Age-related macular degeneration (AMD), a leading cause of blindness, initiates in the outer-blood-retina-barrier (oBRB) formed by the retinal pigment epithelium (RPE), Bruch’s membrane, and choriocapillaris. The mechanisms of AMD initiation and progression remain poorly understood owing to the lack of physiologically relevant human oBRB models. To this end, we engineered a native-like three-dimensional (3D) oBRB tissue (3D-oBRB) by bioprinting endothelial cells, pericytes, and fibroblasts on the basal side of a biodegradable scaffold and establishing an RPE monolayer on top. In this 3D-oBRB model, a fully-polarized RPE monolayer provides barrier resistance, induces choriocapillaris fenestration, and supports the formation of Bruch’s-membrane-like structure by inducing changes in gene expression in cells of the choroid. Complement activation in the 3D-oBRB triggers dry AMD phenotypes (including subRPE lipid-rich deposits called drusen and choriocapillaris degeneration), and HIF-α stabilization or STAT3 overactivation induce choriocapillaris neovascularization and type-I wet AMD phenotype. The 3D-oBRB provides a physiologically relevant model to studying RPE–choriocapillaris interactions under healthy and diseased conditions.
Dry AMD initiates by the accumulation of lipid- and protein-rich drusen deposits, triggered by complement pathway activation, in the subRPE region\(^1\). Disease progression to the advanced stage is characterized by geographic atrophy. Disease progression to the advanced stage is characterized by geographic atrophy and neovascularization. Choriocapillaris hyperpolarize, grow under the RPE and pericytes, and form a contiguous capillary network.

### Results
#### Design of 3D-oBRB
Previously, RPE–EC interactions have been studied in coculture systems where the two cell types are separated by a plastic membrane and choroid, we used pure populations of the four key cell types (RPE, ECs, pericytes, and fibroblasts) that constitute the most basic 3D-oBRB (Fig. 1a). The identity of iPSC-derived ECs (iECs), iPSC-derived RPE (iRPE), primary ECs, pericytes, and fibroblasts was confirmed using cell-type-specific markers (ECs positive for CD31, ET2V2, VWF; pericytes positive for NG2, PDGFR-β, COL-I, α-SMA; fibroblasts positive for VIMENTIN, COL-I, and negative for PDGFR-β, α-SMA; and RPE positive for MITF, TYRP1, ZO-1, RPE65, EZRIN consistent with published data; Supplementary Fig. 1)\(^{14–16}\). To aid Bruch's membrane-like tissue formation, a biodegradable scaffold made of thermally fused electrospun poly-(lactic-co-glycolic acid) (PLGA) fibers of 400–500 nm diameter (4–10 µm in thickness) was used with the hypothesis that during scaffold degradation its structure will be replaced by extracellular matrix (ECM) secreted by the RPE and ECs that are cultured on its opposite side (Fig. 1a and Supplementary Fig. 2a,b).

Figure 1a describes the 3D-oBRB manufacturing and maturation timeline. A key step in mediating close interactions between RPE and capillaries was to replace the plastic surface of the snapwell membrane with a 12-mm PLGA scaffold disc (Fig. 1a; Methods). To enhance scaffold hydrophilicity for improved cell and bioink attachment, it was treated with oxygen plasma (5 s cm\(^{-1}\) min\(^{-1}\), 30 min). Improved hydrophilicity was confirmed by the dispersal of a water droplet (Fig. 1a and Supplementary Fig. 2c,d).

### Engineering 3D capillary bed
Previously it has been suggested that high cell density of primary fibroblasts, ECs, and pericytes mixed in hydrogel are required to produce a dense capillary bed with lumens ranging between 5 and 20 µm in diameter\(^2\). To achieve a homogenous bioink that allowed incorporating high cell density, we designed a temperature-sensitive hydrogel by mixing a gelatin-based hydrogel (Novogel, 60 mg ml\(^{-1}\)) with FIBRINOGEN (2.5 mg ml\(^{-1}\)). At 37 °C reduced hydrogel viscosity allowed bioink homogeneity and easier loading in the pipette. Cooling the pipette down to 4 °C increased bioink viscosity allowing the consistent formation of 3D structure. During tissue culture at 37 °C, Novogel dissolved, and FIBRIN continued to provide the 3D architecture needed to support capillary-bed formation. However, FIBRIN degraded after 4 days leading to capillary-bed collapse, as confirmed by the clumping of green fluorescent protein (GFP)-expressing ECs (Supplementary Fig. 3a,b).

The addition of recombinant APROTININ (25 µg ml\(^{-1}\)), a known fibrinolysis inhibitor\(^{16}\), prevented tissue collapse giving fibroblasts time to secrete ECM enabling a stable capillary network (Supplementary Fig. 3c,d).

On the basis of literature evidence, we sought to use a high density of mixed ECs, pericytes, and fibroblasts (12, 6, and 0.6 million fibroblasts, ECs, and pericytes per milliliter of bioink, respectively)\(^{15–20}\). Previously it has been suggested that bioprinting of ECs in a bioink improves tubulogenesis\(^2\). We asked if ECs in our bioink showed different abilities for tubulogenesis after bioink deposition versus bioprinting. First, to optimize bioprinting of the bioink, printed tissue was treated with VEGF (85 ng ml\(^{-1}\)) for 3 or 7 days. In tissues with just 3 days of VEGF treatment, capillary sprouting was evident in printed ECs, but these sprouts did not form a contiguous capillary network and disintegrated by day 7 (arrow heads in Supplementary Fig. 4a). In comparison, 7-day VEGF treatment increased capillary angiogenesis resulting in anastomosis across two printed stripes (arrow heads in Supplementary Fig. 4b and Supplementary Video 1). To perform angiogenesis quantification, we developed a MATLAB-based algorithm (Methods; see Supplementary Information). Quantification revealed 1.5–2× higher angiogenesis on the edges of the printed tissues and 5–10× more angiogenesis in the center of the acellular structure after 7 days VEGF treatment as compared to 3 days treatment (Supplementary Fig. 4a–e), establishing the use of exogenous VEGF for 7 days.

By comparison, dispensing of the same (2:1 fibroblast:EC ratio) bioink without bioprinting, performed under identical VEGF conditions, failed to produce confluent vascular networks by day 7. Interestingly, the 1:1 fibroblast:EC ratio containing bioink formed a confluent capillary network, and 1:2 ratio bioink clearly formed tubular extension from individual GFP-ECs that showed signs of merging on day 7. However, angiogenesis and confluency of these tubular extensions was not evident (compare Supplementary Fig. 5a–c).

Overall, this comparison showed that dispensing of the bioink without bioprinting leads to tubulogenesis if using a lower number of fibroblasts, although the process of angiogenesis and the ability to visualize a patent lumen was clearer in bioprinted tissues. An additional advantage of bioprinting was the ability to control a striped pattern of printing that provided a system for quantification of angiogenic structures, something that was not feasible with regular deposition of the bioink (compare Supplementary Figs. 4 and 5).

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**Fig. 1 | Design of 3D-oBRB.** a, Bioprinting workflow with human ECs and RPE. b–d, Angiogenesis in GFP-expressing primary ECs on day 3 (b), day 5 (c) and day 7 (d) after printing. Arrow heads mark angiogenic sprouts in the acellular zone that anastomose between two printed structures. Scale bars, 500 µm. e–h, Comparative angiogenesis in GFP-positive ECs (green; f, primary ECs (CD31, magenta; g), and iPSC-derived ECs (iECs, CD31, magenta; h)) and nuclei (blue). n = 5 (f), n = 3 (g), n = 4 (h). Scale bars, 500 µm. i, Hematoxylin and eosin (H&E) images of 10-µm-thick cross section of iECs-derived vascular tissue (day 7). Vasculature is marked with black arrow heads, and ECM components are marked with green arrow heads (n = 3). Scale bar, 50 µm. j, TEM images of iECs-derived vascular tissue at day 7. White arrow head shows a capillary, blue arrow head marks a pericyte, and magenta arrow head labels fibroblast (n = 5). Scale bar, 300 nm. k, Projection view of confocal images stained with CD31 (ECs; magenta) and NG2 (pericytes; green). n = 3. Scale bar, 20 µm.

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Further analysis of bioprinted tissues revealed VEGF application likely caused migration of individual ECs that did not incorporate into capillaries22 (circle in Supplementary Fig. 6a,b). To prevent this undesirable EC migration, we supplemented tissues with recombinant ANGIOPOIETIN-1 (ANG-1; 100 ng ml⁻¹), a well-known vessel stabilizing factor23. ANG-1 initially slowed down capillary sprouting (arrow heads IN Figure 6e).

* NG2/CD31

** ANG-1

*** VEGF

**** Aprotinin

††† GFP

‡‡‡ Primary ECs

§§§ iECs

** Coating top side of PLGA scaffold with vitronectin

Incubate tissue for 2 hours at room temperature and then at 37 °C

GFP-ECs

Angiogenesis (branching number/mm)

Day 2 to 42: tissue growth/maturation

Week 1

Tubulogenesis

1/2 EC:RPE medium +Aprotinin +ANG-1 +VEGF

Week 2

ECM production

1/2 EC:RPE medium

Week 3

RPE cell growth

1/2 EC:RPE medium

Week 4-6

RPE maturation

1/2 EC:RPE medium

Day 42: mature 3D-oBRB tissue model
in Supplementary Fig. 6a,c), but it did not disrupt capillary formation. In fact, by day 8 the number of capillaries increased in ANG-1 treated Group 2 as compared to without it (Group 1) (Supplementary Fig. 6b,d), likely because it reduced migration of individual ECs. Reduced EC migration allowed more precise analysis of angiogenesis kinetics.

To monitor the time course of angiogenesis, we analyzed GFP-ECs on days 3, 5, and 7 after printing. By day 3, capillaries grew in a gradient with 5–6 capillary peaks per millimeter close to the printed stripe edge and no peaks in the center of the acellular zone (Fig. 1b,e and Supplementary Fig. 7a). By day 5, the capillary gradient between stripe edges and the center of acellular area had shallowed with 8 peaks per millimeter on the edges and 4 peaks per millimeter in the center (Fig. 1c,e and Supplementary Fig. 7b). By day 7, no statistically significant difference between edges (9 peaks per millimeter) and center (10 peaks per millimeter) was evident (Fig. 1d,e and Supplementary Fig. 7c). A comparative analysis of capillary networks derived from GFP-ECs, non-GFP-ECs, and iECs revealed similar capillary confluency and angiogenesis throughout the tissue (Fig. 1f–h and Supplementary Fig. 7d,e). Together, this confirmed robustness of our bioprinting protocol across different ECs. Histological analysis (hematoxylin and eosin staining) of tissue sections confirmed capillaries with lumen of 5–20 µm (black arrow heads) and interstitial spaces filled with cells and ECM (green arrow heads; Fig. 1i). Transmission electron microscopy (TEM) of tissue cross sections confirmed iECs derived capillaries (white arrow head) with a pericyte (blue arrow head) wrapped around, and fibroblasts (pink arrow head) within interstitial spaces (Fig. 1j). This observation was further confirmed in confocal images where pericytes were found to localize at abluminal side of iEC-derived intact capillaries (Fig. 1k, Extended Data Fig. 1 and Supplementary Video 2). To further confirm the roles played by individual cellular components in the bioink, we tested different combinations of cell types for bioprinting (Extended Data Fig. 2). In the absence of pericytes and fibroblasts, ECs did not undergo complete angiogenesis. Rather GFP-ECs formed a monolayer surrounding printed structure much like how it was seen with the bioink deposition (Extended Data Fig. 2a,b). Similar results were seen when ECs were mixed with pericytes only (Extended Data Fig. 2c,d). When ECs were mixed only with fibroblasts, we noticed angiogenesis in GFP-ECs but the migration of angiogenic sprouts in the acellular zone was slower...
as compared to when all three cell types were combined (compare Extended Data Fig. 2c–h). This data demonstrated that fibroblasts are important in initiating angiogenesis whereas pericytes are critical for stabilizing the vascular network, as has been suggested previously\(^2\). Overall, this data shows that our bioink composed of Novogel, FIBRINOGEN mixed with fibroblasts, ECs, and pericytes (2:1:0.1 ratio) forms a robust capillary network with both primary and iPSC-ECs.

### Development of a complete 3D-oBRB

To complete the 3D-oBRB structure, iPSC-derived RPE (iRPE) cells were seeded and matured\(^1\) on the side of the scaffold opposite to the printing surface. VITRONECTIN (50 µg/ml\(^3\)) coating was performed for RPE attachment\(^4\). TEM confirmed iRPE cells initiated polarization and pigmentation within 3 weeks after seeding (Fig. 2a). By 6 weeks, known structural features of RPE maturation and polarization were evident: dense apical processes, tight junctions between neighboring cells, apically located stage IV melanosomes, and basal infoldings—a critical native-RPE feature that cannot be reproduced in RPE grown on plastic substrates\(^2\). Similar to the native-RPE, and as reported previously for RPE grown on PLGA scaffolds\(^6\), RPE basal infoldings were in continuum with the ECM that had replaced the scaffold and formed a Bruch’s membrane-like structure\(^7\) (Fig. 2b), suggesting that RPE basal surface maturation in 3D-oBRB was not negatively affected by a confluent capillary network on the opposite side. Immunostaining for the tight junction marker E-CADHERIN and the apical process marker EZRIN further confirmed RPE monolayer junctional maturity and polarization (Fig. 2c and Supplementary Video 3). Concurrent with RPE maturation, capillary confluency was evident in CD31 immunostained 3D rendered tissue images (Fig. 2d and Supplementary Video 3). High expression of FELS, a fenestration marker\(^8\), colocalized with CD31 suggested the formation of fenestration in our 3D-oBRB model (Fig. 2e). Histological analysis revealed the structure of the entire 3D-oBRB with a 2–4 µm thick Bruch’s membrane-like acellular ECM region sandwiched between an RPE monolayer on top and a 40–50 µm thick vascular tissue with capillaries running along various tissue planes (Fig. 2f). These results were further corroborated by scanning electron microscopy of a 3D-oBRB side view (Fig. 2g) that shows a 2.5× higher-resolution data with almost an identical pattern to the hematoxylin and eosin staining. To confirm functionality of the 3D-oBRB tight junctions, we measured tissue resistance to current flow (trans-epithelial resistance (TER)), produced by functional tight junctions between neighboring RPE cells. TER of the tissue with vasculature and no iRPE layer was 53.4 ± 1.36 Ohms cm\(^2\). The presence of the iRPE monolayer increased the tissue TER to 740.4 ± 155.99 Ohms cm\(^2\), comparable to the TER of iRPE monolayer without the vascularized tissue 873.7 ± 67.82 Ohms cm\(^2\) (Fig. 2h). We asked if the presence of RPE changed the architecture of tubulogenesis that occurred with bioprint deposition (Supplementary Fig. 8). RPE presence stabilized the network of tubules with 2:1 ratio of EC and fibroblast and allowed them to get more confluent by 5 weeks of culture, similar to what was seen with bioprinting (Fig. 2d,e). However, patent lumenzation was not seen in these tubules with bioprint deposition (compare Supplementary Fig. 8a–c with Fig. 2d,e). Overall, TEM, immunostaining, and barrier resistance confirmed the formation of a functional 3D-oBRB with polarized RPE monolayer and functionally lumenezied capillaries in bioprinted tissues.

### RPE cells induce capillary fenestration in 3D-oBRB

Strong FELS expression in fully mature 3D-oBRB (Fig. 2e) suggested the presence of fenestration in capillaries. To better characterize the fenestration process, we performed a temporal analysis of FELS expression in 3D-oBRB constructs (Extended Data Fig. 3a–d). CD31 co-immunostaining revealed minimal FELS expression in capillaries for the first 2 weeks after printing (Extended Data Fig. 3a,b). Coincidental with iRPE monolayer polarization (Fig. 2a), FELS expression became prominent starting week 3 (Extended Data Fig. 3c,d). To confirm if FELS expression led to fenestration in fully mature 3D-oBRB, we performed ultrastructural analysis of capillaries (Fig. 3a,b). TEM revealed 50–80 nm thinned areas in the EC membrane reminiscent of fenestration in native choroidal capillaries (arrow heads in Fig. 3b). To further confirm if FELS expression was affected by the RPE presence, we cultured the vascular tissue with (Fig. 3c,d,g) or without the RPE (Fig. 3e–g). CD31 and FELS co-immunostaining revealed a confluent capillary bed, higher capillary number and thickness, and 2.5× higher capillaries area coverage in tissues that contained the RPE as compared to tissues that lacked the RPE monolayer where vasculature collapsed (Fig. 3d,f,g). Overall, this work provides the first direct evidence that human RPE cells induce fenestration in anEC-derived capillary-network.

RPE-dependent fenestration formation in our 3D-oBRB suggested that iECs acquire a choroidal fate. To further investigate the phenotypic change in iECs within the 3D-oBRB, we compared the transcriptome profiles of iECs in two-dimensional (2D) monocultures (without RPE) to iECs within the 3D-oBRB cultured with and without the RPE, using single-cell RNA sequencing (scRNA-seq). Data obtained from 3D-oBRB derived from two different healthy iPSC lines was averaged to determine EC, pericytes, fibroblasts, and RPE expression profiles. Clustering samples by uniform manifold approximation and projection (UMAP) plots revealed two heterogenous EC populations in 2D ECs and in ECs derived from 3D capillaries without the presence of RPE (3D no RPE), but only one dominant population in 3D-oBRB derived ECs (3D with RPE) (Fig. 3h–t). scRNA-seq revealed downregulation of multiple cell proliferation, actin remodeling, and EMT genes; notable examples include cell cycle regulators CDK17 and SMCA; tumorigenesis-related genes EDN1 and EGFL7; and migration-inducing and actin remodeling genes CYTOR, SMAD1, KRT18, CNFNF, SMURF2, ROBO4, and MMP1; and an upregulation of EC maturation markers IGFBP3, SFRP1, and WNT5A in both 3D ECs without the RPE or with the RPE (Fig. 3h–k, Supplementary Figs. 9–11, and Supplementary Table 3). Expression of EC maturation markers SLC2A1, ADAM, and TTR increased only in ECs with the RPE (Fig. 3j, Supplementary Fig. 11, and Supplementary Table 3). Interestingly, while a smaller increase in expression of capillary-maturation markers such as CA12, PDGFRα, VEGFA, and CCN4 was noted in 3D ECs without the RPE, a larger expression increase was seen in 3D ECs with the RPE (Fig. 3m–o, Supplementary Fig. 11, and Supplementary Table 3). Furthermore, the expression of genes involved in RPE-choroidal homeostasis such as APOE and to a lesser extent TIMP3 (Fig. 3p,q), and the expression of genes involved in Bruch’s membrane formation, (for example, COL1A1, COHAS, ELN, COL9A2, and ECM1) was increased in 3D ECs with the RPE as compared to 3D ECs without the RPE or 2D ECs (Fig. 3r–t, Supplementary Fig. 12, and Supplementary Table 3). Interestingly, the expression of pro-inflammatory markers (F2LR1 maturation (k,l), capillary maturation (m,n), choroidal phenotype (o,p), and Bruch’s membrane deposition (q,t). Statistical significance was attributed to values of \( P < 0.05 \) as determined by unpaired \( t \)-test and exact \( P \) values are available in Supplementary Tables 3 and 4. Selected genes demonstrated average fold change >1.0 between 2D and 3D-oBRB culture as well as an adjusted \( P < 0.05 \) following differential expression analysis in Seurat. Data depicts results from \( n = 10.624 \) cells (2D iECs), and \( n = 1.399 \) cells (3D-oBRB iECs without RPE), and \( n = 1.728 \) cells (3D-oBRB iECs with RPE). Statistical analysis was performed using a non-parametric Wilcoxon rank sum test, adjusted for Bonferroni correction from all features in the dataset in Seurat.
and GIMAP8) was decreased in both 3D EC populations with or without the RPE; the expression of anti-inflammatory markers NDRG1, PTGDS and BNIP3, and PTGS1 was higher in 3D ECs in the presence of RPE as compared to the other two EC populations (Supplementary Fig. 13 and Supplementary Table 3). Overall, this data suggests that ECs undergo maturation when cultured in 3D tissues, and the presence of

![Image](https://doi.org/10.1038/s41592-022-01701-1)
RPE further matures them into choriocapillaris, induces fenestration, anti-inflammatory phenotype, and stimulates the formation of ECM relevant for the Bruch’s membrane.

Unbiased scRNA-seq analysis of pericytes revealed that, similar to ECs, pericytes within 3D tissues with the RPE became more homogenous in phenotype. The expression of proliferation and migration markers (for example, BIRC5, CCNB1, CDC20, HMG1, HMG2, KRT7, and KRT19) decreased significantly, whereas the expression of capillaries maturation (EGRI, VEGFA, and VEGFB) and hypoxia response (NFkB1A, JUN, and FOSB) genes increased significantly in pericytes within 3D cultures. Within 3D-oBRB, the increased expression was more dominant in the presence of RPE than in its absence (Supplementary Figs 14 and 15 and Supplementary Table 3). This data suggests a role for pericytes in maturation of capillaries network in the 3D tissue and their ability to sense and respond to the presence of RPE.

Unexpectedly, and similar to ECs and pericytes, fibroblasts also responded strongly to the presence of RPE. RPE presence changed fibroblasts to a more homogenous population, converted them into a less fibrotic and less migratory fate (for example, the presence of RPE significantly reduced expression of MKD, ELF6, BANFI, KRT5, KRT18, and MMP1 in fibroblasts); stimulated the expression of genes involved in Bruch’s membrane formation including COLBA2 and ELN and genes involved in choroidal homoeostasis such as APOE, CA12, CRIVAB, CXCL8, and TTR (Supplementary Figs 16 and 17 and Supplementary Table 3), as has been suggested previously in animal models. Overall, our scRNA-seq data helped uncover an intricate crosstalk between RPE and all three cells of the choroid, and underscore the relative importance of individual choroidal cell types in supporting angiogenesis, the matrix around capillaries, and the overall tissue health—similar to previous observations. In fact, a comparative analysis of our scRNA-seq data and published datasets suggested similar expression of several key genes in the three choroidal cell types (Supplementary Fig. 18). This discovery has implications for better understanding of choroidal changes in the eyes of AMD patients.

Formation of Bruch’s membrane in 3D-oBRB

Scanning electron microscopy (SEM) suggested the formation of a Bruch’s membrane-like structure between the RPE and the capillary-bed (Fig. 2g). In higher-resolution images of RPE/Bruch’s membrane region, we confirmed strong immunostaining for ECM components of native Bruch’s membrane including FIBRONECTIN, LAMININ (LMN), COLLAGEN IV (COL IV), and ELASTIN (ELN) within the 3D-oBRB (Fig. 4a–d). Three-dimensional rendered images of the subRPE zone and the CC region, immunostained for LAMININ, ELASTIN, and COLLAGEN IV, clearly showed the presence of a dense and homogenous ECM deposition, reminiscent of a Bruch’s membrane (Extended Data Fig. 4a–f and Supplementary Video 4). Colocalization for ELASTIN and LAMININ with COLLAGEN IV revealed that the three ECM proteins of the Bruch’s membrane are present in different layers, ELASTIN is located closest to the RPE, whereas LAMININ and COLLAGEN IV are present deeper in the Bruch’s membrane (Extended Data Fig. 4g and Supplementary Video 6). A comparative analysis of 2D and 3D tissues showed 15x higher COLLAGEN IV expression and 4x higher ELASTIN expression in 3D-oBRB as compared to 2D-iRPE, suggesting increased production of Bruch’s membrane-related proteins in 3D-oBRB (Fig. 4e–i). To determine the cellular origin of this increased production of Bruch’s membrane related proteins in 3D-oBRB, we analyzed their gene expression in the scRNA-seq data. To our surprise, scRNA-seq data revealed that RPE, endothelial cells, and fibroblasts contributed to different components of the Bruch’s membrane. Interestingly, RPE cells preferentially produced COL1A1, COL1A2, COL3A1, FBN1, and FNI; endothelial cells formed COL4A5, ELN, COL9A2, and ECM1; and fibroblasts produced COL8A2 and ELN proteins (Figs. 3 and 4, Supplementary Figs 12, 17, and 19, and Supplementary Table 3), supporting previous data. Overall, combined SEM, TEM, histology, immunostaining, 3D-rendering, and scRNA-seq provided strong evidence in support of a Bruch’s membrane-like structure present in between RPE and CC in our 3D-oBRB.

So far, our data suggested that RPE cells significantly alter the gene expression of the three cell types of the choroid. Next, we asked if RPE cells also changed phenotype in the 3D-oBRB. scRNA-seq analysis of the RPE showed that unlike ECs, pericytes, and fibroblasts, the maturation state of RPE did not change significantly when compared between 2D cultures and 3D-oBRB. The expression of maturation related markers such as TYRPI, APC, SLC2A1, and TIMP3 continued to be expressed at similar levels in both 2D and 3D RPE populations (Fig. 4j–n and Supplementary Table 3). Interestingly, the expression of certain solute carriers like SLC6A20, Bruch’s membrane genes (COL1A1, COL1A2, COL3A1, FBN1, and FNI), and complement inhibitory genes CFI and CD59 increased in 3D samples as compared to 2D samples (Fig. 4o–t, Supplementary Fig. 19, and Supplementary Table 3). Genes involved in exosome formation and transport, MYH10 and VMP1 (Supplementary Fig. 19 and Supplementary Table 3) were also increased in 3D RPE, as compared to 2D cultures. A comparative analysis of our scRNA-seq data and published datasets suggested similar expression of several key genes in RPE cells (Supplementary Fig. 20), further supporting physiological relevance of our work. This data suggested that RPE cells change minimally, and changes seen in RPE monolayer further facilitate interactions with cells of the choroid including. All together this data shows that the 3D-oBRB resembles in its anatomical and molecular features to a native oBRB, and RPE influences the fate of all three cell types within the choroid. We hypothesize that formation of the Bruch’s membrane-like structure in our model further enabled close interactions between RPE and choriocapillaris consistent with the function of this tissue in vivo.

3D-oBRB recapitulates choroidal phenotypes seen in dry and wet AMD

To validate our 3D-oBRB as a physiologically relevant model, we asked if it could replicate key disease phenotypes of AMD. Drusen—an oxidized protein, lipid, and lipoprotein rich hallmark of dry AMD—accumulates under the RPE and within the Bruch’s membrane; complement-competent human serum (CC-HS), has recently been linked to in vitro drusen formation and linked to in vitro drusen formation and linked to in vitro drusen formation. Treatment of 3D-oBRB with 5% CC-HS induced drusen-like lipoprotein (APOE-positive) and lipid (Nile Red-positive) deposits within the Bruch’s membrane region, as confirmed by histological analysis of 3D-oBRB in en face view (Fig. 5a, d and Extended Data Fig. 5a–f), cross-section view (Fig. 5g and Extended Data Fig. 5g). In 3D-oBRB, APOE-positive deposits were observed around capillaries and the overall tissue health—similar to previous data. This data further suggested that RPE cells significantly change phenotype in 3D-oBRB; RPE cells further matured them into choriocapillaris, induces fenestration, anti-inflammatory phenotype, and stimulates the formation of ECM relevant for the Bruch’s membrane.

So far, our data suggested that RPE cells significantly alter the gene expression of the three cell types of the choroid. Next, we asked if RPE cells also changed phenotype in the 3D-oBRB. scRNA-seq analysis of the RPE showed that unlike ECs, pericytes, and fibroblasts, the maturation state of RPE did not change significantly when compared between 2D cultures and 3D-oBRB. The expression of maturation related markers such as TYRPI, APC, SLC2A1, and TIMP3 continued to be expressed at similar levels in both 2D and 3D RPE populations (Fig. 4j–n and Supplementary Table 3). Interestingly, the expression of certain solute carriers like SLC6A20, Bruch’s membrane genes (COL1A1, COL1A2, COL3A1, FBN1, and FNI), and complement inhibitory genes CFI and CD59 increased in 3D samples as compared to 2D samples (Fig. 4o–t, Supplementary Fig. 19, and Supplementary Table 3). Genes involved in exosome formation and transport, MYH10 and VMP1 (Supplementary Fig. 19 and Supplementary Table 3) were also increased in 3D RPE, as compared to 2D cultures. A comparative analysis of our scRNA-seq data and published datasets suggested similar expression of several key genes in RPE cells (Supplementary Fig. 20), further supporting physiological relevance of our work. This data suggested that RPE cells change minimally, and changes seen in RPE monolayer further facilitate interactions with cells of the choroid including. All together this data shows that the 3D-oBRB resembles in its anatomical and molecular features to a native oBRB, and RPE influences the fate of all three cell types within the choroid. We hypothesize that formation of the Bruch’s membrane-like structure in our model further enabled close interactions between RPE and choriocapillaris consistent with the function of this tissue in vivo.

Fig. 4 | RPE maturity in 3D-oBRB. a–d. Cross sections of 3D-oBRB immunostained for Bruch’s membrane proteins FIBRONECTIN (a), LAMININ (b), COLLAGEN IV (COL IV) (c), and ELASTIN (d). Nuclei were stained with DAPI, n = 3 (a,d), n = 8 (b,e). Scale bars, 10 µm. e–h. Two-dimensional RPE monoculture (e,g) and 3D-oBRB (f,h), co-immunostained for COL IV (green) or ELASTIN with ZO-1 (red), and nuclei (blue). n = 3. Scale bars, 30 µm. i. Fluorescence mean intensity presented in arbitrary units (AU) showing comparison of ELASTIN and COL IV immunostaining in 2D-iRPE and 3D-oBRB models. n = 3. Data are presented as mean ± SEM. Two-way ANOVA and post hoc Tukey’s multiple comparison was used for statistical analysis. j–t, UMAP plots from scRNA-seq of 2D-iRPE and RPE
Data Fig. S5–x), and 3D rendered images (Supplementary Video 5). CC-HS treatment also led to RPE atrophy in 3D-oBRB (Fig. 5b,e and Extended Data Fig. 5s–x). Consistent with RPE atrophy, capillary degeneration was evident with CC-HS treatment (Fig. 5c–g and Extended Data Fig. 5g–r,s–x). A time-dependent choroidal thinning is evident in tissue cross sections (Extended Data Fig. 5s–x). Lastly, these structural changes in the 3D-oBRB led to a loss in its barrier resistance, as confirmed by a 20× drop in TER (Fig. 5h). Overall, these results validated...
3D-oBRB as a physiologically relevant dry AMD model including subRPE drusen deposits, loss of barrier resistance, RPE atrophy, and capillary degeneration.

To develop a wet AMD model, we tested a chemical and a genetic approach. It is thought that hypoxia in the back of the eye leads to stabilization and nuclear translocation of the transcription factor HIF-1α in the RPE. HIF-1α increases the expression and secretion of VEGF, which leads to wet AMD. However, there is no direct evidence supporting this hypothesis for human ocular tissues. We sought to recreate wet AMD using RPE-specific hypoxia in the 3D-oBRB model. Treatment of the 2D-IRPE with ML228 (2 μM), a known HIF-1α activator, led to HIF-1α activation in <5% of cells by 48 h; by 96 h elevated HIF-1α protein levels were evident in 25% of cells (Extended Data Fig. 6a,b,d,e). Continued treatment of ML228 for 2 weeks resulted in HIF-1α activation in majority of IRPE cells and dropped IRPE monolayer TER close to zero; in comparison the TER of vehicle-treated cells was not changed (Extended Data Fig. 6c,f,g). Consistently, HIF-1α expressing cells also lost their epithelial phenotype, as confirmed by the loss of typical hexagonal morphology, unorganized ZO-1 expression, and an increase in cell size (Extended Data Fig. 6e,f,h).

Treatment of mature 3D-oBRB (only on the apical side) with ML228 produced outcomes similar to seen in 2D-IRPE, including disruption of tight junctions and a threefold drop in tissue TER (Fig. 5i–n). Unlike 2D-IRPE, in the 3D-oBRB model the TER did not drop to zero suggesting a protective effect of the capillary bed on RPE barrier resistance (compare Extended Data Fig. 6g and Fig. 5o). Activation of HIF-1α in the RPE of a mature 3D-oBRB initiated subclinical (type-I) CNV-like response with capillaries hyperproliferating towards the IRPE monolayer while the activation of HIF-1α only in the choroid and both RPE and choroid resulted in degeneration of CC, as confirmed by image-based analysis of z-planes and a threefold drop in tissue TER (Fig. 5j–o). This result provided an independent validation that perturbation of typical hexagonal morphology, unorganized ZO-1 expression, and an increase in cell size (Extended Data Fig. 6e,f,h).

To complement our chemical CNV model with an independent and genetically induced model, we focused on a different pro-angiogenic, pro-inflammatory transcription factor, STAT3. Its activation and nuclear translocation in the RPE has also been suggested as a pro-CNV event. We tested if STAT3 overexpression in the RPE would lead to higher proliferation of capillaries in our 3D-oBRB. Towards this goal, we generated an iPSC line with overexpression of STAT3 (Supplementary Fig. 21a, b, uncropped blots in Supplementary Data 2). Differentiated RPE derived from this transgenic iPSC line led to significantly higher nuclear localization of STAT3 and higher activation, as compared to RPE derived from wild-type iPSCs (compare Extended Data Fig. 8a,e and Supplementary Fig. 21b). When analyzing the subRPE and choroidal regions of wild-type and STAT3-overexpressing 3D-oBRB, we observed significantly higher capillaries proliferation and type-I CNV-like growth towards the RPE, both in en face views (Extended Data Fig. 8a–h) and cross-sectional views (Extended Data Figs. 8i–j). The TER of STAT3-overexpressing RPE was significantly lower as compared to that of wild-type RPE (Extended Data Fig. 8k). This result provided an independent validation that perturbation of pro-angiogenic and pro-inflammatory pathways in the RPE can induce CNV-like response in CC.

To determine if the type-I CNV-like response seen in our hypoxic 3D-oBRB model was VEGF induced, we measured VEGF secretion in ML228-treated 3D-oBRB. ML228 treatment led to a fivefold increase in the apical VEGF secretion (Fig. 5p), while basal VEGF secretion did not change with ML228 treatment (Fig. 5q), despite the fact that fourfold higher VEGF-A levels were seen by scRNA-seq in ECs cultured as 3D tissues with the RPE as compared to 2D ECs (Fig. 3o). Quantification of VEGF immunostaining revealed that most of the basally secreted VEGF accumulated around capillaries, likely binding to its cognate receptor—leading to a CNV-like phenotype (Fig. 5r). Overall, the above data confirmed that our 3D-oBRB is able to recapitulate both dry and wet AMD phenotypes.

**Bevacizumab halts CNV in the 3D-oBRB disease model**

To validate our 3D-oBRB as a physiologically relevant platform also applicable for clinical and drug discovery approaches, we asked if we could replicate the efficacy of an anti-VEGF monoclonal antibody (Bevacizumab), used for the treatment of wet AMD. We treated STAT3-overexpressing 3D-oBRB, and co-treated 2D-IRPE and 3D-oBRB with ML228 and a clinical dose (0.3 mg ml−1) of Bevacizumab. Bevacizumab had little, if any, effect reversing ML228-induced 2D-IRPE atrophy (Fig. 6a–d). However, in 3D-oBRB Bevacizumab co-treatment with ML228 partially recovered RPE epithelial phenotype as confirmed by regained epithelial morphology in F-ACTIN-stained and ZO-1-immunostained images (arrow heads in Fig. 6a–g,j–n) and doubled TER values of ML228 and Bevacizumab co-treated samples (Fig. 6d). Reconstructed tissue microsections revealed a noticeable reduction in CNV with Bevacizumab and ML228 co-treatment as compared to ML228 treatment alone (arrow heads in Fig. 6i,k,m and Supplementary Video 7). Image-based quantification of vascular density in each focal plane showed a threefold higher capillary density within 5 μm of the iRPE monolayer in ML228-treated 3D-oBRB (Extended Data Fig. 7a–k) and Supplementary Video 6). It also showed thinning of the Bruch’s membrane ECM. This data suggests that RPE-specific hypoxia in 3D-oBRB induces type-I CNV-like response in CC.

To complement our chemical CNV model with an independent and genetically induced model, we focused on a different pro-angiogenic, pro-inflammatory transcription factor, STAT3. Its activation and nuclear translocation in the RPE has also been suggested as a pro-CNV event. We tested if STAT3 overexpression in the RPE would lead to higher proliferation of capillaries in our 3D-oBRB. Towards this goal, we generated an iPSC line with overexpression of STAT3 (Supplementary Fig. 21a, b, uncropped blots in Supplementary Data 2). Differentiated RPE derived from this transgenic iPSC line led to significantly higher nuclear localization of STAT3 and higher activation, as compared to RPE derived from wild-type iPSCs (compare Extended Data Fig. 8a,e and Supplementary Fig. 21b). When analyzing the subRPE and choroidal regions of wild-type and STAT3-overexpressing 3D-oBRB, we observed significantly higher capillaries proliferation and type-I CNV-like growth towards the RPE, both in en face views (Extended Data Fig. 8a–h) and cross-sectional views (Extended Data Figs. 8i–j). The TER of STAT3-overexpressing RPE was significantly lower as compared to that of wild-type RPE (Extended Data Fig. 8k). This result provided an independent validation that perturbation of pro-angiogenic and pro-inflammatory pathways in the RPE can induce CNV-like response in CC.
seen in Bevacizumab-treated tissues as compared to untreated tissues (compare Extended Data Figs 8e–hand 9d–h). Again, similar to ML228 and Bevacizumab co-treated samples, TER of STAT3-overexpressing and Bevacizumab-treated 3D-oBRB improved slightly, suggesting Bevacizumab may ameliorate ML228- or activated-STAT3-induced TER degrading effects on the RPE. Overall, these results suggest that hypoxia or STAT3-induced type-I CNV seen in our 3D-oBRB is primarily VEGF induced and can be suppressed by the clinically used drug VEGF (AU).
**Fig. 6** | Bevacizumab treatment suppresses wet AMD in 3D-oBRB. 

a–c, RPE monoculture treated for 2 weeks with DMSO (vehicle, a), ML228 (2 µM) (b), and ML228 (2 µM) + Bevacizumab (0.284 mg ml⁻¹) (c), immunostained for HIF-1α (yellow), ZO-1 (green), and nuclei stained with Hoechst (blue). Scale bars, 30 µm. 

d, TER measurement comparison between 2D-iRPE and 3D-oBRB for ML228 and ML228 and Bevacizumab co-treated samples. TER values are normalized to vehicle-treated 3D-oBRBs. 2D-iRPE, n = 3; 3D-oBRB, n = 6. Data are presented as mean ± s.e.m. Unpaired t-test was used for statistical analysis, P = 0.037.

e–g, Maximum-intensity projection images of RPE of 3D-oBRB, immunostained with HIF-1α (yellow), nuclei stained with Hoechst (blue) and F-ACTIN with Phalloidin (cyan). g, Recovered RPE are marked with arrow heads in, n = 3. Scale bars, 30 µm.

h, Vascular area fraction was calculated from CD31-positive area in each z stack. n = 5. ANOVA and Tukey’s multiple comparisons were used for statistical analysis.

i–n, Side view of 3D reconstructed images of 3D-oBRB tissues treated with vehicle (DMSO) (i), ML228 (k), and ML228 + Bevacizumab (m), immunostained with ZO-1 (green) and CD31 (magenta). Arrow heads in k mark hyperproliferating capillaries (n = 4) that are not seen in i and m. j, l, Maximum-intensity projection images of RPE of 3D-oBRB, immunostained with ZO-1 (green). Recovered RPE are marked with arrow heads in (n), n = 4. Scale bars, 25 µm.
Bevacizumab. All together, these results confirm physiological relevance of our model to study human RPE–choriocapillaris interactions and validate its utility for discovering new AMD drugs.

Discussion

3D-oBRB recapitulated key native features that have not been previously replicated in vitro. (1) a fully mature and polarized RPE monolayer with basolateral foldings that are critical for metabolite transport and membrane trafficking. It has been reported previously that the basolateral foldings are lost in diseases such as choroideremia and AMD, and affect RPE–choriocapillaris interactions, underscoring the importance of our 3D-oBRB for studying both monogenic and polygenic eye diseases. (2) A Bruch's membrane-like structure that facilitated RPE–choroid interactions, allowing RPE cells to modulate gene expression in all three cells of the choroid. Bruch's membrane hydraulic conductivity ensures a free flow of nutrients, metabolites and cytokines between the two tissues, with age and in AMD, loss of Bruch’s membrane hydraulic conductivity disrupts communication between these two tissues and contributes to outer retina degeneration. Our 3D-oBRB replicates degenerative changes in the Bruch’s membrane (APOE deposits and angiogenic invasion) and allows the possibility of discovering how such changes contribute to disease progression. (3) A confluent capillary-bed with a patent lumen, fenestration, and choroid-specific gene expression. Loss of fenestration was seen in advanced AMD eyes and reported to be associated with AMD risk alleles. Our model provides direct evidence that fenestration is dependent on the presence of healthy RPE and a possibility of understanding the role of AMD risk alleles via the use of patient iPSCs.

These features were made possible by the introduction of several innovative bioengineering attributes that have not been previously combined in generating an engineered eye tissue. (1) A biodegradable scaffold provided two bioactive surfaces allowing RPE monolayer growth on one side and bioink printing on the opposite side. Scaffold micropores allowed RPE, ECs, and fibroblasts to deposit ECM leading to the formation of a Bruch's membrane-like structure creating a native-tissue-like architecture. The mixture of GELATIN and FIBRIN provided a temperature-sensitive hydrogel with high fluidity at room temperature for developing a homogeneously concentrated bioink of ECs, pericytes, and fibroblasts and low fluidity at 10 °C for relatively easier bioprinting. FIBRIN maintained the 3D tissue structure until fibroblasts secreted sufficient ECM to provide substrate for a confluent capillary-bed. (3) Combination of relevant cell types at appropriate ratio: 2:1 fibroblast to ECs and 1:10 pericytes to ECs allowed for formation of a confluent capillary-bed. (3) Combination of relevant cell types at appropriate ratio: 2:1 fibroblast to ECs and 1:10 pericytes to ECs allowed easier angiogenesis through a native-like ECM structure and sustained long-term capillaries of the correct lumen size (20–50 μm). RPE, ECs, and pericytes cells provided VEGF for capillary growth, confluence, fenestration, and choroidal phenotype. Interestingly, irrespective of the EC origin (retinal or iPSC), the 3D-oBRB environment was able to induce fenestration in choriocapillaris, suggesting plasticity in the EC fate.

Our data complements the recent work that developed RPE/EC and RPE/capillaries co-cultures and illustrates a continued evolution in technology. For instance Benedito et al. mainly focused on RPE-EC co-cultures, whereas Manian et al. used MSCs to help form CC. Our work has taken those approaches further by combining pure cultures of four different cell types, with bioprinting using a thermally modulating hydrogel and the use of scaffolds (a detailed comparison of the three studies provided in Supplementary Protocol).

Our goal was to develop a model that closely mimics the 3D-oBRB of the eye. However, like other in vitro systems, our model also has some limitations. For instance, even though the Bruch’s membrane derived in this system, has a layered pattern of ELASTIN, LAMININ, and COL IV location, it does not have the typical penta-lamellar structure that has been reported for the embryonic Bruch’s membrane and does not represent embryonic development of the oBBR. However, RPE, ECs, and fibroblasts that are known to contribute to the embryonic Bruch’s membrane also contribute key Bruch’s membrane proteins in our tissue. Our model contains key components of the oBBR but lacks other cell types of the choroid, especially cells of the innate immune system and melanocytes. But our modular approach easily allows the possibility of increasing the complexity of this tissue. Another limitation of the 3D-oBRB is the lack of an active perfusion through the choriocapillaris. This along with the observation that we are mimicking type-I CNV, particularly limits the assessment of choriocapillaris dropout seen adjacent to type II CNV regions in patients. Future efforts are focused on developing a fully perfusable system. Future efforts will also test this 3D-oBRB as a potential transplant for dry AMD where both RPE and choriocapillaris degeneration is seen. Although, this type of 3D-oBRB can also be developed without a bioprinter, our data suggests that bioprinting readily allows for formation of a patent lumen and provides a system for quantification of angiogenic structures. With a recent steep drop in bioprinter prices, we believe this limitation can be easily overcome and the use of simple quality controls such as TER and capillary confluency will allow easy replication of this work in other labs. Overall, our work provides a versatile, physiologically and clinically relevant 3D-oBRB that has relevant intricate and complex interactions between various cell types of the choroid with the RPE both under healthy and diseased conditions.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41592-022-01701-1.

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Methods

Study design

Human primary cells, iPSC-derived RPE and ECs were commercially obtained. One control iPSC was used which was previously published and was used under NIH Institutional Review Board approved study protocol 11-E1-0245. The use of cadaver eyes was exempt from the NIH Institutional Review Board approval per 45 CFR 46 guidelines of U.S. Government. Human cadaver eyes were obtained from the Advancing Sight Network (Alabama Eye Blank).

Tissue culture medium

RPE maintenance medium (RPE-MM) components: modified Eagle’s medium alpha-modified medium (Sigma-Aldrich, M-4526) supplemented with N-2 (1% v/v; Gibco, 17520-048), glutamine, penicillin and streptomycin (1% v/v; Sigma-Aldrich, G1146), non-essential amino acids (5 ml, Sigma-Aldrich, M7145), taurine, hydrocortisone and triiodo-thyronin cocktail dissolved in Dulbecco’s phosphate-buffered saline (PBS) (125 mg, 10 µg, 0.0065 µg, Sigma-Aldrich, T-0625, H-0396, T-5516) and heat-inactivated fetal bovine serum (FBS) (5% v/v, Sigma-Aldrich, F4135). iPSC endothelial cell medium (iCell Media) consists of VasuLife basalm medium (Thermo Fisher, LT-0003), growth factors from the VEGF LifeFactors supplement kit (LifeLine Cell Technologies, LS-1020), and iCell endothelial cell medium supplement kit (Cellular Dynamics International, MI019). FBS and gentamycin were excluded from the LifeFactors supplement kit. All medium was filtered through sterile 0.22-µm pore filters before use.

Cell culture

Human microvascular pericytes (Angio-Proteomie, cAP-0029) were cultured on 75-cm² flasks (Thermo Fisher, 156499) coated with quick coat solution (3 ml, Angio-Proteomie, cAP-01). Pericytes were passaged up to three times before bioprinting. iCell endothelial cells (Cellular Dynamics International, R1022 and ECC-010-100-001) and GFP-expressing human retinal endothelial cells (Angio-proteomie, cAP-0010GF) were cultured on 75-cm² Flasks (Thermo Fisher, 156499) coated with FIBRONECTIN (Gibco, 33016-015) or quick coat solution (Angio-proteomie, cAP-01) in iCell medium with medium changes occurring every other day. Cells were passaged at >70% confluence by incubating in 0.25% Trypsin-EDTA (1×, Gibco, 25200-056) for 5 min at 37 °C, resuspended in iCell complete endothelial medium and centrifuged at 200 RCF for 5 min. Cells were passaged up to two times before bioprinting.

To collect primary fetal choroidal fibroblasts, human fetal eye globes were dissected, and choroid tissue was scraped using blunt forceps into 10 ml of 0.25% Trypsin-EDTA at 37 °C. Vial was vigorously shaken to complete tissue separation until no visible tissue parts were observed. Cells were collected by centrifugation at 950g for 5 min, resuspended in fresh 15% FBS containing RPE-MM and seeded onto 75-cm² Flasks. Medium change was performed three times a week using 5% FBS containing RPE-MM until cells reached confluency for use in experiments or were cryopreserved.

iPSC-RPE (Cellular Dynamics, iCell-RPE) were seeded at a density of 2.5 x 10⁵ cells per well on a VITRONECTIN-coated transwell membrane (0.5 mg ml⁻¹; Thermo Fisher, A31804; Corning, 3407). Medium was changed every other day. The RPE was transferred to a sterile Hamilton Gastight syringe (Hamilton, 1750), sealed and placed at 4 °C for 12–15 min with the dispensing side facing up. NovoGen MMX Bioprinter (Organovo) or 3D Discovery (regenHU) was used to extrude the bioink onto scaffolds. One hundred and seventy microliters of the 5 mg ml⁻¹ THROMBIN (10 U ml⁻¹) every 2 days with APEL-2Li supplemented with VEGF-165 (50 ng ml⁻¹) and SB431542 (10 µM, Tocris). After 7 days of differentiation, cells were treated with CTS TrypLe (Thermo Fisher) and stained with CD31 antibody tagged with microbeads (Miltenyi Biotec) as the manufacturer recommended. LS columns were used to purify CD31-positive population and plated on FIBRONECTIN coated flasks (10 µg ml⁻¹) in EGM2 complete medium (Millipore) supplemented with VEGF-165 (50 ng ml⁻¹), bFGF (20 ng ml⁻¹), and CTGF (10 ng ml⁻¹) all from R&D Biosystem. After 1 week of culture, iPSC-EC were cryopreserved until used for 3D bioprinting.

Biodegradable scaffold and bioink

Scaffolds used to support 3D-oBRB were assembled in commercially available transwell inserts (Corning, 3407). The transwell membranes were removed and replaced with 12-mm diameter circular sheets of biodegradable PLGA scaffold (Polysciences, 052218-6; Biorad). The thickness of PLGA scaffolds was between 4–10 µm, fiber diameter between 400–500 nm, pore sizes ranging from 800 nm to 1 µm, and inherent viscosity between 0.6–1.0 dl g⁻¹. For snapwell and 24-transwell format (Corning, 3407 and 3470), transwell frames were diced into Kwik-Cast silicone gel (World Precision Instruments, KWIK-CAST). After removing membrane, and the PLGA scaffolds were attached to the frames. For HTS 24-transwell format, preassembled transwell with PLGA scaffold can be purchased (Biosurfaces, WP24-001/100). Afterward, O-rings (inner diameter = 10.4 mm, outer diameter = 14.5 mm), 3D printed using an Ultimaker2+ 3D Printer (Ultimaker) treated on its inner surface using Kwik-Cast gel, were attached to the basal side of the modified transwells. The gel solidified in 10 min. Immediately attaching the scaffolds to the transwell frame. The scaffolds were coated on the apical side with VITRONECTIN and incubated for an hour at room temperature and an additional hour at 37 °C. VITRONECTIN was aspirated, and 4 ml cell-culture-grade water was added to the bottom of the scaffold in well plates and incubated at 37 °C for 2 h. Following incubation, the water was aspirated, and the scaffolds were dried overnight.

On the day of bioprinting, a 2.5 mg ml⁻¹ FIBRINOGEN solution (Sigma-Aldrich, F3879) was prepared in Dulbecco’s PBS (calcium and magnesium free, Thermo Fisher, 14190144) to encapsulate cells during choroid printing. APIOTININ (0.075 U ml⁻¹, Sigma, A4529) was added to the FIBRINOGEN solution, and the entire solution was filtered through a sterile 0.22-µm filter. Following filtration, a 300-ng sample of Novogel 2.0 (Organovo 3D Bioprinting Solutions, NVG-2.0) or Gelatin powder (0.3g; Sigma-Aldrich, G1890) was added to 5 ml of the FIBRINOGEN solution. The solution was incubated in a 37 °C water bath to dissolve the Novogel or Gelatin powder. After dissolution, the completed 2.5 mg ml⁻¹ FIBRINOGEN solution was incubated at 37 °C for 1 to 2 h before cell encapsulation. A 5.0 mg ml⁻¹ FIBRINOGEN solution was prepared identically to the 2.5 mg ml⁻¹ solution above without the addition of Novogel or Gelatin powder. This solution was placed at 4 °C until bioprinting.

Bioprinting of ‘choroid’ and 3D-oBRB maturation

Immediately before bioprinting, scaffolds were treated in a Plasma Etch PE-50 oxygen-plasma etcher at maximum power (150 W, Plasma Etch) for 30 min under 10 CC min⁻¹ O₂ flow to sterilize and improve surface hydrophilicity. Fibroblasts, endothelial cells, and pericytes were trypsinized from cell culture flasks. Cells were mixed at the following densities in the 2.5 mg ml⁻¹ fibrinogen solution: 1.0 to 2.0 x 10⁶ fibroblasts per milliliter; 5.0 to 7.0 x 10⁵ ECs per milliliter; and 5.0 to 7.0 x 10⁴ pericytes per milliliter, centrifuged at 500 r.c.f. for 4 min and resuspended in 2.5 mg ml⁻¹ FIBRINOGEN solution using sterile spatula (Corning, 3004) to prevent formation of air bubbles. Cell suspension was transferred to a sterile Hamilton Gastight syringe (Hamilton, 1750), sealed and placed at 4 °C for 12–15 min with the dispensing side facing up. NovoGen MMX Bioprinter (Organovo) or 3D Discovery (regenHU) was used to extrude the bioink onto scaffolds. One hundred and seventy microliters of the 5 mg ml⁻¹ THROMBIN (10 U ml⁻¹)
was then added directly to the printed structure to form a supporting structure between printing lanes. Gel solidified within 25 min. Printing medium, 1:2 ratio of iCell medium and serum-free RPE-MM plus 1.6% heat-inactivated FBS, TROMBIN (0.5–1 U ml⁻¹, Sigma, T6884), rh ANG-1 (100 ng ml⁻¹, R&D Systems, 923-AN), VEGF-165 (85 ng ml⁻¹, R&D systems, 293-VE-500), and APRORTIN, was added to the well (0.5 ml apically, 3.5 ml basally) and placed at room temperature for 2 h. Tissues were placed in a 37 °C, 5% CO₂ incubator overnight.

RPE cells (2.5 × 10⁶) were seeded on the apical side of the scaffold on day 7 after bioprinting in RPE-MM. Tissues were fed on the apical and basal sides using the printing medium without THROMBIN, from day 2 to day 7. On day 7, the apical side of the tissues were fed using RPE-MM, while the basal sides of the tissues were fed using vascular growth medium with APRORTIN, excluding ANG-1 and VEGF. On day 14, APRORTIN was removed from vascular growth medium. On day 21, RPE-MM was supplemented with Prostaglandin E2 (100 µM). From day 29 onward, basal side contained vascular maintenance medium.

Detailed protocol is available in Supplementary Information.

Modeling wet and dry AMD
For wet AMD, ML228 (2 µM) was added to the apical side of RPE for 14 days or co-treated with Bevacizumab (0.284 mg ml⁻¹, Genentech) after 4 days of single ML228 treatment. For dry AMD, CC-HS (5%) was added to both apical and basal sides of the tissue with medium changes everyday for 14 days.

TER measurements and tissue fixation
TER was measured using EVOM2 Epithelial Volt/ohm meter (World Precision Instruments, 300532) and an Endohm-245NAP well container (World Precision Instruments). Tissues were fixed in 4% paraformaldehyde in 1× PBS at 4 °C overnight, washed 3× for 10 min in 1× PBS and processed for histological analysis. Samples were permeabilized using in 0.5% Triton X-100 (Thermo Fisher, 85112) solution in 1× PBS, punched, transferred to a 24-well plate, and blocked in 5% goat serum, 0.1% Triton X-100 and 1% bovine serum albumin (BSA) in 1× PBS for 1 h at room temperature, washed 3× for 10 min in 1× PBS.

Microscopy
Time-lapse images of GFP-labeled ECs were performed using an EVOS Auto FL (Thermo Fisher). Images for quantification were taken using Leica TCS-SP8 (Leica) and Zeiss LSM710 (Zeiss) confocal microscopes. Three-dimensional reconstructed images were taken by SP8 confocal microscope (Leica). TEM images were taken using a JEM-1010 microscope (JEOL).

Paraffin sectioning
Fixed tissue were permeabilized for 30 min in 0.5% Triton X-100 (Thermo Fisher, 85112), punched out, blocked in 5% goat serum, 0.1% Triton X-100 and 1% BSA in 1× PBS for 1 h at room temperature, washed 3× for 10 min in 1× PBS and processed for paraffin embedding and sectioning.

Vibratome sectioning
Fixed tissues were embedded in 6.8% w/v Type 7-A Agarose (Sigma-Aldrich, A0701), cut into 100 µm sections on the Leica VT1200S vibratome (Leica), and processed as described above.

Immunostaining
Primary antibodies were diluted in 1× PBS containing 0.1% Triton X-100 and 1% BSA and 3D-oBRR tissues, or iPSC-RPE monolayers were incubated at 4 °C overnight. All antibodies are listed in Supplementary Table 2. Blocking controls were performed using primary antibody buffers without the use of primary antibodies and just using secondary antibodies. Samples were washed 3× for 10 min in 1× PBS. Secondary antibodies were diluted (PBS containing 0.1% Triton X-100 and 1% BSA, and incubated with Hoescht 33342 nuclear stain in the dark at room temperature for 4 h. Samples were washed three times for 10 min in 1× PBS. Cryosectioned, Parafilm-sectioned and whole tissue samples were mounted using Fluoromount G (Southern Biotech, 0100-01).

Angiogenesis quantification
Six-line probes, numbered 1 to 6, were created in the acellular spaces between printed stripes. Fluorescence intensity peaks with a threshold (100 in 8-bit gray scale) that overlapped with the line probes were quantified in each image using MATLAB version 2019b (Mathworks). Counted peaks from each probe were averaged for each image. An angiogenesis index was developed using MATLAB to quantify mean vessel length perpendicular to the printed stripes. Regions of interest were defined in acellular spaces as a space within 250 pixels of a line probe generated through the center of the spaces. Acellular spaces that overlapped with out-of-focus endothelial cell clusters were excluded from analysis. Vessels were defined as objects that were greater than 20 pixels in length and expanded to within a mean distance of 85 pixels of the line probe. Angiogenesis index was calculated using maximum feret angle of each blood vessel relative to the line probe and maximum feret diameter of each blood vessel.

FELS immunostaining quantification
CD31-labeled images taken 1 week after bioprinting were contrasted by isolating the brightest 80% of pixels (Leica TCS-SP8 and Zeiss LSM710) from the background using ImageJ. For the rest of the time points, CD31-labeled images were contrasted with isolating the brightest 20% of pixels from the background. FELS-labeled images were contrasted with separating the brightest 5% of pixels from the background at all time points. The area ratios were taken between FELS-positive area per image and the combined area of CD31- and FELS-positive signal to quantify the area of CD31 that was co-occupied by FELS.

VEGF immunostaining quantification
z-stacks of the VEGF-A-stained cryosectioned tissue slices were analyzed using ImageJ. Images were converted into maximum-intensity projections before quantification. For image quantification, a Gaussian Blur Filter (radius 4.0 pixels) was applied to reduce noise in the images. The average pixel brightness along the thickness of the slice was quantified for each slice to identify areas of high VEGF concentration in the slices. Phalloidin-647 staining was used to identify the apical (RPE) and basal (choroid) ends of the tissue slices.

Cytokine quantification
Apical and basal media were collected at 48-h, 96-h, and 2-week time points and frozen at −80 °C until the assay was performed. Once thawed, the samples were centrifuged at 950 r.c.f. for 5 min to remove possible cell debris from sample supernatant. The assay was carried out using a Milliplex MAP Kits per the manufacturer’s instructions (Millipore-Sigma, HCYTMAG).

Vascular density quantification
Three-dimensional reconstruction of tissues was conducted on confocal images taken with 20× or 25× magnification, and area fractions of CD31 expression were calculated in each focal plane. For estimating distance from RPE region, the beginning focal plane of vasculature overlapped with out-of-focus endothelial cell clusters were excluded from analysis. Vessels were defined as objects that were greater than 20 pixels in length and expanded to within a mean distance of 85 pixels of the line probe. Angiogenesis index was calculated using maximum feret angle of each blood vessel relative to the line probe and maximum feret diameter of each blood vessel.

Quantification of extracellular matrix deposition
z-stacks of ECM images from the apical side of RPE to the basal side of RPE were taken with the same microscope settings among the
treatment groups. Mean intensity of ELASTIN and COLLAGEN IV expression were measured using ImageJ (1.53q).

**scRNA-seq**

Cell isolation of 2D populations was performed using TrypLE (Gibco). ECs and pericyte cultures were treated at 37 °C for 5 min, fibroblast for 10 min, and RPE cells for 30 min. Cell isolation from 3D-oBRB tissues was performed using 1.5 mg ml⁻¹ Collagenase II (Worthington Biochemical Corporation, 1701-015), 0.5 mg ml⁻¹ DNAse I (Worthington Biochemical Corporation, 54D14997) and 0.2 mg ml⁻¹ Dispase I (Sigma, D4818) in PBS. Tissues were placed in a 50 ml conical tube, with digestion solution (10 ml per 6 tissues), shaken on orbital shaker at 37 °C for 30 min, and cells pelleted at 900g for 5 min. Resuspended cells passed through a 44-μm steril filter (EMD Millipore) to remove cell clumps prior to library preparation. Eleven samples were sent to the NHLBI genomic core for scRNA-seq (Supplementary Table 1).

**scRNA library preparation**

Single-cell suspensions were loaded on a Chromium Single Cell 3’ Platform (10X Genomics) with v3 reagent to generate single-cell gel bead-in-emulsions (GEMs) and barcoding. GEMs were transferred to PCR 8-tube strips, and GEM-reverse transcription was performed in a C1000 Touch Thermal Cycler (BioRad). GEMs were lysed in recovery buffer, and single-stranded cDNA was cleaned up using silane DynaBeads (Thermo Fisher). cDNA was amplified in a C1000 Touch Thermal Cycler (BioRad) and cleaned using the SRPSelect Reagent (Beckman Coulter). Quality control and quantification were performed using a High Sensitivity D5000 ScreenTape Assay (Agilent) on a 4200 TapeStation System (Agilent). Library construction was performed by fragmentation at 32 °C for 5 min, end repair, and A-tailing at 65 °C for 30 min. Double-sided size selection was done using the SRPSelect Reagent (Beckman Coulter). Adapter ligation was done at 20 °C for 15 min and cleaned up using the SRPSelect Reagent (Beckman Coulter). Sample indexing was done using the 17 Sample Index Plate (Chromium) in a C1000 Touch Thermal Cycler (BioRad) and double-sided size selection done using the SRPSelect Reagent (Beckman Coulter). Quantification was done using a High Sensitivity D1000 ScreenTape Assay (Agilent) on a 4200 TapeStation System (Agilent). Sequencing libraries were quantified by quantitative PCR using the KAPA library quantification kit for Illumina platforms (KAPA Biosystems) on a QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher). The libraries were sequenced on an Illumina NovaSeq6000.

The cellranger software package from 10X Genomics (v.6.1.1) was used to demultiplex and map raw data to human reference genome (GRCh38-2020-A) and generate raw BCL files. Cell line data was analyzed in the Seurat R package⁵⁴ (Dihub; Seurat 4.0.5; R4.1.2). Low-quality cells (<200 genes, <400 UMI, <0.8 gene complexity (log10GenesPerUMI) and >0.2 mitochondrial ratio) were filtered out from analysis. Genes which are expressed in ten or more cells were analyzed. Doublets were removed by running DoubletFinder R package⁴⁹. Filtered data then normalized, scaled and log-transformed with Seurat Normalize Data and Scale Data functions. Data visualizations were made in RStudio (v.2022.02.3+492) and with the ggplot2 package (3.3.5). Gene set enrichment analysis was performed using GSEA v.4.0.3 (Broad Institute), the gene set databases C2 and C5 (MSigDB v.6.2), which are the curated set databases most enriched with an adjusted P-value of <0.05 and an average log2 fold change >1.0 to be enriched in EC and RPE scRNA-seq phenotypes for 2D- versus 3D-oBRB cells were coded as 0 and 1, respectively, for a test of enrichment by ranked differential gene expression. Significantly enriched phenotypes were selected on the basis of whether there was a nominal P-value of <0.05 and a false discovery rate <25% between 2D- and 3D-oBRB datasets. log2 ratio of classes was used for the ranking metric and otherwise default parameters were used.

**Gene list construction**

The ECM gene list was created by finding the Gene Ontology annotation for ‘extracellular matrix’ (http://wwwinformatics.jax.org/go/term/GO:0003102), then narrowing that list to genes that are also RPE characteristic genes and genes found in the CYCLOPS database to be expressed in the tissues ‘RPE fetal’ and ‘RPE adult’ at levels >2× than were found in at least 70 other tissues in the database. Selected genes must also exceed a minimum Transcripts Per Million (TPM) threshold of 100. Afterward, the gene list was reduced to those pertaining to ECM proteins, matrix metalloproteinases, tissue-inhibitor matrix metalloproteinases, and ECM crosslinking proteins. EC signature genes were derived from literature searches related to choroidal maturation, arterial and venous specification, as well as angiogenesis. Fibroblast and pericyte gene lists were derived from literature searches as well being based on expression patterns found in adult retina samples.

**UMAP cluster analysis**

Statistical comparisons between 2D- and 3D-oBRB gene expression were performed in RStudio (v.2022.02.3+492) using the Seurat Gene Expression Analysis Package and EnrichR. Single-cell samples were filtered to exclude samples with fewer than 500 transcripts detected, fewer than 200 genes detected, and greater than 5% mitochondrial genome content. Quantified genes needed to be expressed in at least ten cells. Sample complexity was selected to be greater than 0.80 log10 genes per unique molecular identifier. Doublets were excluded using the DoubletFinder package⁴⁹. Afterward, samples were clustered using UMAP. Genes that were considered enriched in 3D-oBRB with RPE EC, pericyte, and fibroblast populations versus corresponding 3D-oBRB no RPE and/or 2D populations had adjusted P < 0.05 and an average log fold change of >1. For RPE populations, genes were considered enriched with an adjusted P < 0.05. Genes of interest were evaluated for biological activity using geneCards⁴⁶.

**Comparison to literature**

To compare our gene expression data to literature sources, key signature genes published in Voigt et al. were selected on the basis of an adjusted P < 0.05 and an average log, fold change >1.0 to be enriched in EC, CC, pericyte, and fibroblasts from human patient retinal samples. Similar selection criteria were used to select key signature genes published in Kasyakova et al. that were enriched in 2D human lung fibroblasts. Gene expression counts of the published enriched genes in 3D-oBRB models were performed using the Seurat gene analysis package. Genes that were found to be enriched within cell types of interest in the 3D-oBRB models compared to other 3D-oBRB cell types were selected and displayed on heat maps that were generated using the Pheatmap package (R package v.1.012).

**Statistical analysis**

All the data analyzed were unpaired (that is, the samples were independent from each other). One-way and two-way ANOVA was performed with Tukey’s post hoc multiple comparisons. Unpaired or paired t-tests were used to find significant differences between two single conditions. All statistical tests were two-tailed (two-sided tests). All statistical analyses were performed using Graphpad Prism (8.2.0; GraphPad Software) or Microsoft Excel. P < 0.05 was considered significant. Data are presented as mean ± s.e.m. (Supplementary Table 4).
Reporting summary
Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability
The scRNA-seq data generated in this study have been deposited in the Gene Expression Omnibus database under accession code GSE214928. These RNA-seq data are openly available without any restriction. All the processed data are available within the article. All the raw data generated in this study are provided in Source Data files. Source data for Fig. 6 are available in a figshare repository at https://doi.org/10.6084/m9.figshare.21300198. Source data are provided with this paper.

Code availability
Angiogenesis quantification was performed on MATLAB version 2019b (Mathworks). The custom MATLAB code is available in Supplementary Information.

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Author contributions
Conceptualization: K.B., M.J.S, and M.F.; methodology: K.B., R.Q., E.N, M.J.S, T.V., I.S., and M.F.; Investigation: R.Q., E.N, M.J.S, C.H., R.S., T.S.P., C.K., C.T., C.W., A.S., R.D., D.B., P.D., K.D., S.M., G. C., M.B., A.M., and F.B.; analysis: Y.-C.C., K.B., R.Q., E.N, and M.J.S.; project administration: R.Q., E.N, and M.J.S; writing, review, and editing: K.B., E.N, M.J.S, and M.F; funding acquisition: K.B. M.F., I.S., and M.J.S.; resources: K.B. and M.F.; supervision: K.B. and M.F.

Competing interests
The authors have an approved patent on this technology in Australia (#AU2017359330B2) and a pending patent in the US (#US20190290803A1).

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41592-022-01701-1.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41592-022-01701-1.

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Extended Data Fig. 1 | Pericytes colocalize to capillaries. a-c, Pericytes immunostained with NG2 (a, green), α-SMA (b, green), and PDGFR-β (c, green) and ECs immunostained with CD31 (magenta). (n = 3), scale bars, 30 µm.
Extended Data Fig. 2 | ECs, pericytes, and fibroblasts are essential for formation of dense and stable capillary-bed. a, b, EC only bioink; c, d, EC + pericyte bioink; e, f, EC + fibroblasts bioink; g, h, EC + pericyte + fibroblasts bioink 7 days (a, c, e, g) and 10 days (b, d, f, h) after bioprinting. Tissues fixed at analyzed for GFP expression (green) (n = 4). Scale bars, 500 µm.
**Extended Data Fig. 3 | Time course of fenestration marker expression in 3D-oBRB.** a–d, 3D vascular growth within tissues fixed at week 1 (a), week 2 (b), week 3 (c), and week 4 (d). Tissues were immunostained with FELS (green) and CD31 (magenta). Scale bars, 50 µm. n = 3.
Extended Data Fig. 4 | ECM protein expressions in 3D-oBRB. a–f, 3D reconstructed images of tissues immunostained for CD31 (magenta, a–f), nuclei (blue, a–f), LAMININ (LMN, green a, b), ELASTIN (ELN, green, c, d), COLLAGEN IV (COL IV, green, e, f), viewed at the subRPE level (a, c, e) and choriocapillaris level (b, d, f). N = 3. g, cross section of 3D-oBRB immunostained for Bruch’s membrane proteins LAMININ (yellow) and COLLAGEN IV (COL IV, magenta). Nuclei stained with DAPI (cyan). N = 3. Scale bar, 10µm.
Extended Data Fig. 5 | Time course study of dry AMD phenotype induction in 3D-oBRB. a-l, Nile red (yellow), anti-CD31 (magenta), and Hoechst (nucleus, blue) stained en face images of RPE (a-f), RPE-proximal choriocapillaris (g-l), and RPE-distal choriocapillaris (m-r) from 3D-oBRB treated with complement incompetent human serum (CI-HS, a, c, e, g, i, k, m, o, q) complement competent human serum (CC-HS, b, d, f, h, j, l, n, p, r) for 2, 4, and 7 days. Scale bars, 60 µm (a-l), 300 µm (m-r) n = 3. s-x, Nile red (yellow), anti-CD31 (magenta), and Hoechst (nucleus, blue) stained lateral view 3D rendered images of 3D-oBRB, treated with CI-HS (s, u, w) or CC-HS (t, v, x).
Extended Data Fig. 6 | ML228 treatment on 2D-iRPE and 3D-oBRB.

a-f, RPE monoculture at 48 hr (a,d), 96 hr (b,e), and 2 weeks (c,f) from the beginning of ML228 (2 μM; 96 hr) treatment, immunostained with HIF-1α (magenta), ZO-1 (green), and Hoechst (blue). DMSO used to dissolve ML228 was used as the vehicle. Scale bars, 30 μm. (n = 3)
ge, TER measurement of 2D-iRPE without or with ML228 treatment (n = 3). Data are presented as mean values +/− SEM. Two-way ANOVA, Tukey’s multiple comparisons test were used for statistical analysis.
h, ZO-1 staining based morphometry analysis of individual cell area in vehicle and ML228-treated samples was performed. (n = 1644, number of cells). Data are presented as mean values with standard deviation. ANOVA and Tukey’s multiple comparisons test were used for statistical analysis.
Extended Data Fig. 7 | Activation of HIF-1α in RPE induces type-I CNV-like phenotype in 3D-oBRB. a–h, immunostaining of tissues with anti-CD31 (magenta), anti-HIF-1α (yellow) antibodies, and staining for F-ACTIN (cyan), and nuclei (blue). Tissues were treated with either vehicle (DMSO, a,e), or ML228 (2 µM) on apical side of RPE only (b,f), or basal side of choroid only (c,g), or both sides (d,h). Red arrowheads indicate type-I CNV-like phenotype, white arrowheads indicate examples of HIF-1α translocation to the nuclei. (n = 3), scale bars, 50 µm. i, TER measurements normalized to ctrl. (n = 6). Data are presented as mean values +/- SEM. ANOVA and Tukey’s multiple comparisons test. j, k, pseudo 3D-projected side views of 3D-oBRB immunostained with anti-CD31 (magenta), anti-COL IV (yellow), anti-LAMININ (cyan) antibodies, and nuclei (blue). Tissues were treated with either vehicle (DMSO, j), or ML228 (2 µM) on apical side of RPE only (k). Arrowheads indicate type-I CNV-like phenotype in ML228-treated (k) tissues with choriocapillaris (CC) penetrating through the Bruch’s membrane (BM). (n = 3), scale bars, 10 µm.
Extended Data Fig. 8 | STAT3 overexpression in the RPE induces type-I CNV-like phenotype in 3D-oBRB. a–l, en face views (a–h), and pseudo 3D-projected side views (i, j) of 3D-oBRB immunostained at week 6 with anti-CD31 (magenta), anti-STAT3 (yellow) antibodies, and stained for nuclei (blue), containing wildtype iRPE (a–d, i), STAT3 overexpressing iRPE (e–h, j). BM = Bruch’s membrane, CC = choriocapillaris. N = 3. Scale bars, 50 µm (a–c, e–g), 10 µm (i, j). k, TER measurements normalized to control from 2D-iRPE monoculture, (N = 8). Data are presented as mean values +/- SEM. Unpaired t-test was used for statistical analysis.
Extended Data Fig. 9 | Bevacizumab suppresses type-I CNV-like phenotype in 3D-oBRB. a-c, Images of deep choroidal regions of (a) vehicle, (b) ML228, (c) ML228 + Bevacizumab treated 3D-oBRB, immunostained for CD31 (red) and stained for Hoechst (blue). Scale bars, 350 µm. (n = 4). d–h, en face views (d–g), and pseudo 3D-projected side views (h) of STAT3 overexpressing 3D-oBRB treated with Bevacizumab immunostained at week 6 with anti-CD31 (magenta), anti-STAT3 (yellow) antibodies, and stained for nuclei (blue). BM – Bruch’s membrane, CC – choriocapillaris. N = 3. Scale bars, 50 µm (d–g), 10 µm (h). i, TER measurements normalized to control of STAT3 overexpressing 3D-oBRB and STAT3 overexpressing 3D-oBRB treated with Bevacizumab. (n = 4). Data are presented as mean values +/- SEM. One-way ANOVA and Tukey’s multiple comparisons test were used for statistical analysis.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection  Commercial imaging softwares associated with Leica (TCS-SP8) and Zeiss (LSM710) microscopes were used for data collection

Data analysis  ImageJ (1.53q), MATLAB (2019b), Prism (8.2.0), and Excel (16.0.13001.20266) were used for data analysis. Details of custom codes is provided in the Methods section. Program code is available from the corresponding author upon request.

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The scRNAseq data generated in this study have been deposited in the GEO database under accession code GSE214928. These RNAseq data are openly available without any restriction. All the processed data are available within the article. All the raw data generated in this study are provided in the Supplementary Information/Source Data files. Source data file for figure 6 is available in a figshare repository at 10.6084/m9.figshare.21300198. Angiogenesis quantification code is available in supplementary information.
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| Sample size | No predetermined sample size calculations were performed. To ensure data reproducibility, minimum of three independent experimental replicates were performed. Statistical analysis was performed using two-way ANOVA or unpaired t-test. Both of these methods require a minimum of three samples in each treatment group. |
| Data exclusions | No data was excluded from the analysis |
| Replication | All experiments were replicated by independently by the first three authors of the paper. |
| Randomization | 3D-oBRB were randomly selected into different treatment groups for dry and wet AMD modeling experiments. The data and analysis for this study is objective and not prone to researcher's bias. |
| Blinding | Blinding was done during the treatment of different groups. One operator prepared cell culture medium for the treatment and the other operator treated samples. For analysis, sample names were masked when possible. |

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| [ ] Dual use research of concern | |

#### Antibodies

- **Primary antibodies:** Mouse anti-PLVAP (FELS, 1:50, Abcam, Cat# AB81719); Rabbit anti-CD31 (1:50, Abcam, Cat# AB28364); Mouse anti CD31 (1:50, Agilent, Cat# M0823); Rabbit anti-Laminin (1:50; Abcam, Cat# AB11575); Mouse anti E-cadherin (1:100; Abcam; Cat# AB40772); mouse anti-ZO-1 (1:100; Thermo Fisher Scientific, Cat# 33-9100); rabbit anti-ZO-1 (1:50; Thermo Fisher Scientific, Cat# 61-7300) Mouse anti-Collagen-IV (1:50; Abcam, Cat# AB6311); Rabbit anti-Elastin (1:50 Abcam Cat# AB21610); Rabbit anti-APOE (1:50; Abcam, Cat# AB52607); Rabbit anti-VEGF (1:50; Thermo Fisher Scientific, Cat# F807); Rabbit anti-VWF (1:100; Dako, Cat#GA52761-2); Mouse anti-CD31 (1:100, Dako, Cat# M0823); Rabbit anti-ETV2 (1:100, Abcam, Cat# AB181847); Mouse anti-aSMA (1:200, Sigma Aldrich, Cat# F3777); Mouse anti-NG2 (1:100, Thermo Fisher, Cat# 14-6504-B2); Rabbit anti-PDGFR-b (1:100, Abcam, Cat# AB32570); Rabbit anti-Collagen-I (1:100, Thermo Fisher, Cat# PAS-95137); Rabbit anti-Vimentin (1:100, Abcam, Cat# AB92547); Rabbit anti-Fibronectin (1:100, Abcam, Cat# AB2413)
- **Secondary antibodies:** Alexa-fluorTM Goat anti Rabbit 647 (1:2000, Thermo Fisher, Cat# A21244); Goat anti Rabbit 594 (1:2000, Thermo Fisher, Cat# A11012); Goat anti Rabbit 555 (1:2000, Thermo Fisher, Cat# A23732); Goat anti Rabbit 488 (1:2000, Thermo Fisher, Cat# A11008); Goat anti mouse 647 (1:2000, Thermo Fisher, Cat# A32728); Goat anti mouse 594 (1:2000, Thermo Fisher, Cat# A11032); Goat anti mouse 555 (1:2000, Thermo Fisher, Cat# A21422); Goat anti mouse 488 (1:2000, Thermo Fisher, Cat# A32723);

**Validation**

Validation statement is described in each manufacturer’s website including antibody information and citations. Blocking controls were performed using primary antibody buffers without the use of primary antibodies and just using secondary antibodies.
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
Human placental microvascular pericytes (Angio-Proteomie, Cat# cAP-0029, Boston, MA), iCell endothelial cells (Cellular Dynamics International, Cat# R1022), iCell Retinal Pigment Epithelial Cells (Cellular Dynamics International, Cat# R1101), Choroidal fibroblasts (Alabama Eye Bank), Primary Human Retinal Microvascular Endothelial Cells (Cell systems, Cat# ACBRI 181), GFP Expressing Human Retinal Microvascular Endothelial Cells (Angioproteomie, Cat# cAP-0010GFP)

Authentication
All cell lines were authenticated with immunostaining with markers. Data is provided in Fig. S1.

Mycoplasma contamination
All cell lines were routinely tested to be negative for mycoplasma contamination

Commonly misidentified lines
n/a
(See ICLAC register)

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
n/a

Wild animals
n/a

Field-collected samples
n/a

Ethics oversight
n/a

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics
Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write “See above.”

Recruitment
Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

Ethics oversight
Identify the organization(s) that approved the study protocol.

Note that full information on the approval of the study protocol must also be provided in the manuscript.