Altered mechanobiology of Schlemm’s canal endothelial cells in glaucoma

Darryl R. Overby1,2, Enhua Zhou1,2, Rocio Vargas-Pinto1, Ryan M. Pedrigi2, Rudolf Fuchshofer2, Sietse T. Braakman3, Ritika Gupta4, Kristin M. Perkumase, Joseph M. Sherwooda, Amir Vahabikashia, Quynh Dangb, and D. R. O. wrote the manuscript.

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Significance

Glaucoma is an eye disease that is characterized by increased intraocular pressure (IOP) and causes nerve damage, leading to vision loss. The main cause of glaucoma is unknown, but it is believed to be related to increased blood flow resistance in the eye’s drainage system, called the trabeculum. This study investigated the mechanobiology of Schlemm’s canal (SC) endothelial cells in normal and glaucomatous eyes. The authors found that SC cells from glaucomatous eyes have altered mechanobiology, which could lead to increased resistance and increased IOP. These findings provide new insights into the mechanism of glaucoma and could lead to the development of new therapies.

Results

The authors investigated the mechanobiology of SC cells in normal and glaucomatous eyes by using atomic force microscopy and in vitro assays. They found that the SC cells from glaucomatous eyes have altered mechanobiology, with increased stiffness and decreased pore formation. These changes in mechanobiology could lead to increased resistance and increased IOP. The authors also found that the mechanobiology of SC cells is altered in glaucomatous cell strains, which could explain the increased resistance.

Discussion

The authors discuss the implications of their findings for the development of new therapies for glaucoma. They suggest that the altered mechanobiology of SC cells in glaucomatous eyes could be a target for new therapeutics that target cell stiffness. The authors also mention the potential for these findings to be generalized to other diseases that are associated with increased resistance and increased IOP, such as hypertension.


The authors declare no conflict of interest.

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1D.R.O., E.H.Z., and R.V.-P. contributed equally to this work.

2Present address: Ophthalmology, Novartis Institutes of BioMedical Research, Cambridge, MA 02139.

3To whom correspondence should be addressed. Email: m-johnson@northwestern.edu.

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Increased flow resistance is responsible for the elevated intraocular pressure characteristic of glaucoma, but the cause of this resistance increase is not known. We tested the hypothesis that altered biomechanical behavior of Schlemm’s canal (SC) cells contributes to this dysfunction. We used atomic force microscopy, optical magnetic twisting cytometry, and a unique cell perfusion apparatus to examine cultured endothelial cells isolated from the inner wall of SC of healthy and glaucomatous human eyes. Here we establish the existence of a reduced tendency for pore formation in the glaucomatous SC cell—likely accounting for increased outflow resistance—that positively correlates with elevated subcortical cell stiffness, along with an enhanced sensitivity to the mechanical microenvironment including altered expression of several key genes, particularly connective tissue growth factor. Rather than being seen as a simple mechanical barrier to filtration, the endothelium of SC is seen instead as a dynamic material whose mechanical effects on pore formation—in the glaucomatous eye, this process becomes impaired. Together, these observations support the idea of SC cell stiffness—and its biomechanical effects on pore formation—as a therapeutic target in glaucoma.

Aqueous humor flows across the inner wall endothelium of Schlemm’s canal (SC) and generates a transendothelial pressure gradient from the cellular base to the cellular apex. From a biomechanical perspective, the direction of this gradient is remarkable considering that the endothelium of the systemic vasculature experiences a pressure gradient in precisely the opposite direction. In the healthy eye, this basal-to-apical transcellular pressure gradient is of sufficient magnitude to partially separate the SC cell from its supporting basement membrane, inflate dome-shaped structures known as giant vacuoles, and generate cellular mechanical strains exceeding 50% (Fig. 1) (1). The formation of giant vacuoles leads to substantial thinning of the SC endothelial cell and is thought to be associated with formation of pores that provide an outflow pathway across the SC endothelium (2). Although reported dysfunction of the pore formation process might be expected to affect outflow resistance and elevate intracocular pressure (IOP) (3, 4), mechanisms for such dysfunction have never before been established, in large part because SC cells from healthy eyes are so difficult to isolate technically, but also because isolated SC cells from the glaucomatous eye are a resource that has been exceedingly scarce. Here for the first time to our knowledge we show that the process of pore formation differs substantially between cells from the healthy versus the glaucomatous human eye and show, further, that this difference depends upon cytoskeletal stiffness that is altered in the glaucomatous SC cell, likely owing to altered substrate sensitivity and gene expression in these cells. Specifically, stiffer glaucomatous cells impede pore formation and thereby elevate IOP.

Materials and Methods

Pore Formation in SC Cells Is Altered in Glaucomatous Cell Strains. To examine pore formation in SC cells, we used an in vitro monolayer perfusion system to mimic the biomechanical and filtration environment of SC endothelium in vivo (5). As described previously, SC cells were isolated from normal and glaucomatous human donors and extensively characterized (Materials and Methods) (6, 7). When perfused in a basal-to-apical direction pores formed in SC cell monolayers, with pores passing transcellularly through individual SC cells or paracellularly between neighboring SC cells, consistent with the two pore types observed along the SC endothelium in situ (Fig. 2A) (8). The density of pores (pores per cell area) increased significantly with perfusion pressure (P < 3 × 10−5; Fig. 2B), and porosity (pore area per cell area) showed a similar dependence upon pressure (P < 0.003;...
Glaucomatous SC Cells Demonstrate Elevated Subcortical Stiffness.

We reasoned that it would be more difficult for a pore to form in stiffer SC cells. To investigate this possibility, we measured the stiffness of SC cells isolated from normal and glaucomatous human donors (Materials and Methods) using atomic force microscopy (AFM) using both sharp tips (20-nm tip radius) and rounded tips (4.5 μm and 10 μm) (10). In other cellular systems, AFM measurements using a sharp tip characterize the cell cortex, whereas larger, spherical tips probe the subcortical cytoskeleton (10). Here we use the term “cortex” to refer to the actin-dense region of the cell lying immediately beneath the plasma membrane, and the term “subcortical cytoskeleton” to refer to the internal cytoskeleton underlying the cortex (11). For all tip geometries, elastic moduli were found to be similar between nuclear and peripheral regions of the cell, and there was no systematic variation between Young’s modulus and donor age (SI Text). Cell stiffness measured with sharp tips was 10-fold higher than that measured with the larger, spherical tips (Fig. 3B), consistent with the prominent actin-rich cell cortex found in SC cells and other endothelia (Fig. 3A).

Measured with a sharp AFM tip, we found no difference in stiffness between normal versus glaucomatous SC cells (P > 0.85; Fig. 3B). Cortex thickness as measured by structured illumination microscopy was similar between normal (400 ± 20 nm, n = 3 cell strains) and glaucomatous SC cells (380 ± 60 nm, n = 2). However, when measured with the larger, spherical AFM tips, we found systematic differences in stiffness between glaucomatous SC versus normal SC cells (Fig. 3B and C). With a 4.5-μm tip, the modulus of glaucomatous SC cells was 1.47 ± 0.29 kPa (n = 5 cell strains; m = 128 measurements), whereas that of normal SC cells was measured as 1.01 ± 0.12 kPa (n = 6; m = 104) (P < 0.12). Using a 10-μm tip, the modulus of glaucomatous SC cells was 1.24 ± 0.11 kPa (n = 5; m = 120), whereas that of normal SC cells was 0.79 ± 0.10 kPa (n = 6; m = 153) (P < 0.02). Relative to the normal SC cells, glaucomatous SC cells revealed substantially elevated subcortical stiffness. Both cortical and subcortical SC cell stiffness were greatly reduced by latrunculin-A, consistent with an important role for actin in determining stiffness (Fig. 3A); however, there was no difference in the relative decrease in cell stiffness following latrunculin between normal and glaucomatous SC cells (SI Text), suggesting that perhaps another constituent of the subcortical cytoskeleton [e.g., intermediate filaments (11)] may be altered in glaucomatous SC cells.

For two normal and three glaucomatous SC cell strains in which both cell stiffness and pore density were measured we examined the relationship between these parameters. Subcortical stiffness (10-μm spherical tip) was related inversely to pore density (P < 0.002; Fig. 3D) and porosity (P < 0.012; SI Text). A relationship was apparent between subcortical stiffness and pore density for both pore subtypes; however, subcortical stiffness showed a more significant correlation with transcellular pore density (P < 0.02) compared with paracellular pore density (P < 0.07) (SI Text). These data do not establish causality but do strongly support the idea that increased subcortical cell stiffness and decreased pore formation go hand in hand.

On Increasingly Stiffer Gels, Both Normal and Glaucomatous SC Cells Stiffen.

We asked next what might cause this stiffness difference. One possibility is mechanotransduction of the mechanical properties of the SC cell microenvironment (12, 13). We thus investigated how substrate stiffness might influence SC cell

Fig. 1. Aqueous humor flow pathway. (Left) Schematic of anterior segment of eye showing the direction of aqueous humor flow in red. (Center) Enlargement of the iris-cornea angle (boxed region in left panel) to show the conventional outflow pathway. (Right) Transmission electron micrograph of endothelial cells forming the inner wall of SC; aqueous humor crosses the endothelium through pores to enter the lumen of SC. V, giant vacuoles. C is reproduced with permission from ref. 41.

Fig. 2. Pore density in perfused SC monolayers. (A) Representative image of transcellular and paracellular pores in normal (SC52) and glaucomatous SC (SC62g) cells. (B) Pore density increases in monolayers formed from three nonglaucomatous SC cell strains with transcellular (basal-to-apical) pressure drop in one SC cell strain (SC62g) perfused in the apical-to-basal direction (AB), pore densities are similar to unperfused controls at 0 mmHg. (C) Pore density is reduced in glaucomatous compared with normal SC cells following perfusion at 6 mmHg in the basal-to-apical direction. Bars are SEM.
showed that normal SC cells stiffened in response to increased substrate stiffness \((P = 10^{-6}; \text{Fig. 4 C and D})\) and were 131% stiffer when cultured on the stiffest gel compared with the softest gel. Glaucomatous SC cells showed a much greater stiffening response \((P = 0.011 \text{ comparing normal versus glaucoma), increasing by 371% over the same range of substrate stiffness (Fig. 4 E and F).}\) Thus, similar to other endothelial cells, SC endothelial cells stiffen in response to increasing substrate stiffness. Compared with the healthy SC cell, the glaucomatous SC cell exhibits a strikingly enhanced stiffening response.

**Expression of Glaucoma-Related Genes Is Dependent upon Substrate Stiffness and Exaggerated in Glaucomatous Cell Strains.** In endothelial cells and fibroblasts, substrate stiffness is known to modulate gene expression \((17-19)\). Using real-time quantitative PCR as a function of substrate stiffness in normal and glaucomatous SC cells \((\text{Materials and Methods})\), we examined the expression levels of 13 genes \((\text{Table 1})\) previously linked to mechanosensing, glaucoma, ECM remodeling, or TGF-β/β/2/connexin tissue growth factor (CTGF) signaling.

The mRNA expression of \(\text{Col}1\) was up-regulated by up to 20-fold with increasing substrate stiffness for both normal and glaucomatous cells \((P < 10^{-6})\), with no significant difference between normal and glaucomatous cells \((P > 0.4)\) \((\text{Fig. 5 A})\) \((\text{see Materials and Methods for statistical treatment})\). Significant

stiffness and gene expression. Because of the need to examine a large number of cells on substrates of a variety of stiffnesses, we used optical magnetic twisting cytometry \((\text{OMTC})\) \((\text{Materials and Methods})\) to study SC cells isolated from normal and glaucomatous human donors \((\text{Materials and Methods})\).

Grown on rigid substrates, we found no difference in stiffness between normal and glaucomatous SC cells strains \((\text{SI Text})\), and, as expected, these results were consistent with the AFM findings using a sharp tip described above \((11, 14)\). We also examined how SC cells grown on rigid substrates responded to drugs with known effects on outflow resistance. Similar to our finding previously reported for normal SC cells \((15)\), we found in glaucomatous SC cells that every agent that we examined decreased outflow resistance also decreased cell stiffness, and every agent that increased outflow resistance also increased cell stiffness \((\text{SI Text})\).

We then examined the influence of substrate stiffness on the cells. The physiological substrate of the SC cell is the trabecular meshwork, and its compressive Young’s modulus has been reported to be substantially increased in glaucoma \((16)\). Normal and glaucomatous SC cells were cultured on collagen-coated polyacrylamide gels of tunable stiffness \((\text{Materials and Methods})\) with Young’s moduli ranging from 1.1 kPa to 34.4 kPa, the former mimicking normal trabecular meshwork and the latter mimicking glaucomatous trabecular meshwork \((\text{albeit not modeling the complex geometry of the basement membrane and juxtanacilcular connective tissue that underlie the SC cells})\).

With increasing gel stiffness SC cells exhibited more prominent actin stress fibers and vinculin-containing focal adhesions \((\text{compare Fig. 4 A and B})\), suggestive of increased cytoskeletal contractility and/or elevated cell stiffness. OMTC measurements
increases with increasing substrate stiffness were also seen for
SPARC (P < 10−6), TGM2 (P < 10−3), ACTA2 (P < 10−3), MMP2
(P < 10−3), PAI1 (P < 0.005), BMP4 (P < 10−3), and GREM1 (P < 10−2). (Fig. 5 B, C, H, J, L, and M). Marginally statistically
significant increases (0.01 < overall P < 0.05) in TPM1 and
TGFβ2 were observed with increasing substrate stiffness (Fig. 5
D and F). These results indicate that normal and glaucomatous
SC cells share some common molecular responses to elevated
substrate stiffness.

We also identified three genes that were differentially modu-
lated by substrate stiffness in glaucomatous compared with normal
SC cells. PTGS2 had a marginally significant negative association
with substrate stiffness in glaucomatous cells (overall P < 0.03)
but not in normal cells (Fig. 5K). Importantly, CTGF and DCN
were more strongly up-regulated by elevated substrate stiffness
in glaucoma SC cells (P < 0.05; P < 10−3, respectively) than
in normals (Fig. 5 E and G). Of note, the absolute increase in
CTGF gene expression in glaucoma cell strains, compared
with normals (P < 0.05), was the highest of all of the genes
investigated (Fig. 5N). Other genes with higher expression in
glaucoma SC cells included TGF-β2 (P < 0.05) and PAI1
(P < 0.01). Genes with lower expression in glaucoma SC
cells included DCN (P < 0.05) and BMP4 (P < 0.01).

Together, these data demonstrate that SC cells modulate their
gene expression in tandem with substrate stiffness and that
Glaucomatous SC cells have altered substrate sensitivity that affects
key genes, particularly CTGF and DCN. In a mouse model of
glaucoma, CTGF has been associated with increased stress fiber
formation, IOP elevation, and glaucomatous optic neuropathy (20).
Here we establish a link between the expression of these same genes and changes of substrate stiffness.

Discussion
The cause of the elevated pressure and increased outflow re-
sistance characteristic of glaucoma is unknown despite being a
topic of investigation for over 140 y (21). Recent studies have
focused on the role of decreased extracellular matrix perme-
ability (22) or increased extracellular matrix stiffness (16) in the
glaucomatous process. Our studies here suggest that the cells of
the inner wall of SC may play a fundamental role in generating
increased outflow resistance in the diseased eye. The density
of pores in glaucomatous eyes is lower than in normal eyes
(3, 4). Pores in the inner wall endothelium of SC are thought
to modulate aqueous outflow resistance through a hydrodynamic
interaction with the flow of aqueous humor passing through the
trabecular meshwork (23, 24). Thus, decreased pore density is
expected to increase the resistance to outflow of aqueous humor
from the eye and thereby increase IOP, a characteristic of many
cases of glaucoma. Moreover, in the glaucomatous eye the ul-
trastructure and material properties of the trabecular meshwork
that supports the SC cell are altered (16, 25, 26). Because SC
cells from glaucomatous human eyes comprise a scarce experi-
mental resource, an innate limitation of this study is that the
differences reported between normal versus glaucomatous SC
cells may be inherent to the disease process itself or may arise
instead from chronic exposure to drugs used to treat the disease.
Although we cannot distinguish between these possibilities, we
do establish that these glaucomatous SC cells exhibit elevated
subcortical cell stiffness, enhanced sensitivity to the mechanical

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
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<tbody>
<tr>
<td>ACTA2</td>
<td>Alpha smooth muscle actin (SMA)</td>
</tr>
<tr>
<td>Col1A1</td>
<td>α-1 type I collagen</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>DCN</td>
<td>Decorin</td>
</tr>
<tr>
<td>MMP2</td>
<td>Matrix metalloproteinase-2</td>
</tr>
<tr>
<td>PAI1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PTGS2</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein acidic and rich in cysteine</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>Transforming growth factor-β2</td>
</tr>
<tr>
<td>TGM2</td>
<td>Tissue transglutaminase</td>
</tr>
<tr>
<td>TPM1</td>
<td>Tropomysin α-1 chain</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone morphogenetic protein 4</td>
</tr>
<tr>
<td>GREM1</td>
<td>Gremlin 1</td>
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</table>
microenvironment, and altered gene expression, notably CTGF, which has been shown to lead to ocular hypertension and glaucomatous optic neuropathy in mice (20). Furthermore, we have demonstrated that these altered material properties of the glaucomatous SC cells render them less able to form pores and thus presumably lead to increased IOP.

To lower IOP in glaucoma, two classes of new drugs are currently in clinical trials—Rho kinase inhibitors and actin depolymerizers (27, 28)—both of which lower outflow resistance (29, 30). The exact site of action in the conventional outflow tract of these drugs in lowering IOP in glaucoma is unknown, but it is interesting to note that both classes cause cell stiffness to decrease (15). We demonstrate here that both normal and glaucomatous SC cells alter their stiffness when treated with drugs that alter outflow resistance. These findings emphasize the importance of cell stiffness and the contractile state to the modulation of aqueous humor outflow resistance and control of IOP. The mechanosensitivity of SC cells thus represents an interesting therapeutic target for restoring the function of the conventional outflow pathway. Specifically, targeting SC cell stiffness is likely to provide an efficacious therapeutic approach to lower IOP for glaucoma therapy, with minimal off-target effects.

Materials and Methods

In the past, the comparison between normal and glaucomatous tissues and cells has been hindered by the lack of fresh human donor eyes. Our work included SC cells from nine normal and four glaucomatous donors, representing the largest collection of such samples to date (Table 2).

SC Cell Isolation and Culture. Human SC cells were isolated from cadaveric ocular tissues provided by the Midwest Eye Bank, National Disease Research Interchange, or Life Legacy within 36 h of death with enucleation occurring less than 6 h after death. Isolation of cells from donor eye tissue was done according to techniques developed and optimized previously (6). Briefly, after enucleation, cells were washed with buffered saline. Cells were seeded at confluence (4.5 × 10^4 cells/cm²) on the gels, grown in low-glucose DMEM with 1% FBS for 3 d, and switched to DMEM containing 1% (wt/vol) API, and a variable percentage of bisacrylamide (0.04, 0.1, 0.2, 0.5, and 1.3%; wt/vol) (18, 35–37). These API gels were positively charged and electrostatically absorb ECM proteins, including collagen 1 (36). Previous work confirmed the absorption of fibronectin and collagen to be independent of gel stiffness (38). Gels were cast between two glass plates to achieve a final thickness of about 0.8 mm. Disks 5 mm in diameter were punched out of gel sheets using surgical punches, transferred into 96-well plates, and stored in PBS. These gels were soaked in 10 µg/mL collagen 1 overnight (PureCol; Advanced BioMatrix) before cell plating. Young’s moduli of the gels were measured using AFM to be 1.1, 2.5, 4.2, 11.9, and 34.4 kPa for bisacrylamide concentrations of 0.04, 0.1, 0.2, 0.5, and 1.3%, respectively; the Young’s modulus scaled roughly linearly with cross-linker concentration (SI Text). SC cells at passages 5–6 were seeded confluent (3 × 10^5 cells/cm²) on the gels, grown in low-glucose DMEM with 1% FBS for 3 d, and switched to DMEM with 1× ITS (Sigma-Aldrich) overnight before OMTc testing using PLL-coated ferrimagnetic beads (4.5- or 10-μm diameter) were magnetized with a strong magnetic pulse in the horizontal direction and twisted with a much weaker magnetic field in the vertical direction. This vertical field, which oscillates at 0.77 Hz, imposed a sinusoidal torque on each bead. The torque was automatically adjusted to drive a net axial motion of about 60 nm. The bead motion was quantified by image analysis. The ratio of magnetic torque to bead motion defines the apparent stiffness (g*) measured by each bead (34). g* is a complex number and we report the modulus g = |g*|, which has units of pascals per nanometer.

Table 2. Summary of SC cell strain donor ages used in the present study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cell strain no.</th>
<th>Donor age, y</th>
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<tbody>
<tr>
<td>Normal</td>
<td>SC41</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>SC51</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>SC52</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>SC58</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>SC60</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>SC61</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>SC67</td>
<td>44</td>
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<tr>
<td></td>
<td>SC68</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>SC71</td>
<td>44</td>
</tr>
<tr>
<td>Glaucoma</td>
<td>SC57g</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>SC59g</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>SC62g</td>
<td>66</td>
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<tr>
<td></td>
<td>SC63g</td>
<td>78</td>
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<tr>
<td></td>
<td>SC64g</td>
<td>78</td>
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</tbody>
</table>

In addition, SC cells from normal donors were grown in low-glucose DMEM with 1% FBS for 3 d and switched to DMEM with 1× ITS (Sigma-Aldrich) overnight before OMTC testing using PLL-coated ferrimagnetic beads (4.5- or 10-μm diameter) were magnetized with a strong magnetic pulse in the horizontal direction and twisted with a much weaker magnetic field in the vertical direction. This vertical field, which oscillates at 0.77 Hz, imposed a sinusoidal torque on each bead. The torque was automatically adjusted to drive a net axial motion of about 60 nm. The bead motion was quantified by image analysis. The ratio of magnetic torque to bead motion defines the apparent stiffness (g*) measured by each bead (34). g* is a complex number and we report the modulus g = |g*|, which has units of pascals per nanometer.

For AFM measurements, we used subconfluent normal or glaucomatous SC cells at passage 4 or 5 with sharp pyramidal tips or spherical tips of 1-μm diameter (31, 32). Cells were magnetized with a strong magnetic pulse in the horizontal direction and twisted with a much weaker magnetic field in the vertical direction. This vertical field, which oscillates at 0.77 Hz, imposed a sinusoidal torque on each bead. The torque was automatically adjusted to drive a net axial motion of about 60 nm. The bead motion was quantified by image analysis. The ratio of magnetic torque to bead motion defines the apparent stiffness (g*) measured by each bead (34). g* is a complex number and we report the modulus g = |g*|, which has units of pascals per nanometer.

Fabrication of Substrates with Varied Stiffness and Testing Procedure. Spherical gels were cast between two glass plates to achieve a final thickness of about 0.8 mm. Disks 5 mm in diameter were punched out of gel sheets using surgical punches, transferred into 96-well plates, and stored in PBS. These gels were soaked in 10 µg/mL collagen 1 overnight (PureCol; Advanced BioMatrix) before cell plating. Young’s moduli of the gels were measured using AFM to be 1.1, 2.5, 4.2, 11.9, and 34.4 kPa for bisacrylamide concentrations of 0.04, 0.1, 0.2, 0.5, and 1.3%, respectively; the Young’s modulus scaled roughly linearly with cross-linker concentration (SI Text). SC cells at passages 5–6 were seeded confluent (3 × 10^5 cells/cm²) on the gels, grown in low-glucose DMEM with 1% FBS for 3 d, and switched to DMEM with 1× ITS (Sigma-Aldrich) overnight before OMTC testing using PLL-coated ferrimagnetic beads (4.5- or 10-μm diameter) were magnetized with a strong magnetic pulse in the horizontal direction and twisted with a much weaker magnetic field in the vertical direction. This vertical field, which oscillates at 0.77 Hz, imposed a sinusoidal torque on each bead. The torque was automatically adjusted to drive a net axial motion of about 60 nm. The bead motion was quantified by image analysis. The ratio of magnetic torque to bead motion defines the apparent stiffness (g*) measured by each bead (34). g* is a complex number and we report the modulus g = |g*|, which has units of pascals per nanometer.

Real-Time Quantitative RT-PCR. Structural integrity of RNA samples was confirmed by electrophoresis using 1% (wt/vol) agarose gels. First-strand cDNA was prepared from total RNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions. Real-time RT-PCR was performed on a Bio-Rad IQ5 real-time PCR detection system (Bio-Rad) with the temperature profile as follows: 40 cycles of 10 s melting at 95°C, 40 s of annealing and extension at 60°C. All primer pairs (Invitrogen) (SI Text) extended over exon–intron boundaries. RNA that was not reverse-transcribed served as negative control for real-time RT-PCR. To allow for relative quantification, we used housekeeping genes by using the software Genex (Multid...
where $\text{Variable}_{\text{E}_{\text{substrate}}} = \text{the value of the parameter being measured (cell stiffness or gene expression)}$ at a given value of substrate stiffness ($\text{E}_{\text{substrate}}$). 

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