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As the endothelial cell reorients, its tensile forces stabilize

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Running head: Cell reorientation and stabilization of cytoskeletal tension

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Abstract

When adherent cells are subjected to uniaxial sinusoidal stretch at frequencies close to physiological, their body and their contractile stress fibers realign nearly perpendicularly to the stretch axis. A common explanation for this phenomenon is that stress fibers reorient along the direction where they are unaffected by the applied cyclic stretch and thus can maintain optimal (homeostatic) tensile force. The ability of cells to achieve tensional homeostasis in response to external disturbances is important for normal physiological functions of cells and tissues and it provides protection against diseases, including cancer and atherosclerosis. However, quantitative experimental data that support the idea that stretch-induced reorientation is associated with tensional homeostasis are lacking. We observed previously that in response to uniaxial cyclic stretch of 10% strain amplitudes, traction forces of single endothelial cells reorient in the direction perpendicular to the stretch axis. Here we carried out a secondary analysis of those data to investigate whether this reorientation of traction forces is associated with tensional homeostasis. Our analysis showed that stretch-induced reorientation of traction forces was accompanied by attenuation of temporal variability of the traction field to the level that was observed in the absence of stretch. These findings represent a quantitative experimental evidence that stretch-induced reorientation of the cell's traction forces is associated with the cell's tendency to achieve tensional homeostasis.

1. Introduction

A ubiquitous phenomenon observed in various cell types is that in response to unidirectional cyclic stretch of the substrate, the cell body realigns globally and the cytoskeletal stress fibers realign locally. If the sinusoidal stretching frequency falls in the range of heart pulsatility, the realignment is away from the stretch axis (Hayakawa et al., 2001; Wang et al., 2000, 2001; Kaunas et al. 2005, 2006; Kurpinski et al., 2006). The prevailing interpretation of these observations has been that realigning contractile stress fibers away from the direction of stretch axis reduces variability of forces carried by stress fibers, which enables the cell to maintain cytoskeletal contractile stress (or tension) stable - a phenomenon known as tensional homeostasis (Brown et al., 1998). It has been argued that tensional homeostasis is essential for normal physiological functions of tissues, such as the endothelium and the epithelium, and provides protection against diseases, including atherosclerosis and cancer (cf. Chien 2007; Paszek et al., 2007; Butcher et al., 2009; Humphrey 2008a,b).

Several theoretical models were advanced to explain mechanisms that govern stretch-induced reorientation of cytoskeletal stress fibers and stabilization of tensile forces carried by the fibers (cf. De et al., 2007; 2008; Kaunas et al., 2011; Pirentis et al., 2011). However, a direct experimental verification of tensional homeostasis associated with the reorientation is lacking. That is, the evidence that after stretch-induced reorientation is completed, the cytoskeletal stress returns to the state it had prior to stretch application has not yet been produced.

In 2012, we studied how cellular traction forces in isolated endothelial cells change in response to a slow, non-sinusoidal, cyclic, uniaxial stretch. We observed that for 10% strain amplitudes the traction field reoriented in the direction perpendicular to the stretch axis. In contrast, in the absence of stretch, the traction field did not reorient (Krishnan et al., 2012). Here

79 we carried out a secondary analysis of those data to investigate whether the traction field
80 reorientation is associated with the cell's tendency to achieve tensional homeostasis. In
81 particular, we analyzed how temporal fluctuations of the traction field around its mean value
82 changed during reorientation. Results of our analysis indicated that those fluctuations became
83 attenuated once the traction field reorientation was completed, suggesting that the cell achieved
84 the state of tensional homeostasis.

85 **2. Materials and Methods**

86 *2.1. Definition and Quantification of Tensional Homeostasis*

87 Tensional homeostasis in cells has often been identified with the cell's ability to recover
88 its baseline tension in response to applied stretch. Many experimental studies of tensional
89 homeostasis have been centered around measurements of time-dependent changes of some scalar
90 metric of cytoskeletal tension following static, quasi-static or transient stretch application
91 (Brown et al., 1998; Mizutani et al., 2004; Trepat et al., 2007; Ezra et al., 2010; Webster et al.,
92 2014; Weng et al., 2016). If tension would return to its baseline value, it would indicate that the
93 cell has the ability to achieve tensional homeostasis. While we learned a lot from those studies,
94 these static force metrics could not account for the fact that cytoskeletal tension is innately
95 dynamic and that it exhibits temporal variations around the set point (Plotnikov et al., 2012;
96 Krishnan et al., 2012; Canović et al., 2016; Zollinger et al., 2018). Thus, the ability of cells to
97 achieve and maintain tensional homeostasis must also include the ability to attenuate excessive
98 tensional fluctuations.

99 During recent years, we have studied dynamic aspects of tensional homeostasis. In those
100 studies, we defined tensional homeostasis as the ability of cells to maintain a consistent level of

cytoskeletal tension with low temporal fluctuations (Canović et al., 2016; Zollinger et al., 2018; Li et al., 2020). In order to quantitatively assess tensional homeostasis in single cells and in multicellular clusters, we measured cellular traction forces (Canović et al., 2016; Zollinger et al., 2018). We used the magnitude of the cell traction moment (M), at a given time (t), as a scalar metric of the traction field (Butler et al., 2002). Note that M is directly proportional to the product of the mean normal stress of the cytoskeleton (i.e., mean tension) times the cell volume. To the extent that cell volume changes very little during the experiments, M is indicative of the cytoskeletal tension.

Here and in our previous studies of tensional homeostasis (Canović et al., 2016; Zollinger et al., 2018), we have used the coefficient of variation (CV_M) to quantify temporal fluctuations of $M(t)$. It is indicative of the extent of temporal variability of cytoskeletal tension relative to its mean. Mathematically, CV_M is defined as the standard deviation [$\sigma(M)$] of $M(t)$ divided by its corresponding time-average ($\langle M \rangle$) over the observation time, i.e.,

$$CV_M = \frac{\sigma(M)}{\langle M \rangle}, \tag{1}$$

Based on our definition of tensional homeostasis, the smaller the value of CV_M, the closer is the cell to the state of tensional homeostasis.

Here we hypothesize that if the observed stretch-induced traction field reorientation were associated with the cell's tendency to achieve and maintain tensional homeostasis, then $M(t)$ should exhibit greater temporal fluctuations during reorientation than after the reorientation was completed.

2.2. Traction Microscopy

We used data from our previous measurements of traction forces of single human umbilical vein endothelial cells (HUVECs) exposed to pure uniaxial cyclic stretch (Krishnan et al., 2012). Briefly, cells were cultured on the soft polyacrylamide gel substrates. Stretch was then applied by a parallel plate indentation of the gel that created a pure uniaxial strain on the gel surface to which cells adhered. Trapezoid strain pulses (1 s loading, 3 s hold, 1 s unload) of 10% strain amplitudes were applied each 49 s over 2 h. Traction forces were measured in the plane of the gel substrate during unloading using constrained Fourier Transform Traction Cytometry (Butler et al. 2002). We also measured tractions in cells that were not exposed to cyclic stretch (time control measurements), using the same sampling rate as in the case of stretch application. From these measurements, we calculated the traction moment matrix (**M**), as a first moment of the traction field. The trace of **M** is equal to M (Krishnan et al., 2012). By calculating eigenvalues and eigenvectors of **M** at different time points, we could quantify reorientation of the traction field with time. As a metric of reorientation, we used the angle between the larger eigenvalue and the axis perpendicular to the stretch axis (Krishnan et al., 2012).

For comparison between different cells, for each cell we normalized $M(t)$ with its time average value $\langle M \rangle$. We considered data from $n = 10$ cells that were subjected to stretch and from $n = 5$ cells from the time control measurements.

3. Results

Time lapses of $M(t)/\langle M \rangle$ exhibited erratic fluctuations in both stretched and unstretched cases (Fig. 1). In the case of applied stretch, these fluctuations appear to be more prominent during the first hour of stretching, when the traction field reoriented, than during the second

144 hour, when the traction field maintained its perpendicular orientation relative to the stretch axis
145 (Fig. 1a). In the absence of stretch, no such difference was obvious (Fig. 1b).

146 During stretch application, the traction field reoriented away from the stretch axis.
147 Within 1 h from the onset of stretch, the traction field aligned perpendicularly to the stretch axis,
148 and did not change this orientation thereafter (Fig. 2). In the absence of stretch, cells did not
149 exhibit reorientation (Krishnan et al., 2012).

150 To quantitate the extent of traction field fluctuations, we computed, according to Eq. 1,
151 values of CV_M for each cell during the first hour and during the second hour of stretching. We
152 found that in each but one stretched cell, CV_M was greater during the first hour than during the
153 second hour of stretching (Table 1). On average, the value of CV_M obtained for the first hour was
154 significantly greater than the value obtained for the second hour (Fig. 3a). We also found in all
155 but one unstretched cell that CV_M was higher during the first hour than during the second hour
156 (Table 1). However, on average the values of CV_M obtained for the two time intervals were not
157 significantly different (Fig. 3b). Importantly, when we compared the average value of CV_M
158 obtained during the second hour of stretching with the average value of CV_M obtained in the
159 absence of stretch for 2-h observation, we found no significant difference (Fig. 3a).

160 We also compared values of $\langle M \rangle$ during the first hour and during the second hour of
161 stretching. In seven cells, $\langle M \rangle$ was smaller during the first hour than during the second hour and
162 in three cells it was the opposite (Table 2). However, on average, the difference between values
163 of $\langle M \rangle$ during these two periods was not significant (Fig. 4).

164 **4. Discussion**

165 Results of our analysis demonstrated that stretch-induced reorientation of the traction

166 field of single HUVECs was closely associated with attenuation of temporal fluctuations of the
167 traction field. This finding supported our hypothesis that the traction field reorientation was
168 linked to the cell's ability to achieve and maintain tensional homeostasis. Because traction
169 forces arise in response to cellular contraction, our results are also supportive of the notion that
170 previously observed stretch-induced realignment of contractile stress fibers away from the
171 stretch axis is driven by the cell's tendency to achieve tensional homeostasis. Although the
172 cycling frequency in our experiments was low (~0.02 Hz), it has been shown by Tondon and
173 colleagues (2012) that stress fiber realignment is more responsive to the strain rate than to stretch
174 frequency per se. These authors applied a similar waveform as we did at 0.01 Hz, and they
175 observed stress fiber realignment in the perpendicular direction.

176 The mechanistic underpinning of the above results may be explained as follows. Based
177 on Eq. 1, one would expect that higher values of CV_M during the first than during the second hour
178 of stretching (Fig. 3a) may be due to lower values of $\langle M \rangle$ and/or due to higher variability
179 (variance) of $M(t)$ during the first hour. Data shown in Fig. 4, indicate that during the first hour
180 of stretching, $\langle M \rangle$ was on average somewhat lower than during the second hour, but that this
181 difference was small and not statistically significant and therefore, it may not account for the
182 observed difference in CV_M. This, in turn, suggests that the decrease in CV_M during the second
183 hour is most likely a result of a decrease of the variance of $M(t)$. This decrease of the variance
184 was probably caused by reduced variability of traction forces due to their realignment along the
185 direction perpendicular to the stretch axis, where they were little affected by the cyclic stretch.
186 Since traction forces are innately dynamic, they continued to exhibit temporal fluctuations after
187 their realignment was completed. However, those fluctuations were not significantly different
188 from the fluctuations observed in the absence of stretch (Fig. 3a).

From the physiological point of view, the ability of endothelial cells to organize their contractile cytoskeleton in order to achieve and maintain tensional homeostasis in the presence of cyclic stretch is important for normal physiological function of the endothelium, where cells are subjected to sustained periodic circumferential stretch of blood vessels walls due to pulsatile blood pressure. It has been shown that stable tension in the endothelium downregulates pro-inflammatory and proliferative pathways and thus it is atheroprotective (cf. Chien 2007).

Since endothelial cells and epithelial cells *in vivo* form monolayers, future studies of tensional homeostasis during cyclic stretch may be focused on measurements of traction field reorientation of multicellular aggregates. While we have shown that multicellularity can, by itself, promote tensional homeostasis in endothelial cells (Canović et al., 2016), it would be of considerable interest to find out how cyclic stretch applied to multicellular clusters may affect homeostasis during and after reorientation of the traction field of the cluster.

In conclusion, this study demonstrated that reorientation of traction forces in response to cyclic uniaxial stretch was accompanied by attenuation of temporal fluctuations of the traction field. This represents a quantitative experimental evidence that stretch-induced reorientation of cellular traction forces is associated with the cell's tendency to achieve tensional homeostasis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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212

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283 Table 1. Values of CV_M of individual HUVECs obtained during the first hour and during the

284 second hour of the observation time from n = 10 stretched cells and from n = 5 unstretched cells.

285

CV_M			
Stretch		No stretch	
0-1 h	1-2 h	0-1 h	1-2 h
0.5502	0.2407	0.1588	0.142
0.8421	0.4145	0.2875	0.2068
0.5915	0.4348	0.2027	0.1314
0.3978	0.1321	0.0834	0.1934
0.8027	0.2617	0.091	0.0714
0.5312	0.1542	-	-
0.2769	0.0744	-	-
0.1687	0.3187	-	-
0.5217	0.4481	-	-
0.3213	0.0711	-	-

286

287 Table 2. Values of $\langle M \rangle$ obtained during the first hour and during the second hour of stretching of

288 individual HUVECs. Data are from $n = 10$ cells.

289

$\langle M \rangle$ (pN·m)	
0-1 h	1-2 h
2.9536	6.8648
6.2751	9.1697
9.1741	14.8168
58.2902	46.3523
22.2446	29.6931
45.0282	59.5743
10.1569	13.2528
8.3184	5.9886
13.0464	9.2556
8.9594	12.8069

290 **Figure Captions**

291 Figure 1. Time lapses of the traction moment (M) normalized by its time-average ($\langle M \rangle$) in the
292 presence of stretch of 10% strain amplitudes (n = 10 cells) (a) and in the absence of stretch (n = 5
293 cells) (b) obtained from traction microscopy measurements in single HUVECs (Krishnan et al.,
294 2012). Each color corresponds to a different cell.

295 Figure 2. Stretch-induced reorientation of the traction field. In response to applied uniaxial
296 cyclic stretch, the angle of orientation of the traction field of single HUVECs aligns with the axis
297 (0°) perpendicular to the direction of stretch within 1 h from the onset of stretch. Each color
298 corresponds to a different cell, consistent with colors from Fig. 1a. [Adapted from Krishnan et
299 al. (2012).]

300 Figure 3. The average coefficient of variation of the traction moment (CV_M) obtained during the
301 first observation hour (0-1 h) and during the second observation hour (1-2 h) in the presence of
302 stretch (n = 10 cells) (a) and in the absence of stretch (n = 5 cells) (b). Data are mean ± standard
303 error; * indicates statistical significance ($p < 0.05$) and NS indicates no significance. Statistical
304 comparison using the two-tailed paired t-test indicates that in the presence of stretch the average
305 CV_M is significantly greater during the first hour than during the second hour of stretching ($p <$
306 0.0031) (panel 3a). No significance was observed in the absence of stretch (p = 0.669) (panel
307 3b). The average value of CV_M calculated during the second hour of stretching is also compared
308 with average value of CV_M observed in the absence of stretch for 2 h using the two-tailed t-test
309 (panel 3a). No significant difference between these two values was found (p = 0.322).

310 Figure 4. The average traction moment ($\langle M \rangle$) calculated during the first hour (0-1 h) of stretching
311 and during the second hour (1-2 h) of stretching (n = 10 cells). Statistical comparison using two-

312 tailed paired t-test indicates no significant (NS) difference ($p = 0.327$). Data are mean ± standard

313 error.

314

Figure 1

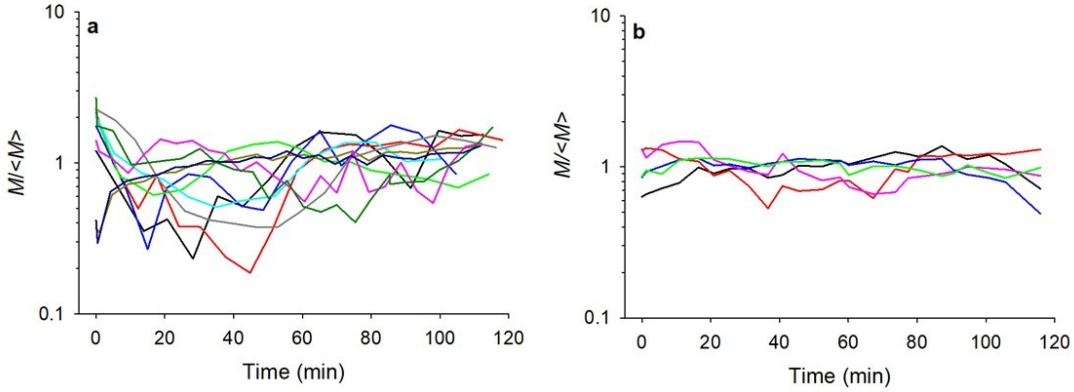

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Figure 2

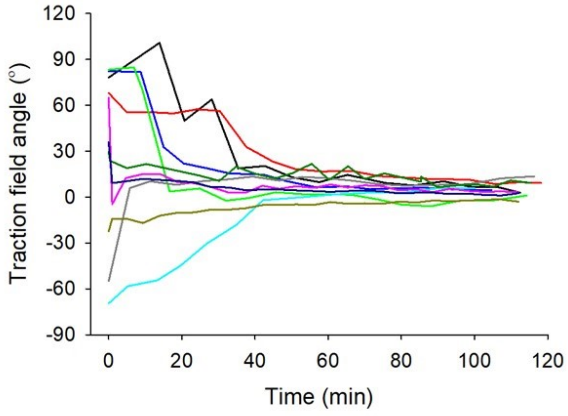

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Figure 3

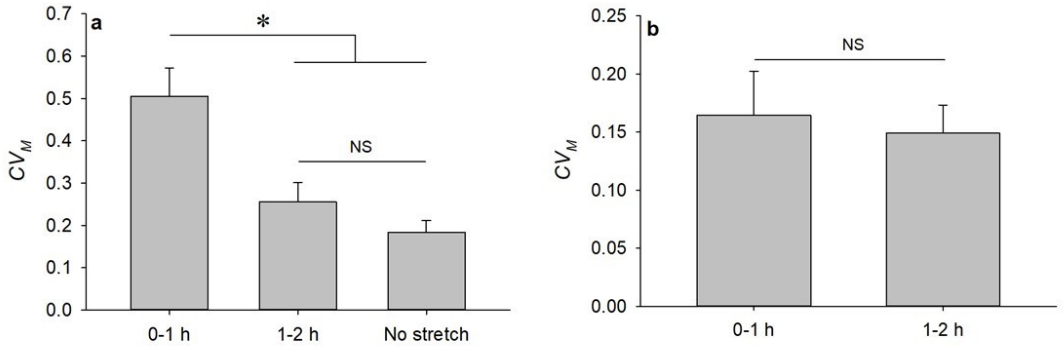

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Figure 4