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Biomechanical Strain as a Trigger for Pore Formation in Schlemm’s Canal Endothelial Cells

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Abstract

The bulk of aqueous humor passing through the conventional outflow pathway must cross the inner wall endothelium of Schlemm’s canal (SC), likely through micron-sized transendothelial pores. SC pore density is reduced in glaucoma, possibly contributing to obstructed aqueous humor outflow and elevated intraocular pressure (IOP). Little is known about the mechanisms of pore formation; however, pores are often observed near dome-like cellular outpouchings known as giant vacuoles (GVs) where significant biomechanical strain acts on SC cells. We hypothesize that biomechanical strain triggers pore formation in SC cells. To test this hypothesis, primary human SC cells were isolated from three non-glaucomatous donors (aged 34, 44 and 68), and seeded on collagen-coated elastic membranes held within a membrane stretching device. Membranes were then exposed to 0%, 10% or 20% equibiaxial strain, and the cells were aldehyde-fixed 5 minutes after the onset of strain. Each membrane contained 3–4 separate monolayers of SC cells as replicates (N = 34 total monolayers), and pores were assessed by scanning electron microscopy in 12 randomly selected regions (~65,000 μm² per monolayer). Pores were identified and counted by four independent masked observers. Pore density increased with strain in all three cell lines (p < 0.010), increasing from 87±37 pores/mm² at 0% strain to 342±71 at 10% strain; two of the three cell lines showed no additional increase in pore density beyond 10% strain. Transcellular “I-pores” and paracellular “B-pores” both increased with strain (p < 0.038), however B-pores represented the majority (76%) of pores. Pore diameter, in contrast, appeared unaffected by strain (p = 0.25), having a mean diameter of 0.40 μm for I-pores (N = 79 pores) and 0.67 μm for B-pores (N = 350 pores). Pore formation appears to be a mechanosensitive process that is triggered by biomechanical strain, suggesting that SC cells have the ability to modulate local pore density and...
filtration characteristics of the inner wall endothelium based on local biomechanical cues. The molecular mechanisms of pore formation and how they become altered in glaucoma may be studied in vitro using stretched SC cells.

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**Introduction**

The endothelium lining the inner wall of Schlemm’s canal (SC) contains micron-sized pores that are putative pathways for aqueous humor outflow across an otherwise continuous cell layer containing tight junctions. Pores may pass transcellularly through individual cells (known as “I-pores”) or paracellularly through borders between neighboring cells (known as “B-pores”) (Epstein and Rohen, 1991; Ethier et al., 1998). The density of I- and B-pores is reduced in primary open angle glaucoma (POAG) (Allingham et al., 1992; Johnson et al., 2002), leading to the possibility that impaired pore formation may contribute to obstruction of aqueous humor drainage through the conventional outflow pathway. Very little is known, however, about the mechanisms of pore formation or the factors that determine pore diameter and density within the inner wall of SC.

SC endothelium experiences significant biomechanical loads due to the basal-to-apical (backwards) direction of aqueous humor flow and pressure drop across the inner wall (Ethier, 2002; Overby et al., 2009). The direction of this pressure drop pushes SC cells away from their underlying basement membrane and the supporting juxtacanalicular tissue (JCT). As a result, SC cells form large dome-like outpouchings, known as giant vacuoles (GVs) (Tripathi, 1972; Johnstone and Grant, 1973; Pedrigi et al., 2011), where the instantaneous biomechanical strain acting on SC cells may exceed 50% (Ethier, 2002; Overby, 2011). Pores are often associated with giant vacuoles, and while giant vacuoles and pores are thought to be driven by IOP (Grierson and Lee, 1974; 1977; Lee and Grierson, 1975; Ethier et al., 1998), the precise mechanism of pore formation remains unknown.

We hypothesize that biomechanical strain triggers pore formation in SC cells. To test this hypothesis, SC cells were seeded on elastic membranes that were stretched by 0%, 10% or 20% and aldehyde-fixed in the stretched state. Scanning electron microscopy was used to image SC cell monolayers in order to count pores, measure pore diameter, and classify I-versus B-pores. In vitro pore density and diameter were analyzed as a function of strain, and in vitro pore data were compared against in situ pore data acquired from previous studies of human donor eyes (Ethier et al., 2006). The imaging, identification and classification of pores were done by masked observers who did not know the identity of the samples nor the magnitude of applied strain until after the pore classification was finalized.

**Methods**

**SC Cell Isolation and Culture**

This study examined 3 primary SC cell “lines” from non-glaucomatous human donors, aged 34 (SC58), 44 (SC67) and 68 (SC65) years. SC cells were isolated using the cannulation technique of Stamer et al. (Stamer et al., 1998) and characterized based on expression of VE-cadherin and fibulin-2 (Perkumas and Stamer, 2012). SC cells between passage 3 and 5
were used for all experiments. Although primary cell lines are typically referred to as cell “strains”, we refer to these as cell “lines” to avoid confusion with the mechanical “strain” applied to the cells.

Cells were cultured in low glucose DMEM containing 25 mM HEPES buffer (Gibco 12320, Life Technologies Co, USA), 10% fetal bovine serum (Hyclone SH30070.03, Thermo Scientific, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (P4333, Sigma Aldrich, UK). SC cells were cultured in 5% CO$_2$ in a humid incubator at 37°C, and passaged prior to confluence using trypsin-EDTA (T4049 Sigma-Aldrich, UK).

**Membrane Stretching Device**

Three membrane stretching devices were machined based on the design of Lee et al. (Lee et al., 1996). Briefly, these devices use a coaxial arrangement of threaded cylinders to pull an elastic membrane over an annular indenter (Figure 1A), thereby imposing equibiaxial strain (i.e., a strain magnitude that is equal in all directions (Ethier and Simmons, 2007)) to the membrane when the outer cylinder is turned with respect to the inner cylinder. The cells were seeded on the upward-facing surface of the membrane, and the membrane and inner cylinder delineate a compartment to hold culture medium. The devices were autoclaved prior to use, and each stretching device was kept sterile after cell seeding by covering it with a lid from a 100 mm plastic petri dish.

To confirm that the membrane strain was equibiaxial and to account for subtle machining variations between devices, each stretching device was calibrated by measuring the two-dimensional Green-Lagrange strain tensor ($\mathbf{E}$) as a function of the number of turns of the coaxial cylinders. $\mathbf{E}$ is a mathematical construct that captures the finite strain (or relative elongation) occurring in each coordinate direction at each point on a membrane undergoing a large deformation ((Humphrey, 2002); see Appendix A). To measure $\mathbf{E}$, a grid of 12 fiducial markers was drawn on the membrane (Supplemental Figure A.1A), and the displacement of these markers was recorded using a CCD camera in steps of ~0.5 whole turns. $\mathbf{E}$ was then calculated for each set of 4 neighboring markers based on the change in length of the 6 line segments connecting these 4 markers according to

$$
\left( \delta_{ij} \right)^2 - \left( \delta_{ij,0} \right)^2 = \delta_{ij} \cdot 2 \mathbf{E} \cdot \delta_{ij,0} \quad \text{(Equation 1)}
$$

where $\delta_{ij}$ and $\delta_{ij,0}$ are the vectors describing the line segments between markers $i$ and $j$ ($i ≠ j$) in the deformed and undeformed states, respectively, expressed in polar coordinates with respect to the center of the stretching device. Applying Equation 1 to each of the 6 line segments yields a system of 6 equations that can be optimized (in a least-squares sense) to determine the 3 unique strain components of $\mathbf{E}$ for the region outlined by the 4 markers. Specifically, the unique strain components of $\mathbf{E}$ are the 2 normal strains in the circumferential ($E_{cc}$) and radial directions ($E_{rr}$) and the shear strain ($E_{cr}$). To qualify as equibiaxial strain, $E_{cc}$ and $E_{rr}$ must be identical and uniform across the membrane while $E_{cr}$ must equal zero everywhere across the membrane. Our calibrations in Figure 1B are consistent with this definition. The measured values of $E_{cc}$ and $E_{rr}$ were also in excellent agreement with the theoretical values.
agreement with the expected analytical solution for the applied strain (red dotted line in Figure 1B; see Appendix A for derivation). Performing the calibration on three different membranes gave repeatable results for each stretching device (data not shown). This characterization demonstrates that the stretching devices consistently impose equibiaxial strain onto the membranes and therefore onto any cells that are firmly adherent to those membranes.

Membrane Coating

Cells were seeded on commercially available 0.25 mm thick, transparent, polydimethylsiloxane (PDMS) elastic membranes (70P001-200-010, Specialty Manufacturing Saginaw, USA). Because SC cells do not firmly adhere to bare PDMS, even in the presence of serum (Figure 2A, left panel), the membranes were covalently coated with collagen type I following the protocol by Wipff et al. (Wipff et al., 2009), (Figure 2B). Membranes were first plasma oxidized for 90 seconds at 70 Pa and 70 W (Plasma Prep 5, GaLa Instrumente, Germany) to introduce hydroxide groups onto the PDMS surface. The membranes were then incubated in 10% APTES ((3-aminopropyl)triethoxysilane, 440140, Sigma Aldrich, UK) in ethanol for 45 minutes at 55°C, followed by incubation in 3% freshly prepared glutaraldehyde (TAAB, UK) in PBS (D8537, Sigma Aldrich, UK) for 30 minutes at room temperature. The membranes were then incubated for 1 hour with 50 μg/mL collagen type I (PureCol, Nutacon, The Netherlands) at 37°C. Membranes were washed twice with PBS after each step. SC cell attachment and spreading appeared similar between tissue culture plastic and PDMS membranes coated using the above procedure (Figure 2A, middle and right panels).

Stretch Experiments

For each cell line, 4 cloning rings (10x10 mm, SciQuip, UK) were adhered to the membrane of each of the 3 stretching devices using autoclaved silicone grease. The area inside each cloning ring (0.50 cm²) was seeded at 16,000 cells/cm², and the cloning rings were removed 6 hours after seeding. The cells seeded within the cloning rings were all obtained from the same cell supply, such that the resulting 4 cell monolayers per membrane gave 4 repeated samples from each same cell line at each strain level. The cells were cultured on the membrane for 3 days prior to the onset of strain, and strain was applied simultaneously for each of the 3 membranes.

To apply the strain, the stretching device was turned by the number of turns required to achieve the desired strain level (i.e., 10% or 20% equibiaxial strain), which was determined based on the calibration curve for each stretching device (e.g., Figure 1B). Unstretched (0% strain) controls were treated identically but without engaging the stretch device. Immediately prior to fixation, cells were washed with PBS at 37°C and then fixed exactly 5 minutes after the onset of strain using fixative at 37°C (2.5% glutaraldehyde, 2% formaldehyde (TAAB, UK) in PBS). After 1 hour in fixative, the cells were washed twice and stored in PBS to await processing for scanning electron microscopy (SEM). Throughout the fixation process, the membrane was maintained in the prescribed stretched state, as described next.
Scanning Electron Microscopy

It was necessary to perform SEM and pore counting with the cells maintained in the stretched state. Otherwise, if the membrane were removed from the stretching device and allowed to return to 0% strain after the cells were fixed, the cells would become compressed and wrinkled, damaging the delicate morphological features that allow identification and classification of pores (Supplemental Figure 1). To maintain the membrane in a stretched state, molded PDMS support stubs (6 mm thick, 8 mm diameter, Sylgard 184, Dow Corning, UK) were irreversibly bonded to the bottom surface of the membrane below each circular region containing cells. The irreversible bond was created by briefly exposing the bottom-facing surface of the membrane and support stub to a corona (BD-20AC, Electro Technic Products, USA) that increased surface energy, allowing the PDMS polymers of each interface to interdigitate once pressed together (Haubert et al., 2006). This process required dry and clean surfaces to avoid impurities at the interface that might lead to failure of the bond. The membrane surrounding each stub was then cut with a razor blade and removed, such that the cells on the membrane were maintained in a stretched state by the support stub. Each cell layer supported by a stub thus resulted in one specimen that was processed for SEM. In 2 of 36 specimens, the adhesion between the stub and the membrane failed during processing and these specimens were discarded (SC58 at 10% strain and SC65 at 20% strain, which had 3 instead of 4 specimens each).

Specimens were processed for SEM by incubating with 2% tannic acid (TAAB, UK) and 2% guanidine hydrochloride (50933, Sigma Aldrich, UK) in PBS for 2 hours at room temperature, followed by a triple wash in PBS and post-fixation for 1 hour with 1% osmium tetroxide (TAAB, UK). Specimens were thoroughly washed with deionized water for 5 minutes, dehydrated in a series of ethanol dilutions (25%, 50%, 75%, 95% and 3 times in 100% for 5 minutes each) and air-dried. Each specimen was mounted on an aluminum stub using carbon paste (TAAB, UK) and sputter coated with gold palladium for 75 s at 11 mA (Polaron SC7620, Quorum Technology, UK). Specimens from SC67 were imaged by author STB using a JEOL JSM 6390 (JEOL, Japan), while specimens from SC58 and SC65 were imaged by author ATR using a Hitachi S-3400N VP SEM (Hitachi, USA). The specimens were coded such that the microscopist was masked to the identity of each specimen throughout the processing and imaging steps.

Pore Imaging and Counting

Imaging and pore counting were performed with all specimens masked such that the cell line and the applied strain were unknown to the observers. The key was broken only after all pore classifications had been finalized for each cell line. For each specimen, 12 regions of interest (ROIs) occupying ~5,400 μm² each were positioned using a random number generator programmed in Microsoft Excel that approximated a uniform random distribution over each cell layer. A post-hoc power analysis determined that 12 ROIs was sufficient to detect strain-induced differences in pore density with a power exceeding 90% (Appendix B). Each ROI was imaged at 1,500x magnification, and, if more than approximately one quarter of the ROI was covered by cracks in the cell layer, then that ROI was discarded and a new random ROI was selected. Within each 1,500x image, any gap, opening or pore-like structure within the cell layer was re-imaged at 10,000x magnification. All 10,000x images
were prescreened by STB to filter images with obvious artifacts (e.g., tears, ruptures, or broken openings in the cell layer). To mask the remaining images, each 10,000x image was assigned a random filename and distributed electronically to four observers (CRE, DRO, RMP, STB) who independently marked each 10,000x image to identify pores and classify pore types (see below). Images from each cell line were marked as a group that included specimens at 0%, 10% and 20% strain. Any disparity in marking between observers was discussed in a panel meeting until a majority consensus was reached. Following the panel meeting, the filenames of the images were unmasked and the pore density and diameter were measured for all the pores agreed on in the panel meeting.

Because the specimens were imaged in the stretched state, specimens with larger strain encompassed a smaller original undeformed area per ROI. To normalize for this effect, the number of pores counted in stretched specimens was multiplied by the areal increase relative to unstretched specimens. During equibiaxial strain, the area increases by \((1 + \varepsilon)^2\), where \(\varepsilon\) is the applied strain. This results in a multiplication factor of \(1.10^2 = 1.21\) for specimens at 10% strain and by a factor of \(1.20^2 = 1.44\) for specimens at 20% strain. This normalization ensures that all pore counts are referenced to the same unstretched area between specimens, regardless of the applied strain.

In order for a gap or opening in a cell monolayer to be considered as a pore, it should be elliptical with a smooth perimeter. Because the cell monolayer was flat (as opposed to the inner wall that is highly vacuolated in situ), the PDMS membrane could often be seen through the pore, so the interior of most pores did not appear as dark as typically observed in situ. Candidate pores were excluded when located near to damaged areas of the cell, when overlapping cell processes contributed to the appearance of a pore, or when part of the pore fell outside the borders of the 1,500x image representing the sample ROI.

Pores were classified as B-pores when a cell border was seen to intersect its perimeter. When no cell borders were observed in the close vicinity of a pore, it was classified as an I-pore. Sometimes, part of the pore was concealed by part of a cell, hindering unambiguous classification. In such cases, pores were classified as unknown (U-pores), following previous convention (Ethier et al., 1998).

### Statistical Tests

Counting of relatively sparse events over time or space, such as the number of pores in an SC cell layer, may be best modeled as a Poisson random process. This is in contrast to a Gaussian random process that would otherwise neglect the discreteness of sparse pores and assume that pore counts are normally distributed. The probability distribution of a Poisson random process is described by a single parameter, \(\lambda\), that is equal to both the mean and the variance of the distribution, with the best estimate of \(\lambda\) equal to \(k/m\), where \(k\) is the number

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1The pore perimeter was outlined using image processing software (ImageJ). The resulting pore area, \(A\), was used to calculate the pore diameter, \(D\), according to \(D = 2 \sqrt{\frac{A}{\pi}}\).

2The logarithmic-normal distribution can be described by the geometric mean (\(\mu^*\)) and the multiplicative standard deviation (\(\sigma^*\)). (Limpert et al., 2001), \(\mu^*/\sigma^*\) and \(\mu^*\cdot\sigma^*\) then form the lower and upper bound of the 68% confidence interval. E.g. the 68% confidence interval of the diameter of all pores is [0.29, 1.31] \(\mu\)m. Note that the interval is asymmetric around \(\mu^*\) and that \(\sigma^*\) is dimensionless.
of pores counted over \( m \) specimens (Table 1). When \( \lambda \) is greater than approximately 10, the
distribution of a Poisson process can be approximated by a Gaussian normal distribution
with a variance and mean equal to \( \lambda \) (Govindarajulu, 1965). Therefore, a Poisson process
can be thought of as a more general case for analyzing the statistics of pore counting. A
single value of \( \lambda \) was calculated for each cell line at each strain level, and the \( k/m \) ratio in the
stretched specimens was multiplied by the relative area increase (1.21 for 10% strain and
1.44 for 20% strain) such that all values of \( \lambda \) were referenced to the same unstretched cell
area (see above). To test whether pore density increases with strain, \( \lambda \) was compared pair-
wise between 0%, 10% and 20% strain within each cell line using the E-test
(Krishnamoorthy and Thomson, 2004), which is the Poisson equivalent of the Student’s t-
test, programmed in MATLAB (v 2014A, Mathworks, Natick, MA, USA). To test whether
pore density was different between cell lines, \( \lambda \) was compared pair-wise between cell lines at
a given strain using the E-test. Reported \( p \)-values (representing the probability that the null
hypothesis of no difference is true) are the highest calculated \( p \)-values across the three cell
lines when examining the effect of strain or across the three strains when examining the
effect of cell line. Reported pore densities were characterized as \( \lambda \pm \sqrt{\lambda} \) divided by the
sampled area per specimen (analogous to mean \( \pm \) SD). The sampled area was 64232 \( \mu \)m\(^2\) for
SC58 and SC65, and 65510 \( \mu \)m\(^2\) for SC67. Similar pair-wise comparisons were performed
for porosity (total pore area/undeformed sample area), except that a one-way analysis of
variance (ANOVA) test was used because pore area, in contrast to pore density, is a
continuously distributed random variable. Pore diameter distributions were tested for
normality using a Shapiro-Wilk test with the null-hypothesis that the data follow a normal
distribution, and a two-sample Kolmogorov-Smirnov test was used to compare between any
two pore diameter distributions using SPSS (v 21, IBM Corp, Armonk, NY, USA). The
significance threshold was defined to be \( p < 0.05 \).

Results

Cell and Pore Morphology

By SEM, SC cells appeared as a nearly confluent monolayer with occasional gaps or cracks
through which the PDMS membrane was visible. Presumably, the cracks occurred during
SEM processing because there was no detectible difference in cracking between the
stretched and unstretched specimens.

Transendothelial pores were observed in cultured SC cells, passing both between (B-pores)
and through (I-pores) individual cells. Pores were approximately 0.6 \( \mu \)m in diameter and
rarely exceeded 3 \( \mu \)m. While pores generally had a smooth elliptical perimeter, the
morphology of the pores observed in vitro appeared somewhat more irregular compared to
pores observed in situ (Figure 3). In particular, thin membrane protrusions or processes often
extended from the pore perimeter, occasionally bridging across the pore interior, giving the
appearance of a bumpy or non-elliptical perimeter. The “bridging” cell processes were
distinct from the overlapping processes described in the exclusion criteria above because the
bridging processes emerged from the pore perimeter itself, while the overlapping processes
extended far beyond the pore along the cell membrane. In total, the 4 masked observers
identified 431 pores in 34 specimens from 3 cell lines (Table 1).
Effects of Strain on Pore Density and Porosity

Increasing strain led to an increase in total pore density (total pore count/undeformed sample area) across all three cell lines between 0% and 10% strain (p ≤ 0.0003) and between 0% and 20% strain (p ≤ 0.001; Figure 4A). At zero strain, total pore density was 87±37 pores/mm$^2$, increasing to 342±71 pores/mm$^2$ at 10% and 344±72 pores/mm$^2$ at 20% strain averaged across all 3 cell lines. Total pore density appeared to plateau between 10% and 20% strain in two cell lines (SC58 and SC65; p ≥ 0.13), but it increased further still in one cell line between 10% and 20% strain (SC67; p = 0.0004). Total pore density was significantly different between cell lines exposed to 10% or 20% strain (p ≤ 0.005), except between SC65 and SC67 at 20% strain (p = 0.06). The highest total pore density was exhibited by SC58, followed by SC65 and then SC67. Similar results were obtained for porosity (total pore area/undeformed sample area) that increased between 0% and 10% strain (p = 0.039) and between 0% and 20% strain (p = 0.009), but not between 10% and 20% strain (p = 0.39) (Supplemental Figure 2).

Paracellular B-pores comprised the majority of all pores, accounting for 76% of total observed pores, while I-pores accounted for 17% of total pores, with the remaining 7% being U-pores (Table 1). Between 0% and 10% strain, B-pore density increased from 76.1 ± 34 to 259 ± 61 pores/mm$^2$ (p ≤ 0.0070; Figure 4B) and I-pore density increased from 6.5 ± 7.8 to 64 ± 30 pores/mm$^2$ (p ≤ 0.038; Figure 4C). Between 10% and 20% strain, B-pore density and I-pore density increased in one cell line (SC67; p ≤ 0.013) but not in the other two cell lines (SC58 and SC65; p ≥ 0.10). I-pore porosity increased between 0 and 10% strain (p = 0.0084), and B-pore porosity increased between 0 and 20% strain (p = 0.014). There was no difference in I-pore or B-pore porosity between 10% and 20% strain (p ≥ 0.34).

The pore density and porosity results presented above were normalized by the original unstretched sample area (see Methods). Note that this normalization increases the measured pore density and porosity in the stretched specimens, and may thereby augment the apparent increase in pore density and porosity with increasing strain. To examine whether this normalization on its own could explain the observed results, the statistical analysis was repeated on the raw data without correcting for the change in membrane area. For each of the three cell lines, a statistically significant increase was observed in uncorrected pore density between 0% and 10% or 20% strain for total pores, I-pores and B-pores (p ≤ 0.008). For porosity, a statistically significant increase was observed in I-pore and total pore uncorrected porosity between 0% and 10% or 20% strain (p ≤ 0.047), but the influence of strain on B-pore uncorrected porosity rose above the significance threshold (p = 0.068).

Effects of Strain on Pore Diameter

For a given cell line at a given strain, the cumulative distribution function (CDF) describing I- or B-pore diameter was better approximated by a logarithmic-normal, rather than a Gaussian-normal, distribution (Figure 5). A Shapiro-Wilk test applied separately to each I- or B-pore diameter distribution for each cell line at each strain level yielded significant differences between the empirical CDF and the Gaussian-normal distribution in all 13 cases (10$^{-8}$ < p < 0.04) that contained more than 10 pores each. After logarithmic-transformation...
of the pore diameter, however, this difference was eliminated in 12 of the 13 cases (0.97 > p > 0.10), suggesting that I- and B-pore diameter distributions more closely follow a logarithmic-normal, rather than a Gaussian-normal, distribution. Importantly, this suggests that logarithmic transformation of pore diameters is necessary to obtain Gaussian-normally distributed data required for ANOVA and many other statistical tests.

Accounting for the logarithmic-normal distribution, the typical pore diameter for SC cells in culture was 0.62 μm ± 2.1 (geometric mean ± multiplicative SD; N = 431)², with I-pore diameter (0.41 μm ± 1.9, N = 79) tending to be smaller than B-pore diameter (0.70 μm ± 2.1, N = 350). Neither I-pore nor B-pore diameter appeared to be affected by strain (p = 0.93 and p = 0.12, Supplemental Figure 3). The pore diameter distributions for the three strain levels could therefore be aggregated to derive a single pore diameter distribution for each cell line and pore type (see Figure 6). The empirical CDF of the aggregated pore diameters was fit to an idealized logarithmic-normal CDF that yielded the best estimate of the geometric mean and the 68% confidence interval (CI) of the logarithmic-transformed pore diameter (Table 2). From this, a probability density function (PDF) describing the distribution of pore diameters can be generated and interpreted similar to a histogram (Figure 6). I- and B-pore diameters were smaller and more tightly distributed for SC65 compared to SC67, and this difference was statistically significant (p < 0.003, ANOVA), suggesting that each SC cell line may have a unique “set point” for pore diameter.

Comparison with In Situ Pores

The total pore density in stretched SC cells (mean: 343 pores/mm²) was 2- to 6-fold less than the total inner wall pore density previously reported in situ (range: 150 – 2960, mean ~800) (Ethier et al., 1998; Johnson et al., 2002; Ethier et al., 2006). The majority of this difference was attributable to I-pores that showed a much lower pore density in culture (46 I-pores/mm²) versus in situ (416 ± 85 I-pores/mm², mean±SEM) (Ethier et al., 2006), while B-pore density was more consistent between in culture (191 B-pores/mm²) and in situ (342 ± 110 B-pores/mm²).

Pore diameter in cultured SC cells closely approximated the pore diameter measured in the inner wall in situ, based on data from a prior study of 5 perfusion-fixed, non-glaucomatous human eyes (Table 2) (Ethier et al., 2006). More specifically, the empirical CDFs describing pore diameter in culture generally fell between the maximum and minimum limiting pore diameter CDFs from the in situ data, for both I- and B-pores (Figure 7).

Apart from the inner wall of SC, similar pores or pore-like structures have been described in vascular endothelia associated with inflammation, filtration and leukocyte diapedesis. Pore formation in vascular endothelia during diapedesis is a dynamic event, where cell membrane processes known as ventral lamellipodia extend and close the pore within minutes of leukocyte passage (Martinelli et al., 2013). Although we were unable to visualize the dynamics of pore formation or closure in SC cells by SEM, structures resembling ventral lamellipodia were occasionally observed extending from the pore perimeter in cultured SC cells (Figure 8).
**Discussion**

This study demonstrated that cultured SC cells form transendothelial pores similar to those that occur along the inner wall of SC in vivo. Furthermore, formation of both transcellular I-pores and paracellular B-pores appeared to be stimulated by biomechanical strain, as evidenced by increasing pore density (but not pore diameter) with increasing strain applied to the elastic membrane to which the cells were adhered. These data suggest that pore formation is a mechanosensitive process that is partly regulated by the magnitude of biomechanical strain experienced by SC cells.

The inner wall of SC resides within a demanding biomechanical environment where the basal-to-apical pressure drop across the inner wall imposes significant forces and deformations on SC cells. In vivo, such deformations are realized during giant vacuole formation or during “ballooning” of the inner wall following detachment from the underlying juxtacanalicular tissue (Johnstone and Grant, 1973; Grierson and Lee, 1977; Battista et al., 2008). Inner wall deformation increases with IOP, imposing biomechanical strain on SC cells ranging from 6 – 30% at 15 mmHg to 51 – 228% at 30 mmHg (Ethier, 2002; Overby, 2011), although these estimates are sensitive to assumptions that are difficult to validate in vivo. Nonetheless, the magnitude of the strain experienced by SC cells likely exceeds the strain necessary to fatally damage most other cell types (~50%) (Tschumperlin and Margulies, 1998), suggesting that SC cells must be somehow better adapted to withstand the large strains and demanding biomechanical environment of the inner wall in vivo.

In this study, cellular strain was applied using a membrane stretching device originally designed by Lee et al. (Lee et al., 1996). The imposed membrane strain was an idealized “equibiaxial” strain where the magnitude of strain (representing the relative change in the distance between any two points on the membrane) was the same in all directions. While this clearly neglects the complex deformations and locally varying strain fields experienced by SC cells in vivo, this simplified approach allowed us to examine the relationship between pore formation and cellular strain in a precisely controlled manner. This approach assumes that the strain applied to the SC cells was equal to the strain applied to the membrane, which requires that the cells remain firmly adhered to the membrane during the period of applied strain.

**The Influence of Strain on Pore Formation**

With increasing membrane strain, we observed an increase in I-pore and B-pore density, suggesting that both pore types are mechanosensitive in SC cells. When strain increased above 10%, however, 2 of the 3 cell lines showed no further increase in pore density for either pore type, suggesting that there may be a strain threshold necessary to initiate pore formation or that there may be a non-linear relationship between pore density and strain that saturates between 0% and 10%. At 0% strain, there was a small but non-zero pore density, suggesting that there may be a baseline population of pores that form independently of applied strain or form in response to endogenous tension within the cell or cell monolayer. Similar conclusions regarding the influence of strain on pore density were reached regardless of whether the pore counts were normalized by the stretched or unstretched cell area.
Despite both pore types increasing with strain, I-pores appeared to be more sensitive to strain, exhibiting a 10-fold increase in I-pore density compared to B-pores that exhibited a 3-fold increase between 0% and 10% strain. It is unclear why I-pores would be more strain-sensitive, but this may be related to differences in the underlying mechanisms of I- and B-pore formation. B-pores, we hypothesize, result from local disassembly and widening of inter-cellular junctions, following mechanisms similar to those described for paracellular “gap” formation during inflammation and diapedesis (Carman et al., 2007; Carman, 2009; Overby, 2011). I-pores, we hypothesize, result from fusion of the apical and basal cell membranes that may come into apposition as the cytoplasm thins under applied strain, with caveolae, vesicles, or “mini-pores” (Inomata et al., 1972; Overby, 2011; Herrnberger et al., 2012) acting as potential nucleation sites populated with fusigenic proteins or lipids. Each hypothetical mechanism may exhibit a different dependency upon strain, or strain may differentially affect the rate of pore formation, possibly contributing to differences in the strain dependence between I- and B-pore density. Alternatively, immature or discontinuous inter-cellular junctions, that are typical of cultured endothelial cell monolayers (Albelda et al., 1988) could have partially disturbed B-pore formation without significantly affecting I-pores.

The finding that pore diameter was relatively unaffected by strain leads to two important conclusions. First, the increase in pore density or porosity with increasing strain cannot be readily explained by a widening of intercellular gaps or openings in the cell monolayer. This also suggests that our pore counting technique effectively excludes gaps or openings that would otherwise contribute to an apparent increase in “pore” diameter in response to strain. Second, there must be a mechanism to maintain a relatively constant pore diameter and oppose tensional forces tending to expand the pore with increasing strain. Excessive widening of a pore would damage the SC cell and compromise the barrier function of the inner wall. It is therefore tempting to speculate that there must be a supporting structure for SC pores, possibly involving cytoskeletal filaments such as those surrounding the smaller ‘fenestrae’ pores present in filtration-active vascular endothelia (Braet and Wisse, 2002). Indeed, the decoupling of the cell membrane from the cortical actin network, as can be induced by pathogenic bacteria, was shown to facilitate transcellular pore formation (Gonzalez-Rodriguez et al., 2012). The same study also described a limiting maximum transcellular pore size on the order of a few μm.

Comparison with In Situ Pores

Pores observed in cultured SC cells were of similar size and shape as pores observed along the inner wall in situ, but the cultured pores tended to be slightly more irregular with bumpy edges, imperfect elliptical geometries, and bridge-like processes extending from the pore perimeter. The reason for the irregular morphology of cultured pores may be related to non-physiological culture conditions, such as the absence of flow, weakly developed cell junctions, or altered substrate compliance or surface chemistry. Alternatively, the irregular pore morphology may be related to the dynamics of pore formation or closure that are captured instantly upon fixation in culture, compared to a slower ‘time-lapse’ capture over an extended fixation process as may occur in situ. Despite these differences, the reasonable morphological similarity and the similar diameter distributions between pores in culture...
versus pores in situ suggests that the cell stretch model may capture many of the in vivo mechanisms of pore formation in SC cells.

Despite similarities in pore size, the total pore density in stretched SC cells was 2- to 6-fold less than the pore density typically reported for the inner wall in situ, with the majority of this difference attributable to fewer I-pores in culture. The reason for this discrepancy is unclear, but one should recognize that the comparison is complicated by the fact that the biomechanical strain acting on the inner wall in situ is largely unknown and likely varies regionally. The cell stretch model, in contrast, uses well-controlled, uniform strain fields. Furthermore, the measured in situ pore density is likely sensitive to fixation conditions, particularly for I-pores that increase in proportion to the volume of fixative perfused through the outflow pathway (Ethier et al., 1998). It is unknown whether fixation itself had any influence on the measured pore density in cultured SC cells, but because the fixation process was identical between samples, fixation alone cannot explain the observed dependence of pore density on strain.

Schlemm’s canal endothelium is not the only endothelium in the body that forms pores. Arachnoid villi, involved in drainage of cerebrospinal fluid, form transendothelial pores (Tripathi, 1977). Toxins such as anthrax and substance p induce transcellular and paracellular “pores” in vascular endothelia (McDonald et al., 1999; Maddugoda et al., 2011) and leukocytes pass through transcellular or paracellular “pores” to enter or leave the bloodstream (Carman et al., 2007; Woodfin et al., 2011). Following paracellular diapedesis, actin rich processes known as ventral lamellipodia extend and close a paracellular “pore” over minutes, while actin-dependent “pore” closure has been described following transcellular diapedesis (Martinelli et al., 2013). Structures resembling ventral lamellipodia were occasionally observed surrounding pores in cultured SC cells, suggesting that similar mechanisms may be involved in pore formation and/or closure between SC and vascular endothelial cells.

**SC Endothelium as a “Smart” One-Way Valve**

The hypothesis that cellular strain induces pore formation implies that the SC endothelium can function as a “smart” one-way valve that adjusts its local porosity to accommodate local demands in filtration. For instance, if at any point along the inner wall, local filtration demands were high and local transendothelial conductivity were low, then the local pressure drop across the endothelium would increase and impose biomechanical strain on SC cells, realized either through giant vacuole formation or bulging or ballooning of the inner wall. With strain as a trigger, pore formation would occur preferentially in those regions with larger filtration demand and sufficient strain. Such a hypothetical mechanism would allow the inner wall to adapt to non-uniform or segmental outflow conditions based on local biomechanical cues. This mechanism would also allow the inner wall to reduce its local porosity in regions of low strain or under conditions of low or reversed pressure drop, thereby allowing the inner wall to function as a one-way valve to oppose retrograde flow into the trabecular meshwork (Johnstone and Grant, 1973). The one-way valve function of the inner wall may be particularly important for maintaining the blood-aqueous barrier by...
preventing reflux of blood or serum proteins from SC into the anterior chamber under conditions of hypotony or elevated episcleral venous pressure.

In conclusion, this study has shown that SC cells retain their pore-forming ability in culture and that pore formation is a mechanosensitive process that is triggered by biomechanical strain. This mechanism may allow the inner wall to function as a “smart” one-way valve that may modulate its local porosity to accommodate local demands for aqueous humor filtration while also maintaining the blood-aqueous barrier by preventing blood and serum proteins from entering the anterior chamber. It is tempting to speculate that altered biomechanical stiffness of SC cells or their surrounding tissue, as may occur in glaucoma (Last et al., 2011; Camras et al., 2012), may disrupt SC valve function and contribute to reduced SC pore density as observed in glaucomatous eyes (Allingham et al., 1992; Johnson et al., 2002). The cell stretch model therefore provides an opportunity to investigate the molecular mechanisms of pore formation in SC cells so as to deepen our understanding of the physiology and pathophysiology of the inner wall with respect to glaucoma.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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We thank Prof. Ralph Knöll (Imperial College London) for lending his cell stretching device and Prof. Boris Hinz (University of Toronto) for providing a detailed protocol for the covalent coating of PDMS. We acknowledge Dr. Joseph Sherwood (Imperial College London) for helpful advice regarding the statistical analysis.

**References**


Appendix A: Analytical Solution for the Strain Applied by the Membrane Stretching Device

The stretching device pulls an elastic silicone membrane over a cylindrical indenter, thereby imposing a uniform equibiaxial strain field on the membrane (Ethier and Simmons, 2007). Due to the cylindrical geometry, the problem is axisymmetric, and the strain can be calculated analytically by considering the change in membrane dimensions between the stretched and unstretched states. Let Δ represent the distance between the inner and outer cylinders, R represent the radius of the inner cylinder, and h represent the depression of the inner cylinder with respect to the outer cylinder (Supplemental Figure A.1B). In the undeformed state, the length of the line segment connecting the center of the membrane to the membrane edge is equal to R + Δ. In the deformed state, this length increases to R + \sqrt{\Delta^2 + h^2}. Assuming no friction at the contact point between the membrane and the
indenter, the stretch ratio, $\lambda$, representing the ratio of the deformed to undeformed length can therefore be written as

$$\lambda = \frac{R + \sqrt{\Delta^2 + h^2}}{R + \Delta}$$ \hspace{1cm} \text{Equation A. 1}$$

The Green-Lagrange strain ($\varepsilon_{GL}$) can be written in terms of $\lambda$ according to:

$$\varepsilon_{GL} = \frac{\lambda^2 - 1}{2}$$ \hspace{1cm} \text{Equation A. 2}$$

Finally, $h$ can be related to the number of turns ($n$) of the indenter according to:

$$h = n \varphi$$ \hspace{1cm} \text{Equation A. 3}$$

where $\varphi$ is the pitch of the threads on the indenter. Combining Equations A. 1–3 produces the analytical solution shown in Figure 1. B.

Appendix B: Post-hoc Power Analysis of Pore Sampling

Because pores are relatively infrequent, measurements of pore density are necessarily subject to sampling errors. To examine whether 12 regions-of-interest (ROIs) were sufficient to detect differences in pore density in response to strain, we performed a post-hoc statistical power analysis using MATLAB (v2014a, Natick, MA, USA). The analysis used data from the cell line with the lowest pore density (SC67; Figure 4), including the 12 pore density measurements from each of 12 ROIs from each of 4 specimens at 0% and 4 specimens at 10% strain. (We also performed the analysis for 0% versus 20% strain, but because the pore density increase was larger at 20%, this was a less conservative analysis). The number of ROIs used to calculate the overall pore density of each specimen was then varied between 5 and 11, where for each number of ROIs 10,000 realizations were analyzed by randomly selecting the given number of ROIs from the available 12. For each realization, we calculated the average pore density for the 4 specimens and performed an E-test to detect for statistical differences between the stretched and unstretched specimens. The statistical power of the pore density measurements, $\beta$, was equal to the percentage of realizations for which the E-test yields a significant difference ($p<0.05$), and $\beta$ was plotted against the number of ROIs evaluated.

With increasing number of ROIs, there was an increase in the statistical power for detecting a difference in pore density between stretched and unstretched specimens (Supplemental Figure B.1). Comparing between 0% and 10% strain, a statistical power of $\beta = 0.8$ was reached at 8 ROIs (Supplemental Figure B.1A). For the analysis between 0% and 20% strain, $\beta = 0.8$ was reached at less than 5 ROIs (Supplemental Figure B.1B). Therefore, 12 ROIs per specimen was adequate to test the hypothesis that pore density increases with strain with a statistical power exceeding 90%.
Highlights

• Human Schlemm’s canal (SC) cells were exposed to 0%, 10% or 20% biomechanical strain.
• Transcellular “I” and paracellular “B” pores were observed in cultured SC cells.
• I- and B-pore densities increased with strain.
• I- and B-pore diameters were unaffected by strain.
• I- and B-pore diameters closely matched in situ SC pore diameters.
Figure 1.
The membrane stretching device used to apply equibiaxial strain to adherent Schlemm’s canal endothelial cells. A) A schematic vertical cross-section through the device originally described by Lee et al. (1996). A silicone elastic membrane is clamped into the device and cells are seeded on the upward-facing surface of the membrane. The membrane is stretched by turning the threaded membrane holder (grey) along the outer cylinder (white), thereby pulling the membrane over the indenter (black) to impose equibiaxial strain. B) A representative calibration curve obtained from one of the three cell stretching devices used in this study. The measured components of the Green-Lagrange strain tensor ($E_{cc}$, $E_{rr}$, $E_{cr}$) are plotted against the number of turns of the membrane stretching device. The normal components of the strain tensor in the circumferential ($E_{cc}$) and radial ($E_{rr}$) directions increase with each turn and remain statistically identical, whereas the shear component ($E_{cr}$) remains close to zero, indicating that equibiaxial strain is applied to the membrane. The calibration closely follows the analytical solution (Appendix A). Error bars show the standard deviation of each strain component measured over 5 different regions on the membrane.

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Figure 2.
Method to covalently coat the elastic PDMS membrane with collagen. A) Representative brightfield images (scale bar 50 μm) showing the attachment and spreading of Schlemm’s canal (SC) cells on bare and collagen-coated PDMS relative to tissue culture plastic. SC cells do not adhere to bare PDMS but attach and spread on collagen-coated PDMS similar to as when on tissue culture plastic. B) The diagram summarizes the method to covalently coat the PDMS membrane with collagen, after Wipff et al., 2009. PDMS is exposed to oxygen plasma to create hydroxide groups on the surface. The hydroxide groups are then reacted with APTES to present an amino group that can be cross-linked to the amino groups of collagen using glutaraldehyde. PDMS, poly(dimethylsiloxane); APTES: (3-aminopropyl)triethoxysilane.
Figure 3.
Scanning electron micrographs of pores in Schlemm’s canal (SC) endothelial cells. The top two rows are representative images of pores observed in cultured SC cells following stretch, while the bottom row is representative of pores observed in the inner wall in situ following perfusion at 8 mmHg (ostensibly normal human eye, unpublished data). Transcellular “I” pores, paracellular “B” pores and unknown “U” pores are indicated by yellow text. White arrowheads mark the border between adjacent cells. Note that the pores and cell surfaces observed in culture are more irregular than those observed in situ. Unmarked openings were considered to be artifacts. The cell line and strain-level are specified in the upper left of each micrograph. Scale bars: 1 μm.
Figure 4.
Pore density increases from 0% to 10% Green-Lagrange strain in cultured Schlemm’s canal (SC) cells for total pores (p ≤0.0003, E-test; panel A), paracellular “B” pores (p ≤0.0070; panel B), and transcellular “I” pores (p ≤0.038; panel C). Each symbol (circle, plus or cross) denotes a pore density measurement from a single specimen (based on 12 regions-of-interest per specimen), while the lines connect the mean pore density at each strain level for each cell line as given in Table 1. The difference in total pore, I-pore and B-pore density between 10% and 20% strain is not statistically significant for SC58 or SC65 (p ≥0.10), but is statistically significant for SC67 (p ≤0.013). Note that all vertical axes are presented on the same scale and that symbols are shifted slightly on the horizontal axes so as to avoid overlap (bottom braces). Pore density data are normalized by the original (undeformed) membrane area, as described in Methods. Statistically significant changes in pore density between 0% and 10% or 20% strain are indicated by (*) for p < 0.05, (†) for p < 0.01 and (‡) for p < 0.001, where the reported p-values are the maximum p-values across all three cell lines.
Figure 5.
The cumulative distribution function (CDF) describing pore diameter (red) plotted with the best-fit predictions of the CDF from a logarithmic-normal (solid black line) and a Gaussian-normal (dashed black line) distribution. The logarithmic-normal distribution better represents the empirical CDF describing pore diameter than does the normal distribution. This is true for both transcellular “I” pores ($p = 0.97$ vs. $p = 0.04$, Shapiro-Wilk; left panel) and paracellular “B” pores ($p = 0.44$ vs. $p < 10^{-5}$, right panel). Pore data taken from SC58 at 20% strain.
Figure 6.
The pore diameter distributions for transcellular “I” pores and paracellular “B” pores across all three Schlemm’s canal (SC) cell lines. Because pore diameter was insensitive to strain, all measured pore diameters were aggregated to determine the empirical cumulative distribution function (CDF) for each cell line (circles, right axes). Each circle represents an individual pore diameter measurement. Each empirical CDF was then fitted to the theoretical CDF of the logarithmic-normal distribution (dashed curves, right axes), and that fit was used to estimate the best-fit logarithmic-normal probability distribution function (PDF) or “histogram” describing pore diameter for each cell line (solid curves, left axes). These data reveal differences in the pore diameter distributions between different SC cell lines, with SC65 tending to have smaller (p < 0.03, ANOVA) and more tightly distributed pore diameters relative to SC58 and SC67.
Figure 7.
Empirical cumulative distribution functions (CDF) describing pore diameter for Schlemm’s canal (SC) cells in culture (thin lines) versus the inner wall in situ (thick lines). In situ data were taken from a prior study of 5 ostensibly normal human eyes perfusion fixed at 8 mmHg (Ethier et al., 2006), but only the maximum and minimum CDFs are shown to represent the physiologically observed range of pore diameters along the inner wall in situ. For both transcellular “I” pores (A) and paracellular “B” pores (B), the pore diameters observed in cultured SC cells are broadly consistent with the pore diameters measured along the inner wall in situ. The CDFs of pore diameter in culture are reproduced from Figure 6.
Figure 8.
A comparison between the morphology of pores observed in cultured Schlemm’s canal (SC) cells and “pores” or transendothelial “tunnels” observed in vascular endothelial cells. A) In SC cells, protrusions (yellow dotted curves) and processes (yellow arrows) were occasionally observed extending from the perimeter of paracellular “B” pores. Images are scanning electron micrographs (SEMs) from SC58, with the cell borders indicated by white arrowheads. B) In vascular endothelial cells from a prior study (Martinelli et al., 2013), closure of transcellular “pores” and paracellular “gaps” often occurs by ‘ventral lamellipodia’ (white dotted curves) that extend across the cell surface (blue arrowheads) to cover and close the “pore” or “gap”. Note the similarity between the ventral lamellipodia in Panel B and the cellular protrusions and processes about B-pores in SC cells shown in Panel A. Panel B represents a time-lapse confocal fluorescence image sequence with the elapsed time in minutes displayed in the upper left of each frame. Reproduced with permission of the authors and the publisher. ©2013 Martinelli et al., Journal of Cell Biology. 201: 449–465. doi: 10.1083/jcb.201209077. C) In SC cells, protrusions (yellow dotted curves) were occasionally observed extending from the perimeter of transcellular “I” pores. Images are SEMs from SC67 (left and middle) and SC65 (right). D) In vascular endothelial cells from a prior study (Maddugoda et al, 2011), closure of transcellular “pores” or “tunnels” was often associated with actin-rich membrane waves that extended from the “pore” perimeter, as visualized in cells transfected with green fluorescent actin. Inset on the right shows time-lapse images from the start (top) to end (bottom) of closure. Note the similarity between the shape of the actin-rich protrusions in vascular endothelial cells and the protrusions surrounding I-pores in SC cells in Panel C. Reproduced from with permission of the authors and the publisher ©2011 Maddugoda et al., Cell Host & Microbe. 10: 464–474, doi: 10.1016/j.chom.2011.09.014. Scale bars: 1 μm (AμC), 5 μm (B) and 10 μm (D).
Table 1

Pore statistics measured in three SC cell lines at 0%, 10%, and 20% equibiaxial strain. k is equal to the number of pores counted over m specimens for each cell line at each strain level. Note that k is the raw pore count and is not corrected for changes in membrane area. The Poisson parameter λ is equal to \( \frac{k}{m} \) multiplied by 1.21 for samples stretched 10% and by 1.44 for samples stretched 20% (see methods). The mean and the standard deviation of the pore density were calculated as \( \lambda \pm \sqrt{\lambda} \) divided by the analyzed area in each specimen: 64232 μm² for SC58 and SC65, 65510 μm² for SC67.

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<th>k</th>
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The pore diameter (geometric mean and 68% confidence interval) measured in Schlemm’s canal cells in culture relative to the pore diameter measured along the inner wall in situ (data from a prior report (Ethier et al., 2006)). Because pore diameter appeared unaffected by strain, pore diameter distributions from the three strain levels were aggregated together for each cell line to give the data shown.

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