The Rho family GEF Asef2 regulates cell migration in three dimensional (3D) collagen matrices through myosin II

Léolène Jean¹, Lijie Yang², Devi Majumdar¹, Yandong Gao², Mingjian Shi¹, Bryson M. Brewer², Deyu Li², and Donna J Webb¹,3,*

¹Department of Biological Sciences and Vanderbilt Kennedy Center for Research on Human Development; Vanderbilt University; Nashville, TN USA; ²Department of Mechanical Engineering; Vanderbilt University; Nashville, TN USA; ³Department of Cancer Biology; Vanderbilt University; Nashville, TN USA

Keywords: guanine nucleotide exchange factor, myosin II, microfluidics, Rac, Rho family GTPases, type I collagen

Abbreviations: Collagen I, type I collagen; 2D, 2-dimensional; 3D, 3-dimensional; DMEM, Dulbecco’s Modified Eagle Medium; ECM, extracellular matrix; GEF, guanine nucleotide exchange factor; MyoII, non-muscle myosin II; PAK, p21-activated kinase; PBD, p21-binding domain; PBS, phosphate buffer saline; PDMS, polydimethylsiloxane; UV, ultra-violet.

Cell migration is fundamental to a variety of physiological processes, including tissue development, homeostasis, and regeneration. Migration has been extensively studied with cells on 2-dimensional (2D) substrates, but much less is known about cell migration in 3D environments. Tissues and organs are 3D, which is the native environment of cells in vivo, pointing to a need to understand migration and the mechanisms that regulate it in 3D environments. To investigate cell migration in 3D environments, we developed microfluidic devices that afford a controlled, reproducible platform for generating 3D matrices. Using these devices, we show that the Rho family guanine nucleotide exchange factor (GEF) Asef2 inhibits cell migration in 3D type I collagen (collagen I) matrices. Treatment of cells with the myosin II (MyoII) inhibitor blebbistatin abolished the decrease in migration by Asef2. Moreover, Asef2 enhanced MyoII activity as shown by increased phosphorylation of serine 19 (S19). Furthermore, Asef2 increased activation of Rac, which is a Rho family small GTPase, in 3D collagen I matrices. Inhibition of Rac activity by treatment with the Rac-specific inhibitor NSC23766 abrogated the Asef2-promoted increase in S19 MyoII phosphorylation. Thus, our results indicate that Asef2 regulates cell migration in 3D collagen I matrices through a Rac-MyoII-dependent mechanism.

Introduction

Cell migration is critical to many biological processes, such as tissue morphogenesis, tissue homeostasis, as well as tissue regeneration and wound repair.¹ Much of our knowledge to date has been obtained from studying cells migrating on 2D substrates. Migration on 2D substrates can be described as a 4-step cycle that includes extension of lamellipodia at the leading edge, assembly of cell-extracellular matrix (ECM) adhesions, forward translocation of the cell body, and retraction of the rear of the cell.²,³ While some aspects of cell migration on 2D substrates are recapitulated in 3D environments, the specific mechanisms and regulation of migration can be dependent on the dimensionality of the matrix.⁴ Indeed, recent studies with 3D cell culture models, which more closely mimic the microenvironment of tissues, have identified differences in cell morphology and modes of migration when compared with cell migration on 2D substrates.³⁻⁸ For example, cells migrating in 3D matrices typically adopt a more elongated morphology and extend dendritic-like protrusions instead of the broad lamellipodia observed with cells migrating on 2D substrates.⁵,⁸ Cells migrating in 3D matrices can interact with the ECM across their entire surfaces, unlike cells on 2D substrates which typically only attach to the ECM at their ventral surfaces. These differences in cell–ECM interfaces as well as ECM topography may be integral to the changes in morphology and modes of cell migration seen in 3D environments.

Although many of the key signaling mechanisms that modulate cell migration on 2D substrates are known, understanding the signaling pathways that regulate 3D migration is only in its infancy. Available data, however, point to the Rho family of small GTPases, including Rac, Rho, and Cdc42, as having important roles in regulating cell morphology and modes of migration in 3D environments.⁵,⁷,⁹,¹⁰ For instance, active Rac drives cells toward an elongated, mesenchymal morphology that is important for fibroblast migration in 3D collagen matrices.⁵,⁹ In contrast, active Rho signaling induces tumor cells to adopt a more rounded morphology that allows for an “amoeboid” mode of migration through 3D matrices.⁵

The activity of these Rho GTPases, which is central to their function in migration, is controlled by GEFs and GTPase-
activating proteins (GAPs). GEFs facilitate the exchange of GDP for GTP, which serves to activate the GTPases, whereas GAPs stimulate their intrinsic GTPases activity, returning the small GTPases to an inactive state. Active Rho GTPases can interact with downstream effectors to propagate the signal and illicit biological responses. Because Rho GTPase activation is controlled by GEFs, these proteins have been increasingly receiving attention as important regulators of cell migration. Despite this recent interest, we still have a great deal to learn about the role of GEFs in modulating cell migration, especially their function in regulating cell migration in 3D environments.

Asef2 is a newly identified Rho family GEF that has been shown to regulate migration on 2D substrates. Our previous work demonstrated that Asef2 impairs cell migration on 2D collagen I by increasing activation of Rac, which subsequently enhances MyoII activity. MyoII is an actin motor protein whose major function in cells is to generate contraction by sliding actin filaments relative to each other. MyoII contractility (activity) is controlled by phosphorylation within its regulatory light chains (RLC). Specifically, phosphorylation of serine 19 (S19) is crucial for activation of the MyoII motor domain, and phosphorylation of threonine 18 further increases MyoII activity. Although MyoII activity is classically thought to be mediated by Rho signaling, our previous data reveal that activation of Rac by Asef2 modulates MyoII activity, and this is an important regulatory mechanism for cell migration on 2D substrates. However, the role of Asef2 and the contribution of Asef2-Rac-MyoII signaling to cell migration in 3D environments is unknown.

In this study, we demonstrate that Asef2 inhibits HT1080 cell migration in 3D collagen I matrices. Asef2 increases Rac activity, which subsequently enhances S19 MyoII phosphorylation. Inhibition of MyoII activity negates the Asef2-mediated effect on migration, suggesting that Asef2 regulates migration in 3D collagen I matrices via a Rac-MyoII-dependent mechanism.
Results and Discussion

Asef2 impairs migration in 3D collagen I matrices via MyoII

The microfluidic devices that we have developed offer a controlled, reproducible platform for generating 3D environments (Fig. 1). Therefore, we used these devices to examine the migration of HT1080 cells stably expressing GFP or GFP-Asef2 (less than 3-fold over endogenous)12,13 (Fig. 2A) in 3D collagen I matrices. When GFP and GFP-Asef2 cells were mixed with a collagen I solution and loaded into the cell chambers of the microfluidic devices, the cells embedded in 3D matrices adopted an elongated morphology that is characteristic of cells in 3D environments8,20 (Fig. 2B). The migration speeds of GFP-Asef2 cells embedded in 3D collagen I matrices was decreased significantly compared to GFP (control) cells (Fig. 2B). Next, we used 2 Asef2 short hairpin RNAs (shRNAs) to further demonstrate that Asef2 regulates migration in 3D collagen I matrices. We had previously shown that these Asef2 shRNAs decrease endogenous expression of Asef2 by approximately 65% in HT1080 cells.12,13 Transfection of Asef2 shRNA 1 or Asef2 shRNA 2 into HT1080 cells caused a significant increase in the migration speeds of these cells in 3D collagen I matrices compared to that observed with a non-targeting shRNA (NT shRNA) or Asef2 shRNAs. Three days later, these cells were used in migration assays. Phase contrast images are shown where the blue line traces the migration path of an individual cell. Right, quantification of the migration speeds for cells transfected NT shRNA or Asef2 shRNAs. Error bars represent SEM for 26–34 cells from 3 independent experiments (*P < 0.0001). (D) GFP and GFP-Asef2 cells were treated with 50 μM blebbistatin or DMSO (vehicle control) for 3 h at 37°C prior to being used in 3D migration assays. The average migration speed for GFP and GFP-Asef2 cells, subjected to the indicated treatments, was quantified. Error bars represent SEM for 50 cells from 3 independent experiments (*P = 0.003; **P < 0.0001).

Figure 2. Asef2 hinders migration in 3D collagen I matrices. (A) Left, GFP-Asef2 cells were immunoblotted for Asef2. The ~100 kDa band represents GFP-Asef2, whereas the ~75 kDa band denotes endogenous Asef2. Right, quantification of the amount of Asef2 in GFP-Asef2 cells. Error bars represent SEM from 3 separate experiments (*p = 0.04). (B) GFP and GFP-Asef2 cells in 3D collagen I matrices in microfluidic devices were imaged with time-lapse microscopy. Left, time-lapse images show GFP and GFP-Asef2 cells migrating within the 3D matrix (arrows). Right, the migration of individual cells was tracked, and the migration speed was calculated. The average migration speeds for GFP and GFP-Asef2 cells is shown. Error bars represent SEM for 50 cells from 3 independent experiments (*P < 0.0001). (C) Left, wild-type HT1080 cells were transfected with a non-targeting shRNA (NT shRNA) or Asef2 shRNAs. Three days later, these cells were used in migration assays. Phase contrast images are shown where the blue line traces the migration path of an individual cell. Right, quantification of the migration speeds for cells transfected NT shRNA or Asef2 shRNAs. Error bars represent SEM for 26–34 cells from 3 independent experiments (*P < 0.0001). (D) GFP and GFP-Asef2 cells were treated with 50 μM blebbistatin or DMSO (vehicle control) for 3 h at 37°C prior to being used in 3D migration assays. The average migration speed for GFP and GFP-Asef2 cells, subjected to the indicated treatments, was quantified. Error bars represent SEM for 50 cells from 3 independent experiments (*P = 0.003; **P < 0.0001).

Asef2 augments Rac activity in 3D collagen I matrices

Figure 3. Asef2 augments Rac activity in 3D collagen I matrices. Left, GFP and GFP-Asef2 cells were transfected with FLAG-Rac1 cDNA. Twenty-four hours later, these cells were embedded in 3D collagen I matrices and incubated overnight. Then, the matrices with embedded cells were homogenized, and active Rac was pulled down from homogenized samples. Total Rac is shown as a control. Right, quantification of active Rac levels in homogenized samples of GFP and GFP-Asef2 cells in 3D matrices from at least 3 separate experiments is shown. Error bars represent SEM (*P < 0.005).
To determine if Asef2 similarly regulates migration in 3D collagen I matrices, we treated GFP and GFP-Asef2 cells with blebbistatin, an inhibitor of MyoII ATPase.\textsuperscript{21,22} When GFP cells were incubated with blebbistatin, an increase in migration speed was observed (Fig. 2D). This augmentation of migration by blebbistatin treatment has been previously reported by us as well as by other groups.\textsuperscript{13,23-25} Importantly, the migration speed of GFP-Asef2 cells was significantly decreased compared with that observed with GFP cells, and blebbastatin treatment abolished this Asef2 effect on migration (Fig. 2D). This result suggests that Asef2 regulates migration in 3D collagen I matrices through MyoII.

**Asef2 increases Rac activity in 3D collagen I matrices**

In our previous study, we showed that Asef2 enhanced Rac activity when cells were plated on 2D collagen I; the augmented active Rac, in turn, increased phosphorylation of MyoII on S19.\textsuperscript{13} To determine whether Asef2 also increased the amount of Rac in cells in 3D collagen I matrices, we utilized a Rac activity assay. In this assay, the p21-binding domain (PBD), from p21-activated kinase (PAK), is tagged with glutathione-S-transferase (GST) and used to pull down the active form of Rac. PAK is an effector that only binds to the active form of Rac.\textsuperscript{26-28} When GFP and GFP-Asef2 cells were embedded in collagen I matrices, and Rac activity was assessed, an almost 3-fold increase in the amount of active Rac was seen in GFP-Asef2 cells compared to that observed in GFP cells (Fig. 3). This result indicates that Asef2 induces activation of Rac in 3D collagen I matrices.

**Asef2 promotes S19 phosphorylation of MyoII through Rac**

Because our results demonstrate that MyoII activity is important for the Asef2-mediated modulation of cell migration in 3D collagen I matrices (Fig. 2D), we next examined the effect of Asef2 on MyoII S19 phosphorylation. Phosphorylation of S19 within the RLC of MyoII is critical for its activation; therefore, phosphorylation of this amino acid can be utilized to gauge MyoII activity.\textsuperscript{18,29} GFP and GFP-Asef2 cells were embedded in 3D collagen I matrices and subsequently immunostained with an antibody that recognizes MyoII phosphorylated at S19. The amount of S19 phosphorylated MyoII (p-S19 MyoII) was significantly increased in GFP-Asef2 cells compared to GFP cells (Fig. 4A), whereas the amount of total MyoII was similar in these cells (Fig. 4B). Moreover, we observed a 1.4 ± 0.2-fold (n = 2 separate experiments) increase in the amount of p-S19 MyoII in GFP-Asef2 cells compared to GFP cells, as determined by In-cell Western analysis, which was performed as previously described.\textsuperscript{13} In contrast, the amount of total MyoII was not significantly different in GFP and GFP-Asef2 cells. These results demonstrate that Asef2 enhances the amount of active MyoII in cells in 3D collagen I matrices.
Because we have previously shown that Asef2 increases the level of active MyoII on 2D collagen I via Rac, we investigated the contribution of Rac to the Asef2-promoted increase in MyoII activity. GFP and GFP-Asef2 cells, embedded in 3D collagen I matrices, were treated with the Rac inhibitor NSC23766, and S19 MyoII phosphorylation was assessed. Treatment of GFP cells with NSC23766 resulted in a decrease in S19 phosphorylated MyoII (p-S19 MyoII) (Fig. 4A), suggesting that inhibition of basal Rac activity diminished the amount of active MyoII. Interestingly, treatment of GFP-Asef2 cells with NSC23766 abolished the Asef2-promoted increase in active MyoII (Fig. 4A). The level of total MyoII was similar in GFP and GFP-Asef2 cells and was not affected by NSC23766 treatment (Fig. 4B). These results suggest that Asef2 enhances MyoII activity through a Rac-dependent mechanism in cells in 3D collagen I matrices.

Our results indicate that Asef2 activates Rac and inhibits the migration of HT1080 cells in 3D collagen I matrices. Others Rac GEFs, including DOCK3 and P-Rex, have also been shown to regulate the migration of tumor cells in 3D environments, pointing to the importance of GEFs in modulating 3D migration. The DOCK3-stimulated activation of Rac promoted an elongated, mesenchymal-like morphology of melanoma cells and suppressed “amoeboid” migration. We observed a similar elongated morphology with Asef2-expressing HT1080 cells in 3D collagen I matrices. The effect that the Asef2-mediated activation of Rac has on amoeboid movement is currently unknown, but it will most likely depend on the effectors of Asef2-Rac signaling. In this context, Asef2 activation of Rac could recruit and target different effectors to the signaling complex to regulate cell morphology and migration in 3D matrices. Identification of the Asef2-Rac effectors that modulate 3D migration represents an exciting avenue for future study.

In our study, Asef2-Rac signaling increases MyoII activity, which is critical for the impaired migration that we observe in 3D collagen I matrices. Indeed, our data demonstrate that treatment with the MyoII inhibitor blebbistatin abolished this Asef2-mediated effect on migration and resulted in an increase in migration speed. Others have reported that blebbistatin treatment decreases migration in 3D environments. The migration speed of human foreskin fibroblasts in cell-derived 3D matrices and human breast adenocarcinoma cells in 3D Matrigel were reduced following blebbistatin treatment. Our studies were performed with HT1080 cells migrating in 3D collagen I matrices, whereas the previous experiments were done with other cell types migrating in 3D cell-derived matrices or 3D Matrigel. Therefore, the differences in matrices as well as cell types could contribute to the differential effect observed on migration with blebbistatin treatment. Future studies will be needed to determine the reasons for this discrepancy.

Conclusions

Our data suggest that Asef2 regulates cell migration in 3D collagen I matrices through Rac and MyoII. Asef2 increased activation of Rac in 3D collagen I matrices, which, in turn, enhanced MyoII activity. MyoII activity was critical for the Asef2 impaired migration in 3D collagen I matrices. Activation of Rac has not been previously demonstrated to induce MyoII activity in 3D matrices; thus, it will be interesting to examine the effect of Rac activation by other GEFs on MyoII. These results are consistent with our previous observations on 2D collagen I. Therefore, these data point to a common mechanism by which Asef2 modulates migration on 2D collagen I substrates and in 3D matrices.

Materials and Methods

Fabrication and assembly of microfluidic devices

The microfluidic cell culture devices were fabricated using standard soft-lithography techniques with polydimethylsiloxane (PDMS) replica molding (Ellsworth Adhesives, Germantown, WI) as previously described. To generate the devices, 2 layers of PDMS with the desired channels and cell chambers were bonded to a microscope coverslip. The bottom PDMS layer consisted of a single large cell culture chamber (5 mm long, 4.6 mm wide, and 500 μm in height) and inlet/outlet media channels that were 500 μm wide and 500 μm in height. Inlet and outlet holes for 3 media reservoirs and 2 gel reservoirs, which were 4 mm and 3.5 mm in diameter, respectively, were punched through the PDMS to facilitate media/gel loading and removal. The large cell culture chamber was connected to the inlet/outlet media channels through vias formed by 1 mm diameter holes that were punched through the bottom PDMS layer. Four separate connecting chambers (100 μm in height) in the top PDMS layer were aligned with the 1 mm holes in the bottom PDMS layer, allowing media to flow through the cell chamber.

The 2-layer design with separate inlet/outlet channels for the media and gel mixture allows for direct access of the nutrient media to the cell culture gel without perfusion through the long gel-loading channels filled with polymerized gel. This scheme facilitates a sufficient supply of fresh culture media to the cells in the culture chamber to maintain their long-term health. Note that although media could flow freely in this vertically layered channels, the collagen I gel mixture did not flow backward through the small vias during loading due to the larger fluidic resistance of the small holes compared with the cell chamber and the loading scheme of using negative pressure to withdraw the gel-cell mixture through the outlet well of the gel-loading channel.

To assemble the device, a pre-polymer PDMS solution was mixed with a curing agent at a ratio of 10:1 and poured over the molds for the bottom layer. After the PDMS polymerized, it was peeled from the mold, and holes were punched through the PDMS for the media wells. The surface of the glass coverslips (No. 1, VWR Vista Vision, Suwanee, GA) as well as the bottom PDMS layer were treated with oxygen plasma and then bonded together. The top PDMS, after oxygen plasma treatment, was subsequently aligned and bonded to the bottom PDMS layer. Pyrex cloning cylinders (10 mm × 10 mm) (Fisher Scientific,
Pittsburg, PA) that served as media reservoirs were glued to the punched holes using the liquid PDMS mixture. The assembled microfluidic device was placed in an oven at 70°C for 1 h to cure the glue and then sterilized under ultraviolet (UV) light for 1 h.

Reagents and plasmids

Phospho-MLC (S19) polyclonal antibody (clone 3671) and MLC2 polyclonal antibody (clone 3672) were purchased from Cell Signaling (Beverly, MA). FLAG-M2 monoclonal antibody was obtained from Sigma-Aldrich (St. Louis, MO). Asef2 rabbit polyclonal antibody was prepared as previously described.12 Alexa Fluor® 647 anti-rabbit was from Molecular Probes (Eugene, OR). IRDye 680 anti-mouse was obtained from Rockland Immunochemicals (Gilbertsville, PA). Rat tail type I collagen was purchased from BD Biosciences (Bedford, MA). Bovine serum albumin (BSA), blebbistatin, and NSC23766 were from EMD Bioscience (La Jolla, CA). Aqua Poly/Mount mounting solution (Cat # 18606) was purchased from Polysciences, Inc. (Warrington, PA).

Cell culture

HT1080 cells stably expressing GFP or GFP-Asef2 were made using retroviral induction as previously described.12 Cells were passaged in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and penicillin/streptomycin (Invitrogen, Carlsbad, CA). GFP-tagged Asef2 cDNA was generated by cloning full-length Asef2 cDNA into pE GFP-C3 vector as previously described.12 Wild-type Rac1 and GST-tagged PBD were kindly provided by Alan Hall (Memorial Sloan-Kettering Cancer Center, NY). Asef2 shRNAs were prepared by ligating 64-mer oligonucleotides into pSUPER vector as previously described.36,37 The target sequences for Asef2 were previously described in Bristow et al.12 The non-target sequence, 5’-CAGTCGCGTTTGCAGCTTG-3’, was used as a control.38

Preparation and loading of 3D collagen I matrices and cells into microfluidic devices

Collagen I was mixed to a final concentration of 1.5 mg/ml in DMEM or PBS and neutralized with 1N NaOH (23 μl x the volume of collagen I solution) on ice. Cells were dissociated and resuspended in culture media (100,000 cells/ml) on ice. Prior to cell loading, microfluidic devices were equilibrated with culture media. Then, 10 μl of collagen I solution was mixed with 100 μl of the cell suspension, loaded into the cell chamber of the microfluidic devices, and incubated for approximately 30 min at 37°C to allow the collagen I gel with embedded cells to solidify. Subsequently, culture media was flowed through the microfluidic devices overnight at 37°C to permit the embedded cells sufficient time to attach and extend protrusions. Cells were then used in either migration assays or for immunocytochemistry. In some experiments, GFP and GFP-Asef2 cells embedded in 3D collagen I matrices were treated with 200 μM NSC23766 or DMSO (vehicle control) for 5 h at 37°C before performing immunocytochemistry.

Microscopy and immunocytochemistry

Cells in 3D matrices were washed by flowing phosphate buffered saline (PBS; Life Technologies, Grand Island, NY) through the microfluidic devices. Cells were then fixed with 4% paraformaldehyde and 4% sucrose for 20 min at 37°C, washed with PBS, and treated with 0.1M glycine in PBS for 30 min. Cells were permeabilized with 0.5% (v/v) Triton X-100 in PBS for 30 min at 23°C and incubated with 2% BSA with 0.3% Triton X-100 in PBS (blocking solution) for 1 h at 23°C to block non-specific antibody binding. Following blocking, cells were incubated overnight at 4°C with primary antibody, washed with 0.3% Triton X-100 in PBS, and subsequently treated with blocking solution for 1 h at 23°C. Fluorescently-conjugated secondary antibodies, which were diluted in blocking solution, were incubated with cells for 1 h at 23°C. After antibody incubation, cells were washed with 0.3% Triton X-100 in PBS followed by PBS and finally sterile ddH2O. Three drops of Prolong Gold Antifade reagent (Invitrogen, Carlsbad, CA) were added to the devices before imaging.

Images were collected using a Quorum WaveFX spinning disk confocal system with an inverted Nikon Eclipse Ti microscope, which was equipped with a Hamamatsu ImageEM-CCD camera and a PlanApo 60X TIRF objective (NA 1.49). For excitation of GFP, a 491 nm laser line was used, and GFP was imaged using a 525/50 emission filter (Semrock, Rochester, NY). Alexa Fluor 647 was excited with a 642 nm laser line and imaged using a 700/75 emission filter (Semrock, Rochester, NY). The background-subtracted, average fluorescence intensities (normalized to cell areas) of phosphorylated (S19) MyoII and total MyoII amounts were obtained with MetaMorph software.

Migration in 3D collagen I matrices

Prior to imaging, SFM4MAb™ media (Hyclone, Logan, UT) supplemented with 2–5% FBS was added to the microfluidic devices and flowed through the 3D collagen I matrices. Images were acquired every 5 min for 6 h. At each time point, sequential z-planes images were collected in 5 μm increments, and the z-stack with the best focal plane was selected for each x, y coordinate. Cell migration within the 3D matrices was tracked using MetaMorph software (Molecular Devices, Sunnyvale, CA), and the migration speed was calculated by dividing the total migration distance (μm) by the time. To ensure that the selected cells were embedded in the 3D matrices, the z coordinates where the gels contacted the glass coverslips (bottom of the gels) were determined, and images were collected 50–200 μm above this point. Live-cell imaging in 3D matrices was performed on a Quorum WaveFX spinning disk using a 10X ADL objective (NA 0.25). Some cells were pre-treated with 50 μM blebbistatin or DMSO (vehicle control) for 3 h at 37°C prior to imaging.

Rac activity assay in 3D Collagen I matrices

Rac activity in cells embedded in 3D collagen I matrices was assessed as previously described with some modification. GFP and GFP-Asef2 stable cells were transfected with 8 μg FLAG-Rac1 cDNA (per 100 mm culture dish). Twenty hours later, 5 x 10⁶ cells were mixed with DMEM and collagen I (final concentration 1.5 mg/ml). Cell-collagen gel suspensions (600 μl...
volume) were placed in 12 well plates and incubated overnight at 37°C. The gels were then homogenized in 50 mM Tris, 100 mM NaCl, 2 mM MgCl2, 10% glycerol, 1% NP-40 with a protease inhibitor cocktail, pH 7.5 (lysis buffer) by repeated passage through 1 ml syringes with 26 gauge needles. Active Rac in homogeneous samples was determined as previously described for 2D assays. Briefly, a small fraction of each homogenized sample was kept to measure the amount of total Rac. The remaining sample was incubated with GST-PBD, which was attached to glutathione sepharose beads, for 1 h at 4°C with mixing. After washing 3 times with lysis buffer, bound protein was eluted from the sepharose beads using Laemmli sample buffer and analyzed by Western blot. The amount of active Rac that was pulled down was normalized to total Rac for each sample.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
1. Vicente-Manzaneque M, Horwitz AR. Cell migration: an overview. Methods Mol Biol 2011; 769:1-24; PMID:21748665; http://dx.doi.org/10.1007/978-1-61770-676-3
2. Laffrenburger DA, Horwitz AF. Cell migration: a physically integrated molecular process. Cell 1996; 84:359-69; PMID:8605859; http://dx.doi.org/10.1016/S0092-8674(00)81280-5
3. Vicente-Manzaneque M, Webb DJ, Horwitz AR. Cell migration at a glance. J Cell Sci 2005; 118:4917-9; PMID:16254237; http://dx.doi.org/10.1242/jcs.026626
4. Doyle AD, Petrie RJ, Kurys ML, Yamada KM. Dimensions in cell migration. Curr Opin Cell Biol 2013; 25:642-9; PMID:23853950; http://dx.doi.org/10.1016/j.ceb.2013.06.004
5. Petrie RJ, Gavara N, Chadwick RS, Yamada KM. Non-polarized signaling reveals two distinct modes of 3D cell migration. J Cell Biol 2012; 197:439-55; PMID:22547408; http://dx.doi.org/10.1083/jcb.201201124
6. Baker BM, Chen CS. Deconstructing the third dimension: how 3D culture microenvironments affect cellular cues. J Cell Sci 2012; 125:3015-24; PMID:22797912; http://dx.doi.org/10.1242/jcs.079569
7. Sahai E, Marshall CJ. Differing modes of tumour cell invasion have distinct requirements for RhoROCK signalling and extracellular proteolysis. Nat Cell Biol 2008; 10:221-5; PMID:18435014; http://dx.doi.org/10.1038/ncb1540
8. Cukierman E, Pankov R, Yamada KM. Cell interactions with the extracellular matrix: lessons from cell migration at a glance. J Cell Sci 2005; 118:4917-9; PMID:16254237; http://dx.doi.org/10.1242/jcs.026626
9. Sanz-Moreno V, Gadea G, Ahn J, Paterson H, Marra F. The Raf/MEK/ERK pathway drives invasion of breast tumor cells in 3D microenvironments. PLoS One 2013; 8:e53982; http://dx.doi.org/10.1371/journal.pone.0053982
10. Petrie RJ, Gavara N, Chadwick RS, Yamada KM. Non-polarized signaling reveals two distinct modes of 3D cell migration. J Cell Biol 2012; 197:439-55; PMID:22547408; http://dx.doi.org/10.1083/jcb.201201124

Acknowledgments
We thank Alan Hall for generously providing us with reagents. Any opinion, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

Funding
This work was supported by NIH grants GM092914 (DJW) and CA155572 (DJW and DL) and by the National Center for Research Resources Grant S10RR025524 (DJW). LJ was supported by Ruth L Kirschstein National Research Service Award (NRSA) GM108407 from NIH. In addition, this material is based upon work supported by the National Science Foundation Graduate Research Fellowship under grant no. DGE-0909667.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.

No potential conflicts of interest were disclosed.
demonstration of its applications to neurobiology and cancer biology. Biomed Microdevices 2011; 13:539-48; PMID:21424383; http://dx.doi.org/10.1007/s10544-011-9525-9

34. Whitesides GM, Ostuni E, Takayama S, Jiang X, Ingber DE. Soft lithography in biology and biochemistry. Annu Rev Biomed Eng 2001; 3:335-73; PMID:11447067; http://dx.doi.org/10.1146/annurev.bioeng.3.1.335

35. McDonald JC, Whitesides GM. Poly(dimethylsiloxane) as a material for fabricating microfluidic devices. Acc Chem Res 2002; 35:491-9; PMID:12118988; http://dx.doi.org/10.1021/ar010110q

36. Wegner AM, Nebhan CA, Hu L, Majumdar D, Meier KM, Weaver AM, Webb DJ. N-WASP and the Arp23 complex are critical regulators of actin in the development of dendritic spines and synapses. J Biol Chem 2008; 283:15912-20; PMID:18430754; http://dx.doi.org/10.1074/jbc.M801555200

37. Zhang H, Macara IG. The PAR-6 polarity protein regulates dendritic spine morphogenesis through p190 RhoGAP and the Rho GTPase. Dev Cell 2008; 14:216-26; PMID:18267090; http://dx.doi.org/10.1016/j.devcel.2007.11.020

38. Saito T, Jones CC, Huang S, Czech MP, Pilch PF. The interaction of Akt with APPL1 is required for insulin-stimulated Glut4 translocation. J Biol Chem 2007; 282:32280-7; PMID:17848569; http://dx.doi.org/10.1074/jbc.M704150200

39. Yamazaki D, Kurisu S, Takenawa T. Involvement of Rac and Rho signaling in cancer cell motility in 3D substrates. Oncogene 2009; 28:1570-83; PMID:19234490; http://dx.doi.org/10.1038/onc.2009.2

40. Knaus UG, Bamberg A, Bokoch GM. Rac and Rap GTPase activation assays. Methods Mol Biol 2007; 412:59-67; PMID:18453105; http://dx.doi.org/10.1007/978-1-59745-467-4_5