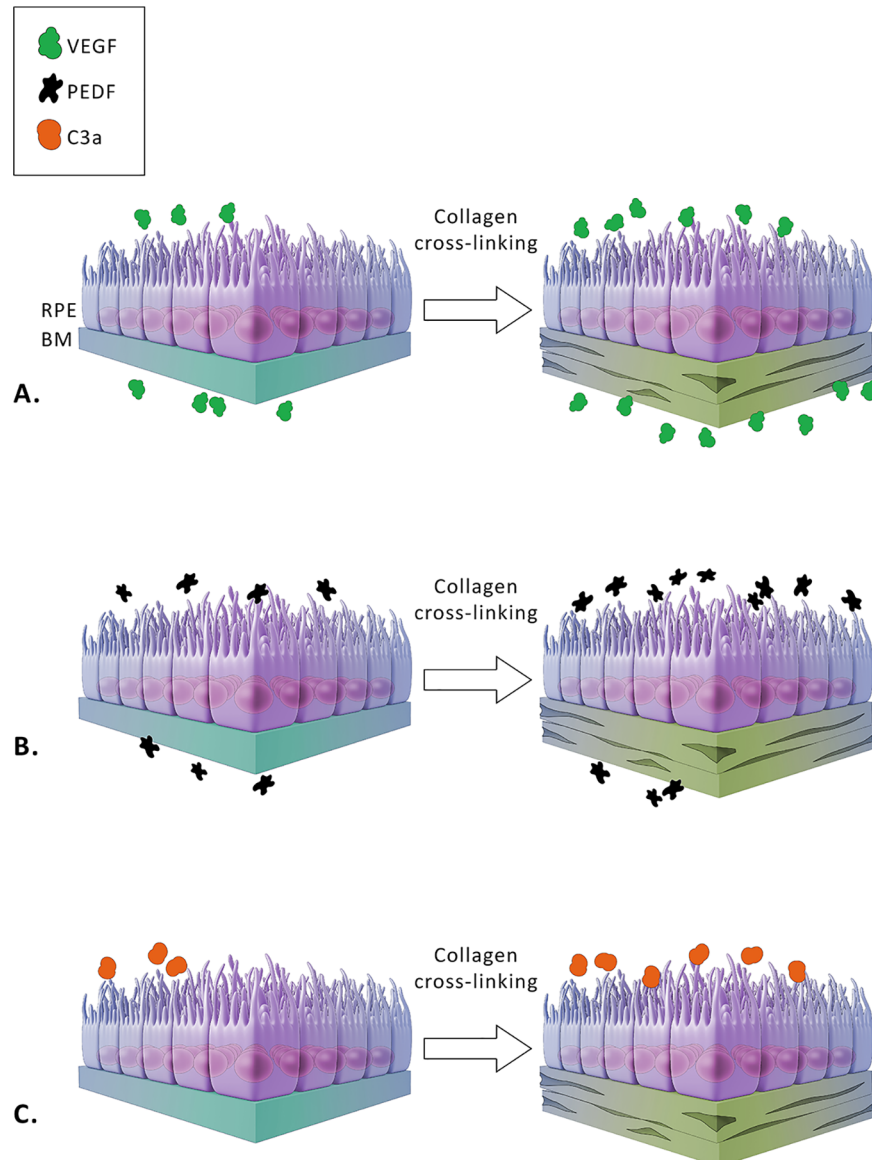
Fig 7. Effects of nitrite modification on VEGF, PEDF and C3a in iPSC-derived RPE cells. Release of VEGF, PEDF and c3a from patient-derived iPSC-RPE cells on normal versus "aged" extracellular matrix is represented diagrammatically. VEGF release is higher basally than apically on normal basement membrane (**A**). Cross-linking of collagen in the basement membrane increased release of VEGF both apically and basally in iPSC-derived RPE cells while preserving this polarity (**A**). PEDF release is higher apically than basally on normal basement membrane (**B**). Cross-linking of collagen in the basement membrane increased release of PEDF apically, and preserved this polarity (**B**). Cross-linking of collagen in the basement membrane increased release of C3a in iPSC-derived RPE cells (**C**). The aging of Bruch's membrane increases VEGF (apical and basal) and PEDF (apical and basal) release in the RPE cells as well as C3a production. Increased release of these factors may contribute to the pathology at the site of RPE cells seen in exudative and advanced dry age-related macular degeneration (AMD) or geographic atrophy.

data suggest that treatments for AMD will certainly need to consider the state of BM. Models of retinal degenerative disease such as those of 'aging' could prove valuable to study complex diseases such as AMD that include both the contribution of genetic mutation and environmental risk factors [66, 81, 82].

Conclusion

In order to completely understand AMD pathophysiology, and for cell-based therapies for AMD to be viable, the issue of BM damage must be addressed. This study demonstrates that nitrite modification of the ECM increased VEGF and PEDF release in iPSC-derived RPE cells cultured onto this surface. Nitrite modification also increased the production of C3a in iPSC-derived RPE cells. This approach may be beneficial as a tool for disease modeling by mimicking the structural changes on BM and observing iPSC-derived RPE cell behavior on this surface [13, 83].

Acknowledgments

The authors would like to thank Emma Vought for her contribution of artwork and Luanna Bartholomew, Ph.D., for critical review of the manuscript.

Author Contributions

Conceptualization: MF LDP HB.

Data curation: HB MF JG.

Formal analysis: HB MF.

Funding acquisition: MF LDP.

Investigation: HC EM MF JG HB.

Methodology: LDP MF.

Project administration: MF LDP.

Resources: MF LDP.

Supervision: MF LDP.

Validation: MF LDP.

Visualization: MF HB LDP.

Writing – original draft: MF HB.

References

1. Wiradjaja F, DiTommaso T, Smyth I. Basement membranes in development and disease. Birth Defects Res C Embryo Today. 2010; 90(1):8–31. https://doi.org/10.1002/bdrc.20172 PMID: 20301220

2. Nystrom A, Bornert O, Kuhl T. Cell therapy for basement membrane-linked diseases. Matrix Biol. 2016.

3. Pauleikhoff D, Harper CA, Marshall J, Bird AC. Aging changes in Bruch's membrane. A histochemical and morphologic study. Ophthalmology. 1990; 97(2):171–8. Epub 1990/02/01. PMID: 1691475

4. Marshall GE, Konstas AG, Reid GG, Edwards JG, Lee WR. Type IV collagen and laminin in Bruch's membrane and basal linear deposit in the human macula. Br J Ophthalmol. 1992; 76(10):607–14. Epub 1992/10/01. PubMed Central PMCID: PMC505233. PMID: 1420043

5. Pauleikhoff D, Harper CA, Marshall J, Bird AC. Aging changes in Bruch's membrane. A histochemical and morphologic study. Ophthalmology. 1990; 97(2):171–8. PMID: 1691475

6. Sarks SH, Arnold JJ, Killingsworth MC, Sarks JP. Early drusen formation in the normal and aging eye and their relation to age related maculopathy: a clinicopathological study. Br J Ophthalmol. 1999; 83 (3):358–68. PubMed Central PMCID: PMCPMC1722952. PMID: 10365048

7. Spraul CW, Lang GE, Grossniklaus HE, Lang GK. Histologic and morphometric analysis of the choroid, Bruch's membrane, and retinal pigment epithelium in postmortem eyes with age-related macular

degeneration and histologic examination of surgically excised choroidal neovascular membranes. Surv Ophthalmol. 1999; 44 Suppl 1:S10–32.

8. Curcio CA, Johnson M, Rudolf M, Huang JD. The oil spill in ageing Bruch membrane. Br J Ophthalmol. 2011; 95(12):1638–45. PubMed Central PMCID: PMCPMC3633599. https://doi.org/10.1136/bjophthalmol-2011-300344 PMID: 21890786

9. Abdelsalam A, Del Priore L, Zarbin MA. Drusen in age-related macular degeneration: pathogenesis, natural course, and laser photocoagulation-induced regression. Surv Ophthalmol. 1999; 44(1):1–29. Epub 1999/08/31. PMID: 10466585

10. Tezel TH, Del Priore LV, Kaplan HJ. Reengineering of aged Bruch's membrane to enhance retinal pigment epithelium repopulation. Invest Ophthalmol Vis Sci. 2004; 45(9):3337–48. Epub 2004/08/25. https://doi.org/10.1167/iovs.04-0193 PMID: 15326159

11. Chen M, Muckersie E, Robertson M, Forrester JV, Xu H. Up-regulation of complement factor B in retinal pigment epithelial cells is accompanied by complement activation in the aged retina. Experimental eye research. 2008; 87(6):543–50. https://doi.org/10.1016/j.exer.2008.09.005 PMID: 18926817

12. Strauss O. The retinal pigment epithelium in visual function. Physiological reviews. 2005; 85(3):845–81. https://doi.org/10.1152/physrev.00021.2004 PMID: 15987797

13. Sun K, Cai H, Tezel TH, Paik D, Gaillard ER, Del Priore LV. Bruch's membrane aging decreases phagocytosis of outer segments by retinal pigment epithelium. Mol Vis. 2007; 13:2310–9. Epub 2008/01/18. PMID: 18199972

14. Nozaki M, Raisler BJ, Sakurai E, Sarma JV, Barnum SR, Lambris JD, et al. Drusen complement components C3a and C5a promote choroidal neovascularization. Proc Natl Acad Sci U S A. 2006; 103 (7):2328–33. Epub 2006/02/03. PubMed Central PMCID: PMC1413680. https://doi.org/10.1073/pnas.0408835103 PMID: 16452172

15. Wang Z, Paik DC, Del Priore LV, Burch RL, Gaillard ER. Nitrite-modified extracellular matrix proteins deleteriously affect retinal pigment epithelial cell function and viability: a comparison study with nonenzymatic glycation mechanisms. Curr Eye Res. 2005; 30(8):691–702. Epub 2005/08/20. https://doi.org/10.1080/02713680590968259 PMID: 16109650

16. Fields MA, Cai H, Bowrey HE, Moreira EF, Beck Gooz M, Kunchithapautham K, et al. Nitrite Modification of Extracellular Matrix Alters CD46 Expression and VEGF Release in Human Retinal Pigment Epithelium. Invest Ophthalmol Vis Sci. 2015; 56(8):4231–8. PubMed Central PMCID: PMCPMC4703405. https://doi.org/10.1167/iovs.15-16438 PMID: 26161984

17. Witmer AN, Vrensen GF, Van Noorden CJ, Schlingemann RO. Vascular endothelial growth factors and angiogenesis in eye disease. Prog Retin Eye Res. 2003; 22(1):1–29. Epub 2003/02/25. PMID: 12597922

18. Blaauwgeers HG, Holtkamp GM, Rutten H, Witmer AN, Koolwijk P, Partanen TA, et al. Polarized vascular endothelial growth factor secretion by human retinal pigment epithelium and localization of vascular endothelial growth factor receptors on the inner choriocapillaris. Evidence for a trophic paracrine relation. Am J Pathol. 1999; 155(2):421–8. Epub 1999/08/06. PubMed Central PMCID: PMC1866848. https://doi.org/10.1016/S0002-9440(10)65138-3 PMID: 10433935

19. Wong TY, Chakravarthy U, Klein R, Mitchell P, Zlateva G, Buggage R, et al. The natural history and prognosis of neovascular age-related macular degeneration: a systematic review of the literature and meta-analysis. Ophthalmology. 2008; 115(1):116–26. https://doi.org/10.1016/j.ophtha.2007.03.008 PMID: 17675159

20. Lim LS, Mitchell P, Seddon JM, Holz FG, Wong TY. Age-related macular degeneration. Lancet. 2012; 379(9827):1728–38. https://doi.org/10.1016/S0140-6736(12)60282-7 PMID: 22559899

21. Ablonczy Z, Dahrouj M, Marneros AG. Progressive dysfunction of the retinal pigment epithelium and retina due to increased VEGF-A levels. FASEB J. 2014; 28(5):2369–79. Epub 2014/02/22. PubMed Central PMCID: PMC3986839. https://doi.org/10.1096/fj.13-248021 PMID: 24558195

22. Sunness JS, Gonzalez-Baron J, Bressler NM, Hawkins B, Applegate CA. The development of choroidal neovascularization in eyes with the geographic atrophy form of age-related macular degeneration. Ophthalmology. 1999; 106(5):910–9. Epub 1999/05/18. https://doi.org/10.1016/S0161-6420(99)00509-6 PMID: 10328389

23. Klettner A, Roider J. Constitutive and oxidative-stress-induced expression of VEGF in the RPE are differently regulated by different Mitogen-activated protein kinases. Graefes Arch Clin Exp Ophthalmol. 2009; 247(11):1487–92. Epub 2009/07/16. https://doi.org/10.1007/s00417-009-1139-x PMID: 19603178

24. Klein R, Peto T, Bird A, Vannewkirk MR. The epidemiology of age-related macular degeneration. Am J Ophthalmol. 2004; 137(3):486–95. Epub 2004/03/12. https://doi.org/10.1016/j.ajo.2003.11.069 PMID: 15013873

25. Friedman DS, O'Colmain BJ, Munoz B, Tomany SC, McCarty C, de Jong PT, et al. Prevalence of age-related macular degeneration in the United States. Arch Ophthalmol. 2004; 122(4):564–72. Epub 2004/04/14. https://doi.org/10.1001/archopht.122.4.564 PMID: 15078675

26. Yu Y, Bhangale TR, Fagerness J, Ripke S, Thorleifsson G, Tan PL, et al. Common variants near FRK/COL10A1 and VEGFA are associated with advanced age-related macular degeneration. Hum Mol Genet. 2011; 20(18):3699–709. Epub 2011/06/15. PubMed Central PMCID: PMC3159552. https://doi.org/10.1093/hmg/ddr270 PMID: 21665990

27. van Leeuwen R, Klaver CC, Vingerling JR, Hofman A, de Jong PT. The risk and natural course of age-related maculopathy: follow-up at 6 1/2 years in the Rotterdam study. Arch Ophthalmol. 2003; 121 (4):519–26. Epub 2003/04/16. https://doi.org/10.1001/archopht.121.4.519 PMID: 12695249

28. Marneros AG. NLRP3 inflammasome blockade inhibits VEGF-A-induced age-related macular degeneration. Cell Rep. 2013; 4(5):945–58. Epub 2013/09/10. PubMed Central PMCID: PMC3821550. https://doi.org/10.1016/j.celrep.2013.08.002 PMID: 24012762

29. Tombran-Tink J, Chader GG, Johnson LV. PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. Exp Eye Res. 1991; 53(3):411–4. PMID: 1936177

30. Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu H, Benedict W, et al. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science. 1999; 285(5425):245–8. PMID: 10398599

31. Tong JP, Yao YF. Contribution of VEGF and PEDF to choroidal angiogenesis: a need for balanced expressions. Clinical biochemistry. 2006; 39(3):267–76. https://doi.org/10.1016/j.clinbiochem.2005.11.013 PMID: 16409998

32. Pons M, Marin-Castano ME. Nicotine increases the VEGF/PEDF ratio in retinal pigment epithelium: a possible mechanism for CNV in passive smokers with AMD. Invest Ophthalmol Vis Sci. 2011; 52 (6):3842–53. PubMed Central PMCID: PMC3109060. https://doi.org/10.1167/iovs.10-6254 PMID: 21330654

33. Ablonczy Z, Dahrouj M, Tang PH, Liu Y, Sambamurti K, Marmorstein AD, et al. Human retinal pigment epithelium cells as functional models for the RPE in vivo. Invest Ophthalmol Vis Sci. 2011; 52 (12):8614–20. Epub 2011/10/01. PubMed Central PMCID: PMC3208409. https://doi.org/10.1167/iovs.11-8021 PMID: 21960553

34. Anderson DH, Mullins RF, Hageman GS, Johnson LV. A role for local inflammation in the formation of drusen in the aging eye. Am J Ophthalmol. 2002; 134(3):411–31. Epub 2002/09/05. PMID: 12208254

35. Crabb JW, Miyagi M, Gu X, Shadrach K, West KA, Sakaguchi H, et al. Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. Proc Natl Acad Sci U S A. 2002; 99 (23):14682–7. Epub 2002/10/23. PubMed Central PMCID: PMC137479. https://doi.org/10.1073/pnas.222551899 PMID: 12391305

36. Hageman GS, Luthert PJ, Victor Chong NH, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. Prog Retin Eye Res. 2001; 20 (6):705–32. Epub 2001/10/06. PMID: 11587915

37. Mullins RF, Russell SR, Anderson DH, Hageman GS. Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. FASEB J. 2000; 14(7):835–46. Epub 2000/04/27. PMID: 10783137

38. Wang L, Clark ME, Crossman DK, Kojima K, Messinger JD, Mobley JA, et al. Abundant lipid and protein components of drusen. PLoS One. 2010; 5(4):e10329. Epub 2010/04/30. PubMed Central PMCID: PMC2859054. https://doi.org/10.1371/journal.pone.0010329 PMID: 20428236

39. Anderson DH, Radeke MJ, Gallo NB, Chapin EA, Johnson PT, Curletti CR, et al. The pivotal role of the complement system in aging and age-related macular degeneration: hypothesis re-visited. Prog Retin Eye Res. 2010; 29(2):95–112. Epub 2009/12/08. https://doi.org/10.1016/j.preteyeres.2009.11.003 PMID: 19961953

40. Wang AL, Lukas TJ, Yuan M, Du N, Handa JT, Neufeld AH. Changes in retinal pigment epithelium related to cigarette smoke: possible relevance to smoking as a risk factor for age-related macular degeneration. PloS one. 2009; 4(4):e5304. PubMed Central PMCID: PMC2669185. https://doi.org/10.1371/journal.pone.0005304 PMID: 19390692

41. Kunchithapautham K, Atkinson C, Rohrer B. Smoke-exposure causes endoplasmic reticulum stress and lipid accumulation in retinal pigment epithelium through oxidative stress and complement activation. J Biol Chem. 2014. Epub 2014/04/09. PubMed Central PMCID: PMC4031511.

42. Smith W, Assink J, Klein R, Mitchell P, Klaver CC, Klein BE, et al. Risk factors for age-related macular degeneration: Pooled findings from three continents. Ophthalmology. 2001; 108(4):697–704. PMID: 11297486

43. Taylor HR, Munoz B, West S, Bressler NM, Bressler SB, Rosenthal FS. Visible light and risk of age-related macular degeneration. Transactions of the American Ophthalmological Society. 1990; 88:163–73; discussion 73–8. PubMed Central PMCID: PMC1298584. PMID: 2095019

44. Taylor HR, West S, Munoz B, Rosenthal FS, Bressler SB, Bressler NM. The long-term effects of visible light on the eye. Arch Ophthalmol. 1992; 110(1):99–104. PMID: 1731731

45. Donoso LA, Kim D, Frost A, Callahan A, Hageman G. The role of inflammation in the pathogenesis of age-related macular degeneration. Surv Ophthalmol. 2006; 51(2):137–52. https://doi.org/10.1016/j.survophthal.2005.12.001 PMID: 16500214

46. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007; 131(5):861–72. Epub 2007/11/24. https://doi.org/10.1016/j.cell.2007.11.019 PMID: 18035408

47. Buecker C, Chen HH, Polo JM, Daheron L, Bu L, Barakat TS, et al. A murine ESC-like state facilitates transgenesis and homologous recombination in human pluripotent stem cells. Cell Stem Cell. 2010; 6 (6):535–46. Epub 2010/06/24. https://doi.org/10.1016/j.stem.2010.05.003 PMID: 20569691

48. Hanna J, Cheng AW, Saha K, Kim J, Lengner CJ, Soldner F, et al. Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. Proc Natl Acad Sci U S A. 2010; 107(20):9222–7. Epub 2010/05/06. PubMed Central PMCID: PMC2889088. https://doi.org/10.1073/pnas.1004584107 PMID: 20442331

49. Kerr CL, Cheng L. Multiple, interconvertible states of human pluripotent stem cells. Cell Stem Cell. 2010; 6(6):497–9. Epub 2010/06/24. https://doi.org/10.1016/j.stem.2010.05.014 PMID: 20569683

50. Yamanaka S, Takahashi K. [Induction of pluripotent stem cells from mouse fibroblast cultures]. Tanpakushitsu Kakusan Koso. 2006; 51(15):2346–51. Epub 2006/12/13. PMID: 17154061

51. Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc Jpn Acad Ser B Phys Biol Sci. 2009; 85(8):348–62. PubMed Central PMCID: PMCPMC3621571. https://doi.org/10.2183/pjab.85.348 PMID: 19838014

52. Singh R, Phillips MJ, Kuai D, Meyer J, Martin JM, Smith MA, et al. Functional analysis of serially expanded human iPS cell-derived RPE cultures. Invest Ophthalmol Vis Sci. 2013; 54(10):6767–78. Epub 2013/09/14. PubMed Central PMCID: PMC3799561. https://doi.org/10.1167/iovs.13-11943 PMID: 24030465

53. Meyer JS, Shearer RL, Capowski EE, Wright LS, Wallace KA, McMillan EL, et al. Modeling early retinal development with human embryonic and induced pluripotent stem cells. Proc Natl Acad Sci U S A. 2009; 106(39):16698–703. Epub 2009/08/27. PubMed Central PMCID: PMC2757802. https://doi.org/10.1073/pnas.0905245106 PMID: 19706890

54. Gong J, Fields MA, Moreira EF, Bowrey HE, Gooz M, Ablonczy Z, et al. Differentiation of Human Protein-Induced Pluripotent Stem Cells toward a Retinal Pigment Epithelial Cell Fate. PLoS One. 2015; 10 (11):e0143272. PubMed Central PMCID: PMCPMC4659559. https://doi.org/10.1371/journal.pone.0143272 PMID: 26606685

55. Paik DC, Dillon J, Galicia E, Tilson MD. The nitrite/collagen reaction: non-enzymatic nitration as a model system for age-related damage. Connect Tissue Res. 2001; 42(2):111–22. Epub 2001/11/23. PMID: 11718466

56. Crow JP, Ischiropoulos H. Detection and quantitation of nitrotyrosine residues in proteins: in vivo marker of peroxynitrite. Methods Enzymol. 1996; 269:185–94. PMID: 8791648

57. Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. Exp Eye Res. 1996; 62(2):155–69. https://doi.org/10.1006/exer.1996.0020 PMID: 8698076

58. Sonoda S, Spee C, Barron E, Ryan SJ, Kannan R, Hinton DR. A protocol for the culture and differentiation of highly polarized human retinal pigment epithelial cells. Nat Protoc. 2009; 4(5):662–73. PubMed Central PMCID: PMC2688697. https://doi.org/10.1038/nprot.2009.33 PMID: 19373231

59. Quinn RH, Miller SS. Ion transport mechanisms in native human retinal pigment epithelium. Invest Ophthalmol Vis Sci. 1992; 33(13):3513–27. PMID: 1334477

60. Johnson LV, Leitner WP, Rivest AJ, Staples MK, Radeke MJ, Anderson DH. The Alzheimer's A beta-peptide is deposited at sites of complement activation in pathologic deposits associated with aging and age-related macular degeneration. Proc Natl Acad Sci U S A. 2002; 99(18):11830–5. Epub 2002/08/22. PubMed Central PMCID: PMC129354. https://doi.org/10.1073/pnas.192203399 PMID: 12189211

61. Murdaugh LS, Wang Z, Del Priore LV, Dillon J, Gaillard ER. Age-related accumulation of 3-nitrotyrosine and nitro-A2E in human Bruch's membrane. Exp Eye Res. 2010; 90(5):564–71. Epub 2010/02/16. https://doi.org/10.1016/j.exer.2010.01.014 PMID: 20153746

62. Karwatowski WS, Jeffries TE, Duance VC, Albon J, Bailey AJ, Easty DL. Preparation of Bruch's membrane and analysis of the age-related changes in the structural collagens. Br J Ophthalmol. 1995; 79 (10):944–52. Epub 1995/10/01. PubMed Central PMCID: PMC505298. PMID: 7488585

63. Solberg Y, Rosner M, Belkin M. The association between cigarette smoking and ocular diseases. Surv Ophthalmol. 1998; 42(6):535–47. Epub 1998/06/23. PMID: 9635902

64. Borland C, Higenbottam T. Nitric oxide yields of contemporary UK, US and French cigarettes. Int J Epidemiol. 1987; 16(1):31–4. Epub 1987/03/01. PMID: 3570619

65. Moreira EF, Cai H, Tezel TH, Fields MA, Del Priore LV. Reengineering Human Bruch's Membrane Increases Rod Outer Segment Phagocytosis by Human Retinal Pigment Epithelium. Transl Vis Sci Technol. 2015; 4(5):10. PubMed Central PMCID: PMCPMC4633034. https://doi.org/10.1167/tvst.4.5.10 PMID: 26557417

66. Nguyen HV, Li Y, Tsang SH. Patient-Specific iPSC-Derived RPE for Modeling of Retinal Diseases. J Clin Med. 2015; 4(4):567–78. PubMed Central PMCID: PMCPMC4470156. https://doi.org/10.3390/jcm4040567 PMID: 26239347

67. Tabar V, Studer L. Pluripotent stem cells in regenerative medicine: challenges and recent progress. Nat Rev Genet. 2014; 15(2):82–92. PubMed Central PMCID: PMCPMC4539940. https://doi.org/10.1038/nrg3563 PMID: 24434846

68. Ogata N, Wada M, Otsuji T, Jo N, Tombran-Tink J, Matsumura M. Expression of pigment epithelium-derived factor in normal adult rat eye and experimental choroidal neovascularization. Investigative ophthalmology & visual science. 2002; 43(4):1168–75.

69. Bhutto IA, McLeod DS, Hasegawa T, Kim SY, Merges C, Tong P, et al. Pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) in aged human choroid and eyes with age-related macular degeneration. Exp Eye Res. 2006; 82(1):99–110. PubMed Central PMCID: PMCPMC4932847. https://doi.org/10.1016/j.exer.2005.05.007 PMID: 16019000

70. Ohno-Matsui K, Yoshida T, Uetama T, Mochizuki M, Morita I. Vascular endothelial growth factor upregulates pigment epithelium-derived factor expression via VEGFR-1 in human retinal pigment epithelial cells. Biochem Biophys Res Commun. 2003; 303(3):962–7. PMID: 12670505

71. Amano S, Yamagishi S, Inagaki Y, Nakamura K, Takeuchi M, Inoue H, et al. Pigment epithelium-derived factor inhibits oxidative stress-induced apoptosis and dysfunction of cultured retinal pericytes. Microvasc Res. 2005; 69(1–2):45–55. https://doi.org/10.1016/j.mvr.2004.11.001 PMID: 15797260

72. Tsao YP, Ho TC, Chen SL, Cheng HC. Pigment epithelium-derived factor inhibits oxidative stress-induced cell death by activation of extracellular signal-regulated kinases in cultured retinal pigment epithelial cells. Life Sci. 2006; 79(6):545–50. https://doi.org/10.1016/j.lfs.2006.01.041 PMID: 16504212

73. Kawa MP, Machalinska A, Roginska D, Machalinski B. Complement system in pathogenesis of AMD: dual player in degeneration and protection of retinal tissue. Journal of immunology research. 2014; 2014:483960. PubMed Central PMCID: PMC4168147. https://doi.org/10.1155/2014/483960 PMID: 25276841

74. Fett AL, Hermann MM, Muether PS, Kirchhof B, Fauser S. Immunohistochemical localization of complement regulatory proteins in the human retina. Histology and histopathology. 2012; 27(3):357–64. https://doi.org/10.14670/HH-27.357 PMID: 22237713

75. Parmeggiani F, Romano MR, Costagliola C, Semeraro F, Incorvaia C, D'Angelo S, et al. Mechanism of inflammation in age-related macular degeneration. Mediators Inflamm. 2012; 2012:546786. PubMed Central PMCID: PMC3504473. https://doi.org/10.1155/2012/546786 PMID: 23209345

76. Anderson DH, Mullins RF, Hageman GS, Johnson LV. A role for local inflammation in the formation of drusen in the aging eye. Am J Ophthalmol. 2002; 134(3):411–31. PMID: 12208254

77. Hageman GS, Luthert PJ, Victor Chong NH, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. Prog Retin Eye Res. 2001; 20 (6):705–32. PMID: 11587915

78. Abdelsalam A, Del Priore L, Zarbin MA. Drusen in age-related macular degeneration: pathogenesis, natural course, and laser photocoagulation-induced regression. Surv Ophthalmol. 1999; 44(1):1–29. PMID: 10466585

79. Mullins RF, Aptsiauri N, Hageman GS. Structure and composition of drusen associated with glomerulonephritis: implications for the role of complement activation in drusen biogenesis. Eye. 2001; 15(Pt 3):390–5. https://doi.org/10.1038/eye.2001.142 PMID: 11450763

80. Capuron L, Schroecksnadel S, Feart C, Aubert A, Higueret D, Barberger-Gateau P, et al. Chronic low-grade inflammation in elderly persons is associated with altered tryptophan and tyrosine metabolism: role in neuropsychiatric symptoms. Biol Psychiatry. 2011; 70(2):175–82. Epub 2011/02/01. https://doi.org/10.1016/j.biopsych.2010.12.006 PMID: 21277567

81.   Gamm DM, Phillips MJ, Singh R. Modeling retinal degenerative diseases with human iPS-derived cells: current status and future implications. Expert Rev Ophthalmol. 2013; 8(3):213–6. Epub 2013/09/17. PubMed Central PMCID: PMC3770479. https://doi.org/10.1586/eop.13.14 PMID: 24039627

82.   Brandl C, Grassmann F, Riolfi J, Weber BH. Tapping Stem Cells to Target AMD: Challenges and Prospects. Journal of clinical medicine. 2015; 4(2):282–303. PubMed Central PMCID: PMC4470125. https://doi.org/10.3390/jcm4020282 PMID: 26239128

83.   Grskovic M, Javaherian A, Strulovici B, Daley GQ. Induced pluripotent stem cells—opportunities for disease modelling and drug discovery. Nat Rev Drug Discov. 2011; 10(12):915–29. Epub 2011/11/15. https://doi.org/10.1038/nrd3577 PMID: 22076509