

Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis

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Communicated by Richard O. Hynes, Massachusetts Institute of Technology, Cambridge, MA, May 31, 2006 (received for review December 24, 2005)

Cell migration on 2D surfaces is governed by a balance between counteracting tractile and adhesion forces. Although biochemical factors such as adhesion receptor and ligand concentration and binding, signaling through cell adhesion complexes, and cytoskeletal structure assembly/disassembly have been studied in detail in a 2D context, the critical biochemical and biophysical parameters that affect cell migration in 3D matrices have not been quantitatively investigated. We demonstrate that, in addition to adhesion and tractile forces, matrix stiffness is a key factor that influences cell movement in 3D. Cell migration assays in which Matrigel density, fibronectin concentration, and $\beta 1$ integrin binding are systematically varied show that at a specific Matrigel density the migration speed of DU-145 human prostate carcinoma cells is a balance between tractile and adhesion forces. However, when biochemical parameters such as matrix ligand and cell integrin receptor levels are held constant, maximal cell movement shifts to matrices exhibiting lesser stiffness. This behavior contradicts current 2D models but is predicted by a recent force-based computational model of cell movement in a 3D matrix. As expected, this 3D motility through an extracellular environment of pore size much smaller than cellular dimensions does depend on proteolytic activity as broad-spectrum matrix metalloproteinase (MMP) inhibitors limit the migration of DU-145 cells and also HT-1080 fibrosarcoma cells. Our experimental findings here represent, to our knowledge, discovery of a previously undescribed set of balances of cell and matrix properties that govern the ability of tumor cells to migration in 3D environments.

cell motility | EGF receptor | extracellular matrix | matrix metalloproteinase

Cell movement is a product of a net force generated by the actin-based machinery within a cell and transmitted through membrane adhesions to the extracellular matrix. Studies of movement on 2D surfaces have led to a conceptual (1–3) and computational (4–7) understanding of how signaling from the cytoskeleton and the cell adhesion complexes is coordinated. A significant feature of cell migration on 2D surfaces is a biphasic relationship between cell speed and cellular adhesive forces (7). Movement slows from a maximum at intermediate adhesion strengths as traction forces decrease (low adhesion force) or when cell detachment becomes inhibited (high adhesion force).

However, cells usually migrate in a 3D extracellular matrix of fibers and glycosaminoglycans during embryonic development, pathogen surveillance, and other physiological processes. In contrast to the 2D case, the traction forces generated by a cell moving in a 3D gel are transmitted through cell/matrix attachments over all surfaces of a cell in contact with the surrounding matrix. Consequently, cells take on a spindle or amoeboid shape as they move through the pores of varying sizes and as adhesions through integrin receptors are modulated (8–12). Unfortunately, the conceptual and

computational models developed for migration on 2D surfaces do not account for the full complexity of migration through a 3D gel such as the roles of biochemical factors (e.g., the distribution of cell/matrix attachments over the entire cell), steric factors such as hindrance by fibers of the matrix, and mechanical factors such as stiffness of the matrix fibers (e.g., ref. 13). A recent computational model (14) has predicted that these factors are interlinked and affect migration at multiple levels, for example modulation of ligand density affects pore size, matrix stiffness, and forces generated at the cell–matrix interface.

To understand how these factors are convolved, we investigate here the migration of DU-145 human prostate carcinoma cells (15), both parental and EGF receptor (EGFR)-overexpressors, through 3D fibronectin-constituted Matrigel environments (16, 17). We observe seemingly paradoxical effects of blocking integrin/matrix adhesion using anti-integrin antibodies as the Matrigel density and concentration of added fibronectin are varied, contrasting with expectations from previous understanding for 2D systems. However, our previously undescribed observations can be accounted for in terms of a force-based computational model for cell migration in 3D matrices (14).

Results and Discussion

Because cell/substratum adhesion is a governing parameter of cell migration on 2D surfaces (18) and a predicted parameter of cell migration in 3D (14), we first investigated how modulating matrix ligand and adhesion receptor density affected the migration speed through Matrigel. In 67% Matrigel DU-145 parental cells locomoted at a maximum of 12 $\mu\text{m}/\text{h}$ in the absence of fibronectin, and their speed diminished as additional fibronectin was presented in the 3D matrix (Fig. 1 *a* and *b*). The behavior of EGFR-overexpressing DU-145 cells was qualitatively similar but exhibited faster migration speed by ≈ 2 -fold under all conditions (data not shown). When cell/matrix adhesiveness was reduced by use of an $\beta 1$ integrin-blocking antibody, migration of the parental and EGFR-expressing cells (data not shown) displayed a biphasic character in which the maximum in cell speed shifted to higher fibronectin concentrations as binding to integrin was progressively inhibited (Fig. 1*c*). This behavior is consistent with previous studies of the effects of integrin/substratum binding inhibition in 2D using an integrin-blocking peptide (19). Because these observations suggested that movements on 2D surfaces and in 3D gels displayed a similar balance between low and high adhesive forces, we replotted

Conflict of interest statement: No conflicts declared.

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Abbreviation: EGFR, EGF receptor.

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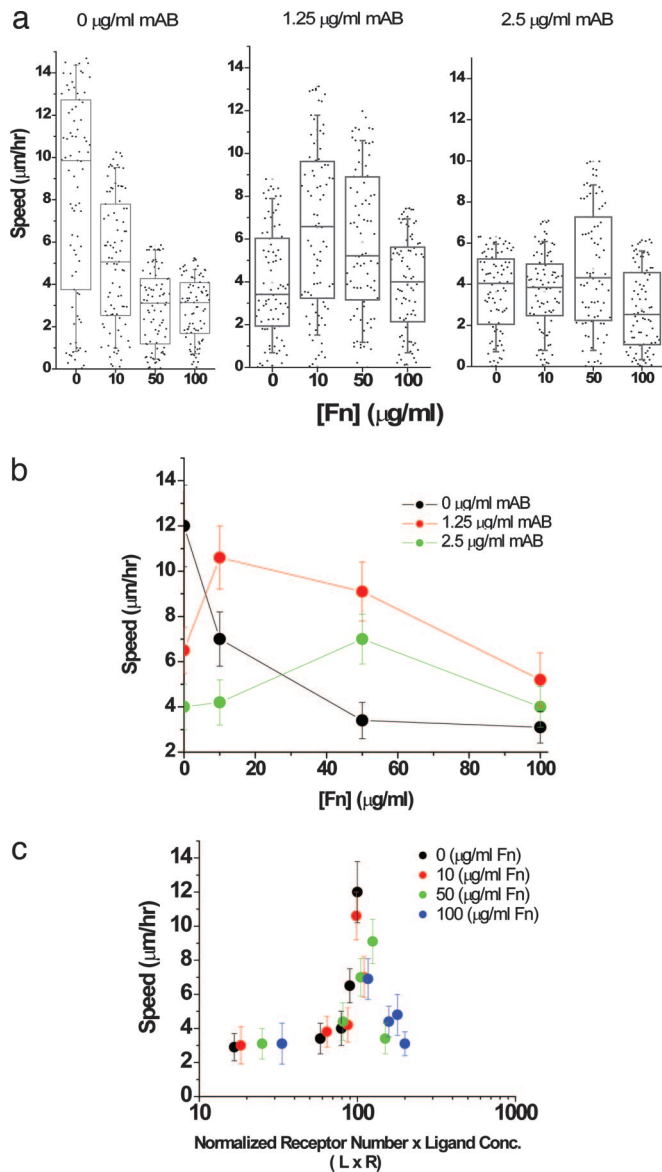


Fig. 1. Maximum cell speed is an optimum between oppositely acting adhesive forces. The adhesivity of DU-145 parental cells to the matrix was modulated by either adding fibronectin to the matrix or inhibiting binding to integrin with 4B4 antibody. (a) Box-and-whiskers plot with all of the raw data overlaid for DU-145 cell migration under specified conditions. The boxes represent 25th and 75th percentile with the median shown by the line bisecting the box. The mean is shown by a black dot inside the box. The whiskers represent 10th and 90th percentiles of the data. Cell speed values of $<3 \mu\text{m/h}$ come from “oscillating” cells or simply active shape changes rather than actively motile cells. These cells are ignored from the cell speed analysis shown in *b*. (b) In 67% Matrigel concentration, cell speed decreases from $12 \mu\text{m/h}$ as fibronectin is included in the matrix. As integrin binding is blocked with 4B4 antibody, cell speed displays a biphasic character in which the maximum in cell speed shifts to higher fibronectin concentrations. (c) Cell speed of “motile cells” (see *Materials and Methods*) over the range of fibronectin concentrations displays a biphasic relationship with an adhesiveness factor, $L \times R$. As the number of available receptors decreases, the loss in integrin activity is compensated by increase in ligand concentration, resulting in a constant relationship between speed and substrate adhesivity. The error bars represent SEM in results from five independent experiments where ≈ 15 – 20 cells were tracked per experiment.

cell speed as a function of an “adhesiveness” parameter, ($[L] \times [R]$), that incorporates the changes in functional ligand and receptor density (Fig. 1c). At a given Matrigel concentration, the variations in fibronectin levels and integrin-binding activity collapse onto a

single curve exhibiting an optimum in cell speed at intermediate adhesiveness levels, consistent with analogous previous 2D plots (18, 19). Thus, at first glance we conclude that similar counterbalancing adhesive forces affect cell migration on 2D surfaces and in 3D matrices, implicating a common force-sensing control of the cell migration machinery.

With this initial foundation, our main goal was to investigate the influence of effects likely present in 3D but generally not in 2D, such as steric (e.g., porosity and/or fiber size) and mechanical (i.e., stiffness or compliance) properties of a gel (20) that are typically convoluted in a single term such as matrix density. Lastly, we determined whether proteolysis was required for migration through steric barriers posed by an extracellular matrix in which the pore size is significantly smaller than cellular dimensions. We experimentally separated the biochemical factors from the steric and biomechanical factors. When DU-145 cells were tracked in matrix of different densities, migration of both the parental and EGFR-overexpressing cells showed a biphasic dependence on Matrigel concentration (Fig. 2), with the EGFR-overexpressing cells again moving at approximately twice the speed of the parental cells under all conditions. This biphasic behavior can again be interpreted in terms of a balance between forward and rearward traction forces as the density of ligand is varied. Our 3D results showing an increase in speed with EGFR overexpression agree with migration experiments in 2D, which have shown an increase in speed with EGFR overexpression in DU-145 cells (17).

In 2D systems, a decrease in traction force by reducing integrin expression (18) or employing an anti-integrin blocking peptide (19) is balanced by shifting the maximum in cell speed to higher matrix (ligand) densities, i.e., a “right shift.” To test whether ligand and receptor levels display a similar relationship in a 3D context, we challenged DU-145 cell migration with various levels of mAb 4B4, a $\beta 1$ integrin-blocking antibody. In striking contrast to the well established results for 2D substrates, the biphasic curve in 3D for both DU-145 parental and EGFR overexpressing cells showed a “left shift” toward lower Matrigel concentrations as binding through $\beta 1$ integrin is inhibited (Fig. 2). In the limit of excess of blocking antibody (10 $\mu\text{g/ml}$ or more), cell speed is reduced to $3 \mu\text{m/h}$ (data not shown). These data therefore indicate that migration in 2D and 3D can exhibit diametrically opposite behaviors upon integrin blocking. Instead of compensating for a decrease in the number of receptors by binding at higher ligand concentrations, cells in a 3D gel paradoxically appear to shift their maximum speed to lower traction forces.

Along with the effects of cell/matrix adhesiveness, our recently published computational model for 3D migration predicts that matrix stiffness also may modulate cell speed (14). To quantify these convoluting factors, we measured the viscoelastic properties of gels assembled at different Matrigel concentrations (Fig. 3a). Over a range of 50–100% Matrigel, the stiffness of the gel changes linearly 5-fold and is consistent with transmission electron microscopy (TEM) images that show a corresponding increase in network density (Fig. 3c and Fig. 5, which is published as supporting information on the PNAS web site). In contrast to the 5-fold change in stiffness, the ligand concentration can be reasonably assumed to change by 2-fold over the same range of Matrigel concentrations because no other ligands were added. The pore size also varies with change in Matrigel concentration; however, even at 50% gel concentration the average pore size is much smaller than the cellular dimensions (average pore size $\approx 2 \mu\text{m}$ at 50% Matrigel; see Fig. 5).

In contrast, when fibronectin (at 0–100 $\mu\text{g/ml}$) was incorporated within the matrix, the added ligand had a relatively small effect on gel stiffness (Fig. 3b). Although the effect was small, the gel still showed a minor increase in stiffness upon fibronectin addition, suggesting that fibronectin was in fact getting incorporated in the matrix rather than simply being trapped in the holes and crevices of the gel. The changes in Matrigel density here affect the mechanical

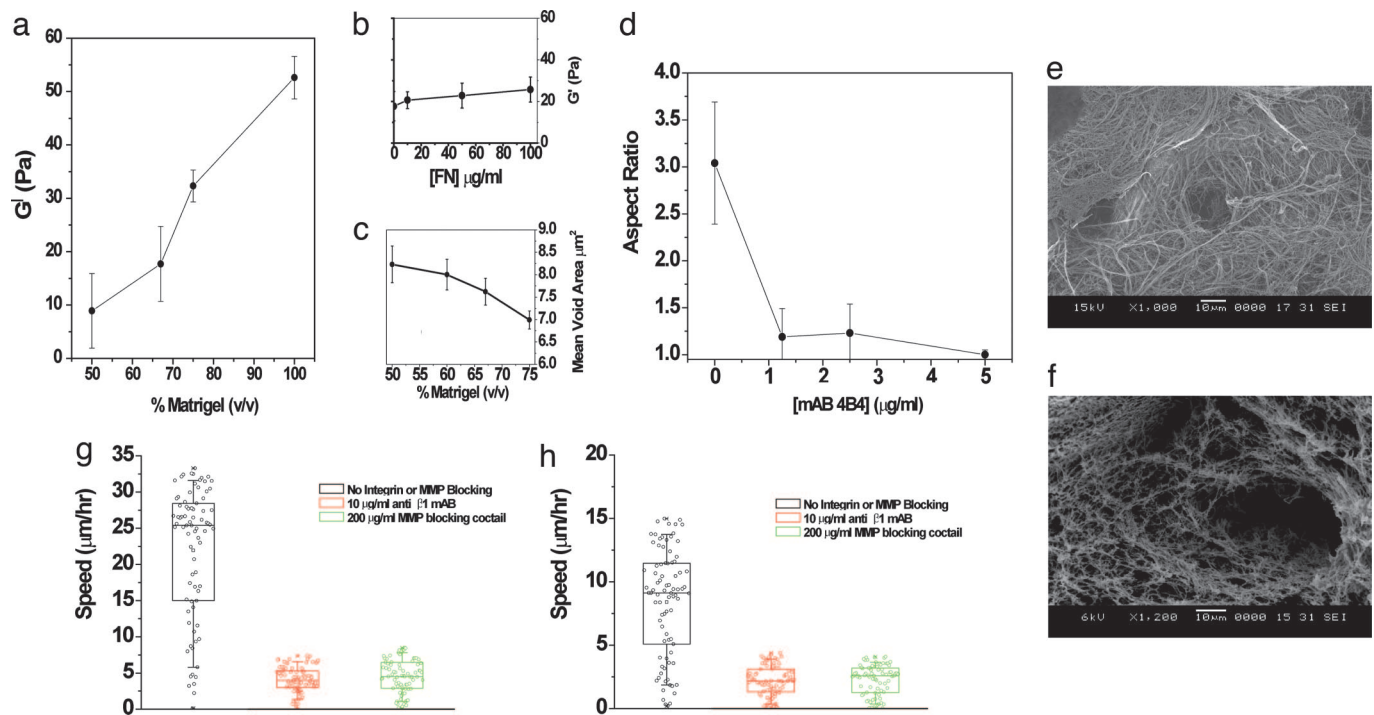


Fig. 3. Matrigel stiffness and steric properties influence migration. (a and b) The stiffness, G' , of Matrigel was measured for different concentrations of matrix (a) and for different amounts of exogenously added fibronectin at 67% Matrigel (b). Between 50% and 100% Matrigel, the storage modulus increases 5-fold. (c) The mean void area decreases as the Matrigel concentration is increased. (d) Aspect ratio (length of major axis/minor axis) of DU-145 parental cells as a function of mAb 4B4 concentration. The error bars show SEM for 10 different cells. The results for EGFR-overexpressed DU-145 are similar (data not shown). (e) Scanning electron microscopy images of Matrigel and collagen show starkly different matrix structures at the same resolution. Matrigel stock concentration is ≈ 10 – 12 mg/ml, and collagen I is typically used at a concentration of 1–2 mg/ml. (f) Collagen I structure at a concentration of 2.8 mg/ml. (g) Although HT-1080 cells show plasticity in migration in collagen matrices (10), they are unable to migrate when they are unable to either adhere or proteolyse the matrix (see Movies 1–6). (h) Similar to HT-1080 cells, DU-145 parental cells (EGFR cells show qualitatively similar behavior) show negligible motility in the presence of anti-integrin antibody or matrix metalloproteinase (MMP)-inhibiting mixture (see Movies 1–6).

results demonstrate that movement in 3D requires multiple balances between integrin activity, adhesion ligand density, and matrix stiffness, as well as proteolysis and steric hindrance. At a minimum, as shown here, these include a balance between the following: (i) cell/matrix adhesiveness and cell-generated force; (ii) cell/matrix adhesiveness and matrix stiffness; and (iii) traction force and matrix sterics.

A multidimensional landscape plot (Fig. 4) of our experimental data, illustrates that migration speed is a joint function of two key system variables: Matrigel density (representing matrix stiffness and sterics) and fibronectin level (mainly representing ligand density). In the absence of integrin-blocking antibody (Fig. 4a), a maximum in motility lies at an intermediate matrix stiffness. At a constant stiffness, cell speed displays the same biphasic dependence on adhesion as demonstrated for cell migration on 2D surfaces. A decrease in adhesiveness caused by blocking integrin receptors (Fig. 4b) is accompanied by a shift in the maximum to higher ligand concentration and lower matrix stiffness, thus accounting for both the right shift found in Fig. 1 and the left shift found in Fig. 2. The dominating influence of stiffness on cell speed found experimentally (Fig. 4c) is predicted by the computational model (Fig. 4d) for cell movement in 3D matrices (14). At any integrin density, cell speed increases as matrix stiffness decreases, and an optimal speed is predicted at high receptor density. Likewise, the left shift in maximal cell speed to lower matrix stiffness when receptor density decreases is also seen in the direction of the contour lines predicted by the model.

Cell migration for 2D systems is influenced by substrate compliance. Pelham and Wang (21) found that 3T3 fibroblast migration speed on acrylamide substrata was monotonically greater on sur-

faces of decreasing stiffness; this finding could be consistent with the right-hand side of the biphasic curves we show in Fig. 2. Peyton and Putnam (22) reported that maximal migration speed of smooth muscle cells on fibronectin-coated acrylamide substrata occurred on a less stiff surface for a higher fibronectin level, which appears to contrast with our results. However, the differences in migration speed they observed across their various conditions were relatively small, <2 -fold, so possibly smooth muscle cells operate on a much milder parametric landscape, enabling other cell or substratum properties to play a relatively more significant role.

Although we have probed cell migration as a function of bulk matrix stiffness, we realize that matrix heterogeneity and changes in local stiffness of the gel also may play an important role in determining the overall landscape of migration. Recent advances in multiple particle-tracking rheology (23, 24) as well as the use of nested collagen matrices (25, 26) to probe tension in the proximity of cells have shown great promise in improving our understanding of cell–matrix interactions at these length scales.

Finally, an unexplained observation of our experiments is the 2-fold difference in speed between parental and EGFR-overexpressing cells that display the same amount of integrin at their cell surface. Many laboratories are attempting to determine how motility-related signals might be connected (often in two-way fashion) to the proximally governing biophysical mechanisms (22, 27, 28). Because of the great complexity of biochemical signaling networks and of their connections to biophysical mechanisms by which cell functional responses such as motility are conducted (29), we are convinced that construction of cell signal–response models will require multivariate computational analysis (30, 31).

Rheological Measurements. Stainless steel washer molds (8.5-mm inner diameter, 0.9-mm high) on squares of wetted parchment paper (2 × 2 cm) were slightly overfilled with 85 μl of cold, liquid Matrigel mixed with fibronectin (0, 100 $\mu\text{g}/\text{ml}$). Matrigel solutions were allowed to gel for 30 min at 37°C. Gels were cut to the height of the washer with a scalpel blade, and the washer was gently removed. Cylindrical gels were transferred to the plate of an AR1000 rheometer (TA Instruments, New Castle, DE) on the parchment paper, and an 8-mm plate was lowered to the gel surface. Storage moduli (G') of gels were obtained over a range of frequencies from 0.1 to 1 rad/s at 0.1 $\mu\text{N}\cdot\text{m}$ oscillatory torque.

Estimation of Effective Receptor Numbers and Ligand Density. The normalized receptor number was calculated from FACS experiments. FITC-labeled mAb 4B4 was used to label $\beta 1$ integrins. The total number of available $\beta 1$ integrins was maximum at 0 $\mu\text{g}/\text{ml}$ and lowest at 10 $\mu\text{g}/\text{ml}$. Increasing the mAb concentration to 20 $\mu\text{g}/\text{ml}$ increased the percent fluorescence signal by $\approx 3\%$. Further increase of mAb concentration to 30 $\mu\text{g}/\text{ml}$ resulted in no net increase in fluorescence. We calculated the normalized receptor number at each mAb concentration by assuming that the fluorescence signal at 20 $\mu\text{g}/\text{ml}$ corresponded to $<1\%$ available $\beta 1$ integrin receptors ($>99\%$ blocking as further increase in mAb resulted in no net increase in fluorescence signal) and that the background fluorescence value at 0 $\mu\text{g}/\text{ml}$ mAb corresponded to 100% available $\beta 1$ integrin receptors. Thus, at each mAb concentration the normalized receptor number is equal to

Normalized receptor number

$$= 1 - \frac{(\text{Fluorescence signal at given mAb conc} - \text{Fluorescence signal at } 0 \mu\text{g}/\text{ml mAb})}{(\text{Fluorescence signal at } 20 \mu\text{g}/\text{ml mAb} - \text{Fluorescence signal at } 0 \mu\text{g}/\text{ml mAb})}$$

The above formula ensures that at 0 $\mu\text{g}/\text{ml}$ the normalized receptor number has a maximum value of 1, and at 20 $\mu\text{g}/\text{ml}$ the normalized receptor number value is at a minimum.

The concentration of available ligands was calculated by assuming a similar binding constant between integrins and fibronectin as the binding constant between integrins and all of the other integrin binding proteins in Matrigel (laminin, collagen, etc). Thus, the total available ligands was equal to the amount of fibronectin added plus the concentration of Matrigel ($\approx 500 \mu\text{g}/\text{ml}$ at 67% Matrigel).

Mean Void Area Calculation. Matrigel at varying concentrations was imaged by using quick-freeze deep etch (QFDE). The digital

images were binarized by using IMAGEJ (<http://rsb.info.nih.gov/ij>). The digitized binary images were then skeletonized, and the surface area covered by the fibers (dark pixels) was calculated. The mean void area was calculated by taking the reciprocal of the mean covered area in five independently measured gel samples.

Calculation of Aspect Ratio. The aspect ratio of cells in 3D Matrigel environment with varying levels of mAb 4B4 was calculated by collapsing the 3D image onto 2D and approximating the cell shape as an ellipse. The aspect ratio is the ratio of the length of the major axis to the minor axis for this ellipse. For an amoeboid cell (spherical morphology), the aspect ratio $\rightarrow 1$, whereas for mesenchymal cells (elongated morphology) the aspect ratio is >1 .

Computational Model. The details of the model are given elsewhere (14). Briefly, the computational model predicts the speed and persistence of cell migration by calculating the total force, represented as a vector, acting on the cell centroid at each time step ($\Delta t = 600$ s). Total force is divided into adhesive forces (F_{trac}) acting at the front and the rear of the cell, protrusive forces ($F_{\text{protrusion}}$) from the cell in a 3D matrix, and resistive forces (F_{drag}), viscous drag experienced by the cell due to the viscoelastic nature of the extracellular matrix

$$F_{\text{tot}} = F_{\text{drag}} + F_{\text{trac}} + F_{\text{protrusion}} = 0.$$

The model calculates the velocity and position of the cell by using Newton's equations of motion and addresses the relationship between steric resistance and adhesivity for a migrating cell. Our approach assumes that ligand density is proportional to steric hindrance and that at very high ligand concentration, the matrix is too dense for the cells to migrate. Computations were performed in MATHEMATICA 4.1 (Wolfram Research, Champaign, IL). A total of 10,000 simulations of 300 time steps each (to simulate ≈ 48 h of migration) were computed on a PIV cluster (Intel, Santa Clara, CA). The initial location of the cell in the extracellular matrix and the protrusion vectors were determined by using a random number generator.

We thank Prof. Fred Grinnell (University of Texas Southwestern Medical Center, Dallas, TX) for careful reading of the manuscript and members of our laboratories for numerous enlightening discussions. This work was supported by National Institutes of Health Grants R01-GM 57418 (to P.M.) and P01-HL064858 (to R.D.K.); National Science Foundation Grant NIRT 0304128 (to P.M.); the National Institute of General Medical Sciences (NIGMS) Cell Migration Consortium (to D.A.L.); the National Cancer Institute Integrative Cancer Biology Program (to D.A.L.); and NIGMS Grants 1-R01-GM-076689 (to R.D.K.) and GM069668 (to A.W.). M.H.Z. was supported by a Sokol Foundation Fellowship.

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