Glaucoma

Increased Substrate Stiffness Elicits a Myofibroblastic Phenotype in Human Lamina Cribrosa Cells

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PURPOSE. Alteration in the extracellular matrix (ECM) of the optic nerve head (ONH) causes lamina cribrosa (LC) fibrosis and affects the mechanical integrity of the ONH. Increased ECM tissue stiffness drives myofibroblast activation leading to tissue fibrosis throughout the body. Here using primary human LC cells, we investigate the effect of substrate stiffness on profibrotic changes, which might be a key molecular mechanism driving ECM remodeling of the LC in primary open-angle glaucoma (POAG) glaucoma.

METHODS. Primary human LC cells from normal and age-matched POAG glaucoma donors were cultured on substrates with defined mechanical properties of 5 and 100 kPa to replicate the range of mechanical microenvironments that cells may experience in vivo. Cell morphology, spread area, actin stress fibers, vinculin-focal adhesion formation, and z-smooth muscle actin (z-SMA) signal were examined using immunofluorescence staining. The elastic modulus of cells was measured using atomic force microscopy (AFM).

RESULTS. Significantly greater cell spread area along with increased actin filament development, and vinculin-focal adhesion formation (number and size) were found in both normal and glaucoma LC cells cultured on stiff substrates. These changes were positively associated with elevated cell stiffness measured by AFM. Changes in spreading and cytoskeleton organization of glaucoma LC cells were significantly more pronounced than those in normal cells. The transformation to a myofibroblast-like cell phenotype was identified in both LC cells exposed to stiffer substrates, as indicated by an increased z-SMA signal and its localization with the actin stress fibers.

CONCLUSIONS. These findings demonstrated that a stiffer cell microenvironment activates a myofibroblastic transformation in human LC cells, and therefore contributes to LC remodelling and fibrosis in glaucoma.

Keywords: substrate stiffness, myofibroblast transformation, lamina cribrosa cells, biophysical cues, glaucoma

Glaucoma, one of the leading causes of irreversible blindness worldwide, is characterized by an optic neuropathy involving progressive loss of retinal ganglion cell (RGC) axons, excavation or cupping of the optic nerve head (ONH), and associated visual field loss.1 Clinically, primary open-angle glaucoma (POAG), the most common form of glaucoma in the western world, is characterized by elevated IOP and an open trabecular meshwork (TM) drainage angle measured with gonioscopy.2 Elevated IOP results from a reduction in aqueous humour outflow resistance and control of IOP, which ties of outflow pathway cells and tissues to the modulation of aqueous humour outflow resistance and control of IOP which...
play a fundamental role in the initiation of glaucoma and its progression.

Aging is associated with increasing tissue stiffness throughout the body and is associated with ECM accumulation causing tissue fibrosis. Fibrosis is driven by activated fibroblasts (myofibroblasts), which have a phenotype charac-
terized by excessive production of collagenous ECM and increased expression of the contractile protein α-smooth muscle actin (α-SMA). Prolonged myofibroblast activity and excessive contractility lead to tissue stiffening, impaired organ function, and ultimately to organ failure due to tissue fibrosis.

Biomechanical cues are increasingly recognized as essential modulators of myofibroblastic transformation and activation, with decisive roles in regulating cell behaviors and fate. Cells can sense and respond to their microenvironment, translating mechanical signals to biological responses through mechanotransduction. This process is mediated via integrin-dependent focal adhesions that link the surrounding matrix to the actin cytoskeleton. Focal adhesions are therefore also the sites at which forces are transmitted between the cell and substrate and can be detected with fluorescence labeling of specific adhesion molecules such as vinculin, paxillin, and talin. The modulus of the substrate limits the forces exerted by adherent cells in a dynamic equilibrium that controls focal adhesion (FA)-dependent signals. Therefore, ECM elasticity is a basic biomechanical feature that is detected by most cell types, differing from brain to bone, varying with age and disease, and has a strong impact on the actin cytoskeleton and cell intrinsic stiffness.

Schlunck et al. previously used polymer culture substrates with tuneable stiffness to demonstrate that human TM cells exhibited distinct functional behaviors, as indicated by the robust expression of α-SMA and an increased expression of several ECM-related genes that depend on substrate stiffness, within the elasticity range of normal and fibrotic TM tissue. Stiffer substrates facilitate stronger adhesion to matrix proteins which activates stiffness-dependent myofibroblast differentiation, which may be of pathophysiologic relevance in POAG.

The lamina cribrosa (LC) at the ONH is considered a significant location of optic nerve fiber damage in glaucoma. In a series of experiments, Albon et al. demonstrated an increase in both the collagen and noncollagenous (elastins and advanced glycation end products [AGEs]) ECM components in the aging LC. These alterations were accompanied by a loss of compliance (increasing stiffness) of the LC with age. Histologic evidence indicates that characteristic ECM structural changes occur with aging and in the human and monkey ONH in glaucoma with considerable ECM remodeling. Moreover, a recent study using in vivo shear wave elastography observed a stiffer optic nerve and peripapillary scleral tissue in glaucomatous eyes compared with age-matched controls. As the LC stiffens, it is more likely to lose its capacity to respond normally under physiologic conditions resulting in mechanical failure and collapse when the pressure exceeds the yield point. Therefore, it is important to determine whether the biomechanics of LC tissues and interactions with resident cells (such as ONH astrocytes and LC cells) help drive LC profibrotic activities such as tissue stiffening and fibrosis in the LC. The expression of the profibrotic cytokine transforming growth factor β2 (TGF-β2) is elevated in the glaucomatous optic nerve head, and TGF-β2 may be at least partially responsible for this pathologic ONH remodeling.

Our group, and others, have examined the cellular fibrotic phenotype associated with glaucoma in the LC and suggest a strong role for LC cells in glaucoma ONH ECM remodeling due to their ability to express fibrotic/ECM genes following exposure to glaucoma related stimuli. Isolated LC cells from healthy and glaucoma eyes are a resource that provides an opportunity for evaluating the mechanical properties of the LC at the cellular level and also provide insight into understanding the influence of an aberrant LC microenvironment that may affect residing cells and tissue stiffness and dysfunction. Therefore, the aim of this work was to investigate whether the mechanical cues from the cellular microenvironment influence fundamental properties of LC cells. We demonstrate that increased substrate stiffness elicits a myofibroblastic phenotype in human LC cells, which may ultimately contribute to fibrosis and stiffening of the LC in glaucoma.

**Materials and Methods**

**Substrate Preparation and Surface Functionalization**

Silicon elastomer substrates were purchased from ExCellness Biotech SA (Lausanne, Switzerland) with a nominal stiffness of 5 (soft) and 100 kPa (stiff). These stiffness values were selected based on the report by Last et al., which indicated that TM tissue stiffness was 5 kPa in normal eyes and 80 kPa in glaucoma eyes. Substrate stiffness was independently verified using AFM.

Silicone elastomer substrates have been widely used as study models for evaluating cell behavior modulated by the mechanical environment as they are nontoxic, tuneable, optically transparent and user friendly. However, a significant drawback of using untreated substrates as a culture surface for cells is the high hydrophobicity and extremely low cell attachment. To overcome these limitations, the substrate surfaces were functionalized by covalently attaching fibronectin using the cross-linker Sulfo-SANPAH (Pierce Biotechnology, Rockford, IL, USA) as previously described. Sulfo-SANPAH (0.5 M) was dissolved in 200 mM HEPES, pH 8.5, buffer to 50 μM, added to the substrate surface, and treated with UV light for 10 minutes to bind the cross-linker to the gel surface. The substrates were washed three times in 200 mM HEPES, pH 8.5. Subsequently, fibronectin from human plasma (Sigma-Aldrich Corp., St. Louis, MO, USA) was diluted in PBS to 10 μg/mL solution and added to the substrate surfaces and incubated overnight at 4°C. Following protein binding, all substrates were rinsed and remained in culture medium for 1 hour before plating cells.

**AFM Imaging and Substrate Mechanics**

All AFM experiments were conducted at room temperature (RT) using the MFP-3D AFM (Asylum Research, Santa Barbara, CA, USA) combined with a Nikon Eclipse Ti fluorescent microscope (Nikon, Tokyo, Japan). To reduce thermal drift, substrate samples were allowed to equilibrate at RT for 1 hour before collecting data. To assess the coverage of fibronectin on substrates of different stiffness, the topography of coated silicone elastomer surfaces was imaged by AFM using the tapping mode. Topography images were recorded with a resolution of 512 × 512 pixels (maximum scan area, 20 × 20 μm) and a linear scan rate of 0.3 to 0.5 Hz using silicon nitride cantilever probes (DNP10; Tip C, Bruker, United Kingdom) with a nominal spring constant of 0.32 N/m. For a given image area, the reported roughness value is the average root mean square (RMS) roughness (Rq) obtained from five random regions of triplicate specimens. Data were analysed using the Igor Pro (Wavemetrics, Portland, OR, USA) software, which flattening order 3 was applied to all images to correct for tilt and bow before roughness analysis. Mechanical characteriza-
tion was conducted using a glass spherical tip while the thickness of the substrates was between 100 and 150 μm (Supplementary Text).

**Cell Culture**

Primary human LC cells were isolated as previously described. The LC cells (age-matched normal [n = 4] and glaucoma [n = 3] donors) between passages 4 to 8 were used for all experiments. The LC cells were cultured with Dulbecco modified Eagle medium (DMEM; Sigma Chemical, Poole, United Kingdom), supplemented with 10% (vol/vol) fetal bovine serum (Gibco, Paisley, United Kingdom), 2 mM L-glutamine (Gibco), 2 U/mL penicillin, and 2 mg/mL streptomycin (Gibco) at 37°C with 5% CO₂. Growth medium was exchanged every 2 to 3 days. For all experiments, both normal and glaucoma LC cells were plated at low density (2000 cells/cm²) onto fibronectin-coated substrates and left to adhere for 24 hours prior to analysis.

**AFM Force Spectroscopy Measurements on Single Cells**

Soft cantilevers (CSC38-Tip B; Mikromasch, Tallinn, Estonia) with nominal values of f₃ ≈ 10 kHz, k ≈ 0.03 N m⁻¹, and tip radius ≈ 10 nm were used to probe the cells. Force maps arrays of 12 × 12 points were recorded for each cell over a 20×20-μm area of the cell body (avoiding the nucleus and the cell edge) (force curve rate = 1 Hz; max force = 2 nN; max indentation depth = 800 nm to 3 μm). The elastic modulus E (extrapolated from fitting Log₁₀[E] (Distributions) and reported as mean ± relative error) was determined by fitting the force versus indentation curve to the Sneddon Model using Igor Pro (WaveMetrics) and assuming a conical shaped indenter with α = 20° and Poisson’s ratio ν = 0.5, and analysis was restricted to a maximum indentation depth δ = 200 to 500 nm. Data that did not conform to the Sneddon Model were manually excluded from analysis along with any statistical outliers.

**Immunofluorescence and Fluorescent Microscopy**

The phalloidin F-actin cytoskeleton and FAs were visualized using Kit FAK100 (Chemicon International, Temecula, CA, USA) according to the following protocol. Cells were fixed onto substrates for 20 minutes at RT in prewarmed 4% buffered paraformaldehyde (PFA). Cell membranes were permeabilized using 0.1% Triton X-100 in PBS for 5 minutes at RT and rinsed twice. Blocking solution (1% BSA in PBS) was applied for 30 minutes, and the cells were subsequently incubated with the anti-vinculin solution (1:200 in blocking solutions) at RT for 1 hour and rinsed three times with PBS. The cells were incubated at RT for 40 minutes with AlexaFluor 488-conjugated goat anti-mouse secondary antibody (1:200) and tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin (1:100) in PBS, and the cells were rinsed three times with PBS. All images were acquired using a Nikon A1R confocal microscope using the epifluorescence mode. For α-SMA staining, after fixation cells were washed and incubated in blocking buffer (5% BSA, 10% normal goat serum [NGS], 0.3% Triton X-100 in PBS) for 2 hours at RT. Cells were incubated with anti-α-SMA antibody (1:100) at 4°C overnight. Then, the cells were washed three times with PBS and incubated with TRITC-conjugated phalloidin (1:100) and anti-rabbit fluorescent isothiocyanate (FITC)-conjugated secondary antibody (1:300) at RT for 1 hour. Finally, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) (1:1000 in PBS) at RT for 5 minutes and rinsed three times with PBS. Fluorescent signals were detected using the Nikon A1R confocal microscope (Nikon, Tokyo, Japan) with the NIS-Elements AR 4.0 software package (Nikon).

**Cell Area and Shape Quantification**

Cell area was calculated after tracing cell boundaries in fluorescent images manually using Fiji ImageJ software (http://rsb.info.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Cell shape was quantified by determining a cell shape factor S circularity), as defined by: 

\[ S = 4\pi A / P^2 \]

in which A is the cell area and P is the perimeter. The shape factor is equal to 1 for a perfectly round cell and approaches 0 for an elongated cell. Shape factors were determined from two-dimensional (2D) projections of single cells in a fluorescent image using Fiji ImageJ software.

**Vinculin-Stained Focal Adhesion Size and Number Quantification.** Cell images were processed using Fiji plugin ‘Squash’ to detect, delineate, and quantify vinculin-containing focal adhesions. Identical segmentation parameters such as the threshold for the minimum object intensity and regularization were used across all studied images to allow comparison of the results.

**Statistics**

Statistical analyses were performed using SPSS v 20.0 (SPSS, Chicago, IL, USA). Within one experiment, all data sets were analyzed by 1-way ANOVA with post hoc multiple comparison Tukey’s test. All individual stiffness values for a sample were summarized in Igor Pro (WaveMetrics) software to obtain the distribution of log-transformed stiffness values. The bin width was set to 500 Pa for all samples. For data fitting, the multiple-peak fitting package of Igor Pro was used. All data are represented as mean ± relative error. For the data that was not normally distributed, Kruskal-Wallis 1-way ANOVA on ranks was used. For correlation analysis, Pearson correlation coefficient (r) was calculated. The level for statistical significance was set at P < 0.05 for all comparisons.

**RESULTS**

**Fibronectin Coating Topography and Substrate Mechanics**

To assess the possible topographic effects of fibronectin on LC cell phenotypes, the nanoscale topography and roughness of modified PDMS surfaces were characterized by tapping mode AFM imaging. For this analysis, we chose areas devoid of larger fibril bundles. An example of such a region is shown in Figure 1. As a measure of topography of the deposited fibronectin on substrate surfaces, we compared the RMS roughness (Rq) of the substrate surfaces of soft (2.04 ± 1.77 nm) and stiff (1.54 ± 0.44 nm) samples (Fig. 1). Although the standard deviation (SD) of surface roughness is larger on the soft substrates, there is no significant difference between soft and stiff substrate roughness (P > 0.05). Coated substrate surfaces were relatively uniform despite differences in the substrate stiffness, resulting in uniformly favourable surfaces for cell adherence. The mechanical properties of substrates were measured to be 3.13 ± 3.80 and 78.84 ± 35.51 kPa, referred to as “soft” and “stiff” substrates, respectively (Supplementary Fig. S1).

**LC Cell Structure and Morphology Are Substrate Stiffness Dependent**

We then evaluated the formation of F-actin stress fibers by labeling cells with TRITC-conjugated phalloidin. A series of cell morphologic characteristic experiments were performed generated from four normal and three glaucoma human
donors. Fluorescent images (Fig. 2A) reveal that on soft substrates, both normal and glaucoma LC cells were devoid of stress fibers and exhibited a smaller phenotypic appearance. In contrast, LC cells grown on stiff substrates exhibited prominent F-actin filaments and a more spread appearance as shown by cell membrane extensions to achieve a larger cell area.

Perimeter tracing using ImageJ enabled quantification of LC cell areas on substrates with different stiffness. Figures 2B and 2C shows quantification of cell area and cell shape (circularity) generated from one human donor for each diagnosis. The number of cell studied on soft substrates was \( N = 24 \) cells from normal donors and \( N = 19 \) cells from glaucoma donors. On stiff substrates, we studied \( N = 31 \) cells from normal donors and \( N = 19 \) cells from glaucoma donors. The bar graph in Figure 2B illustrates that stiff substrates induce a significant increase in cell area for both normal and glaucoma LC cells (\( P < 0.001 \)). Compared with normal LC cells, glaucoma LC cells exhibited a significantly larger cell area on stiff surfaces (\( P < 0.001 \), stiff). The interaction between cell diagnosis and substrate stiffness indicates that glaucoma LC cells experience a dynamic cellular remodeling on stiff substrates by exhibiting a larger cell area.

![Image of fluorescent images](http://tvst.arvojournals.org/)

**Figure 1.** Surface structure analysis of fibronectin coating on the silicone elastomer with AFM. Surface topography images of fibronectin-coated soft and stiff substrates were produced with AFM in tapping mode and the RMS roughness (Rq) of five randomly selected regions (5 × 5 \( \mu m \)) were compared. Error bars are SD.

**Figure 2.** (A) Fluorescent images of phalloidin (red) and DAPI (blue) staining represent cell morphology changes induced by substrate stiffness. Scale bar denotes 100 \( \mu m \). (B) Bar graph shows cell spread area (\( \mu m^2 \)) of normal (donor 1) and glaucoma (donor 1) LC cells grown on soft (\( N = 24 \) normal cells, \( N = 19 \) glaucoma cells) and stiff (\( N = 31 \) normal cells, \( N = 19 \) glaucoma cells) substrates. (***\( P < 0.001 \)) Error bars indicate SD. (C) Cell shape dependence on substrate stiffness using a single normal and glaucoma donor for illustration purposes. The cell circularity \( S \) for the cell periphery is high for circular shapes and low for more ramified cell boundaries. Stiff substrates significantly decreased cell circularity and more elongated cell morphologies were observed (characteristic of fibroblast-like cells) compared with soft substrates (\( P < 0.05 \)).
Figure 2C shows that stiff substrates significantly decreased cell circularity and more elongated cell morphologies were observed (characteristic of fibroblast-like cells) compared with soft substrates \((P < 0.05)\).

For four normal and three glaucoma LC donors in which both cell area and circularity were measured, we examined the relationship between these parameters. Substrate stiffness significantly affects cell circularity \((P < 0.001)\), such that greater circularity was found in cells grown on the soft substrates than that on stiff substrates (Supplementary Fig. S2; Supplementary Table S1). Donor number and substrate stiffness have a significant interaction \((P < 0.001)\), and donor number is also found to be significant, which is expected but not of particular interest in the current study. These data establish a potential causality among these variables and strongly support the idea that increased substrate stiffness contributes to enlarged cell area and decreased circularity.

**Stiff Silicone Elastomer Substrate Promotes F-Actin and Vinculin-Stained FA Formation**

Cells were plated at a low density \((<2000 \text{ cells/cm}^2)\) to avoid possible confounding effects of cell–cell contact. F-actin and vinculin-contained focal adhesions were labeled with TRITC-conjugated phalloidin and anti-vinculin antibody, respectively. Immunostained images (Fig. 3A) reveal that LC cells demonstrated additional F-actin filaments on the stiff substrate with more FAs. Glaucoma LC cells, in particular, showed a polygonal shape, a significantly larger cell area, and abundant actin filaments connected to larger vinculin-contained FAs located near the cell edges and in the central part of the cell membrane. However, on soft surfaces, there was no evidence of well-organized stress fibers; instead, the F-actin signal was less intense and exhibited poor spatial definition, along with fewer and smaller, ill-defined FAs (Fig. 3A).

Quantification of vinculin-stained FAs was performed by applying available ‘Squassh’ segmentation protocol, which enables automated detection of fluorescently labeled objects. Quantitative Squash output images included in Figure 3A shows the masked and segmented individual vinculin-stained FA using the same segmentation parameters such as the mean object fluorescence intensity and regularization. Within 24 hours of culturing, the number of vinculin-stained FAs formed on the stiff substrates was approximately two- to threefold more than those formed on the soft substrates for normal and glaucoma LC cells, respectively (Fig. 3B). In comparison with the classical FAs of normal LC cells on stiff substrates \((15 \pm 11 \mu\text{m}^2)\), glaucoma LC cells formed more numerous and larger, widely distributed FAs \((20 \pm 17 \mu\text{m}^2\); Fig. 3C). The greatest total FA area was seen in glaucoma LC cells on the stiff substrate, and this corresponded with the largest cell spreading area shown in Figure 2B.

**LC Cells Exhibit Myofibroblastic Phenotype as Substrate Stiffness Increases**

Cells undergoing myofibroblast transformation were identified by immunostaining for α-SMA, which, when organized into actin stress fibers, is a strong indicator of myofibroblast activation. Figure 4 shows representative images and colocalization results for dual-channel images with F-actin (red) and α-SMA (green). After 24 hours in culture, there was no evidence of stress fibers in cells seeded on the soft substrate. Although normal LC cells stained positively for α-SMA in their nuclear or cytosol region, none of the protein was observed near the cell edges and in the central part of the cell. The transformed α-SMA signal localized at the nucleus to a large extent for both normal and glaucoma LC cells. However, a large variation was observed indicating a large proportion of cells showing heterogeneity in α-SMA distribution across the cell body. Figure 4C shows increased stiffness promotes α-SMA signal and translocation to the cytoplasm from the nuclear pool, resulting in its assembly into the stress fibers that overlay with the actin cytoskeleton, in agreement with observations in Figure 4A.

**Effect of Substrate Stiffness on Cell Stiffness Distribution**

Primary LC cells from normal and age-matched glaucoma donors were studied by AFM based nanoindentation where a force was applied to induce cell membrane deformation and enable determination of Young's modulus \((E)\). By simultaneously performing light microscopy of the live cells, we navigated the AFM force probe over defined regions near but not over the nuclei. Individual glaucoma LC cells on the soft substrate were found to exhibit a bimodal distribution with several dominant peaks possibly localizing to different parts of the cell (e.g., lamellipodia and cell body; Supplementary Fig. S3). The elasticity map obtained shows clear differences of \(E\) between the cell body and peripheral regions. The transformed \(\log_{10}(E)\) histogram exhibits a non-Gaussian distribution varying from a minimum value of 2.57 (0.24 kPa) to a maximum value of 4.45 (27.11 kPa) (Supplementary Fig. S5a). All data were pooled and a histogram was generated. After log transformation, the histograms of \(\log_{10}(E)\) showed complex stiffness profiles for cells grown on substrates with different stiffness as seen in Figure 5A. Both normal and glaucoma LC cells exhibited similar \(\log_{10}(E)\) bimodal distributions on soft substrates. The two peaks in the bimodal distribution of normal cells on soft substrates were at 2.93 (0.85 kPa) and 4.12 (13.18 kPa) (solid line). The two peak values in the bimodal distribution of glaucoma cells were centered at 3.05 (1.12 kPa) and 4.24 (17.38 kPa) (dashed line) (Fig. 5B). These bimodal distributions suggest that perhaps there are two different cell subpopulations exhibiting two different cell stiffness profiles (Supplementary Fig. S4).

On stiff substrates, the two peaks in bimodal \(\log_{10}(E)\) distribution in normal LC cells were centred at 3.91 (8.13 kPa) and 4.64 (43.65 kPa), whereas the corresponding peaks for normal LC cells, α-SMA stress fibers were more pronounced in the glaucoma LC cells on the stiff substrate.

Image correlation analysis between colabeled fluorescent molecules of F-actin and α-SMA was used to facilitate pixel-to-pixel comparison based on the Pearson correlation coefficient \((r)\). The stronger linear relationship and higher \(r\) value were identified as having pronounced expression of α-SMA in F-actin stress fibers, which were exclusively seen in glaucoma LC cells on the stiff substrate, indicating higher cell contractility and activation of the myofibroblast phenotype.
FIGURE 3. Organization of actin cytoskeleton and vinculin-stained FA of LC cells is dependent on substrate stiffness and diagnosis. Normal and glaucoma LC cells were cultured on soft (5 kPa) and stiff (100 kPa) substrates for 24 hours and then were labeled with phalloidin, anti-vinculin, and DAPI. (A) Representative epi-fluorescence images show individual channel components of F-actin, FA, Squash output images of masked and segmented FA, and the merged RGB images. Scale bar denotes 100 μm. (B) Scatter plot shows number of vinculin-stained FA per cell. Each point corresponds to an individual cell: 19 cells were analyzed for each diagnosis. (C) Scatter plot showing area of single vinculin-stained FAs. Each point represents a single FA from Squash output images. The solid lines represent the median values, based on measurements of at least 150 FAs per condition.
Figure 4. (A) Representative cell images expressing F-actin (red) and α-SMA (green) proteins as indicated. To estimate the correlation of distribution between F-actin and α-SMA, a “Pearson colocalization coefficients (r)” was built using ImageJ Coloc-2 software. The 2D intensity histogram was applied to visualize the overlapping of red and green pixel intensities, exhibiting a stronger degree of colocalization of actin and α-SMA. A heterogeneous phenotype was observed on the soft substrate (solid square in the top right). Scale bar denotes 100 μm. (B) Scatter plot showing correlation between cell area and normalized integrated fluorescence intensity (to the mean value of glaucoma LC cells on the stiff
substrate), as a function of disease diagnosis. Each point represents a single cell grown on the corresponding substrate: 16 normal cells and 49 glaucoma cells were analyzed. There is a significant correlation between increased α-SMA straining intensity and cell area for both normal ($r = 0.548$, $P < 0.05$) and glaucoma LC cells ($r = 0.511, ***P < 0.001$). (C) Quantification of the ratio of nuclear to cytoplasmic localization of α-SMA staining signals on varying substrate stiffness. Significantly decreased nuclear to cytoplasmic ratio was observed in both normal and glaucoma cells grown on the stiff substrate ($P < 0.05$). The box and whisker plot depicts the values range from minimum to maximum.

**Discussion**

Although matrix stiffness mediates potent effects on the human TM cells leading to the impaired humour outflow pathways, the impact on the pathological LC cells remains largely unknown. Like TM cells, LC cells are also sensitive to the mechanical properties of the ECM, which, however, have not been well studied in vitro. In this study, we established a cell culture system of primary LC cells on silicone elastomer substrates with defined stiffness, soft (~5 kPa) and stiff (~100 kPa), which may mimic the ONH microenvironment during the pathogenesis of cupping. Our results demonstrated that both normal and glaucoma LC cells undergo significant substrate stiffness-dependent morphologic changes, accompanied by an increasing myofibroblastic phenotype (α-SMA signal intensity and localization) as substrate stiffness increases. In comparison with normal cells, glaucoma LC cells exhibited an enhanced sensitivity to the perturbation in the mechanical microenvironment with pronounced changes in cell area and increased α-SMA incorporation into stress fibers.68

Typically, the cell actin network is a profoundly dynamic structure that is responsible for the determination of cell shape and integrity.67 As substrate stiffness increased, significant changes in the α-SMA actin cytoskeleton were accompanied by an enlarged cell area for both the normal and glaucoma LC cells (Fig. 2A). This is in line with previous reports that human TM cells respond to a stiffer environment by reinforcement of stress fibers and enlarged cells due to spreading.31–35 In addition, the importance of the F-actin network in governing cell–ECM interactions and it has been proposed that vinculin is a key player in the regulation and formation of FAs, which have been well characterized in vitro.58,69 In this experiment, we investigated how vinculin-stained FAs vary in response to increased substrate stiffness and evaluate the difference between normal and glaucoma LC cells in cell–matrix adhesions. Our major findings are the increased formation of FAs occur along with the reorganization of actin cytoskeleton induced by increased substrate stiffness. Quantification of vinculin-stained FAs reveals a significantly increased number and total area ($P < 0.05$) were observed in glaucoma LC cells (Fig. 3), implying a more stable and stronger cell adhesion. This observation was paralleled by an increase in α-SMA expression and localization in the actin stress fibers (Fig. 4). These results support the existence of adhesion and contractility relationship in myofibroblast activation and that these expressions of α-SMA in stress fibers on stiff substrates are associated with the formation of large mature FAs.68,70 However, a differential pattern of vinculin-contained FAs driven by matrix stiffness has not been compared between normal and diseased cell types in previous studies. Job et al.71 demonstrated the dense actin-rich polygonal structures identified as cross-linked actin networks (CLANs) were particularly dominant in glaucoma LC cells compared with normal cells, highlighting the complexity of F-actin distribution patterns in vitro. Our results revealed these distinct features observed in glaucoma LC cells may be at least partly responsible for the high efficiency of activated myofibroblasts to adhere and contract surrounding ECM.

The recruitment of FA proteins modulated by matrix stiffness thus provides a stable connection between the cell’s actin cytoskeleton and the underlying matrix, allowing for stronger cell adhesion and increased contractility which further facilitates myofibroblast adhering and contracting the ECM.69,72 The highest correlation coefficients of α-SMA incorporation into F-actin stress fibers was exclusively noted for glaucoma LC cells on the stiff substrate. Indeed, mechanical tension generated on stiffer substrates activates intracellular signaling through a number of pathways including p38 MAPK, Rho-kinase, and FA kinase to upregulate transcription and subsequent incorporation of α-SMA into the actin stress fibers.68,69 Further investigation of these molecular pathways is needed to further elucidate the relationship between stiffness sensing and cell disease state.

Coupled mechanical factors and local cytokine mediators such as TGF-β are considered to be potent stimuli of myofibroblast activation and persistence.70,71 Substrate stiffness may provide the initial stimulus for activation, which is then enhanced by chemical signals, such as TGF-β, resulting in the morphologic and functional maturation of activated state.72 Previous studies in our laboratory have revealed gene signatures in glaucoma LC cells are remarkably different from age-matched normal cells.55 Glaucoma LC cells were found to have upregulated ECM and profibrotic TGF-β gene expression in comparison with normal cells.57 The appearance of the myofibroblast phenotype in the glaucoma LC cells and the stiffer substrate implies that the TGF-β may drive the substrate stiffness-mediated transition to the myofibroblast phenotype. In addition, there is increased expression of the profibrotic cytokine gremlin in the glaucoma ONH, which works with TGF-β2 to generate a feedforward fibrotic ONH environment.75 Further studies on this process are warranted. Importantly, previous work has shown that dense actin-rich polygonal structures identified as CLANs occur in glaucoma TM and LC cells.71,76 The predominant cross-linked F-actin architecture seen in glaucoma LC cells will profoundly impact local cell stiffness in response to mechanical stimuli playing a significant role in the ability to cope with biomechanical stress and strain in the abnormal microenvironments of the ONH in glaucoma.

The impact of this structural reorganization in the cells mechanical integrity remains incompletely understood. We used AFM nanoindentation to quantitatively assess cell stiffness in response to mechanical stimuli. AFM nanoindentation has the extraordinary ability of mapping elasticity with precisely controlled force at the nanoscale, and the use of the sharp tip provides invaluable information of localized cell elasticity given the heterogenous nature of cells surfaces.77 Our results
FIGURE 5. (A) Log distribution of the elastic modulus of (a) normal LC cells on 5-kPa substrate (n = 1000 curves), (b) normal LC cells on 100-kPa substrate (n = 553 curves), (c) glaucoma LC cells on 5-kPa substrate (n = 1251 curves), and (d) glaucoma LC cells on 100 kPa (n = 784 curves). (B) The multipeak fitting package in Igor Pro (Wavemetrics) software was applied to the log10(E) distribution, with peak value depicted on (a) normal LC cells on 5-kPa substrate, (b) normal LC cells on 100-kPa substrate, (c) glaucoma LC cells on 5-kPa substrate, and (d) glaucoma LC cells on 100 kPa (red/blue lines: Gaussian fit of all log-transformed data). (C) A box and whisker plot of the log distribution of E. The Kruskal-Wallis test from nonparametric samples (which does not assume any specific distribution) shows that both normal and glaucoma LC cells stiffen following exposure to increased substrate stiffness. *P < 0.05. NLC, normal lamina cribrosa cells; GLC, glaucoma lamina cribrosa cells.
showed that the log$_{10}(E)$ histograms of LC cells exhibited non-Gaussian distributions on both substrates. Immunohistochemical staining with α-SMA, a marker indicating a myofibroblast-like phenotype, suggested that the specimen may contain two cell subpopulations on the soft substrate (positive and negative for α-SMA), which may give rise to heterogeneity in measured stiffness profiles (Supplementary Fig. S4). Moreover, this heterogeneity in measured stiffness across the cell body and from cell to cell is related to a number of factors including cell cycle,78 cell aging,79 and ligand density.80 Despite these differences of morphology, a notable cell population shift to higher Young’s modulus was observed on the stiff substrate (Fig. 5). Although variation in reported cell stiffness values measured with AFM is large, most studies agree that cells are stiffer when cultured on stiffer substrates.30,81,82 Solon et al. previously reported that fibroblasts adapt the stiffness of their cytoskeleton to that of their substrate, where the average stiffness of the fibroblast increased from 1 to 8 kPa as substrate stiffness increased from 2 to 10 kPa.30 Additionally, Overby et al. revealed SC cells become stiffer in response to increased substrate stiffness, and compared with normal SC cells, glaucoma SC cells exhibit a much greater stiffening response when cultured on the stiffest gel (34.4 kPa) compared with the softest gel (1.1 kPa).13 In our study, the stiffness profiles demonstrate that both the normal and glaucoma LC cells have increased subcortical cell stiffness induced by stiffer substrates ($P < 0.05$). However, the median cell stiffness of normal LC cells was found to be significantly higher than that of glaucoma LC cells on the stiff substrate ($P < 0.05$), and the distribution of the log$_{10}(E)$ histogram was also noticeably broader than that of glaucoma LC cells. This result might indicate differences within the actin cytoskeleton organization between normal and glaucoma LC cells induced by the stiff substrate, and the use of the sharp AFM tip magnifies local strength differences. Such an effect would be understandable when a cell possesses a highly organized cytoskeleton and might provide an explanation for the broad distribution in cell stiffness. Future experiments using alternative tip geometries (sphere or blunt-shaped) may provide further insight into the global versus local effects in cell mechanics. Overall, the data emphasize the importance of heterogeneity in cell stiffness and the use of stiffness profiles helps to fill in crucial knowledge gaps in our understanding about the biomechanics at the single cell level. Moreover, our findings support the hypothesis that cellular mechanical properties may serve as novel biological markers of cell phenotypes reflecting changes in disease progression, and this area warrants further investigation.

We acknowledge that the chosen stiffness values were based on previous work by Last et al.11 profiled from human TM glaucoma research and may not be entirely accurate for human LC in normal and glaucoma conditions. It is quite probable that the tissue stiffness in the LC may be stiffer than the values that we used in this study. However, the use of this culture system with defined stiffness and surface characterization allows us to isolate LC cell response to very specific stiffness stimuli.

**CONCLUSIONS**

This study demonstrated that both normal and glaucoma LC cells are mechanosensitive, where increasing substrate stiffness promotes an enlarged cell area and a change in the cell shape, F-actin network reinforcement, vinculin focal adhesion formation, increased α-SMA concentration and localization, and increased cell stiffness; all phenotypes associated with activated myofibroblasts in both the normal and glaucoma LC cell. Taken together with previous findings, the increased substrate stiffness induces myofibroblast activation in LC cells, which may be centrally involved in the pathogenic fibrotic processes seen in the LC in glaucoma.

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**References**


