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Mechanical compression drives cancer cells toward invasive phenotype

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Contributed by Rakesh K. Jain, November 28, 2011 (sent for review August 10, 2011)

Uncontrolled growth in a confined space generates mechanical compressive stress within tumors, but little is known about how such stress affects tumor cell behavior. Here we show that compressive stress stimulates migration of mammary carcinoma cells. The enhanced migration is accomplished by a subset of “leader cells” that extend filopodia at the leading edge of the cell sheet. Formation of these leader cells is dependent on cell microorganization and is enhanced by compressive stress. Accompanied by fibronectin deposition and stronger cell–matrix adhesion, the transition to leader-cell phenotype results in stabilization of pericellular organization and is enhanced by compressive stress. Our results suggest that compressive stress accumulated during tumor growth can enable coordinated migration of cancer cells by stimulating formation of leader cells and enhancing cell–substrate adhesion. This novel mechanism represents a potential target for the prevention of cancer cell migration and invasion.

Results

**Compression Induces Migration of Breast Cancer Cells and Cytoskeletal Remodeling.** To test this hypothesis, we subjected normal and cancer epithelial cells to defined compression by pressing them against a membrane surface with a weighted piston (Fig. S1A). This geometry is similar to that experienced by cells at the periphery of a mammary acinus; in these 3D structures, uncontrolled growth of cancer cells within the acinus lumen effectively presses cells at the periphery of the acinus against the surrounding basement membrane (Fig. S1B). We subjected five established mammary epithelial cell lines (MCF10A, MCF7, 67NR, 4T1, and MDA-MB-231; listed in order of increasing invasion potential) to constant compressive stress and measured migration rates via a scratch-wound assay (throughout this paper, “wound” refers to the denuded area in our 2D cultures where cells were removed or excluded) (Fig. S1A). Stress levels were similar to those estimated in the native breast tumor microenvironment: 5.8 mmHg (21). At this level, compression did not significantly increase cell proliferation (Fig. S2A). However, compressive stress did enhance the motility of highly aggressive 4T1 and MDA-MB-231 cells, as well as 67NR cells, which have undergone partial epithelial–mesenchymal transition (characterized by loss of E-cadherin and vimentin expression) (27). In contrast, compressive stress suppressed the migration of normal mammary epithelial MCF10A cells and non-invasive, well-differentiated MCF7 cells, which retain certain features of normal mammary epithelium (28) (Fig. 1A). For the MCF10A cells, the slowed migration was associated with a decrease in cell number (Fig. S2A). It should be noted that during the first 16 h of compression, there was slight compaction of the agarose and a corresponding flow of fluid out of the gel. However, the resulting shear forces experienced by the cells were negligible (29, 30) (maximum 3.2 × 10⁻⁵ dyn/cm²; see SI Materials and Methods, Analysis of Fluid Dynamics at the Surface During Compression). These results demonstrate that applied compressive stress (ACS) enhances the migration potential of mammary carcinoma cells independent of any changes in cell proliferation.

**Fig. 1B** shows the dramatic difference in cell morphology at the wound edge between MCF10A and 67NR cells—the two cell lines that exhibited the most prominent inhibition or enhancement of migration, respectively. Compression stimulated changes in cell shape and cytoskeletal organization at the wound edge in 67NR, but not MCF10A cells. Specifically, compressed 67NR cells showed actin stress fibers that were more defined, and microtubule rearrangement (Fig. 1C). This cytoskeletal adaptation to mechanical stimulation suggests that (i) compression-induced 67NR cell motility could be mediated by increased tension in the actin cytoskeleton, thereby inducing formation of stress

The authors declare no conflict of interest.

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fibers, and (ii) the elevated intracellular tension, in turn, induces formation of a microtubule network to ensure mechanical stability (7, 31).

We next investigated whether higher stresses would further enhance the migration of cancer cells. By titrating the piston weight, we determined that stresses >5.8 mmHg significantly triggered apoptosis, with only 40% of cells viable at 58 mmHg (Fig. S2B); there was a corresponding decrease in migration of viable cells at these higher stress levels (Fig. S2C). Furthermore, continuous compression was necessary for enhanced migration: 67NR cells preconditioned with a compressive stress of 5.8 mmHg migrated more slowly after stress removal than continuously compressed cells (Fig. S2D). These results show that moderate levels of continuously applied compressive stress enhance cell motility, whereas excessive stresses trigger cell death and impede cell migration. For the subsequent studies, the compressive stress was maintained at 5.8 mmHg.

ACS Stimulates Formation of “Leader Cells” with Filopodial Protrusions. During wound closure, 67NR cells at the leading edge appeared to guide the directional movement of the cell sheet. This subset of cells acted as leader cells, maintaining connections with their trailing neighbors while extending protrusions into the denuded area (Fig. 1C). In the absence of compression (control; Movie S1), these cells extended in random directions and clustered at the wound periphery. In contrast, compressed 67NR cells demonstrated a clear persistence and directionality in their movement at the leading edge of the cell sheet (Movie S2). Quantitative analysis of cell orientation showed that the compressed 67NR cells preferentially aligned perpendicular to the wound periphery with the leading edges extending into the open area (Fig. 2A). Interestingly, there was a striking difference in the formation of leader cells between the control and compressed cultures. The number of leader cells increased by approximately twofold when the 67NR cells were compressed (Fig. 2B). To further characterize these leader cells, we assessed their size, shape, and polarization in compressed and control cultures. Although leader cells were polarized in both control and compressed conditions (Fig. S2E), those under compression had larger cell-substrate contact areas (Fig. 2C) and longer filopodial protrusions (Fig. 2D).

Compressive Stress Induces Leader-Cell Phenotype in Border Cells, Regardless of Cell Microorganization. Because (i) only cells at the wound edge adopt leader cell phenotype and (ii) leader cell morphology was not observed in sparsely seeded cultures, even when compressed (control, Movie S3; compressed, Movie S4), we hypothesized that the microorganization of cells within the sheet influences leader-cell formation. Specifically, it appeared that the extent of free-cell perimeter (the fraction of cell perimeter not in contact with other cells) was important.

To investigate this possibility, we controlled free-cell perimeter using microcontact printing. Groups of 67NR cells were forced into various defined geometries and their morphological changes were tracked over time. In circle patterns, all cells around the denuded periphery have roughly the same extent of free perimeter. On the other hand, cells at vertices (such as the
points of the rosette) have, on average, more free perimeter (Fig. 3 and Fig. S3). We first confirmed that compression-induced leader-cell formation and enhanced wound closure occurred in the circle pattern formed by microcontact printing, similar to that seen in the scratch-wound assays. As expected, leader cells rarely formed in the uncompressed circle patterns, indicated by a relatively smooth periphery of cell-denuded areas, but were frequent in compressed cultures (Fig. 3A); furthermore, wound closure rates were increased with compression (Fig. 3B), consistent with the scratch-wound cultures. This result also verified that leader-cell formation is not influenced by cell damage in the in vitro scratch assay.

Next, we patterned 67NR cells in a rosette geometry with leader cells predesignated at the tips (Fig. 3C). Interestingly, in the absence of compression, the preformed leader cells (point cells) extended more frequently from the rosette vertices and migrated faster than the other boundary cells (Fig. 3D). In contrast, with compression, there was no preferential location for leader-cell formation—cells extended and migrated from random locations around the rosette, not just from the pattern vertices (Fig. 3C). As a result, there was faster overall migration in these compressed cultures (Fig. 3E).

To quantify the effect of free-cell perimeter on leader-cell formation, we confined colonies of 67NR cells to a square geometry using micro contact printing (Fig. 3F). In these patterns, cells at the corners have more free perimeter than those on the edges, and quantitative comparison is accomplished via the change in shape of the square. Consistent with the rosette data, with no compression, cells at the four corners were more likely to become leader cells; with compression, almost all boundary cells adopted the leader-cell phenotype (Fig. 3F). Quantitative analysis of the patterns confirmed that there was preferential extension from the corners in the control, but not the compressed, cultures (Fig. 3G). Thus, leader-cell formation in uncompressed cultures is sensitive to local cell–cell spatial organization, which determines free-cell perimeter and can influence the dynamics of spreading. The positional advantage disappears with compression, which distends all border cells, likely inducing changes in shape or cytoskeletal tension that mimic those in uncompressed corner cells.

**Actomyosin Contractility Is Required for Migration of Leader Cells, but Not Their Formation.** Previous studies have shown that cell microorganization within a monolayer defines patterns of intracellular tension generated by the actomyosin cytoskeleton (32). Such intracellular tension is important for determining sites of cell proliferation (32) and mammary branching morphogenesis (33) in vitro. To investigate whether similar myosin-dependent intracellular tension is involved in ACS-induced coordinated migration, we altered cellular mechanics using molecular and pharmacological approaches.

Intracellular tension generated by the actomyosin cytoskeleton is regulated in part by signaling through the small GTPase RhoA, its downstream effector Rho kinase (ROCK), and myosin light chain kinase (MLCK). To decrease the actomyosin-mediated contractile tension, we therefore used dominant-negative RhoA (RhoA-T19N) retrovirus, ROCK inhibitor Y-27632, or MLCK inhibitor ML-7. Compression still enhanced wound closure rates of RhoT19N cells compared with uncompressed controls, but the effect was slightly reduced compared with the induction seen in wild-type cells. In addition, inhibition of RhoA activity did not suppress leader-cell formation in the compressed cultures (Fig. 4A). Similar results were seen with Y-27632 and ML-7 treatment (Fig. 4B and C). Our finding that RhoA is not involved in compression-induced leader-cell formation was surprising, considering previous work demonstrating its role in the formation of leader cells during in vitro wound healing (34). It is possible that external mechanical stimulation enables other RhoA-independent signaling mechanisms. Interestingly, when actomyosin contractility was completely blocked by the myosin II ATPase inhibitor Blebbistatin (35), compressive stress was still able to induce leader-cell formation despite compromised cell motility (Fig. 4C). These results indicate that compression can initiate directional protrusions for leader-cell formation independent of actomyosin contractility; however, the contractile machinery is still necessary for sheet migration.

**Cell Adhesion Is Modulated During Compression-Induced Migration.** Cell migration is a coordinated interaction between cells and their surroundings. In our 67NR cells, homotypic E-cadherin adhesion was not necessary for—and did not interfere with—compression-induced migration (Fig. S4). Therefore, we next tested whether compression affects cell–substrate adhesion. We quantified the ability of cells to resist detachment caused by fluid shear forces. Compressed 67NR cells exhibited 2.5-fold higher cell-substrate adhesion than uncompressed cells (Fig. 5A). Analyzing the distribution of fibronectin in the culture, we found that more fibronectin was localized at the cell–substrate interface in the compressed samples than in controls (Fig. 5B and D), which was consistent with the immunostaining pattern of vinculin.
a protein marker of focal adhesions (Fig. 5E). There was also fibronectin between cells, which likely allowed for cohesion of the cell sheet independent of E-cadherin expression. However, the change in fibronectin distribution was not related to altered fibronectin transcription (quantitative PCR analysis; Fig. 5C).

The distinct fibronectin patterns could have been formed by rearrangement of existing extracellular fibronectin or by directed secretion of newly synthesized fibronectin by the cells. To investigate this further, we inhibited all protein translation by treating the 67NR cells with cycloheximide before compression and then monitored migration and fibronectin patterns. We confirmed that cycloheximide-treated 67NR cells still adhered to and migrated on fibronectin substrates, but, in general, speeds were slower compared with the untreated cells (Fig. S5). However, inhibition of protein synthesis abolished the oriented, fibrillar-like pattern of fibronectin seen in the untreated, compressed cultures (Fig. 5F). These results suggest that mechanical compression induces localized secretion of fibronectin by the migrating 67NR cells, and this localized secretion enhances leader-cell formation.

**Discussion**

Uncontrolled proliferation of cancer cells generates mechanical, compressive stresses (19, 20). We hypothesized that such stresses can facilitate tumor progression and found that cells at the periphery of a discontinuous sheet can undergo a phenotypic transformation when compressed. These cells at the sheet boundary became leader cells and participated in coordinated cell migration (collective migration) (36, 37), extending protrusions in the direction of migration and guiding “followers” at their rear. Leader cells have been observed in collective migration during cancer cell invasion, vascular sprouting, wound closure, and embryogenesis (36, 37). Furthermore, there is some evidence that mechanical forces can modulate coordinated migration in vascular morphogenesis: It has been shown that forces exerted by flowing fluids can induce changes in endothelial junction structure (38), differentiation of vascular wall cells (39), and tip cell formation (40). Interestingly, our leader cells are morphologically and functionally similar to endothelial tip cells. Therefore, it is possible that similar mechanisms are involved in the coordinated migration of epithelium and endothelium.

The formation of leader cells in coordinated migration is likely related to the balance of intracellular stresses generated in the cytoskeleton (7, 41). A shift in stress balance due to interactions with other cells (32, 36, 42) or cell distortion as a cell actively exerts pressure, and embryogenesis (36, 37). Furthermore, there is some evidence that mechanical forces can modulate coordinated migration in vascular morphogenesis: It has been shown that forces exerted by flowing fluids can induce changes in endothelial junction structure (38), differentiation of vascular wall cells (39), and tip cell formation (40). Interestingly, our leader cells are morphologically and functionally similar to endothelial tip cells. Therefore, it is possible that similar mechanisms are involved in the coordinated migration of epithelium and endothelium.

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In the absence of exogenous compression, the stress balance—determined by the cell’s position within the monolayer and mediated through actomyosin (32)—affected leader-cell formation. Cells at rosette tips or at the corners of a square pattern have more free-cell perimeter available for extension and formation of new adhesions, resulting in a change in the cell stress balance. We propose that this shift in intracellular forces initiates changes in cytoskeleton and focal adhesions that quickly translate into leader-cell formation.

In contrast, when cells were subjected to applied compressive stress, there was no preferential location for leader-cell formation around the sheet boundary. Even cells that were not in “preferred” positions for self-induced leader-cell formation (i.e., cells in the “edge” positions) became leaders. As the leader cells spread, they secreted fibronectin, facilitating cell-substrate interactions (Fig. 5 B and D). This result is consistent with reports that the persistent movement of leader cells requires cell adhesion to fibronectin (44). Our results are also consistent with the study by Chen et al., who confined individual cells to patterned surfaces and found that cell spreading controls the number of focal adhesions (43). Evidently, the increased free-cell perimeter and surface adhesion caused by compression-induced cell distortion (Fig. 2 C and D) affect cytoskeletal tension and trigger leader-cell formation (Fig. 1C), independent of any preexisting force balance established by the cells themselves. Our observations that (i) ACS-induced coordinated migration is abolished upon removal of the applied stress (Fig. S2D) and (ii) inhibition of actomyosin contractility has no effect on ACS-induced leader-cell formation (Fig. 4) support the concept that the applied stress substitutes for intracellular tension generated by actomyosin contractility to enable and sustain the transformation (Fig. 6).

Indeed, it has been reported that external force application can induce intracellular tension during maturation of focal contacts, independent of Rho/ROCK-dependent actomyosin-driven cell contractility (45). Upon removal of the applied stress, the tension balance could return to normal and the cells revert to nonleader phenotype.

The fact that aggressive carcinoma cells, but not MCF10A cells, respond to compression raises the interesting possibility that cancer cells are somehow “primed” to respond to changes in the mechanical environment. Previous reports have shown that MCF10A cells have a higher apparent elastic modulus than cancer cells (31, 46). This result is consistent with our finding that uncompressed (control) MCF10A cells have a denser network of cytoskeletal structures (particularly microtubules) compared with uncompressed (control) 67NR cells (Fig. 1C). The relatively higher level of cell stiffness may make nontumorigenic MCF10A cells less mechano-sensitive. Our results are consistent with results from 3D cultures, where increased matrix density or stiffness has been shown to trigger malignant transformation (23, 47, 48). It is possible that in these systems, cell growth produces compressive stress that initiates the invasion in a way analogous to our applied compression.

Our results establish a direct link between mechanical stress and enhancement of coordinated cancer cell motility via formation of leader cells. The concept of mechanical induction of tumor invasiveness could open the door to a unique class of targets for blocking mechanical stress pathways and guide the development of approaches for drug screening that take into account mechanical as well as genetic and biological factors. In addition, this work provides unique insight into how physical determinants can influence coordinated migration, a process relevant to other physiological events such as vascular sprouting and wound healing (36, 37).

![Diagram of compression-modulated leader-cell formation and coordinated migration.](image-url)
Materials and Methods

A detailed description of the materials and methods is included in SI/Methods. Briefly, mammary carcinoma (MCF7, 67NR, 4T1, and MDA-MB-231) and normal (MCF10A) cell lines were subjected to 16-h compressive stress at a level of 5.8 mmHg using the in vitro compression device (Figs. S1A and Fig. S6) and their morphology was measured with the scratch-wound assay. Images of cell periphery of the wound were captured with an inverted microscope (Olympus) for analyses of cell orientation and migration. To control free-cell perimeter, 67NR cells were patterned by seeding them on surfaces “stamped” with fibronectin to form circles, squares, and rosettes using microcontact printing, as previously described (49, 50) with minor modifications. For circles and rosettes, the fibronectin (and cells) was excluded from the shape; for squares, the fibronectin (and cells) was confined to the shape. The 67NR cells were also stained with Alexa Fluor-546 phallolidin (Molecular Probes-Invitrogen), anti-vinculin antibody (Sigma), and antiseraum against fibronectin (Sigma) for F-actin, focal adhesions, and fibronectin, respectively. Immunofluorescence images were collected with a confocal microscope (Olympus) and analyzed with ImageJ or Matlab for leader cell frequency and filopodial protrusions as well as fibronectin deposition. The transcriptional expression level of fibronectin was measured by quantitative real-time PCR, using total RNA extracted from the 67NR cells. The effect of compression on the cell-surface adhesion strength was determined by the number of compressed or control cells remaining on the surface after exposure to shear forces. Finally, to determine the role of actomyosin contractility in compression-induced coordinated migration, various chemical inhibitors or molecular modification were used to down-regulate RhoA/ROCK or myosin-associated pathways. Data are presented as mean ± SEM, and P < 0.05 was considered significant in unpaired Wilcoxon–Mann–Whitney tests.

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