**Novel Devices for Studying Acute and Chronic Mechanical Stress in Retinal
Pigment Epithelial Cells**

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Abstract

Choroidal neovascularization (CNV) is a major cause of blindness in patients with age-related macular degeneration (AMD). Overexpression of vascular endothelial growth factor (VEGF), a potent angiogenic protein, by retinal pigment epithelial (RPE) cells is a key stimulator of CNV. Mechanical stress occurs during different stages of AMD and is a possible inducer of VEGF expression in RPE cells. However, robust and realistic approaches to studying acute and chronic mechanical stress under various AMD stages do not exist. The majority of previous work has studied cyclic stretching of RPE cells grown on flexible substrates, but an ideal model must be able to mimic localized and continuous stretching of the RPE as would occur in AMD *in vivo*. To bridge this gap, we developed two *in vitro* devices to model chronic and acute mechanical stress on RPE cells during different stages of AMD. In one device, high levels of continuous mechanical stress were applied to focal regions of the RPE monolayer by stretching the underlying silicon substrate to study the role of chronic mechanical stimulation. In the second device, RPE cells were grown on porous plastic substrates and acute stress was studied by stretching small areas. Using these devices, we studied the effect of mechanical stress on VEGF expression in RPE cells. Our results suggest that mechanical stress in RPE cells induces VEGF expression and promotes *in vitro* angiogenesis. These results confirm the hypothesis that mechanical stress is involved in the initiation and progression of CNV.

1. Introduction

Retinal degeneration due to abnormal neovascularization occurs in many ocular diseases, including age-related macular degeneration (AMD).^{1,2} AMD is classified into two major types: dry (non-neovascular) and wet (neovascular). Dry AMD is characterized by the formation of

lipid deposits (known as drusen) under the retinal pigment epithelium (RPE).^{3,4} In wet AMD, damage to retinal layers is caused by newly-formed blood vessels that invade the retina in a condition known as choroidal neovascularization (CNV), which can lead to irreversible vision loss.^{5,6} The choroid contains the vasculature in the healthy eye and is separated from photoreceptors by two intermediary layers: Bruch's membrane (BrM) and the RPE (Figure 1). Photoreceptors are central to vision and their primary function is to transduce light into neural signals.⁷ RPE cells play an essential role in maintaining photoreceptor function by providing nutrients and growth factors and by phagocytosing photoreceptor outer segments.⁸ Under normal conditions, the RPE also regulates the growth of new blood vessels in the choroid via a repertoire of pro- and anti-angiogenic factors.⁹⁻¹² The initiation and progression of CNV is believed to be driven primarily by the elevated expression of pro-angiogenic proteins in the RPE.^{10,13-15}

Figure 1. Schematic of the photoreceptors-RPE-choroid complex. (A) Healthy retina. (B) Chronic mechanical stress to RPE cells due to drusen formation in dry AMD. (C) Acute mechanical stress to RPE cells due to the invasion of blood vessels during CNV in wet AMD.

Vascular endothelial growth factor (VEGF) is the most commonly implicated potent pro-angiogenic factor in stimulating CNV initiation and progression. Normal levels of RPE-derived VEGF are essential for maintaining the vasculature of the choroid. However, increased secretion of VEGF by RPE cells can result in CNV.^{16–19} Although the exact causes of VEGF overexpression in RPE cells are not completely understood, several factors, such as hypoxia, oxidative stress, inflammation and exposure to blue light are known contributors.^{20–27} Components of drusen, such as amyloid β and complement factors C3a and C5a, have also been shown to induce VEGF expression.^{28,29} More recently, the loss of intercellular junctions (multi-protein complexes involved in cell-cell adhesion) of the RPE was found to elevate VEGF expression by RPE cells.^{30–32}

Another plausible contributor to CNV pathogenesis is excessive mechanical stress on the RPE, which might occur at different rates and in different stages of disease progression. One of the early signs of AMD is the formation of drusen in the BrM and under the RPE.^{33–35} While the components of drusen can induce VEGF expression in RPE cells, the increase in the size of drusen during the course of AMD can also contribute to the AMD pathogenesis by adding a continuous mechanical stress on the overlying layers (chronic stress, Figure 1.B). Drusen deposits grow in size with age and gradually elongate the RPE, often detaching it from its basal membrane.^{4,36,37} Depending on the drusen size and pattern of drusen distribution, up to 53% of dry AMD cases eventually progress to wet AMD.³⁸ The risk of developing CNV has been shown to be higher in eyes with larger drusen, suggesting a role for chronic stretching of the RPE in CNV.^{3,39,40} In the more advanced form of AMD (wet AMD), invasion by newly-formed blood

vessels, a hallmark of CNV, has already begun, so the elongation of the RPE takes place at a faster rate (acute stress, Figure 1.C), suggesting a role for acute stress.^{41–43}

While the growth in drusen size is slow and typically occurs over long periods of time (years),⁴⁴ at certain time points, faster drusen progression (months) may occur.^{44,45} The mechanical stress exerted on the RPE during CNV due to the growth of blood vessels and hemorrhage can occur within hours.⁴⁶ Evaluating the effects of stress exerted to the RPE at different rates is therefore important in understanding the precursors and eventual progression of AMD. However, robust *in vitro* testing of realistic loading scenarios remains a technical challenge. Many previous studies used vacuum-induced equibiaxial stretching of RPE cells cultured on elastic membranes.^{47–49} Other attempts to mimic *in vivo* mechanical stress include using magnetic force, elongating silicon membranes in one direction, and pushing a piston against a plastic culture dish.^{50–52} While these setups replicated many physiological and pathological conditions, they are not ideal for modeling mechanical stress-induced RPE pathologies since they only achieve a limited range of attainable strain and do not provide localized application of high levels of stress as would take place in AMD (Figure 1). The strain experienced by the RPE due to drusen growth and CNV complications, including vascularization and hemorrhage, can exceed 60%, while the strain achieved with commercially available models is limited to a maximum of 30%. Moreover, drusen development occurs chronically over long periods and a realistic model of mechanical stress in AMD must be able to replicate different rates of strain progression. Thus, there is a need for a more realistic model of RPE stretching that can replicate different rates and levels of mechanical stress.

In the present study, we developed two novel *in vitro* devices to accurately model mechanical stress under different modes: In one device, RPE cells were grown on a thin layer of polydimethylsiloxane (PDMS) which was gradually inflated to mimic chronic increase in mechanical stress during drusen formation. In another device, cells were grown on porous membranes of Transwell inserts and a pulse of mechanical stress was added to the membrane to model acute stress due to CNV. These devices provide easy-to-assemble, customizable and reliable platforms for studying mechanical stress in adherent mammalian cells. The elastic properties of the PDMS device allows high levels of stress to be conveyed to cells and the plastic properties of the Transwell membranes are suitable for static stress experiments. We used these two devices to study the expression levels of VEGF from RPE cells under varying rates of mechanical stress and to test the hypothesis that mechanical stretching of RPE cells induces angiogenesis. An *in vitro* tube formation assay was used to monitor changes in the length and junction number of endothelial tubes as an indicator of angiogenic potential of the RPE secretome. Our results showed an increase in VEGF expression and elevated angiogenic potential of RPE secretome due to both chronic and acute mechanical stress.

2. Experimental

2.1 Cell culture

ARPE-19 cells were obtained from American Type Culture Collections (ATCC, Manassas, VA). Cells between passages 12 and 14 were grown in standard tissue culture treated plates (Thermo Fisher Scientific, Visalia, CA) using Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone). Growth media were changed three times per week. Cells were kept in a humidified incubator at 37 °C in 5%

CO₂. ARPE-19 cells were grown on the PDMS film of the chronic mechanical stress (CMS) device and Transwell membranes (Corning, Corning, NY) of the acute mechanical stress (AMS) device for 10 weeks before performing mechanical stress experiments to allow RPE differentiation. To assess the angiogenic potential of RPE secretome, HUVECs (Gibco, Grand Island, NY) were grown in Medium 200 (Gibco) supplemented with Large Vessel Endothelial Supplement (Gibco) or in spent media from RPE cultures for 6 hours at 37 °C in 5% CO₂.

2.2 Fabrication of the chronic mechanical stress (CMS) device

The CMS device was developed to mimic chronic mechanical stress in the RPE. This device was assembled from four components (Figure 2). First, a PDMS block with 100 µm wide microchannels (Figure 3.C) was made using soft lithography (Figure 2.A). Briefly, a photoresist pattern was produced on a silicon wafer using SU-8 2075 (Figure 2.A), and PDMS prepolymer (Sylgard 184, Dow Corning, Midland, MI) was poured over the pattern to achieve a thickness of ~5 mm. PDMS was cured in the oven at 85 °C for 2 hours and then peeled off of the silicon wafer (Figure 2.B). Using a biopsy punch, 28 holes with a diameter of 1.5 mm (3 sets of nine holes, plus one hole for an access port) were punched into the PDMS block (Figure 2.C). Next, PDMS prepolymer was spin coated at 5000 rpm on a silicon wafer using a spin coater (CEE® model 200X) and cured at 85 °C for 30 minutes to make a PDMS film with a thickness of 36.6 ± 1.4 µm. Incubating for 30 minutes at 85 °C was sufficient to cure the PDMS film since the thickness of the PDMS film was significantly smaller than the PDMS block. Both the PDMS block and PDMS film were O₂ plasma treated and the film was permanently bound to the top of the PDMS block to cap the holes (Figure 2.D, 3.A). The capped PDMS block and a glass slide were O₂ plasma treated and the bottom of the block (microchannel side) was bound to the glass

slide (Figure 2.E). PDMS was molded and cured around three plastic cuvettes to produce the cell culture chamber walls. PDMS wells were bound to the top of the device and around the capped holes as described above (Figure 2.F). To apply mechanical stress, air was injected to the microchannels through the access port using a syringe pump to inflate the thin PDMS film (Figure 2.G, 3.D-G).

Figure 2. Fabrication of the CMS device. An SU-8 master was made using photolithography (A) and PDMS was cured from the master (B). After punching 1.5 mm (diameter) holes in the PDMS block (C), a thin PDMS membrane was bound to the top of the PDMS block (D). After attaching the device to a glass slide (E), cells were cultured on the PDMS film (F). Mechanical stress was applied to cells by inflating the PDMS film using a syringe pump (G). (H, I) Fully assembled device.

Figure 3. The CMS device. (A) The device was assembled and connected to a syringe pump using a silicon tubing. (B) Top view of the device showing through holes and the thin PDMS film (Arrow). (C) Bottom view of the device showing microchannels connecting the through holes. (D-G) Injecting air to the device using a syringe pump inflated the PDMS film. (H-K) FEA model of the inflation of the PDMS film in the CMS device. Strain level was highest on top of the inflated PDMS film.

2.3 Fabrication of the acute mechanical stress (AMS) device

The AMS device was made to apply acute mechanical stress to cells cultured on Transwell membranes (polyester, 10 μm thickness). Transwell membranes are composed of permeable plastic sheets that support polarized secretion of proteins from cells cultured on them. Apical-basolateral polarization is an important characteristic of RPE cells and can be achieved when growing cells on Transwell inserts. Four 2.5 μL pipet tips were attached to the plunger tip of a 10 mL syringe using PDMS and the barrel of the syringe was cut at the 3mL mark with a scalpel. The tips of the pipet tips had an outer diameter of $762.25 \pm 13.52 \mu\text{m}$ and an inner diameter of $369.3 \pm 18.54 \mu\text{m}$. The syringe with attached pipet tips was placed on a syringe pump. A 20 mm circle (diameter) was cut from the bottom of the wells of a 6-well plate. 24 mm Transwell inserts

with cells were transferred to the bottomless well plate and the plate was placed on the syringe pump such that the tips of the pipet tips reached the bottom of Transwell membranes. Mechanical stress was applied to Transwell membranes by controlling the movement of pipet tips with the syringe pump (Figure 4.A). Once the membranes were poked with pipet tips (Figure 4.B), they underwent plastic deformation and stretched the cells attached to them (Figure 4.C).

Figure 4. Adding mechanical stress to Transwell membranes using the AMS device. (A) Schematic of the AMS device. The syringe pump pushes the pipet tips against the Transwell membranes placed upside down on a scale. Scale is used to measure the pressure (stress) applied to membranes (B) Stretching a 24 mm Transwell membrane using a pipet tip. (C) Plastic deformation of the Transwell membrane 1 hour after poking the membrane with pipets. (D) FEA model of the AMS device shows the distribution of strain in Transwell membranes when poked with a pipet tip. The maximum strain level was on the side of the deformed area. (E) Transwell membrane breakage due to excessive stress. The mechanical failure occurs on the side of the deformed area (arrow), confirming the FEA results in D.

2.4 Device characterization

To determine how the RPE elongates due to CNV or drusen formation, tomography images of deformed RPE layers from primary literature were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).⁵³ Depending on drusen size and the size of the CNV

area, RPE elongation (measured as the change in the length of the elongated tissue divided by baseline length) ranged from ~10% to 60% in RPE focal regions.^{44,54–56}

Uniaxial and biaxial stress-strain curves for both devices were produced to determine whether these *in vitro* devices can replicate *in vivo* strain levels applied to the RPE during AMD (Figure 5). In the CMS device, air was gradually injected to the device to increase the strain (defined as percent increase in the length and area of the membrane). Changes in the pressure of the inner chamber of the CMS device was recorded during the injections using a manometer (Universal Electronics Inc., Taiwan). Using a stereomicroscope, the deformation of the PDMS film was monitored. The percent change in uniaxial strain was calculated by dividing the change in length of the stretched section of the film by its initial length. Biaxial strain was calculated by dividing the change in the area of membrane's deformed section by its initial area. Area calculation was performed by making 3D models of membrane deformation based on 2D images of the membranes using Ansys SpaceClaim software. Stress relaxation curves were also produced to determine the rate of air leakage from the CMS device by inflating the PDMS film of the device to the maximum achievable strain (72% uniaxial) and monitoring the deflation of the membrane. The pressure applied to Transwell membranes in the AMS device was measured by placing the membranes on a scale and applying mechanical stress using a syringe pump (Figure 4.A). To calculate the pressure added to the membranes, the readings from the scale were divided by the area of the tip of the pipet. Similar to the CMS device, percent strain values were calculated for the AMS device.

Figure 5. Mechanical properties of CMS and AMS devices. (A) Uniaxial stress-strain curve for the PDMS film ($36.6 \pm 1.4 \mu\text{m}$) of the CMS device (B) Biaxial stress-strain curve for the PDMS film of the CMS device. (C) Uniaxial stress-strain curve for the Transwell membrane ($10 \mu\text{m}$) of the AMS device. (D) Biaxial stress-strain curve for the Transwell membrane of the AMS device. Uniaxial (E) and biaxial (F) strain rates for the PDMS film of the CMS device. Uniaxial (G) biaxial (H) strain relaxation curves for the PDMS film of the CMS device. Error bars represent one standard deviation.

2.5 Finite Element Analysis

Finite element analysis (FEA) was performed using Ansys V19.1 to evaluate strain distribution on the PDMS film of the CMS and on the Transwell membrane of the AMS device. Boundary and loading conditions of the FEA models were defined as close to the conditions of the CMS and AMS devices as possible. Changes in the elastic strain levels were analyzed during the addition of 2.7 psi pressure to a $36 \mu\text{m}$ -thick PDMS model (Figure 3.D-K). A 2-parameter

Mooney Rivlin model ($C_{10}=21.03$ psi, $C_{01}=3.63$ psi, $D_1=0$ psi⁻¹) was found to accurately predict the hyperplastic behavior of the CMS device. Plastic strain in the AMS device was simulated by pushing a hollow disk with an inner diameter of 370 μm and an outer diameter of 760 μm (according to the size of the tip of the pipet tips used in the AMS device) on a 10 μm -thick Transwell model to create 1200 psi pressure (Figure 4.D).

2.6 Enzyme-linked immunosorbent assay

Spent media from control and stressed cultures of ARPE-19 cells was collected 8 and 24 hours after adding mechanical stress and assayed for VEGF expression using a human VEGF ELISA kit (Life Technologies, Frederick, MD). Absorbance at 450 nm was measured using a BioTek Synergy 2 plate reader (BioTek, Winooski, VT).

2.7 Immunocytochemistry

Immunocytochemistry (ICC) was performed on ARPE-19 cells grown on Transwell membranes of the AMS device to visualize intercellular junctions. A fluorescein isothiocyanate (FITC) - conjugated anti zonula occludens-1 (ZO-1) antibody (Invitrogen, Carlsbad, CA) was used for this purpose. Nuclei were stained with NucBlue Live ReadyProbes nucleic acid stain (Thermo Fisher Scientific). After staining, samples were imaged using an LSM 710 Carl Zeiss confocal microscope (Jena, Germany). Due to the elastic properties of the PDMS membrane of the CMS device, stretched membranes revert to their original size after the device is disconnected from the syringe pump making confocal imaging of chronically stretched cells not feasible.

2.8 Endothelial tube formation assay

Tube formation assay was performed using an *in vitro* angiogenesis kit (Gibco) according to manufacturer's instructions. Briefly, the bottom of the wells of a 48-well plate were coated with 100 μ L of reduced growth factor Geltrex matrix (Gibco) and incubated at 37 °C for 30 minutes. HUVECs were then diluted in spent media from control or mechanically stressed ARPE-19 cultures to make a concentration of 1.25×10^5 cells/mL. 200 μ L of cell suspensions were seeded on Geltrex matrices and incubated for 6 hours at 37 °C and in a humidified incubator with 5% CO₂ to induce endothelial tube formation. Next, HUVECs were stained with Calcein AM dye (Thermo Fisher Scientific) and imaged using an Eclipse TS100 fluorescence microscope (Nikon Instrument Inc., Melville, NY). Tube length and node numbers were quantified using ImageJ software.

3. Results

3.1 Replicating mechanical stress by *in vitro* devices

Applying different levels of stress to the PDMS film of the CMS device produced up to ~72% uniaxial and ~193.5% biaxial strain (Figure 5.A, B). The maximum strain achieved with the AMS device was ~12 % (uniaxial) and ~12.7 (biaxial) (Figure 5.C, D). Strains greater than the above-mentioned values for each device resulted in punctures to their membranes. Accordingly, to mimic *in vivo* RPE elongation, cells were strained from 0 to 60% (uniaxial) over 24 hours in the CMS device. Using the AMS device, acute mechanical stress (resulting in 10% uniaxial strain) was applied to cells by pushing an array of four 2.5 μ L pipet tips on the bottom of Transwell membranes 5 times, creating 20 bumps in the membrane. The rate of increase in strain level in the CMS device was characterized by measuring the uniaxial and biaxial strain every 2 hours for 24 hours and up to a maximum uniaxial strain of 60% (Figure 5.E, F). To examine

whether there was air leakage from the device, deflation of the PDMS film was monitored for 24 hours. Results showed that the strain dropped to ~6.5% (uniaxial) and ~9% (biaxial) after 24 hours due to air leakage (Figure 5.G, H). FEA results showed that in the CMS device, the top of the inflated PDMS film experiences the maximum strain level (Figure 3.H-K). The sides of the deformed Transwell membranes of the AMS device experienced the maximum strain level (Figure 4.D).

Figure 6. ELISA results show elevated VEGF expression in mechanically stressed ARPE-19 cells. (A) At 8 hours, continuous mechanical stress in the CMS device did not result in significant VEGF induction (n=6). 24 hours of 60% chronic strain resulted in a significant increase in VEGF expression of cells in the CMS device (n=6). (B) 8-hour application of 10% acute strain in the AMS device did not result in significant VEGF overexpression (n=6). (C) 10% acute strain in the AMS device significantly increased apical and basal VEGF expression (n=6). * $p < 0.05$, ** $p < 0.01$, N.S. not significant. Error bars represent one standard deviation.

3.2 Enzyme-linked immunosorbent assay and immunocytochemistry

In both the CMS and AMS devices, at 8 hours, there was no significant change in VEGF expression compared to unstretched controls (Figure 6). At 24 hours, VEGF expression increased significantly in the CMS device due to chronic mechanical stress. In the AMS device, after 24 hours, VEGF expression increased significantly in both the apical and basal chambers of Transwell membranes (Figure 6).

266 According to our ICC results, mechanically stretching the RPE by 10% (uniaxial) in the AMS
267 device disrupts intercellular junctions of ARPE-19 cells and creates breaks in the RPE
268 monolayer (Figure 7). These breaks were located on bumps created by mechanical stress on
269 Transwell membranes. RPE cells in these areas lacked intercellular ZO-1 localization. Losing
270 intercellular ZO-1 localization due to the physical disruption of the RPE monolayer correlates
271 with a statistically significant VEGF overexpression.³² To determine whether the increase in
272 VEGF expression after adding mechanical stress was due to the disruption of intercellular
273 junctions and dislocalized ZO-1, we mechanically stressed a subconfluent (2-day-old) ARPE-19
274 culture using the AMS device and quantified VEGF expression using ELISA. Results showed
275 that 10% uniaxial strain induced VEGF expression both apically and basally in subconfluent
276 cultures lacking intercellular junctions (Figure 8).

AMS (24 Hr subconfluent)

Figure 8. Mechanical stress induces VEGF expression in subconfluent ARPE-19 cells. 24-hour exposure to 10% acute strain in the AMS device significantly induced both apical and basal VEGF expression in subconfluent ARPE-19 cells (n=4). ** p<0.01. Error bars represent one standard deviation

3.3 Endothelial tube formation assay

To determine if the increase in mechanical stress can induce angiogenesis, the angiogenic potential of used media from control and stretched cells in both devices was assessed using an *in vitro* angiogenesis kit. Results demonstrate that continually applying mechanical stress within the CMS device significantly increased the length of endothelial tube-like structures and the number of junctions (nodes). While the apical media from stressed cultures in the AMS device did not significantly induce tube formation, basal media significantly increased both tube length and node number (Figure 9).

Figure 9. *In vitro* angiogenesis assay. (A) Mechanical stress in RPE cells induced endothelial tube formation by HUVECs *in vitro*. (B, C) Endothelial tube length and node number increased when HUVECs were exposed to conditioned media from mechanically stressed RPE cells using the CMS device (n=4; B) and the basal media of the stretched Transwells in the AMS device (n=4; C). Apical media of stretched Transwell cultures failed to change tube length and node numbers significantly (n=4). * p<0.05, ** p<0.01, N.S. not significant. Error bars represent one standard deviation.

4. Discussion

RPE cells *in vivo* are exposed to mechanical stress because of drusen formation and CNV during different stages of AMD. Identifying the effects of mechanical stress on VEGF expression from RPE cells can lead to a better understanding of the causes of the initiation and progression of CNV. In previous works, cyclic mechanical stress on RPE cells has been studied.⁴⁷⁻⁵⁰ However, the pulsatile stretching methodology used in the past cannot replicate chronic and acute mechanical stress in AMD. Further, the maximum tensile strain achieved by the devices employed in previous studies is limited (up to 30%), while the tensile strain exerted on RPE cells during AMD can exceed 60%.^{54-56,44} Therefore, we sought to develop novel mechanical stress devices that accurately mimic mechanical stress on the RPE during drusen formation and CNV.

The devices developed here provide a tunable platform for studying the role that mechanical stress plays in various physiological and pathological conditions. Using these *in vitro* devices, we replicated chronic and acute mechanical stress on the RPE to test the hypothesis that mechanical force can lead to CNV by inducing VEGF expression. Both CMS and AMS devices proved to be reliable, generated reproducible results and were used to study alterations in VEGF expression in response to acute and chronic mechanical stress (Figure 1).

VEGF is a key stimulator of aberrant angiogenesis in AMD.¹⁶ It has previously been reported that cyclic mechanical stress can induce VEGF expression in RPE cells.⁴⁷ However, the mechanical stress exerted on RPE cells due to drusen formation and CNV is not cyclic – it is either static or increases with varying rates. In contrast to previous models of mechanical stress, the devices fabricated here were capable of replicating high levels and different rates of mechanical stress, nearing those experienced *in vivo*. These devices more accurately mimicked the *in vivo* stretching of RPE cells during different stages of AMD to monitor the alterations in VEGF expression. According to our ELISA results, both chronic and acute mechanical stretching of RPE cells is associated with elevated VEGF expression (Figure 6). Besides its role in inducing angiogenesis, VEGF is also involved in stimulating inflammation and vascular permeability.^{57–61} Therefore, according to our results, we determined that the initiation and progression of CNV, and VEGF-induced inflammation in the RPE are, at least in part, due to the mechanical stretching of the RPE.

In ARPE-19 cells, a commercially-available RPE cell line, VEGF expression is polarized and mainly apical.^{62,63} Mechanical stress devices used in previous studies are not capable of

interrogating differences between apical and basal RPE secretion. Using the AMS device, we studied polarized VEGF expression in control and mechanically stressed RPE cells. ELISA results confirmed previous findings with respect to the apical VEGF polarization in ARPE-19 cells, i.e., VEGF expression in 10-week old cultures of ARPE-19 cells was more apical than basal (Figure 6.C). This observation also indicates that ARPE-19 cells grown on Transwell membranes were polarized prior to the experiments. ELISA results also showed that mechanical stress on RPE cells induces VEGF overexpression both apically and basolaterally. While CNV is induced by the basal VEGF secretion towards the choroid, the apical VEGF secretion can also be involved in the pathogenesis of CNV since previous studies have shown that apical VEGF disrupts RPE intercellular junctions and increases permeability in ARPE-19 and primary RPE monolayers.⁶⁴ These conditions can facilitate paracellular VEGF transport to the sub-RPE area towards the choroid, further inducing CNV and facilitating choroidal invasion by creating a VEGF gradient.

Elongation of the RPE is often associated with breaks in the RPE monolayer. In AMD, RPE breaks (also known as RPE tears) occur as a result of CNV formation and follow the detachment of the RPE from its basal membrane.^{65,66} In our experiments with the AMS device, we observed significant breaks in the RPE monolayer and perturbation of intercellular junctions (ZO-1) at stretched sites when a small and acute strain (10% uniaxial) was applied (Figure 7). ZO-1 is a tight junction protein that localizes to intercellular spaces of RPE cultures as early as one week after reaching confluency. Our group and others have previously shown that the disruption of the RPE integrity can induce VEGF expression.³⁰⁻³² Thus, it is possible that the elevation of VEGF expression after adding mechanical stress is partially due to breaks in the RPE monolayer. To

determine whether the increase in VEGF expression was solely a consequence of cell-cell detachment, we evaluated the effect of mechanical stress on VEGF expression in a 2-day old ARPE-19 culture. At 2 days of culture, RPE intercellular junctions are not formed, since ARPE-19 cells require at least 1 week after reaching confluency to start establishing intercellular junctions.³² Therefore, by stretching a subconfluent ARPE-19 culture, we excluded the effect of alterations in junctional structures. Our results showed elevated VEGF expression in mechanically stressed subconfluent cultures of ARPE-19 without intercellular junctions (Figure 8), suggesting that mechanical stress independently induces VEGF overexpression, regardless of the formation of intercellular junctions.

Overexpression of VEGF by RPE cells is known to induce CNV.¹⁶⁻¹⁹ A previous study showed that even a short-term increase in VEGF expression can result in long-term CNV development,¹⁹ suggesting that even temporary mechanical stress on the RPE due to an increase in the size of drusen may trigger CNV and a short-term increase in the size of neovascular regions may accelerate CNV. Our results can also explain the presence of high levels of VEGF in drusen deposits from AMD patients as a result of the mechanical stress experienced by the RPE cells surrounding the drusen.¹⁰

Finally, we used an *in vitro* tube formation assay to explore whether mechanical stress can stimulate the differentiation of endothelial cells to endothelial tube-like structures and to test the hypothesis that elongating RPE cells can induce angiogenesis. Spent media from cells exposed to mechanical stress in the CMS device and from the basal chamber of the AMS device promoted *in vitro* angiogenesis (Figure 8). Interestingly, the elevated apical VEGF secretion (Figure 6A)

368 did not result in a statistically significant increase in angiogenesis, suggesting that anti-
369 angiogenic factors may also be upregulated by mechanical stress apically. Given that the basal
370 secretion of angiogenic proteins by RPE cells is crucial for choroidal angiogenesis, the increase
371 in the angiogenic potential of the basal media supports the hypothesis that mechanical stress may
372 be involved in CNV initiation and progression.

373
374 While mechanical disruption of the RPE is a significant aspect of both dry and wet AMD, it has
375 received limited attention among researchers. This work is the first attempt to develop *in vitro*
376 models of acute and chronic mechanical stress on RPE cells and, for the first time, shows a
377 correlation between RPE stretching and angiogenesis. These findings can help better understand
378 the mechanisms of initiation and progression of CNV and may lead to the development of more
379 effective therapeutic interventions. For example, anti-VEGF treatments, currently used to control
380 the progression of CNV,⁶⁷⁻⁷³ may also be considered as a preventative approach by starting anti-
381 VEGF treatments as soon as growth of drusen is detected to lower VEGF levels and therefore
382 prevent CNV initiation. Treatment strategies can also be optimized by adjusting the dose of anti-
383 VEGF injections according to the amount of stress being exerted to the RPE due to CNV growth
384 (measured by analyzing tomography images), to more effectively control CNV progression.

385
386 CNV is a complex process and is mediated by multiple pro- and anti-angiogenic factors and
387 inflammatory cytokines.^{12,74-76} Given the contribution of mechanical stress in inducing
388 inflammation in various tissues, further research is required to identify inflammatory cytokines
389 that are potentially affected by mechanical stretching of the RPE.⁷⁷⁻⁸⁰ The devices presented in
390 this work can be used for this purpose. In particular, the CMS device can be used to evaluate the

role of different rates of increase in mechanical stress in AMD pathogenesis. According to FEA results, the inflation of the PDMS film in the CMS device can transmit high levels of strain to cultured cells with the highest strain conveyed to cells in the middle of the stressed area (Figure 3.H-K). Although we only used the CMS device for chronic stress experiments, different regimens of stress could be achieved by controlling the rate of air injection. Moreover, the CMS device could be scaled up when a higher cell density is needed or down when smaller samples are desired. However, the non-porous basal membrane (PDMS film) of the CMS device does not allow cell polarization and would need to be modified to support polarized cell growth. Lastly, our results did show that air leakage from the CMS device results in deflation of the PDMS film. While air leakage did not significantly affect our experiments, it could interfere with static stress experiments. Since the device was connected to the syringe throughout our experiments, air leakage from the syringe is suspected to be the main cause of deflation. To prevent leakage after reaching the desired strain, the device could be blocked from the syringe by closing a valve inserted between the device and the syringe.

When polarization of cells is desired, the AMS device could be employed. A major difference between the CMS and AMS devices is that the Transwell membrane of the AMS device can undergo plastic deformation, while the deformation within the CMS device is elastic. While this property makes the AMS device more suitable for static stress experiments, only a limited range of strain levels can be achieved with the AMS device compared to the CMS device (up to ~12% uniaxial in the AMS device compared to ~ 72% uniaxial in the CMS device). Also, to apply mechanical stress with the AMS device, cultured Transwell membranes must be removed from culture medium, which limits the length of the time a membrane can be processed and makes the

AMS device unsuitable for long-term and continuous stretching studies. Further work, such as using a different material with higher plastic deformation properties instead of the Transwell inserts (polyester), is needed to resolve these shortcomings. In addition, according to our FEA results, poking the Transwell membranes resulted in a non-uniform distribution of the strain throughout the membrane, i.e., the sides of the elongated membrane experienced the highest strain (Figure 4.D). The breakage of the membranes from the sides due to high levels of stress (Figure 4.E) and the distribution of cells on the membrane after applying mechanical stress (Figure 7) confirm the findings of our FEA model. Pipet tips of the AMS device could be replaced with needles with a round tip to achieve a more uniform distribution of strain.

The rate of increase in strain level due to drusen formation and CNV has not been studied previously to our knowledge, however in a recent study the rate of increase in the drusen volume has been shown to be ~0.1% per day or slower.⁴⁴ Given that the rate of volume progression in the CMS device was significantly faster (~8.5% per hour), more experiments with exposure times longer than 24 hours will be needed in the future to more accurately replicate chronic mechanical stress during drusen formation.

Besides the role of mechanical stress in inducing growth factor-mediated CNV, the physical damage that it causes on the RPE can directly impair the supportive functions of the RPE, leading to the loss of photoreceptors. Previous studies have shown that RPE injury can lead to retinal degeneration.^{40,81–83} The indispensable element of the visual system, photoreceptors, rely on the RPE for trophic and metabolic support.^{81,84} In future experiments, the devices presented

here can be used to evaluate the effect of mechanical stress on the function of RPE-photoreceptor complex within *in vitro* retinal models.

5. Conclusions

In conclusion, we developed two novel mechanical stress devices to study the role of mechanical stress in CNV in AMD. Our results show that mechanical stress can induce VEGF expression in RPE cells and promote *in vitro* angiogenesis. These results underline the importance of the mechanical stress in CNV pathogenesis.

Author contribution

EV and FF conceived the presented idea. FF designed and carried out all experiments, analyzed the generated data and wrote the manuscript. EV supervised the project, provided funding, contributed to result interpretation and provided critical feedback.

Conflicts of interest

Authors have no conflicts to declare.

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