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Topographical guidance of 3D tumor cell migration at an interface of collagen densities

Francois Bordeleau, Lauren N. Tang, and Cynthia A. Reinhart-King*

Department of Biomedical Engineering, Cornell University, Ithaca, NY, 14853, USA

Abstract

During cancer progression, metastatic cells leave the primary tumor and invade into the fibrous extracellular matrix (ECM) within the surrounding stroma. This ECM network is highly heterogeneous, and interest in understanding how this network can affect cell behavior has increased in the past several decades. However, replicating this heterogeneity has proven challenging. Here, we designed and utilized a method to create a well-defined interface between two distinct regions of High and Low density collagen gels to mimic the heterogeneities in density found in the tumor stroma. We show that cells will invade preferentially from the High-density side into the Low-density side. We also demonstrate that the net cell migration is a function of the density of the collagen in which the cells are embedded, and the difference in density between the two regions has minimal effect on cell net displacement and distance travelled. Our data further indicate that a Low-to-High density interface promotes directional migration and induces formation of focal adhesion on the interface surface. Together, the current results demonstrate how ECM heterogeneities, in the form of interfacial boundaries, can affect cell migration.

Keywords

Metastasis; durotaxis; invasion; matrix; heterogeneity

1. Introduction

The tumor stroma is a complex environment where the extracellular matrix (ECM) exhibits tissue-specific heterogeneous 3D features, organization, rigidity and composition [1, 2]. Notably, the local stromal density around solid tumors is known to increase during cancer progression due to ECM deposition [3–5]. Among the ECM components, collagen I is the most abundant [6], and it is organized into fibers to which cells can attach [1, 7]. The density of this fibrous network can be highly heterogeneous and, as such, loose and dense collagen organization can exist within the same tissue [1]. In the case of breast tumors, densely packed collagen fibers can be organized both parallel and perpendicular to the tumor, depending on the extent of ECM remodeling that has occurred. This heterogeneity in the ECM forms different kinds of interfacial boundaries [3], and yet little is known about how cell behavior is affected by these heterogeneities.

Several approaches have been employed to study the effects of matrix microarchitecture and mechanical properties on cell migration. Early experiments performed on 2D planar substrates of tunable rigidity have revealed the role of durotaxis in driving cell migration [8–10]. When confronted with an interface between a compliant and a stiff substrate, cells will preferentially migrate to the stiffer substrate [9]. However, these experiments do not address

*To whom correspondence should be addressed: Cynthia Reinhart-King, 302 Weill Hall, 526 Campus Rd, Ithaca, NY 14853, Phone: 607-255-8491, Fax: 607-255-7330, cak57@cornell.edu.

the 3D nature of the ECM. Different strategies have been devised to study the effects of 3D architecture on cell migration. These approaches have largely relied on embedding cells within ECM-derived 3D gels such as collagen I, fibrin, basement membrane extract or cell-derived matrix [11, 12]. The use of 3D collagen gels of increasing densities yielding smaller pore size has revealed that microarchitecture provides a steric barrier that limits cell migration [13, 14]. Interestingly, alignment of collagen in 3D provides contact guidance cues which cause cells to migrate along the axis of fiber alignment [7, 15].

There have been several attempts to generate 3D gels containing a heterogeneous interface, including the use of synthetic gel constructs [16, 17] and nested collagen matrices [18, 19]. However, both methods do present limitations. While synthetic constructs are easily tunable, cells are unable to migrate through them [17] unless engineered cleavable sites are introduced [16]. Nested collagen gels can provide an interface, however their fabrication requires both mechanical collagen compaction and significant time in culture [18, 19]. As such, their microarchitecture and mechanical properties are hard to control. Nonetheless, work done with nested collagen matrices has provided valuable information about the effects of interfaces of different ECM composition on cell migration [19]. While such findings have yielded insight into the cell migration process, they also highlight the inherent difficulties in varying the physical properties of a fully 3D microenvironment to study cell migration.

The ability of adherent cells to migrate is dependent on their ability to dynamically regulate cell-ECM linkages at specialized adhesion membrane domains of which focal adhesions (FAs) are the most well-known [20]. While FAs can be readily observed on stiff 2D substrates, it is only recently that FAs have been identified within more physiologically relevant soft 3D ECMs [21–24]. The mechanical properties of the cellular environment have been shown to regulate the activation of specific FA proteins, including focal adhesion kinase (FAK), that act as mechanosensors. As mechanosensors, FAs are known to affect actin organization, cell adhesion and migration [24–28]. Of note, it was observed that increasing the substrate stiffness in either 2D or 3D results in higher FAK phosphorylation levels as well as larger FAs in several cell types [24, 28]. Interestingly, migrating cells exhibit a directional polarization of both FAs and fibrillar actin [26, 29]. Immature FAs are most often found at the leading edge of the cells, whereas more mature FAs are located behind it [20]. While the mechanisms and dynamics behind FA formation and maturation have been well documented in 2D [25, 27, 30, 31], much less is known about their behavior in 3D [23]. Understanding where FA proteins are located and activated when a cell encounters physical heterogeneities is critical to our understanding of cellular migration processes, especially those involved in metastasis.

In the work reported here, we develop and implement a novel and simple approach to address how the presence of an interface between two regions of a 3D collagen gel with known densities and microarchitecture influences the migration of highly metastatic MDA-MB-231 breast adenocarcinoma cells. Using sequential polymerization of separate collagen solutions, a well-defined interface between two different regions of a collagen gel was created. The microarchitecture of the resulting interface was analyzed by confocal reflectance microscopy. Migration experiments were performed using this system by embedding cells in only one of the two collagen regions, and cell migration was monitored using time-lapse microscopy. In addition, we assessed the formation and localization of FA-like structures in cells that were in contact with the collagen interface. Our results indicate that the presence of an interfacial boundary between two regions of different collagen density provides directional cues that guide cell migration, which in turn correlates with induced cell elongation and spreading parallel to the interface.

2. Materials and methods

2.1. Cell culture and reagents

MDA-MB-231 highly metastatic breast adenocarcinoma cells (American Type Culture Collection (ATCC), Rockville, MD, USA) were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen, Grand Island, NY, USA). MCF10A cells mammary epithelial cells (American Type Culture Collection (ATCC), Rockville, MD) were maintained in Dulbecco's Modified Eagle's Media supplemented with 5% horse serum, 20 ng/mL EGF (Invitrogen, Carlsbad, CA), 10 mg/mL insulin, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin (Sigma-Aldrich, St. Louis, MO), and 1% penicillin-streptomycin (Invitrogen). All cells were cultured at 37°C and 5% CO₂. Primary antibodies used were rabbit anti-phospho Y397 FAK (p-FAK, #3283; Cell Signaling Technology, Danvers, MA, USA) and mouse monoclonal anti- α -tubulin (sc-51503, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies used were Alexa 488-goat anti-mouse IgG, Alexa 555-goat anti-mouse IgG and Alexa-594-goat anti-rabbit IgG (Invitrogen). Goat serum and phosphate buffer saline (PBS) were purchased from Invitrogen; Triton X-100 was from JT Baker (Phillipsburg, NJ, USA). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Collagen gel preparation

Type I collagen was extracted from rat tail tendons (Pel-Freez Biologicals, Rogers, AR, USA) via acid-solubilization, purified via centrifugation and lyophilization, and reconstituted in 0.1% acetic acid at 10 mg/mL. 100, 200 or 500 μ l of the 10 mg/mL stock collagen solution was diluted up to 850 μ l by gently mixing with 0.1% acetic acid on ice and neutralized to pH 7.0 with 100 μ l of 1M HEPES. 50 μ l of DMEM containing either 60,000 cells or 0.5 μ m red fluorescent beads (Invitrogen) was then gently mixed with the collagen yielding a final collagen concentration of either 1 mg/mL, 2 mg/mL or 5 mg/mL. For interface experiments, a thin slab of Polydimethylsiloxane (PDMS, Ellsworth Adhesives, Germantown, WI, USA) was placed in the center of a glass-bottom 24-well plate (MatTek, Ashland, MA, USA) as depicted in Fig. 1A. This setup was then sterilized under UV for 15 min. 100 μ l of the first gel solution was seeded into one side of the well in the 24-well plates. Gels were incubated at 37 °C for 30 min for collagen polymerization after which the PDMS slab was gently removed, and a fresh gel preparation was seeded adjacent to the previously polymerized gel to provide the following configuration: 2 mg/ml with beads beside 1 mg/ml with cells (2-1(c)); 5 mg/ml with beads beside 1 mg/ml with cells (5-1(c)); 2 mg/ml with cells beside 1 mg/ml with beads (2(c)-1); 5 mg/ml with cells beside 1 mg/ml with beads (5(c)-1). The setup was incubated at 37 °C for a further 60 min to allow complete polymerization of the two gels after which they were overlaid with 1.5 ml of pre-warmed culture media. The number of embedded cells dying over the course of the experiment was found to be similar to the same cells cultured in regular culture flask (~3%). Dying cells could readily be identified in phase contrast and were not included in the analysis.

2.3. Cell migration

Embedded cells located in the close vicinity of the interface as determined by the presence of the red beads were selected randomly. Care was taken to ensure that these cells were at least 150 μ m away from the bottom of the dish. Individual cell migration was observed with a wide-field digital imaging system (Zeiss Axio Observer Z1, Hamamatsu ORCA-ER camera and Axiovision software v. 4.8.1.0) equipped with an environmental chamber. Phase-contrast images were captured at 30 min intervals over a 24-h period, using a 20X/NA0.5 ph2 dry objective. The cell locomotion was tracked using ImageJ software (v. 1.46, National Institutes of Health, Bethesda, MD, USA). Quantification of cell behavior was obtained from at least three independent experiments and the different migration parameters

are for at least 30 cells. The migration parameters were computed only for the xy imaging plane and cells moving too far out of focus were not analyzed.

2.4. Confocal cell imaging

For actin imaging, the collagen gels were washed once with PBS, fixed for 10 min at RT with 3.7% formaldehyde in PBS, followed by methanol:acetone (3:7) for 5 min at -20°C . For FAK and vinculin imaging, the cells were fixed 10 min at 37°C with 3.7% formaldehyde in PBS, followed by 1% Triton X-100 (JT Baker) in PBS for 1 hr. For microtubule (MT) imaging, the cells were instead fixed 10 min at 37°C with 3.7% formaldehyde in MT stabilization buffer (100 mM PIPES, 5 mM EGTA and 2 mM MgCl_2 , pH 6.8), followed by 0.5% Triton X-100 (JT Baker) and 1% goat serum in PBS for 1 hr. They were then incubated successively, overnight at 4°C with anti-pFAK (1/50 in PBS), anti-vinculin (1/250 in PBS) or anti- α -tubulin (1/250 in PBS) followed with Alexa Fluor® 488 anti-rabbit or anti-mouse IgG antibody at RT for 2 h. Actin was stained with phalloidin-Alexa Fluor® 488 (1/100 in PBS), or with phalloidin-Alexa Fluor® 564 (1/100 in PBS) for co-staining, at RT for 2 h. Images were captured on a Zeiss LSM 700 microscope using the 450, 488 and 543 nm excitation laser lines and a 40X / 1.1 NA objective water immersion objective and operated by Zen software (v. 2010, Carl Zeiss MicroImaging GmbH, Jena, Germany). 3D image reconstruction and optical section slicing were performed with the ImageJ software.

2.5. Quantitative characterization of collagen microarchitecture, radial plots and statistical analysis

Two-dimensional spatial autocorrelations were used as described before [13] to automatically analyze the collagen gels using the Matlab software (The Mathworks, Natick, Massachusetts, USA) and a custom written algorithm to compute the autocorrelation function (ACF). The resulting ACFs were fit to Gaussian surfaces and the $1/e^2$ values describing the diameter of the Gaussian surface was defined as ω_{ACF} . The parameter ω_{ACF} was averaged from 15 different acellular images taken in 3 separate gels following 24 hr migration timelapse. Data processing for the radial plots and the statistical analyses was performed using Matlab Parametric one-way ANOVA with post-hoc Tukey's honest significance test were performed where appropriate. A p value below 0.05 was considered statistically significant. Test for randomness in the directional migration was performed with a Rayleigh randomness test provided in the circular statistic toolbox available for Matlab [32].

3. Results

3.1. Characterization of the 3D interface created by sequential polymerization of two collagen gels

Work by others has shown that a bulk ECM interface could be generated by sequential polymerization of collagen-alginate composite hydrogels [17]. We adapted this sequential polymerization strategy to form a vertical interface between two collagen I gels of a given concentration through a molding process. A PDMS slab was used to mold the first gel to form a vertical boundary (Fig. 1A). The second gel was poured adjacent to the first after removal of the PDMS slab. Using this setup, cells can be embedded in one of the two collagen gels and their interactions with the interface during migration can be monitored (Fig. 1B).

To assess the effect of sequential polymerization on the bulk organization of the collagen at the interface, two different collagen gel pairs with concentrations of 2-1 and 5-1 mg/ml collagen (polymerized sequentially in that pairwise order) were imaged using confocal

reflectance microscopy (Fig. 1C–D). In the two cases, the boundary at the interface between the two gels was easy to identify. To quantify the collagen concentration variation at the interface, a linear profile of the collagen intensity through a cross-section of the interface was generated by integrating the normalized pixel intensity of a 50 by 450 pixel region from confocal reflectance images. The linear profile demonstrates the presence of an interface. The linear profile also indicates that the sequential polymerization process also produces a steep increase in collagen density directly at the interface on the side of the first gel while a slight decrease can be observed on the side of the second gel. Nevertheless, the fibers from the resulting gel are continuous at the interface between the two gels. We further quantified the collagen fiber organization away from the interface using spatial autocorrelation [13] to extract the characteristic collagen dimension length ω_{acf} (Fig. 1E). As expected, ω_{acf} was found to decrease significantly from 1 mg/ml to 5mg/ml collagen gels (Fig. 1F), indicating that adjacent gels were indeed of different feature size. Overall, these results demonstrate that the sequential polymerization of collagen gels generates a discrete interface.

3.2. The interfacial density step provides a physical barrier to cell migration

Prior work has demonstrated that MDA-MB-231 cell migration is influenced by ECM topography, where cells migrate along oriented collagen fibrils [7] or inside pre-formed collagen microtracks. Here, we sought to determine how migration is affected by the presence of an interface between two collagen gels of different density. MDA-MB-231 cells were embedded in several different conditions using the collagen gel configuration quantified above. The 2-1 and 5-1 mg/ml collagen gels were used with cells seeded in the second gel to represent a Low-to-High transition (2-1(c), 5-1(c) respectively). Alternatively, a High-to-Low transition was mimicked by seeding the cells in the denser side of the 2-1 and 5-1 mg/ml collagen gels (2(c)-1, 5(c)-1 respectively). The subsequent cell migration was monitored over the course of 20 hr using time-lapse microscopy (Fig. 2A and B). When confronted by a Low-to-High density interface, we were able to identify only one single cell in all of our experiments that seemed to be in the process of crossing the boundary at the interface, indicating that cells do not generally move from low to high density collagen. However, such an event was readily observed in all experiments performed in the High-to-Low configurations, although the actual fraction of cells migrating across the interface was low (Fig. 2C). This behavior was also observed using 3D reflectance image of fixed samples, where the migration pattern cells can be determined based on the microtrack created by the cell (Fig. 2D–E). Visualization of these microtracks provides additional evidence that cells are capable of crossing the High-to-Low interface (Fig. 2E). Together, these results demonstrate that our experimental approach creates a density-based interface between two collagen gels that can influence cell migration in 3D.

3.3. The presence of an interface boundary does not influence migration distance and track length

Cell migration can be quantified by several parameters, including migration distance and track length. To further characterize cell behavior at the interface, single cell migration was monitored by tracking the cell position relative to the interface every 30 min. Only cells with an initial position less than 40 μm away from the interface were tracked. The resulting cell migration tracks were found to be similar for MDA-MB-231 cells embedded within 1 mg/ml collagen, irrespective of the density of the adjacent gel (Fig. 3A). In these cases, Net Migration Distance and Track Length were not significantly affected by the presence of a higher density interface (Fig. 3C). In contrast, when the cells were embedded in the high-density side of the gel, their migration was significantly affected compared to the Low-to-High configuration (Fig. 3B and C). As expected, cell migration decreases in the 5(c)-1 as compared to the 2(c)-1 condition, as indicated by the significant decrease in Net Migration Distance and Track Length (Fig. 3C).

3.4. The presence of a Low-to-High interfacial boundary induces directional migration

Given that the above data indicate that the presence of the interface did not affect the cellular migration parameters described above per se, we extended our migration analysis to assess whether the presence of the interface influences the directionality of cell migration in relation to the interface. Closer examination of cell migration revealed that a significantly greater number of cells migrated away from the interface in the Low-to-High configuration compared to the High-to-Low gel configuration (Fig. 4A, black bars compared to white bars). Although we observed a small increase in the percent of cells migrating away from the interface between the 2-1(c) and 5-1(c) configurations (from 33% to 45%), it was not found to be significant. Conversely, the cells in the High-to-Low configurations migrated away from the interface in significantly lower number than for the Low-to-High configurations, with 7% and 5% for the 2(c)-1 and 5(c)-1 configurations, respectively (Fig. 4A). To further explore the effects of the interface on guiding cell migration, we assessed the overall direction of cell migration by measuring the angle of the path length obtained above in relation to the interface to test for randomness. As seen in Fig. 4B, the distribution of angles extracted from the migrating cells initially located at the interface show a preferential direction parallel to the interface plane in the Low-to-High configuration. This directional preference could not be observed in the High-to-Low configuration as the migration appeared random. Together, these results suggest that the presence of a Low-to-High collagen density interface provides 3D topographic cues that guide cell migration.

3.5. Cells spread and form adhesions on a Low-to-High interfacial boundary

Since our data indicate that the interface can provide migration guidance, and considering that others have shown that polarization is a key aspect to mechanosensing of substrate stiffness [33], we asked whether cells could adopt a preferential orientation in relation to the interface. Interestingly, numerous cells spread against the interface when presented with a Low-to-High density interface. Our data indicate that over the course of the experiment, between 50 and 70% of cells adopted an elongated shape parallel to the plane of the interface in the Low-to-High configuration (Fig. 5A). Cell elongation in the 5-1(c) configuration was not significantly different than in the 2-1(c) configuration (69% versus 56%). In contrast, cell elongation was observed in less than 20% of the cells in the High-to-Low gel configuration (Fig. 5A). To further characterize the elongation of cells at the interface, we used confocal imaging to create 3D reconstructions of cells located at the interface that were stained with either with actin or MT. We primarily focused on the 5-1(c) gel configuration since both cell elongation and directional migration were observed mainly in the Low-to-High scaffolds. Interestingly, cells presenting an elongated shape appeared to be spreading on interface in a way that is reminiscent of what has been observed in 2D (Fig. 5B), where the cell body aligns parallel to the interface. In addition, a small fraction of cells were elongated perpendicular to the interface, extending in the direction of the low density gel (Fig. 5C). In all of these cases, intense actin bundles were present at the polarized ends of the cell.

Because the spreading of cells on a 2D substrate are known to form an actin cytoskeleton anchored by FAs in a process involving FAK [34], and because FAs in 3D cell culture are increasingly reported in the literature [21–24], we investigated whether cells at the interface could form FA structures. Specifically, we stained cells for vinculin and p-FAK as markers of FAs. Interestingly, we detected well-structured cell-matrix adhesions in the cells at the interface (Fig. 6A). While many of these adhesions appeared along the interface, they did not exclusively appear at the interface. Several of these adhesions appeared to be linked to discrete collagen fibers (Fig. 6B). Nevertheless, the FAs were mainly concentrated at the cell extremities parallel to the interface, indicating their likely involvement in driving directional migration in the plane of the interface.

3.6. Nonmalignant MCF10A cells have similar behavior at Low-to-High interfacial boundary

Because MDA-MB-231 cells are highly invasive cells, we further investigated to see if our finding applied to nonmalignant MCF10A epithelial cells. Interestingly, embedded MCF10A cells in the 2-1(c) configuration were able to spread along the interface over the course of the experiment when in contact with the interface (Fig. 7A). In addition, MCF10A average migration distance was $14.9 \pm 3.1 \mu\text{m}$ over 20 hr and their migration pattern was concentrated along the interface plane (Fig. 7B). In fact, 85% of the MCF10A cells adopted an elongated shape when in contact with the interface while only 3% of cells were observed leaving the interface plane. Accordingly, the distribution of angles extracted from the migrating MCF10A cells initially located at the interface show a preferential direction parallel to the interface plane (Fig. 7C). As shown in Fig. 7D, 3D reconstruction of confocal image sections of MCF10A cells spreading on the interface stained for both p-FAK and actin revealed the presence of well-structured cell-matrix adhesions similar to those observed in MDA-MB-231 cells. Overall, these data correlate with the data obtained with MDA-MB-231 cells, and taken together, the current results suggest that ECM heterogeneities in the form of interfacial boundaries can induce FAs formation and drive directional cell migration.

4. Discussion

Much of our knowledge on the effects of 3D architecture on cell migration comes from studies involving homogeneous 3D ECM scaffolds [13]. However, there are few systems that can recapitulate the heterogeneous nature of the native stroma [7, 16, 17, 19]. Here, we provide a novel and simple method that enables the study of single cell migration at an interface between two collagen gels while independently controlling the 3D architecture through modulation of collagen density. Using this platform, we find that the presence of a discrete interface of densities can inhibit migration, depending on the direction of the density gradient in relation to the cell position. The presence of a positive discrete gradient led to polarized cell spreading and directional migration. Overall, our results indicate that the presence of an ECM interface provides directional cues that can guide cell migration.

The challenge of establishing a good 3D model stems in part from the difficulty in adequately controlling both the mechanical properties and the microarchitecture of 3D ECM scaffolds. Several groups, including ours, have described how one can control various properties of the ECM, including stiffness, pore size and fiber alignment to assess their effects on cell behavior [7, 13, 24, 35–39]. Although considerable efforts have been made to recreate stroma-like heterogeneous microarchitectures, either the lack of information regarding the mechanical properties of the resultant ECM [19] or the inherent limitations of using synthetic ECM constructs [16] impair the extent of the insights we can gain from those approaches. Here, we demonstrate the ability to generate a discrete interface between two collagen gels of different density. However, characterization of the interface itself remains a challenge that will require more advanced image processing software and tools to perform analysis, including the autocorrelation of image sections oriented parallel to the interface plane, to fully appreciate the full extent of collagen organization around the interface. In addition, boundary effects on collagen fiber organization at the interface limit the capabilities of the current system since the interface is always present and its overall spatial organization appears to be independent of the initial collagen gel densities used. Thus, finding new ways to generate artifact-free collagen interface will be required to address this limitation. Nevertheless, cells could interact with the discrete interface and, in some cases, were able to migrate through it.

Our results indicate that cell migration parameters, such as net migration, are not affected by the discrete ECM density change at the interface, but rather by the ECM density of the gels

in which the cells are embedded. This is consistent with findings showing that 3D ECM density and porosity is a major determinant of cell migration speed [13, 24]. Previous studies using nested collagen matrices, which yield a High-to-Low configuration by embedding compressed collagen gels within acellular ECM uncompressed matrices, have shown that the number of fibroblast invading into the acellular gels was independent of the collagen density [19]. Interestingly, our findings suggest that migration of cells through an interface is primarily a one-sided process in favor of cells in the High-to-Low configuration and, even as such events remain rare, the probability that a cell will cross over from a high density to low density gel was not found to be related to collagen concentration. Considering the importance of metalloproteinases (MMPs) for cell migration in 3D scaffolds [40] and since other have shown that scaffold density affects MMPs expression [41], we cannot eliminate the possibility that the absence of cellular invasion in the Low-to-High configuration could be related to a decrease in the expression level of MMPs caused by the reduced scaffold stiffness/density found in the low density portion of the gel. Thus, exploring the role of MMPs at the interface could be crucial. Of particular note, the addition of fibronectin to either the collagen or the fibrin acellular scaffolds used in nested gel experiments is sufficient to increase both cell invasion and migration [19]. Indeed, it is widely accepted that the exact composition of the ECM differentially affects both cell morphology and migration [12, 42]. In light of these observations, one can speculate that, by varying the ECM composition on one side of our system, it could be possible to upregulate cell invasion.

In addition, our results indicate that the presence of the interface induces directional migration. Physical cues from the ECM in the form of either topographical features or durotactic gradients can direct cell migration [9, 43]. The directional migration induced by durotaxis has been shown mainly in 2D systems with mesenchymal cell lines [9, 33, 44]. Interestingly, it was shown previously that two endothelial cells can communicate through the deformation of a soft substrate to mutually influence their migration [45]. Additionally, cells plated on a 2D planar stiffness gradient with a collagen gel overlay invade upward into the collagen only from the softer side of the interface [33]. As such, it is possible that both the physical features of the interface and the deformation of the collagen scaffolds induced by cells further away from the interface may explain why some cells will not stay at the interface but instead migrate toward the low density gel. Moreover, directional migration of cells inside a 3D scaffold can be induced by aligning the fibers within a collagen gel during the polymerization process [7]. Of note, we have shown recently that pre-formed collagen channels mimicking microtracks induce highly directional migration in both invasive and non-invasive tumor cells [46]. Therefore, the local decrease of collagen density we observed directly adjacent to the interface may create a more permissive space for cell migration. Similarly, on the other side of the interface, the increase of collagen density may create a more restrictive migration environment, thus preventing the cells from adequately sensing and spreading on the interface. Conversely, it is possible that the microtracks created by the cells to reach the interface on the denser side already provide a stronger directional cues than the one provided by the interface. These considerations provide a plausible explanation on why the cells in the Hi density ECM side do not tend to orient themselves parallel to the interface or migrate along it. In the future, exploring additional interfacial collagen density combination may provide further insight on these behaviors.

A growing amount of evidence has accumulated in recent years showing the existence of FAs in 3D [21–24]. Consistent with these reports, we were able to observe the presence of FAs in our cells embedded within the 3D gels as indicated by p-FAK and vinculin staining. In fact, cells that appeared to be spreading on the interface presented an intracellular distribution of these adhesions aligned with the plane parallel to the interface. Notably, during the initial steps of cell adhesion on 2D substrates, FAK is rapidly activated, which

allows the binding of partners such as Src and enhances cell spreading and FA maturation [25, 27, 34, 47]. Conversely, FAK activation is needed to establish the front-rear polarity required for directional cell migration, a feat that is achieved by means of spatial control of actin dynamics [26]. Of additional note, FAK is part of the cell mechanosensing system and as such, its activation has been shown to be influenced by the mechanical properties of 2D substrates [28]. When combined with our directional migration data and actin imaging, FAK distribution in cells at the interface indicates that the interface may induce front-rear polarity. The relationship between the spatial regulation of FAK activation, FA formation, and the resulting cell behavior in response to 3D physical heterogeneities remains an interesting area of study.

Here, we use a straightforward and simple approach to address the guiding effect of a 3D interfacial boundary on cell migration. Specifically, we have shown that increasing interfacial ECM density greatly limits the ability of a cell to invade through the boundary, and instead, it promotes guided cell migration. Interestingly, we observe that non-invasive cells tend to remain in contact with the interface while the more invasive cell line tend to migrate away from it. Increased collagen density has been linked to increased invasion potential of tumor cells originating from solid tumor [48]. However, collagen fibers are initially aligned tangentially to the tumor-stroma interface and invading cells are observed in region where collagen fibers are reorganized perpendicular to the interface [48]. Specifically, malignant cells invade in regions of perpendicularly aligned ECM fibers instead of regions of densely packed parallel fibers [3]. These observations, when combined with our own, suggest that tumor cells must break down the tumor-stroma interface before they are able to invade. In addition, immune T cells infiltrating in the tumor are preferentially found in region low ECM density [49], indicating that ECM interfacial boundaries might provide migration cues that affect several cell types. Collectively, our data have significant implications for our understanding of the interaction of cells with physical heterogeneities. Finally, this easily adaptable approach to study cell migration could be used to study the importance of physical heterogeneity on cell migration in the contexts of embryogenesis, angiogenesis and tissue morphogenesis.

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Figure 1. Sequential polymerization of collagen gels generates 3D interfaces

(A) A slab of PDMS is positioned in the center of a well from a 12-well plate to provide the mold for the vertical interface. Gel #1 is then poured on one side of the PDMS slab. After the polymerization of gel #1, the PDMS slab is gently peeled off and gel #2 is poured in its place and allowed to polymerize. (B) For migration experiments, cells are embedded in one of the two collagen regions. (C) The resulting collagen interfaces created by the sequential polymerization process were characterized based on the average relative intensity obtained from confocal reflectance sections of 50 pixels by 450 pixels (actual dimension of 30.5 by 280 μm , $n=6$), for the (C) 2-1 and (D) 5-1 mg/ml collagen gel configurations. (E) Quantitative analysis of collagen gel microarchitecture with image autocorrelation analysis showing representative confocal reflectance images and the associated autocorrelation functions for 1 mg/ml and 5 mg/ml collagen gel. Red outlines represent the $1/e^2$ radii, or μ_{ACF} characteristic lengths. (F) Mean uACF characteristic lengths ($1/e^2$ radii from autocorrelation function). *, $p<0.05$ relative to 1 mg/ml gel and **, $p<0.05$ relative to 2 mg/ml gel.

Figure 2. The interface acts as a migration barrier

Phase contrast images at 0 hr and 20 hr for the (A) Low-to-High and (B) High-to-Low configuration showing the migration of cells initially located at the interface. (C) Quantification of the average percentage of cells able to migrate across the interface ($n=3$ independent experiments) for the Low-to-High (black) and High-to-Low (white) conditions obtained from three independent experiments. Bars denote SE. 3D reconstruction from confocal reflectance images of the interface and the migration track and the corresponding cell highlighted (green and red respectively) after 20 hr showing cell migration on the interface of the (D) Low-to-High 2-1(c) configuration and cell migration through the interface of the (E) High-to-Low 5(c)-1 configuration. Scale bar, 40 μm .

Figure 3. Cell migration depends on embedding collagen density
Cell tracks measured over 20 hr from phase contrast image for the (A) Low-to-High and (B) High-to-Low configuration in relation to the interface plane ($x=0$, y being in the interface plane). (C) Average migration parameters for the net migration displacement (d_{net}) and track length (T_{length}) for the Low-to-High (black) and High-to-Low (white) conditions obtained from three independent experiments. Bars denote SE. *, $p<0.05$ relative to 1-1(c) condition.

Figure 4. A Low-to-High interface provides directional migration cues

(A) Average percentage of cells that migrate away from the interface for the Low-to-High (black) and High-to-Low (white) conditions obtained from three independent experiments. (B) Quantification of migration angles of cells (n=30) initially located at the interface. The dotted vertical line represents the orientation of the interface plane. Bars denote SE. *, $p < 0.05$ relative to 1-1(c) condition.

Figure 5. Cells adopt an elongated shape at the interface

(A) Average percentage of cells that adopted an elongated and spread shape at the interface over the course of 20 hr ($n=3$ independent experiments) for the Low-to-High (black) and High-to-Low (white) conditions obtained from three independent experiments. Bars denote SE. *, $p<0.05$ relative to 2-1(c) condition. 3D reconstruction from confocal image sections of actin (red) and α -tubulin (green) from the 5-1(c) configuration showing (B) cells spread on the Low-to-High interface plane and (C) cells elongated in the direction of the low density gel. The collagen interface was visualized by confocal reflectance (blue). Scale bar, 20 μ m.

Figure 6. Cells in contact with the interface form focal adhesions

3D reconstruction from confocal image sections from the 5-1(c) configuration showing the accumulation of vinculin and p-FAK at the extremities of both (A) cells spread on the interface and (B) cells elongated perpendicular to the Low-to-High interface. The collagen interface was visualized by confocal reflectance. Scale bar, 20 μm .

Figure 7. MCF10A cells in contact with the interface

(A) Phase contrast images at 0 hr and 20 hr for the 2-1(c) Low-to-High configuration showing the migration of MCF10A cells initially located at the interface and (B) the corresponding cell tracks measured over 20 hr from phase contrast image. (C) Quantification of migration angles of MCF10A cells ($n=30$) initially located at the interface. The dotted vertical line represents the orientation of the interface plane. (D) 3D reconstruction from confocal image sections from the 5-1(c) configuration showing the accumulation of p-FAK at the extremities of cells spread on the interface and. The collagen interface was visualized by confocal reflectance. Scale bar, 20 μm .