

## Time-Dependent Migratory Behaviors in the Long-Term Studies of Fibroblast Durotaxis on a Hydrogel Substrate Fabricated with a Soft Band

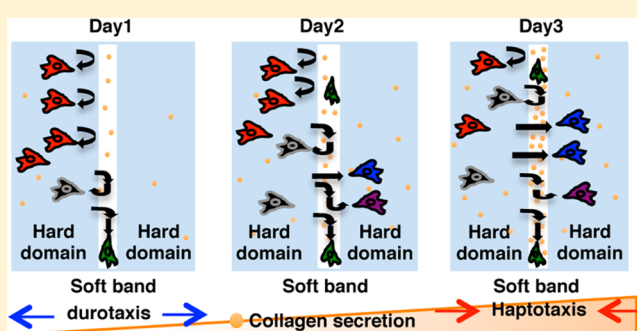
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### S Supporting Information

**ABSTRACT:** Durotaxis, biased cell movement up a stiffness gradient on culture substrates, is one of the useful taxis behaviors for manipulating cell migration on engineered biomaterial surfaces. In this study, long-term durotaxis was investigated on gelatinous substrates containing a soft band of 20, 50, and 150  $\mu\text{m}$  in width fabricated using photolithographic elasticity patterning; sharp elasticity boundaries with a gradient strength of 300 kPa/50  $\mu\text{m}$  were achieved. Time-dependent migratory behaviors of 3T3 fibroblast cells were observed during a time period of 3 days. During the first day, most of the cells were strongly repelled by the soft band independent of bandwidth, exhibiting the typical durotaxis behavior. However, the repellency by the soft band diminished, and more cells crossed the soft band or exhibited other mixed migratory behaviors during the course of the observation. It was found that durotaxis strength is weakened on the substrate with the narrowest soft band and that adherent affinity-induced entrapment becomes apparent on the widest soft band with time. Factors, such as changes in surface topography, elasticity, and/or chemistry, likely contributing to the apparent diminishing durotaxis during the extended culture were examined. Immunofluorescence analysis indicated preferential collagen deposition onto the soft band, which is derived from secretion by fibroblast cells, resulting in the increasing contribution of haptotaxis toward the soft band over time. The deposited collagen did not affect surface topography or surface elasticity but did change surface chemistry, especially on the soft band. The observed time-dependent durotaxis behaviors are the result of the mixed mechanical and chemical cues. In the studies and applications of cell migratory behavior under a controlled stimulus, it is important to thoroughly examine other (hidden) compounding stimuli in order to be able to accurately interpret data and to design suitable biomaterials to manipulate cell migration.



## INTRODUCTION

Developing methodologies to control cell migration has important implications in a range of biomedical applications, for example, designing sophisticated biomaterials for tissue engineering,<sup>1</sup> facilitating wound healing,<sup>2</sup> and inhibiting cancer metastasis.<sup>3</sup> In general, cell migrations can be directionally controlled via intrinsic taxis behaviors triggered by various types of gradient of external stimuli: dissolved chemicals (chemotaxis),<sup>4</sup> light (phototaxis),<sup>5</sup> gravitational potential (geotaxis),<sup>6</sup> electrostatic potential (galvanotaxis),<sup>7</sup> surface topography (contact guidance),<sup>8–10</sup> surface-tethered molecules (haptotaxis),<sup>11,12</sup> and surface stiffness (durotaxis or mechanotaxis).<sup>13–20</sup>

Of the different types of taxis, the latter three are especially useful for the design of functional biomaterial surfaces to manipulate cell migrations on artificial extracellular matrices and scaffolds. Contact guidance enables cells to migrate along a specific topography and/or to reside in a specific, desired

region. Haptotaxis can be applied to recruit cells from millimeter-scale distance toward a region with a higher density of haptotactants, such as cell adhesive proteins. Durotaxis, termed for biased cell movement toward a hard region from a softer region at an elasticity boundary of discretely juxtaposed hard and soft regions on a hydrogel substrate,<sup>13</sup> facilitates repositioning of cells among regions of different elasticities on a mechanically patterned substrate.

While the methods to control contact guidance and haptotaxis have been well established, applications of durotaxis are relatively underdeveloped. The main challenges in the studies and applications of durotaxis include the difficulties in the systematic fabrication of elasticity boundaries with smooth surface topography and the interfering effect from surface

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chemistry-induced taxis, i.e., haptotaxis. The first issue stems from the fact that neighboring hydrogel domains of different elasticities are liable to exhibit different degrees of swelling, causing topographical discontinuity at the boundary. We have recently established protocols, with special attention to focus control, on photolithographic microelasticity patterning of photocurable gelatins to reduce the generation of surface topography at the soft–hard boundary.<sup>15</sup> Using the fabrication methodology, threshold modulus gradient strength to induce durotaxis of fibroblast cells was determined to be 300–400 kPa/50  $\mu\text{m}$  on a gelatinous gel containing a soft base with a modulus value of 10 kPa.<sup>21</sup> Applicability of durotaxis for cell migration manipulation over millimeter-scale distance on the substrate surfaces with smooth topography and minimally required modulus gradient strength was demonstrated in a subsequent publication.<sup>22</sup> The second challenge is attributed to the deposition/absorption of cell adhesive matrix components, typically collagens, secreted by fibroblast cells,<sup>23,24</sup> which can interfere with durotaxis. Cell migratory behaviors especially for fibroblast cells are expected to result from the combined effects of durotaxis and haptotaxis. The interference to durotaxis by the surface-adsorbed proteins and the longevity of durotaxis have not been systematically studied, a knowledge of which is critical to ensure appropriate data interpretation and to establish experimental conditions minimizing the interference by other external stimuli in the design of functional cell-culture substrates.

In the present study, a single soft band with a variable width, from 20, 50, to 150  $\mu\text{m}$ , on a stiffer styrenated gelatinous hydrogel substrate was fabricated employing photolithographic microelasticity patterning to investigate durotaxis of 3T3 fibroblast cells during an extended time period of up to 3 days. The elasticity geometry of the single soft band against a stiff substrate is effective for characterizing time-dependent durotaxis behavior *in situ*. Durotaxis was initially manifested in the strong cell repellency by the soft band during the first day of observation. More cells, however, crossed the soft band over the course of the 3-day culture, which is attributed to the haptotaxis induced by the collagen preferentially deposited on the soft band over time, as characterized by immunofluorescence analysis. Since collagen deposition on the elastic substrate is time-dependent and location/modulus-specific, the observed cell migratory behavior is the compounding consequences of durotaxis and haptotaxis and is time-dependent. The effect of soft-band width and the conditions of time-dependent haptotactic suppression of durotaxis of fibroblast cells were elucidated, and the criteria for designing elastic substrates to utilize durotaxis were established.

## MATERIALS AND METHODS

**Cell Culture.** Mouse fibroblasts (NIH/3T3, Health Sciences Research Resource Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagles medium (DMEM) (Gibco BRL, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), 100 units/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin. Cells were maintained on tissue culture polystyrene dishes at 37 °C under 5%  $\text{CO}_2$  in a humidified incubator.

**Preparation of a Photocurable Sol Solution.** Microelastically patterned substrates were prepared based on our previous report.<sup>15</sup> Photo-cross-linkable styrenated gelatin (StG)<sup>25</sup> (30 wt %; 90% degree of derivatization) and sulfonyl camphorquinone (SCQ, Toronto Research Chemicals, ON, Canada; 3.0 wt % of gelatin) were dissolved in phosphate buffered saline (PBS). The mixed solution was subjected to centrifugation (MX-301, TOMY, Tokyo, Japan) at 14 000 rpm

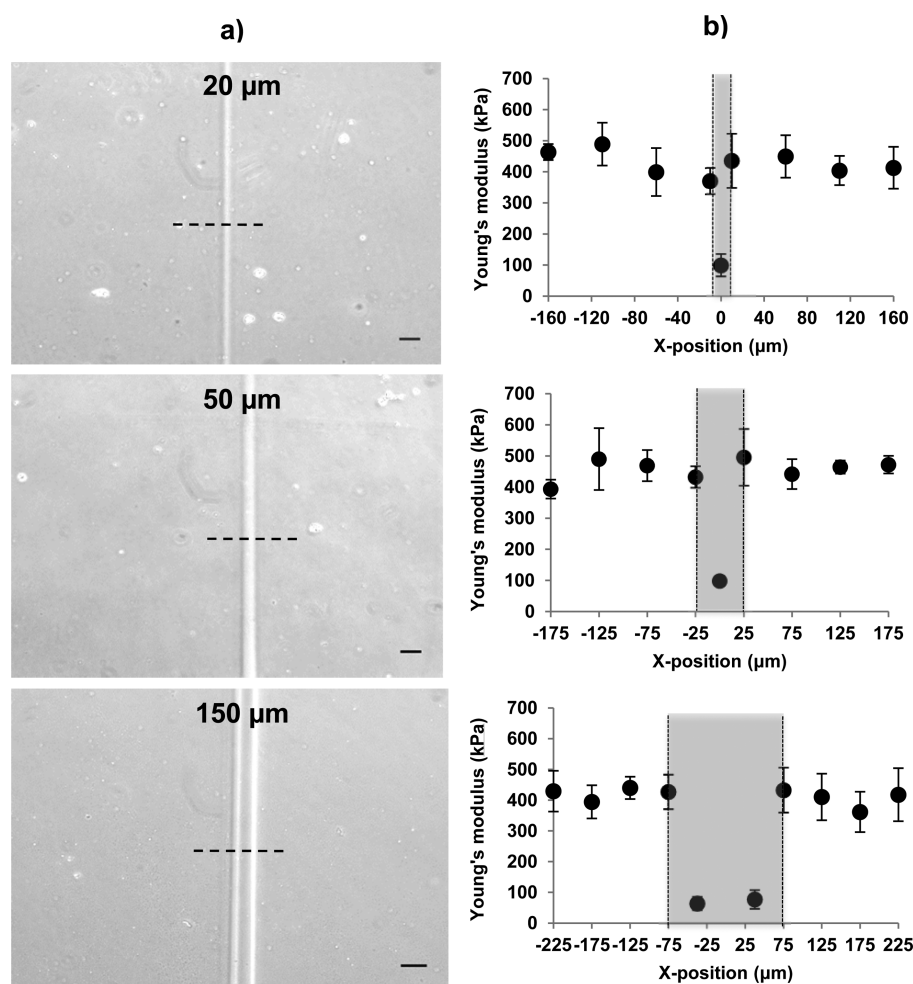
(17800g) for 1 h. The supernatant was subsequently degassed for 1 h to remove dissolved oxygen, conditioned for 10 min using a deforming agitator (MX-201, THINKY, Tokyo, Japan), and stored at –80 °C. The solution was warmed to 45 °C prior to photo-cross-linking.

**Elasticity Patterning of Gelatinous Gels with a Micrometer-Scale Soft Band against a Stiff Background.** A 30  $\mu\text{L}$  StG sol solution was spread between a 18 mm diameter vinyl-functionalized glass substrate and a 18 mm  $\times$  24 mm poly(*N*-isopropylacrylamide) (PNIPAM)-coated glass substrate.<sup>15</sup> Preparation of the sandwiched sol was carried out under a nitrogen atmosphere. Elasticity patterning of the gel was performed using a custom-built, mask-free photolithography system, which was originally developed by Okano et al.<sup>26</sup> A masking image designed in Microsoft PowerPoint on a personal computer was projected onto the sol solution using a liquid crystal display projector (1280  $\times$  800 pixels, EPSON EB-1770 W, 400 mW/ $\text{cm}^2$  at 488 nm). The masking image was reduced by a 2 $\times$  objective lens (NA 0.1, Nikon Corporation, Tokyo, Japan) and elastically copied onto the gel sample. To fabricate a narrow soft band with a width of 20, 50, or 150  $\mu\text{m}$  on a stiffer gel background, the sol solution was irradiated for a prescribed period of time using either a one-step or two-step process. For the 50 and 150  $\mu\text{m}$  wide soft bands, the entire samples were irradiated for 70–90 s followed by an additional 180–200 s irradiation through masking images containing black lines with widths of 0.198 and 0.959 cm, respectively. For the narrowest 20  $\mu\text{m}$  wide soft band, the sample was only irradiated once for 180–200 s through a masking image containing a gradient line (0.039 cm in width) that is black in the middle with increasing transparency toward the edges. After photolithographic patterning, the gel samples were released from the glass substrates coated with PNIPAM, washed vigorously, and immersed in PBS with gentle rocking overnight.

**Time-Lapse Observations.** The migratory behaviors of 3T3 fibroblast cells on the elastically patterned gels were monitored using an automated all-in-one microscope with a temperature and humidity-controlled cell chamber (BIO REVO BZ-9000, Keyence Corporation, Osaka, Japan). Prior to the time-lapse observation, cells were seeded at a density of  $1.0 \times 10^3$  cells/ $\text{cm}^2$  on each gel sample and cultured in DMEM containing 10% FBS under 5%  $\text{CO}_2$  for 7–8 h. DMEM was then replaced with Leibovitz's L-15 (Gibco BRL) containing 10% FBS to adapt the cells for long time observations in a  $\text{CO}_2$ -free environment. Images were captured every 15 min for 3 days. Cell trajectories were manually tracked on four to seven different substrates from three to four separate experiments under each condition using the ImageJ software.

**Characterization of Elasticity Distribution at the Hard–Soft–Hard Boundaries.** Distribution of elastic modulus around the soft band was determined by the microindentation method as previously described.<sup>15,21</sup> The force–indentation curves at different positions on each gel surface were obtained using an atomic force microscope (AFM) (NVB100, Olympus Optical Co. Ltd., Tokyo, Japan; AFM controller and software, Nanoscope IIIa, Veeco Instruments, Santa Clara, CA) using a silicon nitride cantilever with a half-pyramidal tip and a nominal spring constant of 0.02 N/m. Young's moduli of the gel surfaces were evaluated from the force–indentation curves by fitting to the Hertz model.<sup>27–29</sup> Surface elasticity of the gels was monitored throughout cell culture experimentation to detect any changes in the gel physical properties. The elasticity was first measured in PBS. The buffer was then replaced with DMEM culture media, and the samples were incubated at 37 °C overnight. The AFM measurements were again carried out before and after 3-day cell culture in the DMEM media.

**Characterization of Surface Topography of the Patterned Gels.** Surface topographies of the patterned gels were characterized using FITC-labeled albumin. A 50  $\mu\text{L}$  PBS solution containing 3 mg/mL of albumin-FITC was applied onto each gel surface. After incubation at 4 °C overnight, the gels were rinsed several times in PBS. The fluorescent signal of the protein absorbed in the gels was observed using confocal laser scanning microscopy (Zeiss LSM510 META). The cross-sectional analysis was performed with a 20 $\times$  objective lens using Z-stack scanning to obtain slice images of the gel surfaces before and after 3-day cell culture.



**Figure 1.** Young's modulus distribution at the hard–soft–hard boundaries. (a) Phase contrast images of patterned gels each containing a soft band (20, 50, and 150  $\mu\text{m}$ ) that is lighter in color; the scale bars are 100  $\mu\text{m}$ . (b) Surface elasticity measured along the hard–soft–hard boundaries, as indicated by the broken line in (a); modulus changes significantly at the hard–soft and soft–hard boundaries, as defined in the phase contrast images.

**Immunofluorescence Staining of Collagen on Mechanically Patterned Gels.** The 3T3 fibroblasts were cultured on sample gels patterned with a 150  $\mu\text{m}$  soft band for 1 and 3 days. Immunostaining of collagen was then performed as previously described.<sup>30</sup> The samples were fixed with 4% paraformaldehyde, washed 3 times with 1% BSA, and permeabilized and blocked with 10% donkey serum, 1% BSA, and 0.5% Triton-X 100 in PBS. 5  $\mu\text{g}/\text{mL}$  of rabbit anticollagen I (primary antibody, ab34710, Abcam, Tokyo, Japan) and 1/1000-diluted anti-rabbit conjugated Alexa 488 (secondary antibody, Invitrogen, Carlsbad, CA) were then allowed to react with the gel samples overnight. Immunostaining of control samples without cells (the no-cell control) and with cells but without the primary antibody (the secondary-antibody control) was also carried out in parallel. For the no-cell control, the gels were incubated with only culture media, and the reaction was performed in an otherwise identical way. This control was performed to discern whether the deposited collagen comes from the media and/or from cell secretion. The secondary-antibody control was carried out by eliminating the primary antibody and treating the samples with only the secondary antibody after cell culture. Characterization of collagen deposition on gel substrates was performed by confocal laser scanning microscopy using Z-stack scanning with a 40 $\times$  water objective lens. Quantification of the fluorescent signal of collagen I was performed using ImageJ on the selected Z planes corresponding to the topmost surfaces of the soft band and the stiffer background. Pixel intensity of each region was obtained using the plot-profile function in ImageJ by randomly

drawing three lines of 50  $\mu\text{m}$  in length, avoiding the cells, in eight different areas on each of three gel samples for each culture condition.

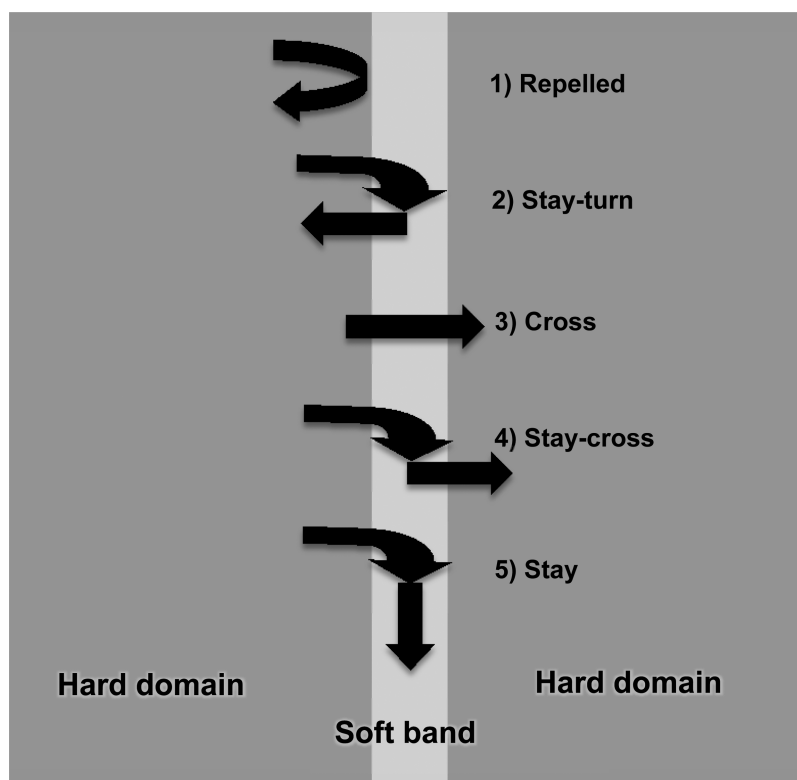
**Time-Lapse Observation of 3T3 Fibroblasts on Collagen-Saturated Patterned Gels.** Gel samples patterned with a 150  $\mu\text{m}$  soft band were incubated in a solution containing 2 mg/mL of collagen I (from rat's tail, Sigma-Aldrich, Tokyo, Japan) in 0.2% acetic acid at room temperature overnight. The gels were rinsed gently in PBS three times for 20 min each time. Cell culture and time-lapse observation for 24 h as well as immunofluorescence staining were performed on these gels as previously described.

## RESULTS AND DISCUSSION

**Fabrication of Microelastically Patterned Gels Containing a Soft Band.** Mask-free, photolithographic micro-elasticity patterning was used in this study for the fabrication of substrates containing a soft band against a stiffer background. For the 50 and 150  $\mu\text{m}$  wide soft bands, a two-step process was adopted: the entire samples were irradiated first prior to irradiation through a masking image. The moduli of the soft band and the stiffer background were dictated by the irradiation time of the first step and the total irradiation time, respectively. For the narrowest 20  $\mu\text{m}$  wide soft band, however, fabrication using the two-step method did not generate a sharp elasticity transition at the hard–soft boundary likely due to the diffusion of radicals from the irradiated region to the masked region.



Scheme 1. Five Cell Migratory Responses from the Hard Domain Approaching the Hard–Soft Boundary



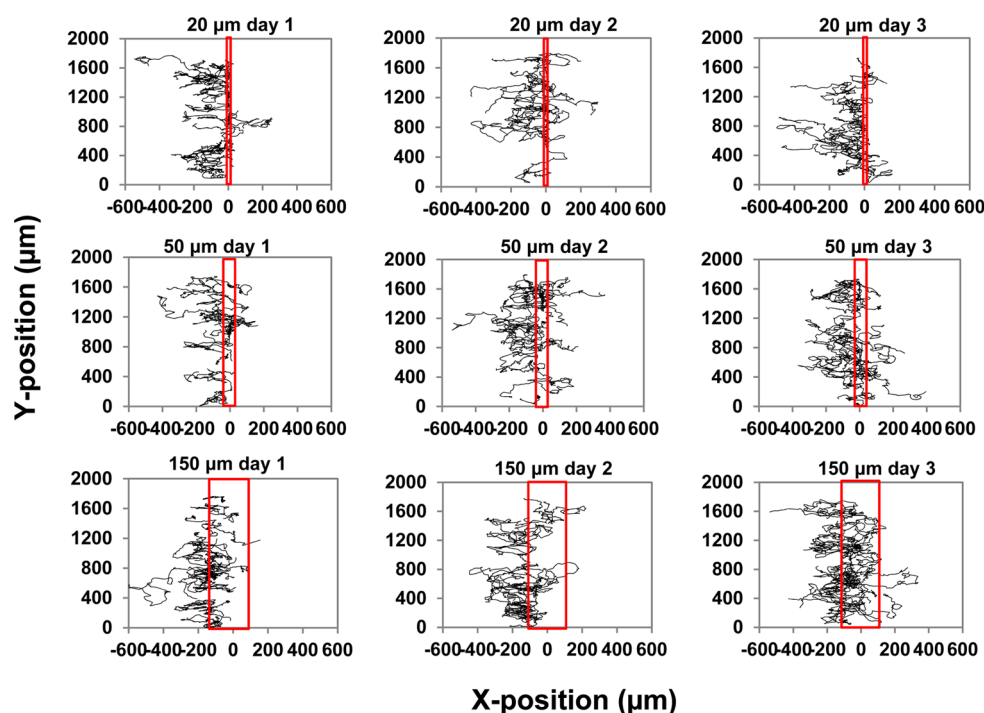
Two adjustments were made to allow the successful fabrication. A gradation line—black in the middle with increasing transparency toward the edges—instead of a solid black line was used as the masking image. Second, the sol solution was only irradiated once through the masking image for 180–200 s.

The patterned gels with significant elasticity difference between the hard background and the soft band were thus fabricated. Phase contrast images of the gel samples and surface elasticity distributions at the hard–soft–hard boundaries are shown in Figure 1. The soft bands appear to be lighter in the phase contrast images (Figure 1a). The actual widths of the soft bands were  $21 \pm 2$ ,  $38 \pm 4$ , and  $129 \pm 10$   $\mu\text{m}$  instead of the intended 20, 50, and 150  $\mu\text{m}$ , respectively. Diffusion of radicals from the irradiated region to the masked band likely results in the narrowing of the fabricated soft bands from the targeted widths of 50 and 150  $\mu\text{m}$ . The gradation masking line was apparently more effective at minimizing radical diffusion and producing a soft band at the targeted width of 20  $\mu\text{m}$ .

For the surface modulus quantifications, AFM measurements were carried out across the hard–soft–hard boundaries at 50  $\mu\text{m}$  intervals (Figure 1b). One point in the middle was measured on each of the 20 and 50  $\mu\text{m}$  bands, whereas two points ( $\sim 50$   $\mu\text{m}$  from each edge) were measured on the 150  $\mu\text{m}$  band. The patterned gels with different soft-band widths had similar moduli of the soft band ( $91 \pm 7$  kPa) and the stiffer background ( $432 \pm 19$  kPa). The resulted modulus difference of greater than 300 kPa between the soft and hard regions is sufficient to induce directional cell migration as shown in our previous study.<sup>15</sup> In addition, comparable moduli of the hard and soft domains among the three patterned gels allow the investigation of the effect of soft-band width on cell migratory behavior. It should be noted that due to differences in fabrication designs and variations in raw materials, it was difficult to achieve comparably low modulus values for the soft

band while maintaining similar stiffness gradient as in the previous study.<sup>15</sup> We acknowledge the likely effect of absolute moduli of the patterned gels on cell migration; however, we do not attempt to make any conjectures in this study.

**Time-Dependent Cell Migratory Responses at the Hard–Soft–Hard Boundaries.** In this study, we are interested in the migratory responses of fibroblast cells starting from the hard domain and approaching the hard–soft boundary during prolonged culture condition. The soft bands with variable widths are chosen to discriminate the time-dependent durotactic character. The different possible responses are shown in Scheme 1. The arrows represent the migratory paths of cells' centroid positions. Only the cells whose bodies physically interact with the soft band are illustrated. Since the soft bands are narrow, almost all cells initiate from the stiffer background. A cell is considered to be “repelled” by the soft band if any part of its body touches the hard–soft boundary prior to retracting back to the hard domain. A cell may be “repelled” by the soft band exemplifying the usual durotaxis behavior, or it may enter the soft band exhibiting the antidurotaxis behavior. Upon crossing the first elasticity boundary and entering the soft band, the cell may move randomly if the soft band is wide enough, “cross” the second elasticity boundary along the stiffness gradient, and finally reach the hard domain on the opposite side. Or the cell may exhibit vertically biased movement in the soft band before crossing to the hard domain on the opposite side (“stay–cross”), returning to the originating hard domain (“stay–turn”), or staying in the soft band for an extended period of time (“stay”). The criterion for “stay–cross”, “stay–turn”, and “stay” is that cells exhibit vertically biased migration in the soft band. Cell residence time in the soft bands was measured from the migration of approximately 10 cells for each bandwidth (data not shown). The residence time for “stay–cross” and “stay–



**Figure 2.** Trajectories of cells from stiffer regions to the soft bands (20, 50, and 150  $\mu\text{m}$ ), which are highlighted by red boxes: each plot contains representative data from at least three separate experiments and tracks  $N = 20\text{--}35$  cells; for each bandwidth, cell trajectories were tracked in day 1 (0–24 h), day 2 (24–48 h), and day 3 (48–72 h); the starting point of each cell is set arbitrarily on the left side of the soft band.

turn” was at least 1 h for the 20 and 50  $\mu\text{m}$  soft bands and at least 4 h for the wider 150  $\mu\text{m}$  soft band. The residence time for “stay” was at least 10 h for all bandwidths. “Cross”, “stay–cross”, and “stay–turn” are all mixed modes, demonstrating distinctive and sequential anti- and usual-durotaxis responses. Their main difference lies in whether there is observed vertical cell movement within the soft band, which is also related to cell residence time in the soft band prior to crossing to the other hard region, reflecting different cell affinity for the soft band. A longer period of “stay” means stronger suppression of durotaxis and enhanced cell affinity for the soft band; i.e., the order of durotactic strength is “repelled” > “cross” > “stay–turn”, “stay–cross”. “Repelled” and “stay–turn” have the same origination and destination but are in different categories of migratory responses in terms of the initial contribution of antidurotaxis to the latter. Another pair that have identical start and end points are “cross” and “stay–cross”. These should also be classified into different categories due to their different degree of cell affinity to the soft band. The final category of “stay” represents complete suppression of durotaxis due to the high adherent affinity of cells to the soft band.

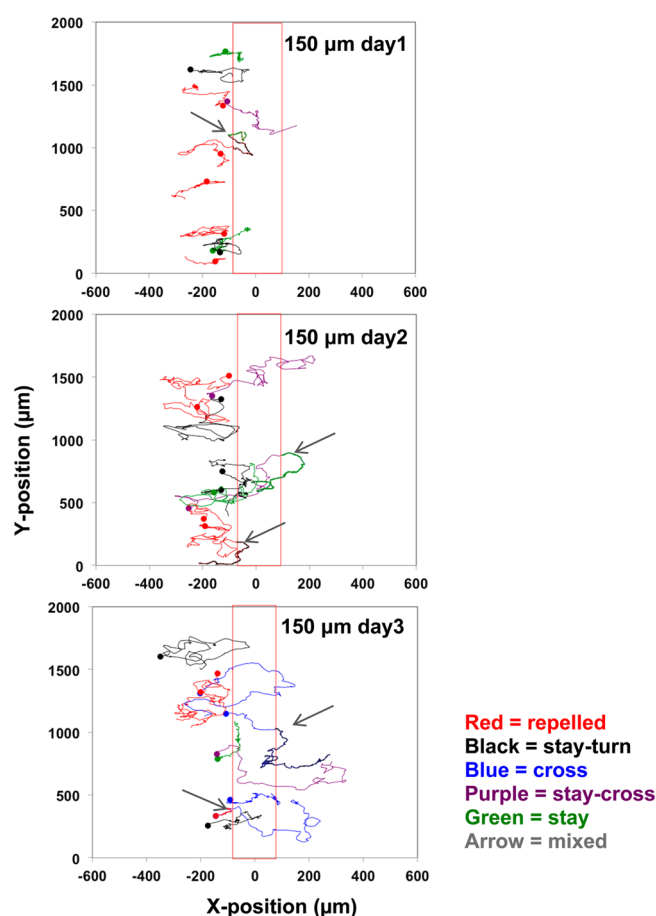
Cell migration studies were carried out on elastically patterned gel substrates for 3 days. The data obtained from the time-lapse movies were divided into first day (0–24 h), second day (24–48 h), and third day (48–72 h). Each plot in Figure 2 shows superimposed cell trajectories from at least three independent experiments; for each tracked cell, the center position of its nucleus was plotted in a continuous line over time. For ease of observation and comparison, all the cells are shown to originate from the hard domain to the left of the soft band.

The overall cell trajectories provide some general trends in terms of cells’ ability to cross the soft band as a function of time and bandwidth. It appears that the cells are more likely to cross

the soft band and end up on the other side over time and that the narrowest 20  $\mu\text{m}$  soft band appears to be somewhat more effective than the wider bands at preventing cells from crossing. This can be attributable to that the 20  $\mu\text{m}$  soft band is too narrow to accommodate the cells, i.e. for cells to enter, spread, and move around in the soft band, relative to the wider bands. These conclusions are based on the net effect of the soft band as a barrier on “an obstacle course” for cells to cross.

The cell trajectory plots, however, do not take into account the individual cell responses toward the soft band. As mentioned earlier, two pairs of cell responses, “repelled” vs “stay–turn” and “cross” vs “stay–cross”, have identical start and end points but represent very different cell migratory behaviors. It is thus necessary to separate the individual cell responses; representative cell trajectory plots with color-coded individual responses are exemplified in Figure 3 for the 150  $\mu\text{m}$  soft band. The most noticeable features are that fewer cells are “repelled” by (in red) and more cells “cross” (in blue) the 150  $\mu\text{m}$  soft band over time.

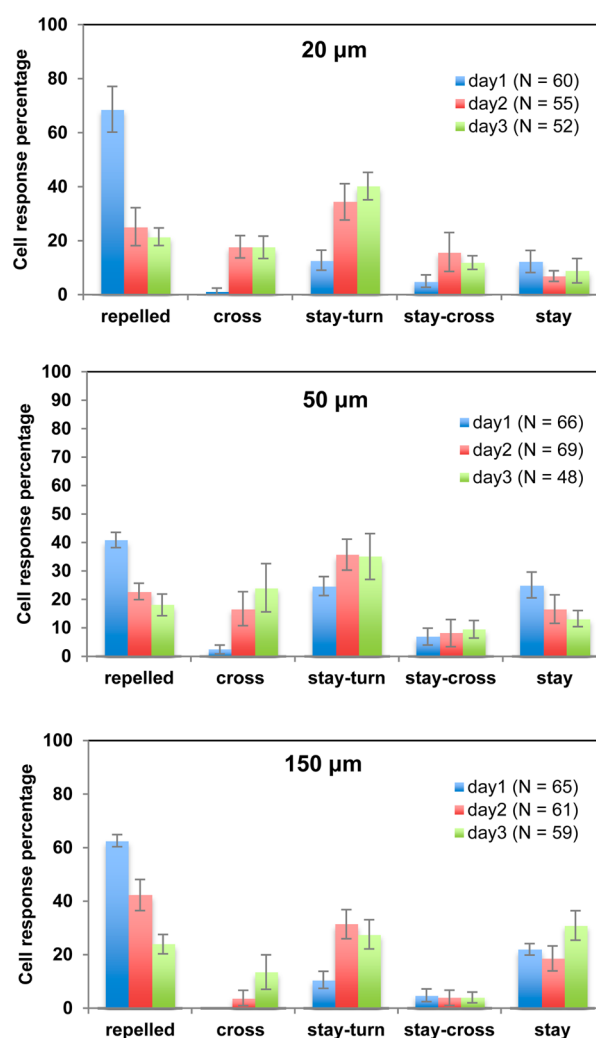
To quantify the cell migratory responses toward the soft band, the frequencies of the five types of cell responses as a function of time are tabulated in Figure 4. Since each cell sometimes interacted with a soft band multiple times resulting in multiple cell responses, frequency of cell responses instead of number of cells or frequency of cells is tabulated here. Cells that were “repelled” decreased significantly from  $69 \pm 19\%$  to  $21 \pm 7\%$ ,  $41 \pm 6\%$  to  $18 \pm 8\%$ , and  $63 \pm 6\%$  to  $24 \pm 10\%$  respectively on gels containing 20, 50, and 150  $\mu\text{m}$  soft bands from day 1 to day 3. It is not clear why the percentage of “repelled” cells by the 50  $\mu\text{m}$  soft band is lower than those by the 20 and 150  $\mu\text{m}$  soft bands on day 1. The reduced cell repellency over time indicates the weakening of usual durotaxis at the first elasticity boundary. On the other hand, durotaxis was recovered at the second elasticity boundary, i.e., “cross”,



**Figure 3.** Representative individual cell migratory responses toward the 150  $\mu\text{m}$  soft bands during day 1, 2, and 3 ( $N = 10$ ). The five color codes represent five different motions. The arrows indicate the trajectories of the cells that had mixed motions.

was observed starting from day 2 for all bandwidths. Cells that “stay–turn” and “stay–cross” tended to increase with time on 20 and 50  $\mu\text{m}$  bands, and cells that “stay” increased on 150  $\mu\text{m}$  bands on day 3. These cell behaviors suggest that the strong-mode durotaxis (“repelled”) is diminished during long-term culture and is replaced by the weak-mode durotaxis (“cross”, “stay–turn”, and “stay–cross”) on 20 and 50  $\mu\text{m}$  bands. In addition, adherent affinity of cells to the soft band, especially the 150  $\mu\text{m}$  bands, became significant manifested in the “stay” response. These bandwidth-dependent differences can be partly attributed to the restriction that the soft-band width places on cell shape. The widths of the soft bands are comparable to the size of cell nucleus ( $\sim 10\text{--}20\ \mu\text{m}$ ), a less spread cell ( $\sim 50\ \mu\text{m}$ ), and a fully extended cell ( $\sim 150\ \mu\text{m}$ ). As such, the tendency for cells to enter, spread, and move around in the soft band is expected to increase as the bandwidth increases. The stronger tendency for cells to stay away from the 20 and 50  $\mu\text{m}$  soft bands, i.e. repelled, cross, stay–turn, and stay–cross, probably results from the lesser likelihood for cells to enter and stay in the narrow soft bands than in the wider 150  $\mu\text{m}$  band.

The most striking result in Figure 4 is that the durotaxis strength changes from strong to weak on the relatively narrow soft bands, and adherent affinity-induced entrapment appears on the relatively wide soft band over time. To the best of our knowledge, this is the first report of time-dependent cell migratory behaviors on elastically patterned substrates. The diminished durotaxis over time may be caused by changes in



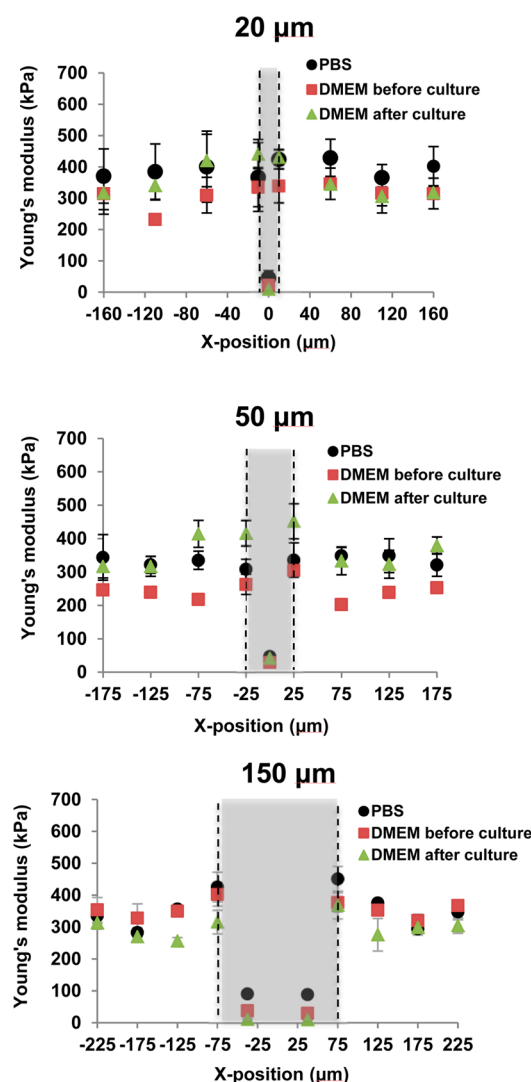
**Figure 4.** Five different cell responses toward the soft bands are tabulated and reported as percentages of the total responses in a time period of 3 days. The total number of cells approached the elastic boundaries under each condition is given in the legend and comes from multiple samples and three to four independent experiments. The error bars represent standard errors.

surface elasticity, surface topography, and surface chemistry. The rest of this section is expended on investigating reason(s) for the time-dependent durotactic behavior.

**Comparisons of Substrate Elasticity and Topography before and after Cell Culture.** Our initial hypothesis was that proteins from culture media and secreted from cells deposit on patterned substrates resulting in changes in surface elasticity, topography, and/or chemistry over time. Cells are sensitive to changes in the microenvironment contributing to the observed time-dependent migratory behaviors. To investigate whether prolonged culture condition affects the physical properties of the gels, surface elasticity and topography at the hard–soft–hard boundaries of the patterned gels were compared before and after 3-day cell culture.

Alteration of substrate stiffness is expected to result from deposition of various extracellular matrix proteins including collagen. Surface elasticity measurements were performed in PBS before cell culture, as the controls, and in DMEM before and after cell culture. The results are shown in Figure 5. There is no significant difference in modulus values before and after

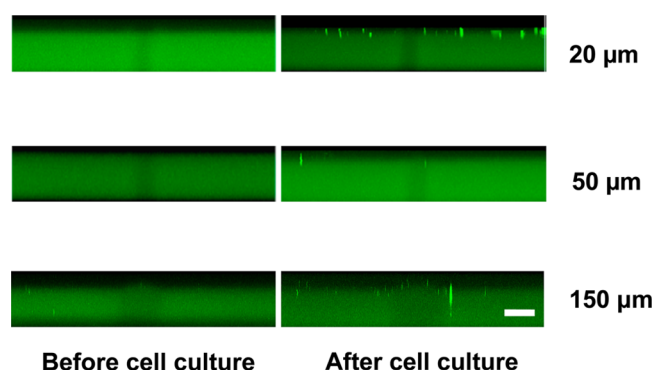
cell culture and the elasticity transition at the hard–soft boundaries remains sharp.



**Figure 5.** Young's moduli of the mechanically patterned gels before and after cell culture. AFM measurements were performed in PBS before cell culture and in DMEM before and after cell culture. The error bars represent standard errors.

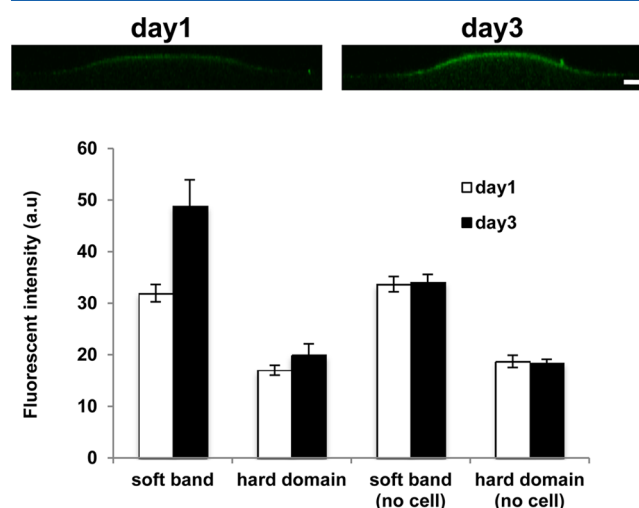
Surface topography was investigated after staining the patterned gels with albumin-FITC. The cross-sectional images obtained from laser confocal microscopy are shown in Figure 6. The 150 μm soft band is slightly more swollen than the rest of the substrate while the hard–soft–hard boundaries on the gels with 20 and 50 μm bands are completely flat. The relatively flat surface topography of the mechanically patterned substrates affirms that the cell repellency by the soft band is the sole result of durotaxis. After cell culture, there is also little difference in surface topography compared to before culture.

**Collagen Deposition at the Hard–Soft–Hard Boundaries.** The lack of changes in both surface elasticity and topography does not rule out protein deposition during cell culture. The extracellular matrix (ECM) molecules regulate various cellular activities including migratory response. It is speculated that even though the deposited proteins are not significant enough to cause physical changes to substrate surfaces, they may impart sufficient changes in surface



**Figure 6.** Confocal laser scanning microscopy images of the patterned gels stained with albumin-FITC, before and after cell culture. The cross-sectional images were obtained from 50 stacks at 1.5 μm intervals. The scale bar is 50 μm.

chemistry to induce adherent affinity of cells to the soft bands, which causes diminished usual durotaxis toward hard regions. In this study, the deposition of ECM molecules, in particular collagen I, were of special interest, as the fibroblasts are known to produce this highly abundant ECM proteins.<sup>23,24</sup> Immunofluorescence staining was carried out using the specific antibody that recognizes native collagen I. The gels containing a 150 μm soft band were used as a model to study the deposition of collagen I on the patterned substrates. In Figure 7, the top



**Figure 7.** Average fluorescent intensities of collagen I on the gels with a 150 μm soft band after 1- and 3-day culture. The upper panel shows the orthogonal view of the patterned gels with cells after 1 day and 3 days. The cross-sectional images were obtained from 30 stacks at 1.5 μm intervals. The scale bar is 20 μm. The lower panel shows the fluorescent intensities (in arbitrary units) of collagen I on the surfaces of the gel samples with and without cell seeding after 1 and 3 days, which were measured on the topmost surfaces of the soft and hard regions in the cross-sectional images. The error bars represent standard errors.

panel shows cross-sectional (z-stacked) fluorescent images of the mechanically patterned gels after cell culture for 1 and 3 days. The lower plot illustrates the average fluorescent intensity of the stained collagen I on the soft and hard regions with or without cells in the culture medium over time.

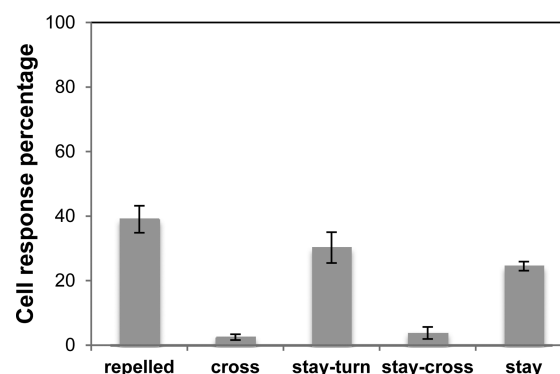
It is noticeable that the fluorescent intensity remains relatively constant on the hard region after 1- and 3-day



exposure to culture medium with and without cells. It should be pointed out that no fluorescent signal was observed on the secondary-antibody control samples (data not shown). This indicates that the deposited collagen I on the hard domain stems solely from the culture medium, and no additional collagen I deposition took place on the hard region after the first day. The situation was noticeably different on the soft band. Overall, the observed fluorescent intensity was higher on the soft band than that on the hard region, indicating preferential deposition of collagen I on the soft domain. The deposited collagen during the first day largely comes from the culture medium since there is little difference in the fluorescent intensity with and without cells. The noticeable increase in the fluorescent intensity on the soft band after 3 days of cell culture implies additional deposition of collagen I secreted from cells. The fluorescent images on the top panel depicting a much stronger fluorescent intensity from the soft band, especially after 3-day cell culture, in agreement with the intensity plot. These data suggest that the initial adsorption of collagen I from culture medium on mechanically patterned gel samples takes place within the first day and is more pronounced on the soft band. Additional deposition of collagen I secreted by cells is only noticeable on the soft band during prolonged cell culture. Overall, a higher density of adsorbed collagens on the soft band than on the hard region is observed on the gel surface.

The reason for this may be attributable to the lower degree of cross-linking of the soft-band portion of the gel. The softer gel matrix with a lower cross-linking density allows more collagens to adsorb/absorb: collagen molecules can adsorb onto the top surface of the softer gel as well as penetrate into its subsurface. Some of the absorbed collagens can protrude at the top surface of the soft gel, while such absorption and partial protrusion of collagens are rather difficult in the case of the harder gel due to the steric hindrance by the highly cross-linked matrix. Since cells can only sense the chemical factors immobilized on the surface of the culture substrates, an increase in collagen density at the top surface of the soft gel can be a significant haptotactant over time.

A control experiment was carried out to test the hypothesis that collagen deposition takes place preferentially on the soft band, which enhances haptotaxis and suppresses durotaxis over time. Patterned gels with a 150  $\mu\text{m}$  soft band were preincubated with collagen I overnight prior to cell culture and time-lapse observation performed for 24 h. Representative cell trajectories are depicted in Figure S1, and the frequencies of five different cell responses are shown in Figure 8. Compared to the data obtained on native samples during the first day of observation (bottom plot in Figure 4, data in blue), a smaller percentage of cells was repelled by the soft band and a larger percentage of cells migrated onto the soft band. Interestingly, the data from the control experiment are almost identical to those obtained during the second day of experiment on native samples (bottom plot in Figure 4, data in red), indicating similar effects of collagen incubation in the control experiment and collagen deposition during cell culture on cell migratory behaviors. The immunofluorescence staining of the samples preincubated with collagen I (Figure S2) supports the notion that collagen deposition is more significant on the soft band in either the presence or absence of the cells. The data from the control experiment directly verify that preferential collagen deposition on the soft band during cell culture significantly contributes to the time-dependent durotactic phenomenon.



**Figure 8.** Percentages of five different cell responses on collagen preincubated gels containing a 150  $\mu\text{m}$  soft band in a time period of 24 h ( $N = 111$  cells). The error bars represent standard errors.

Cell migratory behaviors under coexisting and competing mechanical and chemical cues have been reported in the literature. After seeding fibroblast cells on polyacrylamide gels with two adjoining regions, stiff but with a lower amount of collagen I and soft but with a higher amount of collagen I, preferential haptotaxis toward the softer region with a higher surface density of collagen I was observed.<sup>31</sup> This indicates that the direction of cell taxis is determined in a competitive manner between mechanical and chemical stimuli, and the chemical stimulus outweighs the mechanical cue in the reported study. In our system, collagens from culture medium and secreted from cells preferentially deposited on the surface of the soft bands. Though the amount of the deposited collagens on the soft band is higher than that on the hard domain during the first day of observation, the steep elasticity gradient at the hard–soft boundary apparently prevails, resulting in the majority of the cells being repelled by the soft band. The minority of the cells that are more sensitive to the surface chemistry and less sensitive to the surface elasticity exhibit diminished durotaxis, i.e., multiphasic migrations by randomly moving into the soft region and subsequently turning around, crossing, or staying. On the second and third days, fewer cells are repelled, likely due to the gradual increase of protein deposition on the soft band, which enhance cell affinity to the soft band. The observed time-dependent cell migratory behaviors, the collagen-staining studies, and the control experiment on gels preincubated with collagen I suggest that an increase in deposition of collagen and potentially other proteins on the soft bands provides additional support for cell adhesion and migration across the soft bands over time.

The control of cell migration via mechanical signals from the underlying substrates has gained much attention in the biomaterials field, and the outcomes of these studies have significant implications in tissue engineering and regenerative medicines. In the context of our findings, a soft band on a stiff background demonstrated a strong mechano-repellent effect on cell migration at the early stage of the experimentation. This type of engineered substrate is useful for the further development of cell separation devices based on the variabilities in the mechanical responsiveness of different cell types.<sup>32</sup> Additionally, our mechanically patterned gels can potentially be used as a model system for studying cell migration against micromechanical “obstacles” with a stiffness gradient, mimicking *in vivo* tissue variations that cells encounter. It would be interesting to explore how cells overcome the obstacles and migrate to the target location.



The central finding of our study is the demonstration of the integrated effects of chemical and mechanical stimuli contributing to the time-dependent migration of the fibroblasts. The increase in collagen deposition on the soft band over time is one of the clues that may be responsible for the diminished durotaxis. We do not preclude that other extracellular matrix proteins, such as fibronectin and other types of collagens, either in the secretory or bound form also work in concert to induce cell migration.<sup>33</sup> All these factors should be taken into consideration for the design of biomaterials or medical devices to precisely manipulate cell migration under multiple stimuli.

## CONCLUSIONS

Cell migratory behaviors were examined on gelatinous substrates containing a 20, 50, and 150  $\mu\text{m}$  softer band. The sharp elasticity transition at the hard–soft boundary was fabricated to induce durotaxis. The geometry of the designed mechanical field is well suited for the observation of different cell migratory responses at the hard–soft boundaries. During the early stage of experimentation, most of the cells were strongly repelled by the soft band independent of bandwidth, exhibiting strong durotaxis. During the course of 3 days, cells exhibit diminished durotaxis behaviors likely caused by the preferential deposition of collagen on the soft band. The knowledge of the interference by the surface-adsorbed proteins in the studies of durotaxis and stimulus-triggered cell migration is critical to ensure appropriate data interpretation and to establish experimental conditions minimizing the interference by other compounding stimuli in the design of functional cell-culture substrates.

## ASSOCIATED CONTENT

### Supporting Information

Additional data on gel samples preincubated with collagen I. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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