Anisotropic rigidity sensing on grating topography directs human mesenchymal stem cell elongation

Sum Thai Wong · Soo-Kng Teo · Sungsu Park · Keng-Hwee Chiam · Evelyn K. F. Yim

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Abstract Through mechanotransduction, cells can sense physical cues from the extracellular environment and convert them into internal signals that affect various cellular functions. For example, human mesenchymal stem cells (hMSCs) cultured on topographical gratings have been shown to elongate and differentiate to different extents depending on grating width. Using a combination of experiments and mathematical modeling, the physical parameters of substrate topography that direct cell elongation were determined. On a variety of topographical gratings with different grating widths, heights and rigidity, elongation of hMSCs was measured and a monotonic increase was observed for grating aspect ratio (crosssectional height to line-width ratio) between 0.035 and 2. The elongation was also dependent on the grating substrate rigidity over a range of 0.18–1.43 MPa. A mathematical model was developed to explain our observations by relating cell elongation to the anisotropic deformation of the gratings and how this anisotropy depends on the aspect ratio and rigidity of the gratings. Our model was in good agreement with the experimental data for the range of grating aspect ratio and substrate rigidity studied. In addition, we also showed that the percentage of aligned cells, which had a strong linear correlation with elongation for slightly elongated cells, saturated toward 100 % at higher level of cell elongation. Our results may be useful in designing gratings to elicit specific cellular responses that may depend on the extent of cell elongation.

Keywords Cell alignment · Mechanotransduction · Grating aspect ratio · Cell morphology · Rigidity sensing

1 Introduction

Cells interact with their surrounding extracellular matrix (ECM) by responding to physical and biochemical stimuli. While the ligand–receptor interactions that mediate biochemical cues are extensively studied and well known (Ridet et al. 1977; Mackay et al. 1998), the underlying mechanism for the sensing of physical cues is still an area of active research. Recent studies have focused on the effects of physical cues such as the topography and rigidity of the substrate on cellular responses. Among the various topographical structures used, such as gratings (Kim et al. 2009; Yim et al. 2005; Bettinger et al. 2008), pillars (Saëz et al. 2007; Hu et al. 2010; Sjöström et al. 2011) and wells (Biggs et al. 2009), only anisotropic structures such as gratings (alternating lines...
of grooves and ridges) have been shown to induce cell elongation and alignment. Similar behavior has also been observed for cell clusters when they are cultured on arrays of elliptical pillars with anisotropic rigidity (Saéz et al. 2007). These findings suggest that anisotropy of the substrate rigidity is one of the conditions for inducing cell elongation and alignment. While the influence of grating geometry (height and width) (Crouch et al. 2009; Fraser et al. 2008; Hu et al. 2005; Uttayarath et al. 2005) and substrate rigidity (Saéz et al. 2007; Prager-Khoutorsky et al. 2011) on cell elongation and alignment has been previously reported for a number of cell types, the effect of the anisotropy of substrate rigidity on cell elongation and alignment has only been briefly suggested to be linked to an anisotropy in actomyosin dependent cortical tension (Fouchard et al. 2011). In this paper, we will use a combination of experiments and mathematical modeling to mechanistically understand how anisotropic rigidity sensing directs cell elongation and alignment on gratings.

We will use human mesenchymal stem cells (hMSCs), which are capable of differentiating into multiple mesenchymal lineages such as osteoblasts, myoblasts and adipocytes (Park et al. 2004; McBeath et al. 2004; Kilian et al. 2010). Their lineage specifications have been shown to be influenced by physical cues such as substrate rigidity, geometric constraints and topography. In particular, substrate rigidity has been shown to regulate the differentiation of MSCs into neurons, myoblasts or osteoblasts (Engler et al. 2006). The control of cell shape via deposition of ECM proteins in the form of adhesive islands is also capable of influencing key cellular processes, such as apoptosis (Ferri et al. 2000) and differentiation (McBeath et al. 2004; Gao et al. 2010). Furthermore, topographical cues such as nanogratings have been demonstrated to induce hMSCs to undergo neuronal differentiation while becoming elongated and aligned to the gratings (Yim et al. 2007). In the last example, there is an increase in the level of neuronal markers such as microtubule-associated protein 2 (MAP2) as cells become more elongated. Taken together, constraint of cell shape such as cell elongation may play a significant role in hMSC differentiation. Nevertheless, the mechanism of how cells sense the micro- or nanotopography remains unclear, and hence, it will be important to determine the physical parameters of the substrate topography that will regulate cell shape.

In this study, we have measured hMSC elongation and alignment on gratings with varying rigidity and aspect ratio (crosssectional height to line-width ratio). Based on the experimental results, we hypothesized that hMSCs sense the anisotropy of the rigidity of the gratings and respond by elongating in the stiffer direction along the grating. We have also developed a mathematical model to support this hypothesis, by modeling the individual gratings as beam elements that deflect, tilt or shear when a force is applied to one of the ends. Our model predicted that cell elongation increases with grating aspect ratio. This relationship between cell elongation and grating aspect ratio was validated by measurements of cell elongation on gratings with high grating aspect ratios and substrate materials with different rigidities. In addition, we have also measured cell alignment and found good correlation between cell elongation and the percentage of cells being aligned to the grating axis.

2 Methods

2.1 Preparation of grating and fibronectin line pattern samples

Gratings were replicated on poly(dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning, Midland, MI) by soft lithography using a prepatterned master silicon dioxide grating mold fabricated by photo-lithography. PDMS gratings of various rigidities were made by varying the ratio of PDMS curing reagent. Two ratios, 1:10 and 1:15, were used in this study. The former was used as the default unless otherwise stated. Using the PDMS (1:10) gratings as template, the pattern was transferred onto tissue-cultured polystyrene (TCPS) by heat embossing. Briefly, a piece of flat TCPS was heated to 150 °C on a hotplate and pressed with the PDMS gratings for 3 min. Subsequently, the PDMS was removed after cooling to room temperature. To improve cell adhesion, the top surface of TCPS gratings was coated with fibronectin before use, while PDMS gratings remained uncoated.

Line pattern samples were prepared using microcontact printing (Théry et al. 2006). PDMS gratings samples were incubated with 20 % rhodamine-labeled fibronectin (50 g/ml, Cytoskeleton, Denver, CO) and 80 % fibronectin (1 mg/ml, Biological Industries, Kibbutz Beit Haemek, Israel) for 40 min, after which whose surface was dried with a stream of N2 gas. It was then brought into contact with flat TCPS for 30 s to transfer the fibronectin line patterns via adsorption. In the final step, the uncoated regions in both TCPS gratings and line pattern samples were passivated by incubating with 0.1 % Pluronics F127 (Sigma, St. Louis, MO) for 1 h. A list of all samples used in this study, together with their rigidity and grating/line dimension is shown in Table 1.

2.2 Human mesenchymal stem cell culture

Human mesenchymal stem cells (Poietics™ hMSC, Lonza, Basel, Switzerland) were cultured in Mesenchymal Stem Cell Growth Medium (MSCGM, Lonza). Cells from passages 6–9 were seeded on substrates at a density of $2 \times 10^3$ cell/cm². The hMSCs were fixed and imaged 24 h after seeding.

2.3 Scanning electron microscopy

To check the fidelity of replication, the gratings were sputter coated with 11-nm-thick platinum (JFC 1600 Auto Fine
Table 1 List of samples used in study

<table>
<thead>
<tr>
<th>Substrate material</th>
<th>Young’s modulus $E$ (MPa)</th>
<th>Geometry $w$ (µm)</th>
<th></th>
<th>Height $H$ (µm)</th>
<th>Aspect ratio ($\frac{H}{w}$)</th>
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<tr>
<td>PDMS</td>
<td>1.43 ± 0.26</td>
<td>Theoretical $w$ 1</td>
<td>Measured $w$ 1</td>
<td>Theoretical $H$ 0.35</td>
<td>Measured $H$ 0.34 ± 0.03</td>
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<td>1.42 ± 0.05</td>
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<td>0.85 ± 0.03</td>
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<td>2.03 ± 0.14</td>
<td>0.35</td>
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<td>1.98 ± 0.24</td>
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<td>2.05 ± 0.20</td>
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<td>1.94 ± 0.04</td>
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<td>9.79 ± 0.21</td>
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<td>0.40 ± 0.06</td>
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<td>7.79 ± 0.25</td>
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<td>6.01 ± 0.10</td>
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<td>0.18 ± 0.08</td>
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</table>

In addition to substrate rigidity and dimension of gratings/lines, the corresponding aspect ratio (height/width) is also shown. Height of printed line patterns on TCPS is taken to be zero. The Young's modulus of TCPS substrate is referenced from Tsukruk et al. (2000), while that of PDMS substrate is measured (See Methods). The measured values of aspect ratio of PDMS gratings with higher rigidity were used in curve fitting of our cell elongation model.

2.4 Mechanical characterization of PDMS with different curing ratios

The Young’s modulus of PDMS at different crosslinker to prepolymer weight ratio (1:10, 1:13, 1:15, 1:20) was determined using an Instron testing machine (Model 3345, Instron, Norwood, MA). The gauge length was set at 10 mm, and the samples were tested at a speed of 5 mm/min. Briefly, PDMS sheets of 50 mm in length and 20 mm in width were clamped and deformed with a 1,000 N load cell for the 1:10 and 1:13 PDMS samples and 100 N load cell for the 1:15 and 1:20 PDMS samples. The thickness of the samples was measured at three different points along the length of the PDMS sample with a micrometer screwgauge and averaged. The Young’s modulus was obtained from the slope of the plot of stress versus strain in the linear region. The Young’s modulus for each ratio was calculated as the average measurements from 5 independent samples.

2.5 Fluorescence staining of F-actin and nucleus

Samples were fluorescent stained for F-actin with Oregon Green 488 Phalloidin (Molecular Probes, Invitrogen, Carlsbad, CA) after fixing in 4 % paraformaldehyde (PFA, Sigma-Aldrich, St. Louis, MO) and permeabilized with 0.1 % Triton X-100 (Biorad, Hercules, CA) for 15 min each. The nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, Invitrogen). In between each step, cells were washed twice with phosphate-buffered saline (PBS, 1st BASE, Singapore).

2.6 Immunofluorescence staining of focal adhesions

Samples were stained according to the standard immunofluorescence protocol: Cells were fixed in 4 % PFA, permeabilized with 0.1 % Triton X-100 and blocked with 1 % bovine serum albumin (BSA, VWR, Radnor, PA) and 10 % goat serum (Gibco, Invitrogen) in PBS. Incubation of primary and secondary antibodies was done overnight in 4 °C and for 1 h at room temperature, respectively. The primary antibodies used were mouse anti-human vinculin (1:400, V9131, Sigma). The secondary antibodies used were goat anti-mouse IgG Alexa-Fluor 647 (1:750, Invitrogen). In between each step, cells were washed twice with PBS. The samples were subsequently imaged using confocal microscope (TCS SP5, Leica, Wetzlar, Germany).

2.7 Cell elongation and alignment quantification

Fluorescence images were preprocessed in ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–
2012). Background subtraction was applied with a rolling ball radius of 50 pixels to reduce uneven sample illumination, and local contrast was adjusted such as the percentage of saturated pixels was set to be 0.4%. For each cell, the elongation and alignment with respect to the grating axis are measured by stretching an equimomental ellipse until the error in the areas of the ellipse and the cell is minimized. Cell elongation is defined as the ratio of the major to minor axis of the fitted ellipse less 1. Alignment is defined for cells with elongation greater than 4 as the angle between the major axis and the grating axis. Cells with alignment angle less than 15° are deemed aligned.

3 Results

3.1 Cell elongation and alignment are higher on smaller and stiffer PDMS gratings

hMSCs were cultured on PDMS gratings to study the effect of grating width and substrate rigidity on cell elongation and alignment. The rigidity of the PDMS gratings was varied by changing the ratio of crosslinker to prepolymer. As the ratio of crosslinker to prepolymer was decreased from 1:10 to 1:20, the Young’s modulus of the substrate decreased from 1.43 ± 0.26 to 0.07 ± 0.02 MPa (Fig. S1 in the Supplementary Material). While a ratio of 1:10 was recommended by the manufacturer for normal use, PDMS with a ratio of 1:20 appeared slightly “sticky” due to the high proportion of uncrosslinked prepolymer. Hence, as a trade-off between ease of handling and maximizing rigidity differences, a set of stiff gratings with a ratio of 1:10 and a set of compliant gratings with a ratio of 1:15, corresponding to Young’s modulus of 1.43 and 0.18 MPa, respectively, were used. For each set, gratings widths of 1, 2 and 10 µm were used. All gratings had a constant height of 350 nm. In Fig. 1a, SEM images of the stiff gratings show that the gratings features were replicated with good fidelity. Adsorption of extracellular matrix protein such as fibronectin to the substrate from the serum-containing medium was found to be sufficient to provide cell attachment. At the end of 24 h, the cells adhered to the substrate and elongated along the axis of the gratings (Fig. 1b).

Although the extent of cell elongation varied among gratings of different widths, there was no significant difference in the cell height observed (Fig. S2 in the Supplementary Material). Figure 1c shows that the mean cell elongation, for both the stiff (E = 1.43 MPa) and compliant (E = 0.18 MPa) gratings, decreases monotonically with the grating width from 1 to 10 µm. This is within expectation as it has been shown that cells become elongated on gratings, and the extent of elongation is greater on smaller gratings (Yim et al. 2007). Furthermore, the mean cell elongation is also consistently lower on the compliant gratings compared to the stiff gratings for each of the grating widths, which is consistent with results reported previously (Tzvetkova-Chevolleau et al. 2008). In comparison, the stiff and compliant unpatterned controls...
have the lowest values for mean cell elongation, which are not significantly different from each other. (Additional images of SEM and cell elongation on compliant unpatterned PDMS substrate are given in Fig. S3 of the Supplementary Material). Besides cell elongation, the cell alignment with respect to the grating axis at different grating widths is also quantified. In Fig. 1, the percentage of aligned cells is found to follow a similar trend as the mean cell elongation. From these results, it is clear that both cell elongation and alignment are dependent on both grating width and substrate rigidity.

3.2 Cell elongation and alignment both increase with increasing PDMS grating aspect ratio

Next, we study the effect of the grating aspect ratio on cell elongation and alignment. The aspect ratio is defined as the ratio of the grating height to grating width. In the previous section, the aspect ratio varies from 0.035 (350 nm/10 µm) to 0.35 (350 nm/1 µm). Here, we prepared gratings with the theoretical aspect ratio of unity for each of the gratings of width 1, 2 and 10 µm (The rigidity of this set of gratings is kept constant at 1.43 MPa, which is the Young’s Modulus measured for PDMS with the commonly used curing ratio of 1:10). Fig. 2a shows the SEM images of the cross-section of this set of PDMS gratings. However, due to the high PDMS surface tension preventing complete filling of the grooves, as well as imperfections of the master mold features, the corners of the gratings were rounded resulting in the aspect ratios deviating from unity. The aspect ratios actually measured are 0.6 ± 0.02, 1.04 ± 0.06 and 0.77 ± 0.02 for grating widths of 1, 2 and 10 µm, respectively. The morphology of hMSC cultured on these gratings with aspect ratio close to unity is shown in Fig. 2b, and the corresponding mean cell elongation is shown in Fig. 2c. Compared to PDMS gratings with aspect ratio of 0.035–0.35 in the previous section, this set of gratings induced significantly higher cell elongation for every corresponding width. Likewise, the same trend was observed for cell alignment as shown in Fig. 2c. Among the gratings with aspect ratio close to unity, the 2 µm gratings induce the most cell elongation as the gratings had the highest measured aspect ratio (1.04). Interestingly, cell elongation on the 10 µm gratings was higher than that on 1 µm gratings even though 1 µm gratings induced the most cell elongation in the previous section where gratings of the same height were compared. It was not unexpected as the 1 µm gratings of 350 nm height had the highest aspect ratio (0.35) among the gratings in the group (0.175 for 2 µm gratings, 0.035 for 10 µm gratings). Therefore, cell elongation and alignment are in fact dependent on the single parameter of grating aspect ratio, instead of just grating width alone.

3.3 Cell elongation on line patterns on flat substrates is independent of line-width

To further confirm the role of the grating aspect ratio in determining cell elongation and alignment, we wanted to measure...
hMSCs elongation on line patterns on flat substrates. The flat substrates printed with parallel fibronectin line patterns can be effectively treated as gratings with an aspect ratio of approximately zero, if we assume that the height of the printed patterns is much smaller than the width. This is a reasonable assumption given that the height of the protein monolayer is less than 10 nm (Inerowicz et al. 2002). Therefore, if our hypothesis were true, we will see no dependence of cell elongation on the line patterns regardless of line-width. However, instead of printing line patterns on PDMS substrates, we chose to use TCPS as a substrate as it is a more established method with readily available protocol for micro-contact printing (Saez et al. 2007; Théry et al. 2006). Fluorescence micrographs of the TCPS substrates printed with 1, 2 and 10 µm fibronectin line patterns are shown in Fig. 3a. The fibronectin lines were patterned reproducibly, although there was some unevenness due to minor defects on the PDMS stamp. Through immunofluorescence staining of the focal adhesion protein, vinculin, it can be seen that the passivation coating (Pluronics F127) prevented the formation of focal adhesions between fibronectin lines (Fig. 3b). By confining the initiation and maturation of focal adhesions to within the fibronectin lines (white arrows), the line patterns were effective in presenting guiding cues as zero aspect ratio “gratings” to induce cell elongation and alignment. After one day of seeding hMSC on the line patterns, we observed that the mean cell elongation is fairly constant with values 3.87 ± 0.28, 3.92 ± 0.42 and 4.21 ± 0.72 for line-widths of 1, 2 and 10 µm, respectively (Fig. 3c). In addition, these values were comparable to that on flat TCPS uniformly coated with fibronectin (unpatterned), which was 3.74 ± 0.1. Therefore, these results showed that cell elongation is independent of line pattern width on flat substrates and support our hypothesis that it is the grating aspect ratio that determines cell elongation. Similarly, it was found that cell alignment is also insensitive to line pattern width with the percentage of aligned cells on line-widths of 1, 2 and 10 µm being 30.23 ± 11.15%, 31.15 ± 7.01% and 38.04 ± 12.20%, respectively (Fig. 3c). While it appears that the percentage of aligned cells on the 10 µm line patterns is higher as compared to the smaller line patterns, this is due to experimental variations and there is in fact no significant difference between the groups as indicated by a p-value of 0.72 from a one-way ANOVA test.

3.4 Mathematical model of cell elongation driven by anisotropic grating rigidity

Based on the above results as well as previous works on other cell types (Yim et al. 2005; Bettinger et al. 2008; Diehl et al. 2005), we hypothesized that cell elongation is dependent on the geometrical and mechanical properties of the gratings, namely their aspect ratio and rigidity (Engler et al. 2004; Yeung et al. 2005). Specifically, cells were observed to elongate and align along the length of the gratings, which is also the direction of the highest apparent rigidity. From a mechanical point of view, the gratings act as a substrate with anisotropic rigidity as their geometry results in higher apparent rigidity along the length of the gratings and lower apparent rigidity perpendicular to the gratings, even though the grating is fabricated from an elastically isotropic material (PDMS). In this case, their heights (0.35 µm–10 µm) and...
widths (1 μm–10 μm) are comparable to the dimensions of the hMSCs (approximately 100 μm), whereas their lengths (5–10 mm) are several orders of magnitude larger.

Mathematically, our hypothesis for overall cell elongation e on gratings can be written as

\[ e = e_b + e_g, \]

where \( e_b \) is the basal elongation corresponding to cell elongation induced on a flat substrate that is rigidity dependent and \( e_g \) is the grating-induced elongation corresponding to additional cell elongation induced by grating topographical features that is dependent on the anisotropy of rigidity.

While it has been shown that fibroblast polarization increases with substrate rigidity, we noted that this dependence of cell elongation on substrate rigidity is not linear, but have instead been found experimentally to saturate above a certain rigidity threshold. In fact, this dependence can be approximated by a power law (Fig. 1 in Prager-Khoutorsky et al. 2011). As hMSCs and fibroblasts are stromal cells that share many common features, we assumed that the dependency of hMSC basal elongation on substrate rigidity can adopt the similar power law,

\[ e_b \propto E^\alpha, \]

where \( E \) is the Young’s modulus of the flat isotropic substrate and \( \alpha = 0.23 \) an exponent obtained from the experimental data (Prager-Khoutorsky et al. 2011).

For grating-induced elongation, we hypothesized that its dependency on grating anisotropic rigidity can similarly be written as

\[ e_g \propto \left( \frac{E_x}{E_y} \right)^\alpha \propto \left( \frac{\delta_y}{\delta_x} \right)^\alpha, \]

where \( E_x \) (\( E_y \)) is the apparent rigidity of the grating along (perpendicular to) its length and \( \delta_x \) (\( \delta_y \)) is the deflection of the grating along (perpendicular to) its length under cellular traction. We assumed that this grating-induced elongation is governed by the same underlying mechanism as that of basal elongation, hence the choice for the same power-law form and exponent as in Eq. 2. As the grating length is several orders of magnitude larger than the grating width, this leads to anisotropy in grating rigidity, where the apparent rigidity along the length of the grating (\( E_x \)) is significantly larger relative to the apparent rigidity perpendicular to the grating (\( E_y \)). It follows that as grating deflection is inversely proportional to its apparent rigidity, the deflection along the length of the grating (\( \delta_x \)) resulting from cellular traction is significantly smaller as compared to the deflection perpendicular to the grating (\( \delta_y \)). We hypothesized that cells are able to discern and “measure” the degree of anisotropy in apparent rigidity by comparing \( \delta_x \) and \( \delta_y \).

Next, we derived the functional form for \( e_g \) by first considering \( \delta_y \), the deflection of a single grating in the direction perpendicular to the length of the grating, under a cellular traction force \( F \) (Fig. 4b i and ii). We assumed that the traction force acts over an effective length \( L_{\text{eff}} \) in the direction along the length of the grating. We postulated that this traction force arises from the contractility generated by the cell’s actomyosin machinery and is transmitted to the gratings through focal adhesion complexes. Each focal adhesion complex thus serves as a mechanosensor to “probe” the underlying rigidity of the substrate. We made the following assumptions to simplify the derivation of our mathematical model for the gratings. First, \( L_{\text{eff}} \) is of the typical size of a focal adhesion complex. Second, the grating is modeled as a cantilever beam with length \( H \) and crosssectional area \( w \times L_{\text{eff}} \). Third, \( \delta_y \) is calculated based on a free-standing cantilever beam, neglecting the boundary conditions on both sides of \( L_{\text{eff}} \). In reality, the grating on both sides of \( L_{\text{eff}} \) will act as a constraint limiting \( \delta_y \).

As suggested by Schoen et al. (2010) based on linear elastic theory, the grating deflection \( \delta_y \) can be written as a linear combination of the following contribution (derivation given in Supplementary Material)

\[ \delta_y = \delta_{\text{bending}} + \delta_{\text{shear}} + \delta_{\text{tilt}} \]

\[ = \frac{4F}{EL_{\text{eff}}} \left( \frac{H}{w} \right)^3 + \frac{3.5F}{EL_{\text{eff}}} \left( \frac{H}{w} \right)^2 + \frac{2.82F}{EL_{\text{eff}}} \left( \frac{H}{w} \right)^2, \]

where \( \delta_{\text{bending}} \) is the deflection induced by bending of the gratings, \( \delta_{\text{shear}} \) is the deflection induced by shearing of the gratings, \( \delta_{\text{tilt}} \) accounts for the tilting effect at the base of the gratings, \( E \) is the Young’s modulus of the gratings and \( F \) is the cellular traction force. Experiments on pillar arrays of different stiffness have shown that the saturation force exerted by cells was dependent on substrate rigidity (Trichet et al. 2012). The observed gradual saturation of traction force with higher substrate rigidity (Fig. 2c in Trichet et al. 2012) suggested that this dependency can be approximated by a power law:

\[ F \propto E^\beta, \]

where \( \beta \) is a power-law exponent bounded between 0 and 1.

Finally, we assumed that \( \delta_x \), the grating deflection in the direction along the length of the grating, is inversely proportional to the substrate rigidity only and independent of any grating geometric factors. The justification for our assumption is that \( \delta_x \) is very localized since the grating length is several orders of magnitude larger than the effective length \( L_{\text{eff}} \) of the traction force. Thus \( \delta_x \) can be written as

\[ \delta_x \propto \frac{1}{E}, \]

where \( E \) is the Young’s modulus of the gratings.
Combining Eqs. 1–6, our cell elongation model can therefore be expressed as

$$
e = \left( \frac{E}{E_0} \right)^{0.23} + \left[ \left( \frac{E}{E_1} \right)^{2} \left( \frac{H_w}{w} \right)^{3} + 3.5 \left( \frac{H_w}{w} \right) + 2.82 \left( \frac{H_w}{w} \right)^{2} \right]^{0.23}
$$

(7)

In this form, the model provides a quantitative interpretation on the contribution of substrate rigidity as well as the linear and higher order effects of grating aspect ratio on overall cell elongation. The constants $E_0$ and $E_1$ are characteristic rigidities that are dependent on the substrate material. On the one hand, the basal elongation (first term) is dependent on $E_0$, which corresponds to the substrate rigidity at which unity basal elongation occurs. On the other hand, the grating-induced elongation (second term) is dependent on $E_1$, which corresponds to the local grating rigidity at which unity grating-induced elongation occurs on gratings with aspect ratio of 0.23. This local rigidity arises from the anisotropic geometry of the gratings, and its role in grating-induced elongation is analogous to that of substrate rigidity in basal elongation. In addition, this elongation is modulated by a factor that depends on the grating aspect ratio, as well as a parameter $\beta$ that is dependent on the cell type-specific variation of traction force response to substrate rigidity.

For a given substrate rigidity, this model allows us to predict empirically the cell elongation on gratings of any aspect ratio. In this case, we fitted our model in Eq. 7 to our experimental results on PDMS gratings with Young’s modulus of 1.43 MPa using a nonlinear least square algorithm (Trust-Region-Reflective) and obtained the values of $E_0$, $E_1$ and $\beta$ to be 0.02, 1.03 $\times$ 10$^{-6}$ and 0.55, respectively. In Fig. 4a, we plotted cell elongation versus grating aspect ratio and showed that there is good agreement between the model (solid line) and our experimental data (circles). To further validate our model, we repeated the experiments by preparing a new set of gratings with a higher aspect ratio of 2 (grating width of 2 $\mu$m and height of 4 $\mu$m). The SEM image of the grating crosssection in Fig. 4c showed that the actual aspect ratio of the gratings is 2.01 $\pm$ 0.03. The corresponding cell elongation measured is 18.67 $\pm$ 1.14 (solid circle), which is close to the value predicted by the model. Hence, our model is accurate in reproducing the upward trend in cell elongation as grating aspect ratio increases.

In addition to gratings of different aspect ratios, our cell elongation model is also applicable to gratings of same substrate material with different rigidities. Here, we applied the model in the prediction of cell elongation on a set of more compliant PDMS gratings with a Young’s modulus of 0.18 MPa. In this case, since the substrate material was the same and $E_0$ and $E_1$ are material dependent parameters, the same set of values for $E_0$, $E_1$ and $\beta$ as for the previous stiff PDMS gratings were used. We updated $E$ to reflect the lower substrate rigidity and plotted the theoretical curve (solid line) together with the experimental data (triangles) in Fig. 4a. The model showed good agreement with the small set of data with aspect ratio between 0.04 and 0.36 and a monotonic increase in elongation with grating aspect ratio.

One advantage of this model lies in its versatility in predicting cell elongation on gratings of different materials by the use of material dependent parameters $E_0$ and $E_1$. This point is clearly demonstrated when the model was applied to TCPS gratings. If the same $E_0$, $E_1$ and $\beta$ as that for

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**Fig. 4** a Experimental data and model predictions of cell elongation. Data for PDMS gratings with Young’s modulus of 1.43 MPa are represented as circles, whereas the 2 $\mu$m PDMS sample with 4 $\mu$m height (aspect ratio 2) is represented as solid circle. The corresponding cell elongation predicted by the model as a function of grating aspect ratio is represented by a solid line and shows an upward trend. The model can be applied to gratings of different substrate materials or same substrate material of different rigidities. Data for PDMS gratings with Young’s modulus of 0.18 MPa and TCPS gratings with Young’s modulus of 4,000 MPa are represented as diamonds and squares, respectively. The corresponding cell elongation predicted by the model as a function of grating aspect ratio is represented by a solid line and a dashed line, respectively. The dotted line corresponds to the predicted cell elongation of TCPS gratings without adjusting for effects due to material properties and surface chemistries. b A vertical dashed line in part (a) separates the (i) low aspect ratio region, where grating shear dominates, from the (ii) high aspect ratio region, where grating bending and base tilting dominate. c SEM image showing crosssection profiles of PDMS grating substrates with 2:1 aspect ratio (2 $\mu$m width; 4 $\mu$m height). Actual grating aspect ratio is 2.01 $\pm$ 0.03. Scale bar represents 5 $\mu$m.
PDMS were used in the plot of cell elongation versus grating aspect ratio for TCPS gratings with Young’s modulus of 4,000 MPa (Fig. 4a, dotted line), the predicted cell elongation was linearly correlated with elongation for elongation less than about 6 and saturates to 100% for large elongation (solid circles). A curve, which is given by Eq. 8, fitted to the experimental data with good agreement (solid line). The same trend is observed when the threshold angle is reduced to 5° (solid triangle, dashed line). The morphologies of typical cells with (i) low alignment and low elongation and (ii) high alignment and high elongation are also shown.

3.5 Cell alignment on gratings correlates with cell elongation

For all results up to this point, cells were considered aligned when their alignment with respect to the grating axis was within a threshold angle of 15°. By taking into account the mean elongation and corresponding percentage of aligned cells on all gratings with different aspect ratios from 0 to 2, we noted that there exist a good linear correlation between percentage of aligned cells and elongation for small values of cell elongation less than about 6, whereas for large values of elongation, the percentage of aligned cells saturates to 100% (Fig. 5, solid circles). To quantify this relationship, we fitted a curve of the form

\[ y = y_0 \left(1 - e^{-\frac{x}{x_1}}\right), \]

where \( y \) is percentage of aligned cells, \( x \) is cell elongation, \( y_0 \) is a constant and \( x_0 \) and \( x_1 \) are fitting parameters. Based on the distribution of the data points, this functional form was chosen such that \( y \) approaches \( y_0 \) when \( x \) is large and \( y \) is linear with respect to \( x \) when \( x \) is much smaller than 1. The value of \( y_0 \) is chosen to be 100 to match the saturation value of percentage of aligned cells. After calculating the values for the fitting parameters, we then obtained following equation for the curve

\[ y = 100 \left(1 - e^{-\frac{x}{x_1}}\right) \]  \hspace{1cm} (8)

Figure 5 shows that this curve (solid line) is in good agreement with the observed experimental data. When the percentage of aligned cells is zero, cell elongation in Eq. 8 is approximately 2.87, which is a reasonable value given that we define alignment only for cells with elongation more than 4. At the other end of the curve, the percentage of aligned cells reaches a maximum of 100% when cell elongation is large. The morphologies of typical cells with (i) low alignment and low elongation and (ii) high alignment and high elongation are also shown in Fig. 5.

We verified that this trend remained intact with only a reduction in the initial rate of increase when the cell alignment threshold angle was reduced from 15° to 5°, showing that it is independent of the threshold angle (Fig. 5, solid triangle). In this case, the equation of the fitted curve (dashed line) is given by

\[ y = 100 \left(1 - e^{-\frac{x-4.64}{3.87}}\right) \]  \hspace{1cm} (9)

4 Discussion

In this work, we studied the effect of grating aspect ratio and rigidity on cell elongation and alignment. Over the range of grating aspect ratio (0.035–2) and substrate stiffness (0.18 MPa–1.43 MPa) investigated, we have shown that both cell elongation and alignment are enhanced on smaller and stiffer gratings. The gratings aspect ratio was also found to be an important determinant of cell elongation and alignment. By incorporating the effect of substrate rigidity and aspect ratio based on corresponding experimental data, we have developed a quantitative model that identifies grating anisotropic rigidity as the driving force behind cell elongation.

In past studies that explored the influence of grating geometry and rigidity on cell morphology by varying the grating width or height separately, the results are often inconsistent and not directly comparable due to a lack of reference stan-
dard. For example, while Hu et al. (2005) reported that cell elongation and alignment increase with smaller width and greater height, the opposite effect was observed by another group (Wójciak-Stothard et al. 1995). Although such contradiction might be the result of cell type-specific response, it is nevertheless useful to establish a standard metric for describing grating topography. Toward this end, Crouch et al. (2009) demonstrated that there is a monotonic dependence of cell elongation and alignment on grating aspect ratio, which can be observed in data from a few other studies as well. We investigated the contribution of grating geometry to local rigidity in terms of aspect ratio by fixing the grating width and adjusting its height. For each of the current PDMS grating of a given width, we introduced an additional higher grating of the same width. Instead of considering grating width and height separately, we define the ratio of grating height to width as the aspect ratio. This new set of gratings with higher aspect ratio (ranging from 0.6 to 1.04) induced more elongated and aligned cells as compared to the original set of gratings with a constant height of 350 nm (aspect ratio ranging from 0.035 to 0.35). In addition, cell elongation was greatest on the new 2 \( \mu \text{m} \) grating with the highest aspect ratio of 1.04, even though its width was not the smallest and height was not the greatest. Hence, aspect ratio is a better predictor of cell elongation.

To further investigate the effect of grating aspect ratio on cell elongation and alignment, we used flat TCPS printed with parallel fibronectin line patterns of different widths. As vinculin staining showed that focal adhesions were localized only to the regions deposited with fibronectin, these line patterns effectively served as “gratings” with zero height and aspect ratio. While cell elongation was the highest on the smallest 1 \( \mu \text{m} \) gratings as expected, it was largely independent of widths on the line patterns. In fact, cell elongation on all line pattern substrates was comparable to that on the unpatterned substrate with uniform fibronectin coating. This result suggests that cells are unable to sense and discriminate the different widths among the line patterns on a flat substrate. When deprived of topographical cues from gratings, cell elongation is dependent on only on substrate rigidity (Prager-Khoutorsky et al. 2011). Likewise, cell alignment on line pattern substrates is also insensitive to the widths of line patterns. Even though there was a variation in the size and distribution of focal adhesions formed on fibronectin lines of different widths, this did not translate into a significant difference in the percentage of aligned cells.

We have developed a mathematical model to take into account the grating aspect ratio and substrate rigidity in grating-induced cell elongation and alignment. This model proposed that cell elongation is a consequence of the cell’s ability to sense the local rigidity of the gratings and preferentially spread in the direction of higher rigidity. As can be seen in Fig. 4a, our model shows good agreement with experimental data for PDMS and TCPS gratings of different rigidity. It is interesting to note that the theoretical curve for cell elongation on PDMS gratings with a Young’s modulus of 1.43 MPa shows two distinct regimes (separated by the vertical dash line) with respect to aspect ratio. For high aspect ratios (Fig. 4b i), it can be seen from Eq. 7 that the effects of grating bending and base tilting become important, whereas for low aspect ratios near to zero (Fig. 4b ii), the shearing of the grating is the dominant factor. Between these extremes, cell elongation involves all three modes. The transitional point between the two regimes could be an important consideration in the process of design and microfabrication of topographical gratings. Researchers should consider if the effect of shearing or bending would be more desired before determining the dimension and parameters to be used in the microfabrication, which could be costly and labor intensive in a trial-and-error approach.

Comparing the theoretical and experimental cell elongation data over the entire range of aspect ratios considered, it appears that there is an overestimation at low aspect ratios but underestimation as aspect ratio increases. A possible explanation for this discrepancy is the exclusion of boundary effect in the beams representation of the model. Unlike standalone beam, the deflection of each beam element of the gratings is reduced or enhanced due to the restraining or pulling effects of adjacent beams depending on the grating aspect ratio.

Although our model is similar to that proposed by Crouch et al. (2009) in relating cell elongation to grating aspect ratio, there exist a few critical differences between the two (c.f. Fig. 4A and Fig. 4A in Crouch et al. 2009). First, our model adopts a mechanical approach in identifying anisotropic rigidity on gratings as the driving force behind cell elongation, whereas their model is based on the theory of contact guidance, which relates cell elongation to the relative ease of spreading across grooves versus ridges. Second, our model is derived base on experimental data and can be applied to gratings of different aspect ratios and materials. We have carried out a systematic experimental study of the effect of grating aspect ratio by varying it from zero to one for each of the three different sets of gratings of width from 1 \( \mu \text{m} \) to 10 \( \mu \text{m} \), thus validating the model. In addition, we have also used gratings of different materials (PDMS and TCPS) to investigate the role of substrate rigidity. From our model, we have identified a set of rigidity-related parameters, namely \( E_0 \) and \( E_1 \) in Eq. 7, that suggest a dependency on substrate material properties or surface chemistries. By fitting these parameters using gratings of different substrate materials over a range of aspect ratio, our model is able to provide a quantitative prediction of cell elongation as a function of gratings aspect ratio and substrate rigidity that is adjusted for effects due to material properties. Third, our model shows a slightly different trend and covers a much larger range of gratings aspect ratio. While their model shows that cell elongation reaches a maximum...
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as gratings aspect ratio approaches 0.4, our model shows a monotonic increase in cell elongation up to a gratings aspect ratio of 2 as validated by our experimental data. It is possible that a similar maximum elongation exists for our model, but it was not observed for the range of aspect ratio up to 2.

Although beyond the scope of the present study, we noted that changing the concentration of the ECM protein fibronectin may have an impact on our cell elongation results. It is known that cell adhesion is mediated by the binding interaction between integrins and the RGD sequence of the ECM protein fibronectin. For the establishment of stable integrin adhesions, a critical RGD density is required, in which the RGD ligand spacing cannot exceed 70 nm (Cavalcanti-Adam et al. 2007). Following this, it has been shown the density of ECM proteins actually determines the strength of cell adhesion (Roca-Cusachs et al. 2009) as well as regulates stem cell fate (Trappmann et al. 2012). In our study, we therefore expect that a reduction in the concentration of fibronectin below a threshold will result in a lower number of attached cells and lesser cell elongation on each of the gratings with different widths and rigidities. On the other hand, while a higher concentration of fibronectin on unpatterned substrates will likely have no effect on cell elongation, topographical cues from the smaller gratings may promote the formation of fibronectin fibrils (Ballester-Beltrán et al. 2012) and lead to a higher level of cell elongation. Nevertheless, while we assumed the variation in the fibronectin concentration would not affect the model, there was overall good agreement with the experimental data using the current concentration of fibronectin.

The linear correlation between cell alignment and cell elongation at low elongation and the saturation of percentage of aligned cells at high elongation suggest that there is some dependency between these two mechanisms, even though the causality relationship remains unknown. Moreover, the good agreement between the fitting curve and experimental data also provides additional evidence to support this idea. While previous studies reported that grating height is the more important factor that determines cell alignment (Fraser et al. 2001; Grashoff et al. 2010), they could elongate and align in the direction of greatest applied tension, which will be along the gratings due to anisotropic rigidity. It has been used to induce the formation of aligned populations of polarized cells that is essential to the structure and function of specific tissues (Koo et al. 2011; Franco et al. 2011). As stem cell differentiation has been shown to be dependent on substrate rigidity (Engler et al. 2006; Saha et al. 2008) and cell shape (McBeath et al. 2004; Kilian et al. 2010), it is therefore possible to use gratings with an optimal aspect ratio to induce cell elongation that corresponds to a desired level of differentiation. For example, if nanogratings-induced hMSC neuronal differentiation (Yim et al. 2007) was to be carried out in a clinical setting, it would be necessary to substitute the PDMS used in the original study with a suitable biocompatible material, whose mechanical properties is likely to be different from that of PDMS. In order to achieve similar cellular response, our model can be used to guide the design of gratings to compensate for the difference in substrate rigidity. While previous works have demonstrated the dependence of cell elongation on gratings aspect ratio (Crouch et al. 2009; Hu et al. 2005; Uttayarat et al. 2005), our model provides a quantitative relation between these two entities that can be applied to gratings of different substrate materials or same material with different rigidities.

To date, while there has been intensive research conducted on the effect of topographical cues on cellular behavior, the mechanisms underlying the sensing and response to this stimulus remain unclear. In this respect, our anisotropic rigidity-sensing model may provide some suggestions on the possible mechanisms for gratings-induced cell elongation and alignment. As the primary interface between the ECM and actin cytoskeleton that transduce extracellular forces into downstream biochemical signals, focal adhesions (FAs) are the force sensors that mediate cell-substrate interactions (Balaban et al. 2001; Grashoff et al. 2010). The gratings topography has a direct influence on FA formation by either facilitating (ridges) or disrupting (grooves) integrin binding. During force-dependent maturation of FAs (Balaban et al. 2001), they could elongate and align in the direction of greatest applied tension, which will be along the gratings due to anisotropic rigidity. This initial alignment of FA, which is clearly visible from our immunofluorescence staining of FA protein vinculin in Fig. 3b, could then lead to an overall change in cell morphology following the reinforcement and alignment of actin stress fiber triggered by the actomyosin contractile forces (Zemel et al. 2010). To test this mechanism, we suggest that further work can be done using real-time
imaging to visualize FA formation and actin cytoskeleton remodeling during cell elongation and alignment.

5 Conclusion

In this work, we have studied the effect of topographical gratings on cell elongation and alignment. We have found that both cell elongation and alignment are enhanced on smaller and stiffer gratings. Upon variation of the grating dimension and aspect ratio, however, we found that elongation and alignment were not dependent on line-width alone, but the gratings aspect ratio would be an important determinant of cell elongation and alignment. By incorporating the effect of substrate rigidity and aspect ratio, we have developed a quantitative model that identifies grating anisotropic rigidity as the driving force behind cell elongation. Our model was validated using gratings with a higher aspect ratio and substrate materials with different rigidities. Lastly, we have also shown that there is good correlation between cell elongation and alignment.

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References


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alignment and adhesion. Biomaterials 28(27):3944–3951. doi: 10.1016/j.biomaterials.2007.05.030


