

A flexible network of vimentin intermediate filaments promotes migration of amoeboid cancer cells through confined environments

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Tumor cells can spread to distant sites through their ability to switch between mesenchymal and amoeboid (bleb-based) migration. Because of this difference, inhibitors of metastasis must account for each migration mode. However, the role of vimentin in amoeboid migration has not been determined. Because amoeboid leader bleb-based migration (LBBM) occurs in confined spaces and vimentin is known to strongly influence cell-mechanical properties, we hypothesized that a flexible vimentin network is required for fast amoeboid migration. To this end, here we determined the precise role of the vimentin intermediate filament system in regulating the migration of amoeboid human cancer cells. Vimentin is a classic marker of epithelial-to-mesenchymal transition and is therefore an ideal target for a metastasis inhibitor. Using a previously developed polydimethylsiloxane slab-based approach to confine cells, RNAi-based vimentin silencing, vimentin overexpression, pharmacological treatments, and measurements of cell stiffness, we found that RNAi-mediated depletion of vimentin increases LBBM by \sim 50% compared with control cells and that vimentin overexpression and simvastatin-induced vimentin bundling inhibit fast amoeboid migration and proliferation. Importantly, these effects were independent of changes in actomyosin contractility. Our results indicate that a flexible vimentin intermediate filament network promotes LBBM of amoeboid cancer cells in confined environments and that vimentin bundling perturbs cell-mechanical properties and inhibits the invasive properties of cancer cells.

Cell migration is required for embryonic development, immune surveillance, and wound healing in healthy individuals. However, uncontrolled migration of tumor cells to distant sites is a hallmark of metastasis and is associated with a poor prognosis. In recent years, it has been demonstrated that cells can adopt multiple modes of migration, including mesenchymal, collective, lobopodial, osmotic engine, and amoeboid (1, 2). This is important because blocking metastasis requires each mode of migration to be targeted. Here we determine the role of a well-established regulator of mesenchymal migration, the vimentin intermediate filament cytoskeleton, in regulating amoeboid migration of cancer cells.

The switch from a predominantly keratin to a vimentin expression pattern is a classic marker of epithelial-to-mesenchymal transition. Accordingly, vimentin is known to increase the size and strength of focal adhesions and template microtubules, and during lobopodial migration it is a critical component of the nuclear piston mechanism (3-6). In contrast, the role of vimentin in amoeboid (blebbing) cells has not been determined. Using in vivo and in vitro approaches, it has been shown that highly contractile (metastatic) cancer cells switch from a mesenchymal to a "fast amoeboid" mode of migration in response to physically confining environments, such as those found in microlymphatics/capillaries and perivascular spaces (7–11). Additionally, certain drug treatments, including matrix metalloprotease and tyrosine kinase inhibitors (e.g. dasatinib), induce a switch to bleb-based migration (12-14). Fast amoeboid migration relies on formation of what we termed a leader bleb (7). In confined environments, leader blebs are typically very large and stable blebs containing rapid cortical actomyosin flow (7-10). Although mesenchymal cells utilize integrinextracellular matrix interactions for migration, fast amoeboid or leader bleb-based migration (LBBM)³ only requires friction between the cortical actomyosin flow and the extracellular environment (10). This property likely promotes the invasive properties of cancer cells in vivo.

Because metastasis requires that cells migrate within the confines of tissues, we hypothesized that confined cancer cell migration (*i.e.* LBBM) requires a flexible intermediate filament network. In support of this theory, epithelial cells treated with transforming growth factor β , which promotes a switch from a predominantly keratin to a vimentin expression pattern, undergo fast amoeboid migration (8). Unlike keratin, which stiffens by bundling in response to force (*i.e.* strain stiffens), vimentin remains unbundled and flexible (15). Moreover, photobleaching experiments have shown that vimentin undergoes subunit exchange an order of magnitude faster than keratin and is therefore considered to be more dynamic (16). Recently, a



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This article contains Figs. S1 and S2 and Movies S1–S4.

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³ The abbreviations used are: LBBM, leader bleb-based migration; PDMS, polydimethylsiloxane; LNA, locked nucleic acid; p-RLC, phosphorylated regulatory light chain; OE, overexpression; EGFP, enhanced GFP; HBS, Hepes-buffered saline; ANOVA, analysis of variance.



Figure 1. Vimentin localizes to the cell body of leader bleb–forming cells. *A* and *A'*, localization in cells adhered to fibronectin (*A'*, *left panel*) and uncoated glass (blebbing; *A'*, *center panel*) and confined under PDMS (forming a leader bleb; *A'*, *right panel*) of either endogenous (*A*, adhered) or transiently expressed vimentin (*A'*, vimentin-FusionRed). *B*, percent of confined cells with vimentin localized in the cell body or leader bleb. *C* and *C'*, lysates from A375-M2 and A549 cells probed for endogenous vimentin and pan-keratin. Densitometry (*C'*, n = 3) was used to determine the ratio of vimentin in A375-M2 versus A549 cells. Statistical significance was determined by a one-sample (hypothetical value = 1) Student's t test. Error is S.E. All data are representative of at least three independent experiments. *, $p \le 0.05$.

statin used for lowering blood cholesterol, simvastatin, was identified in a screen for vimentin binding molecules (17). In contrast to other statins, such as pravastatin, simvastatin has been found to directly bind vimentin filaments and induce bundling. In cell-based assays, simvastatin has been shown to block the proliferation of adrenal carcinoma cells, possibly because vimentin bundling inhibits its degradation, required for cell division (18). Importantly, simvastatin binds vimentin with high specificity as opposed to other molecules (*e.g.* withaferin A) that affect other components of the cytoskeleton (19, 20). Here, by combining simvastatin with our recently described approach for confinement of cells, we describe the precise role of a flexible (unbundled) vimentin network in amoeboid human cancer cells (21).

Our data show that the concentration of vimentin and its bundling are potent regulators of mesenchymal and amoeboid migration, mechanics, and survival of human cancer cells in confinement. This work sheds new light on the potential of vimentin as a therapeutic target.

Results

Because a high level of vimentin expression is correlated with hematogenous metastasis within a wide array of melanoma samples, we set out to determine the localization of vimentin in melanoma A375-M2 cells (22). Moreover, this highly metastatic subline has been observed by intravital imaging to undergo amoeboid migration in tumors (23). Using a combination of immunofluorescence and FusionRed-tagged vimentin (vimentin-FusionRed), we determined the localization of vimentin in A375-M2 cells using high-resolution imaging. In cells adhered to fibronectin-coated glass, an isotropic network of vimentin was concentrated near the cell center (Fig. 1*A'*, *left panel*). Similarly, in nonadherent (blebbing) cells on uncoated

glass, vimentin surrounded the nucleus and was excluded from blebs (Fig. 1*A', center panel*). To evaluate the localization of vimentin in cells with leader blebs, we promoted conversion of A375-M2 cells to this morphology by confinement using our polydimethylsiloxane (PDMS) slab–based approach (21). This involves placing cells under a BSA (1%)– coated slab of PDMS held at a defined height above a coverglass by \sim 3- μ m beads. This confinement height has been shown previously to be optimal for stimulating the transition to fast amoeboid migration (8). Using this approach, we found that vimentin was kept entirely within the cell body as opposed to leader blebs (Fig. 1, *A, right panels*, and *B* and Movie S1). This is significant because the cell body resists the motile force generated by the cortical actomyosin flow in leader blebs; thus, vimentin may play an important role in regulating LBBM speed (8).

To directly test the notion that vimentin concentration in the cell body limits LBBM speed, we depleted A375-M2 cells of vimentin using a locked nucleic acid (LNA), which offers enhanced specificity and stability over traditional siRNAs (24). Because of the long half-life of vimentin, cells were incubated with LNAs for 5 days to achieve an \sim 75% reduction in protein levels (Fig. 2B). Moreover, because these cells predominantly express vimentin, they are an ideal (simplified) model for defining the role of intermediate filaments in LBBM (Fig. 1C). Using our PDMS slab-based approach, LBBM was quantitatively evaluated for LNA-treated cells by live imaging over 5 h. Strikingly, in cells depleted of vimentin, the speed of LBBM was increased over the control by \sim 50%, whereas overexpressing an RNAi-resistant form of vimentin-FusionRed (i.e. rescue) had the opposite effect (Fig. 2, E and F, and Movie S2). Directionality over time was unchanged in vimentin RNAi cells (Fig. S1B). Quantitation proved that the leader bleb area, defined as the



single largest bleb within a given frame, is close to double the size of the control (Fig. 2*C*). Interestingly, quantitation of the cell body area found that, in vimentin RNAi cells, the cell body area was decreased by over 25% (Fig. 2*D*). We speculate that this result is consistent with the location of vimentin in these cells, which may limit the degree to which cortical actomyosin is able to contract the cell body. Consequently, more cytoplasm from

the cell body can enter leader blebs, increasing their size. Strikingly, the nucleus in vimentin RNAi cells was observed to undergo large shape changes, which may reflect an increase in the degree of force transmitted to the nucleus from cortical actomyosin (Fig. 2A). A more than 25% increase in the number of vimentin RNAi cells undergoing apoptosis is consistent with reports of nuclear rupture and DNA damage in confined cells







Figure 3. Overexpressing vimentin in confined cells. *A*, montage of an A375-M2 cell overexpressing vimentin and the marker of F-actin, F-tractin, under PDMS. *B* and *C*, quantitative evaluation of leader bleb (*B*) and cell body (*C*) area for cells overexpressing EGFP alone and vimentin-FusionRed. Statistical significance was determined by two-tailed (unpaired) Student's *t* tests. *D*, instantaneous speeds for cells overexpressing EGFP alone (n = 41) and vimentin-FusionRed (n = 41). Statistical significance was determined by an F test. Error is S.E. *E*, cell stiffness for EGFP alone (n = 34) and vimentin-FusionRed-overexpressing (n = 31) cells. Statistical significance was determined by a two-tailed (unpaired) Student's *t* test. In Tukey box plots, *+* and *lines* denote the mean and median, respectively. All data are representative of at least three independent experiments. **, $p \le 0.01$; ***, $p \le 0.001$; NS, not significant.

(Fig. S2*C*) (25, 26). To test the hypothesis that vimentin regulates the stiffness of A375-M2 cells, we used an approach described by the Liu and colleagues (8) that involves compressing cells between two polyacrylamide (1 kPa) gels (Fig. 2*G* and Fig. S1*A*). Using this approach, cell height divided by the diameter, which is a function of the opposing force, is used to define "cell stiffness." After vimentin RNAi, cells were found to be \sim 25% softer than the control (Fig. 2*H*). In contrast, overexpressing an RNAi-resistant form of vimentin-FusionRed (*i.e.* rescue) led to an increase in cell stiffness (Fig. 2*H*). Because cortical actomyosin is also expected to regulate stiffness, we confirmed that the level of active (phosphorylated) regulatory light chain (p-RLC) is not affected by vimentin RNAi (Fig. 2*I*). Thus, we propose that vimentin limits cell migration in confinement by increasing cell stiffness.

Because depleting A375-M2 cells of vimentin had a striking effect on confined migration, we set out to quantitatively evaluate the effect of vimentin overexpression (OE) on LBBM. To this end, we transiently transfected cells with vimentin-FusionRed and performed live imaging (Fig. 3A). Although the leader bleb and cell body area were unaffected, we found an

~35% decrease in LBBM speed after vimentin OE compared with cells expressing EGFP alone (Fig. 3, *B*–*D*). Directionality over time was unchanged in vimentin OE cells (Fig. S2*D*). The survival of confined cells was only slightly affected by vimentin OE (Fig. S1*E*). Using immunofluorescence, transfected cells were found to have an ~2-fold increase in vimentin (Fig. S1*F*). Consistent with this result, cell stiffness was found to be increased by ~50% after vimentin OE (Fig. 3*E*). Supported by our finding that vimentin is entirely localized to the cell body, we speculate that increasing the level of vimentin decreases LBBM speed by stiffening the cell body.

Next we set out to determine whether modulating the architecture of vimentin can impact LBBM. Recently, a potent and selective inducer of vimentin bundling was identified by an image-based screen (17). In this screen, the cholesterol-lowering statin simvastatin was shown to directly bind vimentin and, likely by limiting electrostatic repulsion between filaments, was able to induce filament bundling (17). This result is significant because in large cohort studies, patients taking statins for cholesterol have decreased cancer-associated morbidity (27). Therefore, we treated A375-M2 cells with simvastatin (10 μ M)

Figure 2. RNAi of vimentin promotes rapid leader bleb–based migration. *A*, montage of an A375-M2 cell under PDMS depleted of vimentin by RNAi expressing F-tractin–mEmerald and H2B-FusionRed. *B*, Western blot confirming depletion of vimentin by RNAi in A375-M2 cells. *B'*, densitometry (n = 3) was used to determine the -fold change in vimentin after RNAi. Error is S.E. *C* and *D*, quantitative evaluation of leader bleb (*C*) and cell body (*D*) area in control (*nontargeting*), vimentin RNAi, and rescue (RNAi + Vimentin-FusionRed) cells. Statistical significance was determined by an ordinary one-way ANOVA followed by a post hoc multiple comparisons test. *E*, migration tracks for nontargeting (*left panel*, n = 40) and vimentin RNAi (*right panel*, n = 43) cells under PDMS. *F*, amalgamated instantaneous speeds for nontargeting, Vimentin RNAi, and rescue cells. Data were normalized around the average for nontargeting collected at the time of vimentin RNAi or rescue. Statistical significance was determined by an F test. Error is S.E. *G*, cartoon of the gel sandwich approach for measuring cell stiffness. *H*, cell stiffness for nontargeting (n = 77), vimentin RNAi (n = 30), and rescue (n = 23) cells. Statistical significance was determined by an ordinary one-way ANOVA followed by a post hoc multiple comparisons test. *h*, height; *d*, diameter. *I*, Western blots of endogenous p-RLC (S19) and RLC in nontargeting and vimentin RNAi cells. *I'*, densitometry (n = 3) was used to determine the -fold change in p-RLC after RNAi. Statistical significance was determined by a ne-sample (hypothetical value = 1) Student's t test. Error is S.E. In Tukey box plots, + and *lines* denote the mean and median, respectively. All data are representative of at least three independent experiments. **, $p \le 0.001$; ***, $p \le 0.001$; NS, not significant.



Figure 4. Vimentin bundling inhibits leader bleb- based migration. *A* and *B*, montage of an A375-M2 cell treated with vehicle (*A*, DMSO) or simvastatin (*B*, 10 μ M) expressing F-tractin–mEmerald and vimentin-FusionRed under PDMS. *Arrows* point to areas of collapsed vimentin in the cell body. *C*, quantitative evaluation of vimentin network collapse for vehicle-treated (*n* = 39), simvastatin-treated (*n* = 26), and pravastatin-treated (*n* = 27) cells. Statistical significance was determined by an ordinary one-way ANOVA followed by a post hoc multiple comparisons test. *D*, quantitative evaluation of instantaneous speeds for vehicle-treated (*n* = 28), simvastatin-treated (*n* = 20) cells. Statistical significance was determined by F tests. Error is S.E. *E*, cell stiffness measurements for vehicle (*n* = 77), simvastatin (*n* = 63), and pravastatin (*n* = 62). Statistical significance was determined by a Kruskal-Wallis test followed by post hoc multiple comparisons. *F*, Western blots of endogenous p-RLC, RLC, and vimentin in drug-treated cells. G, percentage of live (drug-treated) cells after 5 h under PDMS. In Tukey box plots, + and *lines* denote the mean and median, respectively. All data are representative of at least three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$; *NS*, not significant.

and performed live imaging of vimentin-FusionRed. Compared with vehicle treatment (DMSO), the vimentin network was observed to progressively collapse in cells treated with simvastatin (Fig. 4, *A* and *B*, and Movies S3 and S4). To quantitatively evaluate this effect, we measured the total area of the vimentin network in vehicle-, simvastatin-, and pravastatin-treated cells. The related statin pravastatin was used for comparison with simvastatin. Using this approach, the average area of the vimentin network was decreased by ~35% in simvastatin-treated

cells, whereas treating cells with pravastatin did not have a significant effect (Fig. 4*C*). Next we quantified the speed of LBBM for vehicle-, simvastatin-, and pravastatin-treated cells. This analysis showed that cells treated with simvastatin were ~60% slower than those treated with vehicle or pravastatin (Fig. 4*D*). To evaluate the effect of simvastatin on cell stiffness, we again used the gel sandwich approach. For cells treated with simvastatin, we observed a small decrease (~10%) in cell stiffness, whereas treating cells with pravastatin had no effect compared





Figure 5. Simvastatin impairs migration and proliferation of cells expressing high levels of vimentin. *A* and *B*, quantitative evaluation of transmigration for drug-treated A375-M2 (*A*) and A549 (*B*) cells through fibronectin-coated (10 μ g/ml) polycarbonate filters with 8- or 12- μ m pores. Statistical significance was determined by a two-tailed (unpaired) Student's t test. Error is S.E. C and D, growth curves for drug-treated A375-M2 (*C*) and A549 (*D*) cells on tissue culture plastic. Error is S.E. *E*, bright-field images of A375-M2 (*top row*) and A549 (*bottom row*) cells 1 day after treatment with vehicle, simvastatin, or pravastatin. All experiments were performed at least three times. *, p \leq 0.05; **, p \leq 0.01; *NS*, not significant.

with vehicle (Fig. 4*E*). Because bundling of vimentin causes the network to collapse into a small area of the cytoplasm, this result might be expected because the gel sandwich assay measures the stiffness of the entire cell. Although we are unable to directly measure stiffness, based on previous studies of bundled intermediate filaments we predicted a local (large) increase in mechanical properties (15). In agreement with this idea, leader blebs appeared to be resisted by the cell body after simvastatin treatment (Fig. 4B and Movie 4). Because the actomyosin cytoskeleton is also expected to affect cell stiffness, we also measured the level of active myosin (p-RLC) in drug-treated cells. By Western blotting, we confirmed that treatment with vehicle, simvastatin, and pravastatin did not affect the level of p-RLC (Fig. 4F). Moreover, the concentration of vimentin was unchanged in these cells (Fig. 4F). Therefore, by inducing vimentin bundling with simvastatin, migration in confined environments is inhibited. As tissue culture cells obtain cholesterol from serum, statin treatment is not expected to alter the level of intracellular cholesterol, which is a critical component of the plasma membrane (28). Therefore, our data support a

model whereby local stiffening of the cell body by vimentin bundling inhibits LBBM.

To evaluate whether simvastatin has a general effect on cancer cell motility, we subjected drug-treated A375-M2 and lung cancer A549 cells, which also express keratin (Fig. 1B), to transmigration assays. Using filters with $8-\mu m$ pores, we found that simvastatin decreases transmigration for A375-M2 cells (\sim 80%) and, to a lesser extent, A549 cells (\sim 15%), whereas pravastatin did not have a significant effect (Fig. 4, A and B). Interestingly, when using filters with larger pores (12 μ m), we did not observe a statistically significant difference in transmigration of A375-M2 cells after simvastatin treatment (Fig. 5A'). Because we observed an \sim 50% increase in the number of apoptotic cells after simvastatin treatment (Fig. 4G), we next determined whether simvastatin had a general effect on cell proliferation. To accomplish this, we counted cells over 5 consecutive days to generate growth curves for A375-M2 and A549 cells. Strikingly, we found that simvastatin but not pravastatin treatment inhibited the proliferation of both cell types (Fig. 5, C and D). However, this effect was significantly more



pronounced for A375-M2 cells, which express high levels of vimentin (Fig. 1*B*). Consistent with vimentin increasing the size and strength of focal adhesions, A375-M2 and A549 cells were frequently less spread 1 day after simvastatin treatment (Fig. 5*E*) (3). Again, this effect was much more pronounced for A375-M2 cells. In contrast, cells treated with pravastatin were not significantly different from vehicle-treated cells (Fig. 5*E*). Similarly, we found that simvastatin, but not pravastatin, inhibited transmigration and proliferation of WM983-B and MDA-MB-231 cells, which express high levels of vimentin (Fig. S2). Altogether, these results suggest that confined migration and proliferation of cancer cells are inhibited by vimentin bundling.

Discussion

Here we describe, for the first time, the contribution of the vimentin intermediate filament network to LBBM. In contrast to mesenchymal migration, we demonstrate that depleting cells of vimentin increases the speed of LBBM. This result may be explained by our observation that vimentin is entirely localized to the cell body, which is thought to resist the motile force produced by leader blebs. In agreement with this concept, leader blebs that have spontaneously separated from the cell body are extremely fast (8). Our measurements found the leader bleb area to be increased after vimentin RNAi, whereas the cell body area was reduced. These results suggest that vimentin limits the compressibility of the cell body by cortical actomyosin; therefore, more cytoplasm can flow into leader blebs to increase their size. Consistent with previous reports, our measurements of stiffness found that vimentin protects the cell against compression (29). Vimentin may be particularly important for protecting the nucleus from compressive force, as several adaptor proteins have been reported to connect it to the nuclear envelope (30-32). Accordingly, vimentin has been reported recently to protect the nucleus from rupture and DNA damage in confinement (33, 34). In line with these results, we found that the nucleus in vimentin RNAi cells undergoes dramatic shape changes during LBBM. Moreover, cells depleted of vimentin were found to undergo apoptosis more frequently. In mesenchymal cells, the density of the vimentin network has been reported to be inversely correlated with the actin retrograde flow rate in lamellipodia (35, 36). Similarly, we speculate that the localization of vimentin in the cell body may be important to not restrict the cortical actomyosin flow in leader blebs. Therefore, our work identifies vimentin as a fundamental regulator of LBBM.

Our results suggest that vimentin increases the stiffness of the cell body to negatively regulate LBBM. To test this model, we used the cholesterol-lowering statin simvastatin, which was identified after a screen of 1,120 biochemically active molecules to specifically bind and induce bundling of vimentin filaments (17). Indeed, using high-resolution live imaging of melanoma cells, we observed the vimentin network to progressively collapse after simvastatin treatment but not with another statin, pravastatin. Because tissue culture cells obtain cholesterol from serum, this effect is not likely to be due to lowering of cholesterol. In line with our model, vimentin bundles led to the cell body being cemented in place, inhibiting LBBM. Using transmigration assays, which involves migration of cells through fibronectin-coated filters, we determined that vimentin bundling could inhibit migration through 8- but not 12- μ m pores. However, this effect appears to be more pronounced in cells that express high levels of vimentin. Additionally, proliferation of cells expressing high levels of vimentin was dramatically inhibited by simvastatin. This result may be due to the loss of adhesion we observed after simvastatin but not pravastatin treatment. Alternatively, these results may be due to defects in the microtubule network, as microtubules and vimentin reciprocally template their growth (4, 5). Moreover, vimentin expression has been reported to reinforce epithelial-to-mesenchymal transition through regulation of intracellular signaling (37, 38). Thus, future work will need to resolve these alternative explanations.

Our results are significant because in large cohort studies, patients taking statins for cholesterol have decreased-cancer associated morbidity (27). However, statins are selectively localized to the liver, the major site of cholesterol production; therefore, this effect is more likely to be due to a decrease in blood cholesterol. Accordingly, rapidly growing cancer cells require high uptake of extracellular cholesterol (39, 40). Therefore, patients are not likely to benefit much from the effect of simvastatin on vimentin. Together with the work of others, our study supports derivatization of simvastatin for generating an entirely vimentin-selective molecule. Notably, mice lacking vimentin exhibit few defects; therefore, systemic administration of a vimentin-selective molecule may be well-tolerated (41–43). Thus, perturbation of vimentin function in cancer cells is an attractive strategy for prevention of metastasis.

Experimental procedures

Cell culture

A375-M2 (CRL-3223), A549 (CCL-185), and MDA-MB-231 cells were obtained from the ATCC (Manassas, VA). WM983-B cells were purchased from Rockland Immunochemicals (Pottstown, PA). All cells were maintained for up to 30 passages in DMEM supplemented with 10% FBS (catalog no. 12106C, Sigma-Aldrich, St. Louis, MO), GlutaMAX (Thermo Fisher Scientific, Carlsbad, CA), antibiotic–antimycotic (Thermo Fisher Scientific), and 20 mM Hepes (pH 7.4). Cells were plated on 6-well glass-bottom plates (Cellvis, Mountain View, CA) directly or after coating with 10 μ g/ml human plasma fibronectin (catalog no. FC010, Millipore, Billerica, MA), as noted in the figure legends.

Pharmacological treatments

Simvastatin (catalog no.1965) and pravastatin (catalog no. 2318) were purchased from Tocris Bioscience (Bristol, UK). DMSO (Sigma-Aldrich) was used to make $1000 \times$ stock solutions for a working concentration of 10 μ M. Prior to imaging under confinement, plates with PDMS slabs were incubated overnight in medium with DMSO, simvastatin, or pravastatin. The following day, this medium was replaced with fresh complete medium containing DMSO, simvastatin, or pravastatin.

Plasmids

Vimentin-FusionRed and H2B-FusionRed were purchased from Evrogen. mEmerald-Vimentin-7 and mEmerald–F-



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tractin–N13 were gifts from Michael Davidson (Florida State University). F-tractin–FusionRed has been described previously (7). 1 μ g of plasmid was transfected using a Nucleofector 2b device (Kit V, Lonza, Basel, Switzerland).

Mutagenesis

The QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used according to the manufacturer's protocol to generate an RNAi-resistant form of vimentin-FusionRed. The following primers were used for PCR: forward, CTGGCACGTCTTGATCTTGAACGCAAAG; reverse, CTTTGCGTTCAAGATCAAGACGTGCCAG. This yielded a single (silent, $C \rightarrow T$) mutation centrally located within the LNA target sequence (GTCTTGACCTTGAACGCAACGCAAA). Clones were verified by sequencing using a commercially available resource (Thermo Fisher Scientific).

LNAs

Nontargeting (catalog no. 4390844) and vimentin (catalog no. 4390824, s14798) LNAs were purchased from Thermo Fisher Scientific. 50 nM (final concentration) LNA was transfected into cells using RNAiMAX (Thermo Fisher Scientific) diluted in Opti-MEM (Thermo Fisher Scientific).

Microscopy

Live high-resolution imaging was performed using a General Electric (Boston, MA) DeltaVision Elite imaging system mounted on an Olympus IX71 stand with a computerized stage; environment chamber (heat, CO_2 , and humidifier); ultrafast solid-state illumination with excitation/emission filter sets for 4',6-diamidino-2-phenylindole, CFP, GFP, YFP, and Cy5; critical illumination; Olympus PlanApo N ×60/1.42 NA DIC (oil) objective; Photometrics (Tucson, AZ) CoolSNAP HQ2 camera; proprietary constrained iterative deconvolution; and vibration isolation table.

Confinement

This protocol has been described in detail elsewhere (21). Briefly, PDMS (Dow Corning, 184 SYLGARD) was purchased from Krayden (Westminster, CO). 2 ml was cured overnight at 37 °C in each well of a 6-well glass-bottom plate (Cellvis). Using a biopsy punch (catalog no. 504535, World Precision Instruments, Sarasota, FL), an 8-mm hole was cut, and 3 ml of serumfree medium containing 1% BSA was added to each well and incubated overnight at 37 °C. After removing the serum-free medium containing 1% BSA, 200 µl of complete medium containing trypsinized cells (250,000 to 1 million) and 2 μ l of beads $(3.11 \ \mu m, Bangs Laboratories, Fishers, IN)$ were then pipetted into the round opening. The vacuum created by briefly lifting one side of the hole with a 1-ml pipette tip was used to move cells and beads underneath the PDMS. Finally, 3 ml of complete medium was added to each well, and cells were recovered for \sim 60 min before imaging.

Leader bleb, cell body, and vimentin area measurements

For leader bleb, cell body, and vimentin areas, freshly confined cells were traced from high-resolution images with the free-hand circle tool in Fiji (RRID: SCR_002285). From every other frame, the percentage of cell body area for leader blebs, percentage of total for cell body areas, and percentage of cell body area for vimentin were calculated in Microsoft Excel (Redmond, WA). Frame-by-frame measurements were then used to generate an average for each cell. All statistical analyses were performed in GraphPad Prism (La Jolla, CA).

Cell migration

To perform cell speed and directionality analyses, we used a Microsoft Excel plugin, DiPer, developed by Gorelik and colleagues and the Fiji plugin, MTrackJ, developed by Erik Meijering for manual tracking (44, 45). For minimizing positional error, cells were tracked every other frame. Brightfield imaging was used to confirm that beads were not obstructing the path of a cell. All statistical analyses were performed in GraphPad Prism.

Cell stiffness measurements

The gel sandwich assay described in detail elsewhere was used with minor modifications (8). Briefly, 6-well glass-bottom plates (Cellvis) and 18-mm coverslips were activated using 3-aminopropyltrimethoxysilane (Sigma Aldrich) for 5 min and then for 30 min with 0.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS. 1 kPa polyacrylamide gels were made using 2 µl of blue fluorescent beads (200 nm, Thermo-Fisher), 18.8 μ l of 40% acrylamide solution (catalog no. 161-0140, Bio-Rad), and 12.5 µl of bisacrylamide (catalog no. 161-0142, Bio-Rad) in 250 µl of PBS. Finally, 2.5 µl of ammonium persulfate (10% in water) and 0.5 μ l of tetramethylethylenediamine was added before spreading 9-µl drops onto treated glass under coverslips. After polymerizing for 40 min, the coverslip was lifted in PBS, rinsed extensively, and incubated overnight in PBS. Before each experiment, the gel attached to the coverslip was placed on a 14-mm diameter, 2-cm-high PDMS column for applying a slight pressure to the coverslip with its own weight. Then both gels were incubated for 30 min in medium before seeding cells in plates. After the bottom gels in the plates were placed on the microscope stage, the PDMS column with the top gel was placed on top of the cells seeded on the bottom gels, confining cells between the two gels (Fig. 2G). After 1 h of adaptation, the height of cells was measured with beads by measuring the distance between gels, whereas the cell diameter was measured using a far-red plasma membrane dye (catalog no. C10046, ThermoFisher). Stiffness was defined as the height divided by the diameter. If drugs were used, gels were first incubated with drug in medium for 30 min before an experiment.

Transmigration

Prior to transmigration assays, polycarbonate filters with 8or 12- μ m pores (Corning, Corning, NY) were coated with 10 μ g/ml fibronectin (Millipore) by air-drying for 1 h. After permitting ~100,000 cells in serum-free medium to attach (1 h), DMSO, simvastatin, or pravastatin was added to each well. Bottom chambers contained 20% FBS in medium to attract cells. After 24 h, cells from the bottom of the filter were trypsinized and counted using an automated cell counter (TC20, Bio-Rad). Transmigration was then calculated as the ratio of cells on the



bottom of the filter *versus* the total. All statistical analyses were performed in GraphPad Prism.

Growth curves

On day 0, \sim 125,000 cells were plated in 6-well tissue culture plates in complete medium with DMSO, simvastatin, or pravastatin. For 5 consecutive days, cells were trypsinized and counted using an automated cell counter (TC20, Bio-Rad). Each day, wells were supplemented with fresh medium and drug until it was their day to be counted. All plots were generated using GraphPad Prism.

Immunofluorescence

After washing with Hepes-buffered saline (HBS), cells in 6-well glass-bottom plates (Cellvis) were fixed on ice with methanol containing 1% paraformaldehyde (Electron Microscopy Sciences) for 20 min. Blocking, permeabilization, antibody incubation, and washing were done in HBS with 1% BSA, 1% fish gelatin, 0.1% Triton X-100, and 5 mM EDTA. A 1:100 dilution of vimentin antibody (catalog no. 5741, Cell Signaling Technology, Danvers, MA) was incubated with cells overnight at 4 °C. After extensive washing, a 1:400 dilution of Alexa Fluor 488-conjugated anti-rabbit secondary antibody (catalog no. A-21206, Thermo Fisher Scientific) was then incubated with cells for 2 h at room temperature. Cells were again extensively washed and then imaged in HBS. To calculate the level of vimentin-FusionRed OE, we used a custom macro in Fiji (RRID: SCR_002285) to automatically quantify vimentin in transfected versus untransfected cells. Transfected cells were identified using FusionRed.

Western blotting

Whole-cell lysates were prepared by scraping cells into icecold radioimmune precipitation assay buffer (50 mM Hepes (pH 7.4),150 mm NaCl, 5 mm EDTA, 0.1% SDS, 0.5% deoxycholate, and 1% Triton X-100) containing protease and phosphatase inhibitors (Roche). Before loading onto 4%-12% NuPAGE Bis-Tris gradient gels (Thermo Fisher Scientific), lysates were cleared by centrifugation. Following SDS-PAGE, proteins in gels were transferred to nitrocellulose membranes and subsequently immobilized by air drying overnight. After blocking in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 1% BSA, primary antibodies against vimentin (catalog no. 5741, Cell Signaling Technology), pan-keratin (catalog no. 4545, Cell Signaling Technology), IQGAP1 (catalog no. 20648, Cell Signaling Technology), p-RLC (catalog no. 3671, Cell Signaling Technology), or RLC (catalog no. 8505, Cell Signaling Technology) were incubated with membranes overnight at 4 °C. Bands were then resolved with HRP-conjugated secondary antibodies and a C-Digit imager (LI-COR Biosciences, Lincoln, NE).

Statistics

All sample sizes were empirically determined based on saturation. As noted in each figure legend, statistical significance was determined by a two-tailed (unpaired) Student's t test, F test, or ordinary one-way ANOVA followed by a post hoc multiple comparisons test. Normality was determined by a D'Agostino and Pearson test in GraphPad Prism (*, $p \leq 0.05;$ **, $p \leq 0.01,$ ***, $p \leq 0.001.$

Data availability

The data that support the findings of this study are available from the corresponding author, J. S. L., upon reasonable request.

Author contributions—S. B. L., S. M. T., M. F. U., and K. W. V. formal analysis; S. B. L., S. M. T., M. F. U., and K. W. V. investigation; J. S. L. conceptualization; J. S. L. supervision; J. S. L. funding acquisition; J. S. L. writing-original draft; J. S. L. project administration.

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A flexible network of vimentin intermediate filaments promotes migration of amoeboid cancer cells through confined environments

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Supplemental figure 1. A. Top, middle, and bottom images of EGFP and the plasma membrane of an A375-M2 cell between two PA (1 kPa) gels containing fluorescent beads. B. Quantitative evaluation of directionality over time for Vimentin RNAi cells. Error is SEM. C. Percent of Vimentin RNAi cells alive after 5 hr under PDMS. D. Quantitative evaluation of directionality over time for Vimentin-FusionRed OE cells. Error is SEM. E. Percent of Vimentin-FusionRed OE cells alive after 5 hr under PDMS. F. Immunofluorescence was used to compare the level of Vimentin in untransfected vs. transfected (Vimentin-FusionRed) cells. Statistical significance was determined by a one-sample (hypothetical value=1) Student's t-test. Error is SEM. All experiments were performed at least three times. * $-p \le 0.05$, ** $-p \le 0.01$, *** $-p \le 0.001$, and **** $-p \le 0.001$



Supplemental figure 2. Sinvastatin impairs the migration and proliferation of WM983-B and MDA-MB-231 cells. A. Western blots of endogenous p-RLC, Vimentin, and Keratin in drug treated WM983-B (left) and MDA-MB-231 (right) cells. B-C. Quantitative evaluation of transmigration for drug treated WM983-B (A) and MDA-MB-231 (B) cells through fibronectin coated (10 μ g/mL) polycarbonate filters with 8 μ m pores. Statistical significance was determined by a two-tailed (unpaired) Student's t-test. Error is SEM. D-E. Growth curves for drug treated WM983-B (C) and MDA-MB-231 (D) cells on tissue culture plastic. Error is SEM. F. Brightfield images of WM983-B (top) and MDA-MB-231 (bottom) cells one day after treatment with Vehicle, Simvastatin, or Pravastatin. All experiments were performed at least three times. * - p ≤ 0.05 , ** - p ≤ 0.01 , *** - p ≤ 0.001 , and **** - p ≤ 0.001

Supplemental movie 1. Time-lapse imaging of an A375-M2 cell under PDMS transiently expressing Vimentin-mEmerald and F-tractin-FusionRed.

Supplemental movie 2. Time-lapse imaging of an A375-M2 cell under PDMS depleted of Vimentin by RNAi expressing F-tractin-mEmerald and H2B-FusionRed.

Supplemental movie 3. Time-lapse imaging of an A375-M2 cell under PDMS treated with Vehicle (DMSO).

Supplemental movie 4. Time-lapse imaging of an A375-M2 cell under PDMS treated with Simvastatin $(10 \ \mu M)$.