

SHORT REPORT

The malignancy of metastatic ovarian cancer cells is increased on soft matrices through a mechanosensitive Rho–ROCK pathway

Daniel J. McGrail¹, Quang Minh N. Kieu¹ and Michelle R. Dawson^{1,2,*}**ABSTRACT**

Although current treatments for localized ovarian cancer are highly effective, this cancer still remains the most lethal gynecological malignancy, largely owing to the fact that it is often detected only after tumor cells leave the primary tumor. Clinicians have long noted a clear predilection for ovarian cancer to metastasize to the soft omentum. Here, we show that this tropism is due not only to chemical signals but also mechanical cues. Metastatic ovarian cancer cells (OCCs) preferentially adhere to soft microenvironments and display an enhanced malignant phenotype, including increased migration, proliferation and chemoresistance. To understand the cell–matrix interactions that are used to sense the substrate rigidity, we utilized traction force microscopy (TFM) and found that, on soft substrates, human OCCs increased both the magnitude of traction forces as well as their degree of polarization. After culture on soft substrates, cells underwent morphological elongation characteristic of epithelial-to-mesenchymal transition (EMT), which was confirmed by molecular analysis. Consistent with the idea that mechanical cues are a key determinant in the spread of ovarian cancer, the observed mechanosensitivity was greatly decreased in less-metastatic OCCs. Finally, we demonstrate that this mechanical tropism is governed through a Rho–ROCK signaling pathway.

KEY WORDS: Ovarian cancer metastasis, Mechanotransduction, Tissue tropism

INTRODUCTION

Ovarian cancer is the fifth leading cause of cancer deaths among women, largely because it is often diagnosed at late stages after metastasis, with a 5 year survival rate of only 30% (Landen et al., 2008). Instead of following the normal metastatic process of intravasation to the vascular system and extravasation at a distal site, ovarian cancer cells (OCCs) are more likely to disseminate through the intraperitoneal fluids. From there, they preferentially accumulate in soft tissues, such as the adipocyte-rich omentum (Nieman et al., 2011). Previous work has suggested that this is because adipocytes act as a rich energy source and actively promote OCC homing through cytokines such as interleukin-8 (Nieman et al., 2011). However, these studies were based solely on chemical factors, whereas the burgeoning field of physical oncology has recently shown that the mechanical environment that a cell experiences can be of equal importance. For instance, pioneering

studies by Weaver and colleagues have demonstrated that increased matrix stiffness can induce a malignant phenotype in mammary epithelial cells by leading to increases in both Rho activation and actomyosin contractility (Paszek et al., 2005), with further studies directly implicating contractility in increasing matrix stiffness and cancer progression (Samuel et al., 2011). Although most of these studies linked increased matrix stiffness to tumor progression, breast cancer metastatic subclones with tropism for soft lung tissue *in vivo* exhibit growth advantages on soft substrates *in vitro* (Kostic et al., 2009). Based on these results, we hypothesized that the preferential accumulation of OCCs in soft tissues might be due to the intrinsic mechanical properties of the environment.

To test this hypothesis, we utilized a series of biophysical and biochemical techniques to understand the response of ovarian cancer cells to a soft matrix similar to adipose tissue and a stiff matrix similar to tumor tissue (Samani et al., 2007; Tse and Engler, 2010), using both the more-metastatic SKOV-3 and the less-metastatic OVCAR-3 human cell lines (Slack-Davis et al., 2009), both of which harbor either mutated or deleted p53, indicative of high-grade serous ovarian carcinomas (Ali et al., 2012; Domcke et al., 2013; Salani et al., 2008). We found that OCCs show increased adhesion on soft microenvironments. After engraftment, OCCs on soft matrices are more proliferative and more resistant to standard chemotherapeutic drugs. In addition to these changes in growth, the cells also displayed increased migratory capacity. Further immunocytochemistry and gene expression analyses revealed a shift from a more epithelial phenotype on stiff substrates to a more mesenchymal phenotype on soft matrices. Cell–matrix interactions were directly probed by using traction force cytometry, which revealed changes in both force magnitude and polarization on softer matrices. Moreover, the use of small-molecule modulators of the Rho–ROCK pathway demonstrates that this signaling cascade plays a key role in determining this tissue tropism. Thus, this study reveals a previously undocumented role for mechanical cues in ovarian cancer metastasis, findings that could lead to new methods to target metastatic disease.

RESULTS AND DISCUSSION**Ovarian cancer cells preferentially engraft, expand and migrate on soft substrates**

Following dissemination from the primary tumor, OCCs frequently engraft onto the mesothelial lining of the soft omentum. To determine whether tissue stiffness plays a role in OCC engraftment, we utilized two model systems. First, we tested the adhesion of OCCs plated onto a monolayer of human mesenchymal stem cells (hMSCs) differentiated into either soft adipocytes ($E \approx 0.9$ kPa) or stiffer osteoblasts ($E \approx 2.6$ kPa) (Darling et al., 2008) – cell types that would both express ligands implicated in mesothelial engraftment, such as VCAM-1 and CD44. We found that OCCs were significantly ($P < 0.001$) more adherent on the softer adipocytes relative to the stiffer

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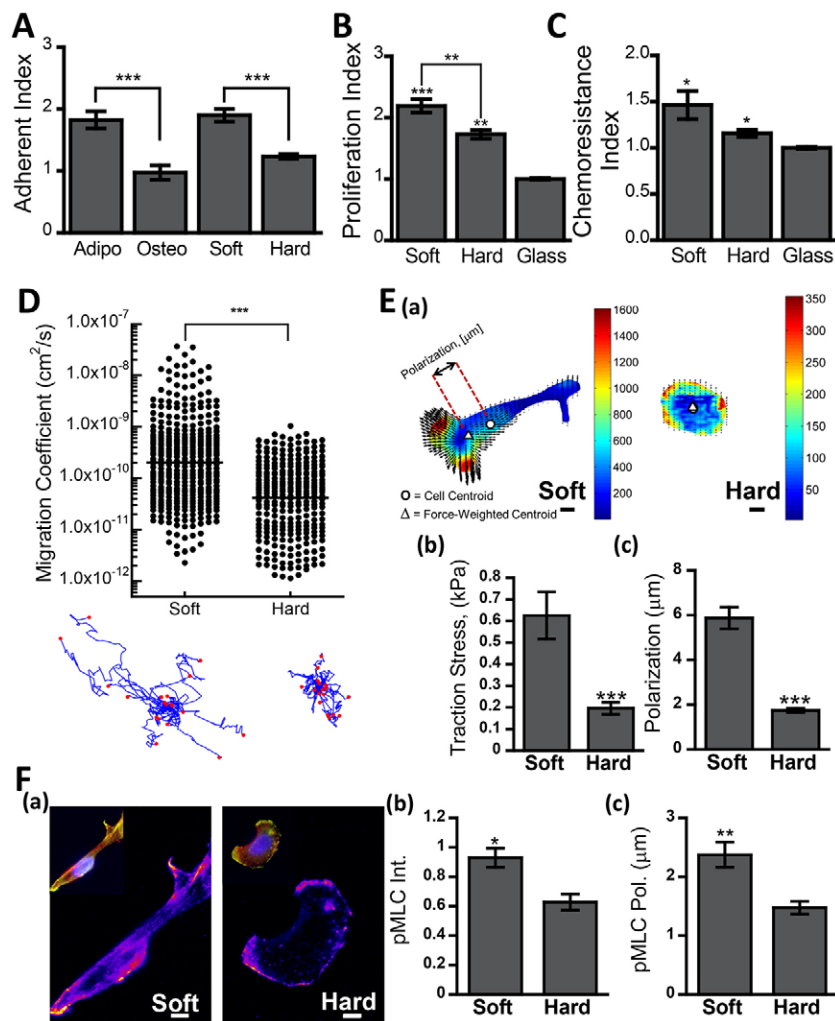


Fig. 1. Metastatic SKOV-3 ovarian cancer cells display a more malignant phenotype on soft matrices.

(A) Measurement of adhesion to soft microenvironments shows that OCCs are more adherent when cultured on soft adipocytes (Adipo) than when cultured on stiffer osteoblasts (Osteo), with nearly identical differences seen between soft (2.83 kPa) versus hard (34.88 kPa) polyacrylamide substrates. (B) After engrafting, cells proliferated more on soft substrates relative to hard substrates, with proliferation on both substrates being significantly greater than that observed on the more rigid collagen-coated glass control. (C) Cells on compliant matrices showed increased viability after a 24-hour treatment with 50 μ M carboplatin relative to their respective DMSO-treated solvent controls. (D) Analysis of cell motility tracks on both soft and hard matrices (shown below the graph) revealed a significantly higher cell-migration coefficient on soft substrates. (E) Heat maps of traction stresses (Pascals) overlaid with black arrows showing cell-induced matrix displacements (a). The cell center of mass is shown by the circle (\circ), and the triangle (Δ) shows the force-weighted center of mass. Quantification of the cell-exerted traction forces shows that OCCs on soft substrates have increased peak traction stresses (b) as well as force polarization (c), defined as the difference between the cell center of mass and the force-weighted center of mass. Scale bars: 10 μ m. (F) Staining for pMLC (a) reveals a corresponding increase in pMLC intensity (b) and polarization (c) in OCCs cultured on soft substrates. Data are shown as the mean \pm s.e.m.; * P <0.05, ** P <0.01, *** P <0.001.

osteoblasts. Next, we repeated this analysis on synthetic polyacrylamide substrates with elastic moduli that were mimetic of either adipose tissue (soft, 2.83 kPa) or tumor tissue (hard, 34.88 kPa), and we found near-identical changes in adhesion (Fig. 1A). Although these substrates were coated with collagen I, additional studies showed equivalent adhesion and spreading on fibronectin (supplementary material Fig. S2). Taken together with the results on cell monolayers, these results suggest that changes in adhesion are not adhesion-ligand dependent.

After OCCs engraft into the secondary site, they must then survive and proliferate. Remarkably, after only 48 hours in culture, there were nearly two times as many cells on the soft substrate as compared with the number of cells on collagen-coated glass, and there were significantly more cells on soft substrate than on hard substrates (P <0.01) (Fig. 1B). We hypothesized that this increase in proliferation might lead to increased levels of chemotherapeutic-induced cell death; however, treatment with carboplatin was significantly less effective on soft substrates (Fig. 1C), similar to results reported previously from a study in which OCCs were cultured in a 3D environment (Loessner et al., 2010). Finally, single-cell motility analysis revealed large increases in migration on soft substrates, as quantified by the calculated cell-migration coefficient (Fig. 1D). Although the overall average migration coefficient increased almost fivefold, the migration coefficient of the fastest 1% of cells

increased by more than 30-fold (supplementary material Fig. S3A). It is possible that the latter cell population represents the small tumor cell subpopulation that is capable of metastasis.

After observing these functional alterations on soft substrates, we sought to investigate the cell–matrix interactions. To do so, we utilized traction force microscopy (TFM) to quantify the force exerted by cells on the underlying substrate (Fig. 1Ea). We found that, when cultured on soft matrices, OCCs exerted more force (Fig. 1Eb), a characteristic that is often indicative of increased metastatic phenotype (Kraning-Rush et al., 2012). Previous work has demonstrated that during invasion into the submesothelial environment, OCCs use myosin-dependent traction force to escape the mesothelial cell layer (Iwanicki et al., 2011). Our results indicate that this key step in ovarian cancer invasion might be exacerbated by the mechanical properties of the omentum. In addition, the cells were more capable of polarizing these forces on the soft substrates (Fig. 1Ec), a crucial step for effective cell migration. These changes in force profiles corresponded to changes in both the overall intensity and polarization of phosphorylated myosin light chain (pMLC) (Fig. 1F).

Culturing OCCs on soft substrates induces a more mesenchymal phenotype

When grown on soft substrates or soft cells, OCCs displayed a more elongated morphology, indicative of a mesenchymal

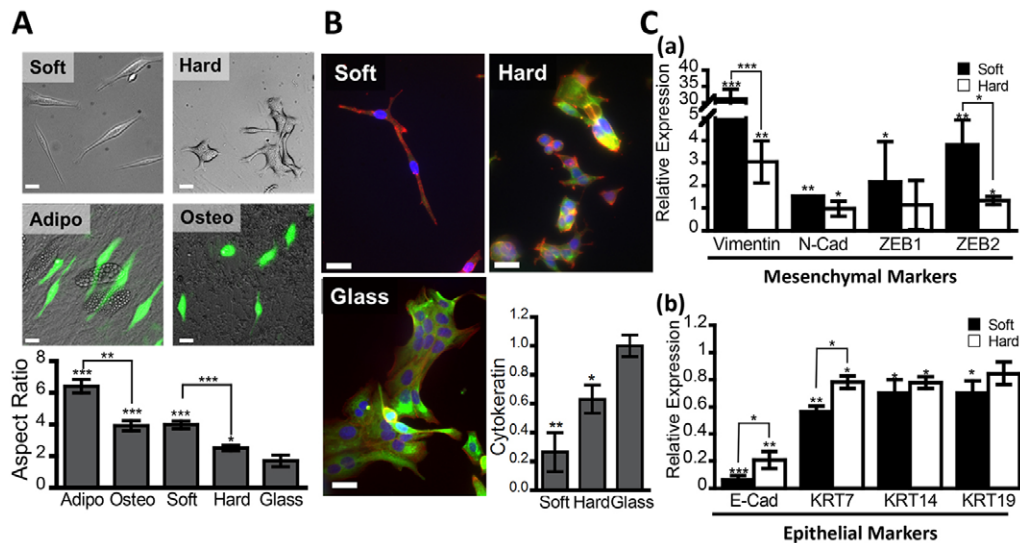


Fig. 2. Increased malignant phenotype correlates with EMT. (A) SKOV-3 cells show morphological elongation consistent with a more mesenchymal phenotype on soft matrices as well as on soft adipocyte (Adipo) monolayers (SKOV-3 cells labeled green with carboxyfluorescein succinimidyl ester (CFSE)), but not on stiffer osteoblast (Osteo) monolayers. (B) Staining cells for pan-cytokeratin (green) and F-actin (red) shows decreased cytokeratin expression on softer substrates. Scale bars: 50 μ m. (C) Gene expression analysis shows both upregulation of mesenchymal markers (a) and downregulation of epithelial markers (b) in cells cultured on hard and soft substrates relative to the expression in cells cultured on collagen-coated glass controls. Data are shown as the mean \pm s.e.m.; * P <0.05, ** P <0.01, *** P <0.001.

phenotype. Cells cultured on adipocyte monolayers showed the most elongation – the length of these cells was nearly six times the cell width (Fig. 2A; supplementary material Fig. S1Ac). This might be due, in part, to soluble factors released by the adipocytes, as polyacrylamide substrates of similar rigidities did not recapitulate this elongation entirely (supplementary material Fig. S3B). The cell aspect ratio was nearly equivalent on osteoblasts and the soft matrix, both of which have similar elastic moduli. However, cells cultured on all evaluated culture substrates showed increased elongation over those cultured on the collagen-coated glass control. Based on the elongated morphology and increased migration, which is indicative of a mesenchymal phenotype, we hypothesized that cells were undergoing epithelial-to-mesenchymal transition (EMT). To test this, we first stained cells for the intermediate filament cytokeratin, which is preferentially expressed in epithelial cells. We found that OCCs cultured on soft substrates displayed approximately a fifth of the amount of cytokeratin (P <0.01) compared with those cultured on collagen-coated glass (Fig. 2B). This finding was validated by using quantitative (q)RT-PCR, which showed increased expression of mesenchymal markers (Fig. 2Ca) and decreased expression of epithelial markers (Fig. 2Cb) when OCCs were cultured on compliant matrices.

Mechanosensitivity is decreased in less-invasive OCCs

After finding that culturing cells on compliant matrices produced an OCC phenotype that was more metastatic, we next questioned whether this mechanosensitivity was decreased in a less metastatic cell line. To do so, we used poorly metastatic OVCAR-3 cells (Slack-Davis et al., 2009). In contrast to their more-metastatic counterparts, these cells showed no significant advantage in adhesion, proliferation, chemoresistance or migration on soft substrates (Fig. 3A). To test whether this was due to altered interactions with the underlying substrate, we repeated the TFM and found a slight increase in traction stresses on soft matrices (Fig. 3B), although the change was much smaller

than that observed in more-metastatic cells. In contrast to other types of cancer that have been studied previously (Kraning-Rush et al., 2012), the peak traction forces exerted by the less-invasive cells were actually higher than those exerted by the more-metastatic cells. However, the more-aggressive SKOV-3 cells showed a larger fold increase in traction forces on soft substrates, suggesting that force modulation based on matrix stiffness might be a more relevant parameter for grading cell invasiveness than the absolute magnitude of the force (Fig. 3Bd). When evaluating changes in epithelial or mesenchymal character of the cells, we found that the less-metastatic OCCs still showed decreases in cytokeratin expression, although, even on soft substrates, these values were still equivalent to those of the SKOV-3 cells on glass (Fig. 3Ca). Gene expression analysis showed similar changes (Fig. 3Cb).

OCC mechanical preference is controlled through a Rho-ROCK pathway

Because Rho and ROCK have both been associated with matrix-stiffness-induced malignancy (Paszek et al., 2005; Samuel et al., 2011) and mechanotransduction (Jaalouk and Lammerding, 2009), we hypothesized that these molecules might play a role in the changes observed in this study. To investigate this, we either treated metastatic OCCs with lysophosphatidic acid (LPA) to activate Rho and ROCK or inhibited ROCK by using the small-molecule inhibitors Y27632 and H1152.

Consistent with previous literature reports that LPA is often increased in ovarian cancer patients and leads to increased cell invasion (Jeong et al., 2012), LPA markedly induced migration on hard substrates with a rigidity similar to that of the primary tumor (Fig. 4Aa). This LPA-induced motility on hard substrates bore great phenotypic resemblance to that seen natively on soft matrices, with changes including increased cell elongation (Fig. 4Ab), increased traction forces and increased force polarization (Fig. 4C). By contrast, on soft matrices, LPA drastically reduced migration and caused cells to collapse to a rounded morphology (Fig. 4Ab). TFM revealed similar peak

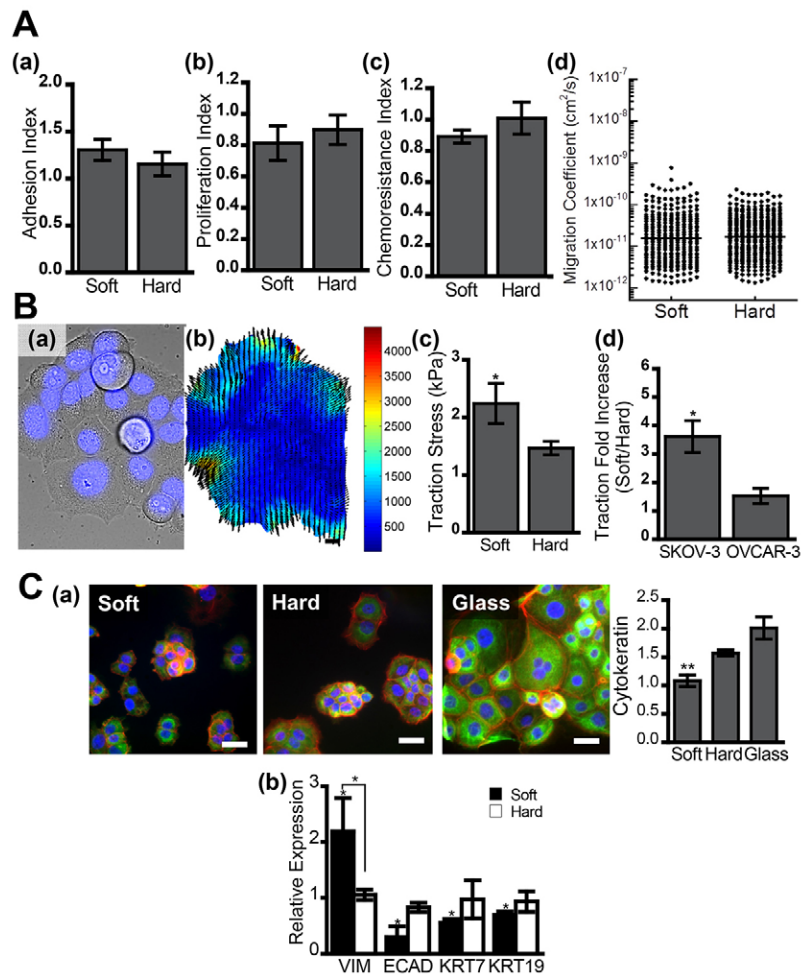


Fig. 3. Less-metastatic OVCAR-3 ovarian cancer cells show decreased mechanosensitivity. (A) The previously observed changes in adhesion (a), proliferation (b), carboplatin resistance (c) and migration (d) were no longer observed in the less-metastatic cells. (B) Imaging of OVCAR-3 cells labeled with NucBlue on stiff substrates (a) and the underlying traction forces (Pascals) overlaid with black arrows to show matrix displacements; scale bar: 10 μm (b). Quantification of peak traction stresses shows a slight increase on soft substrates (c). The fold increase in traction force on soft substrates relative to hard substrates is significantly larger in SKOV-3 cells relative to OVCAR-3 cells (d). (C) OVCAR-3 cells show decreased changes in their epithelial or mesenchymal character as assessed by quantitative image analysis of OVCAR-3 cells labeled for F-actin (red) and pan-cytokeratin (green) (values are normalized to SKOV-3 on glass substrate; scale bars: 50 μm) (a) and by expression of EMT-associated genes (b). Data are shown as the mean ± s.e.m.; * $P < 0.05$, ** $P < 0.01$.

traction stresses for LPA-treated OCCs (Fig. 4B,Ca), although the decreased spread area (supplementary material Fig. S4A) did result in a significantly lower total force exertion on soft matrices (supplementary material Fig. S4B). We hypothesize that, on the soft matrix, the Rho activation by LPA led to hypercontractility and subsequent cell collapse, such as that observed in neuronal cells, suggesting that collapse upon exposure to soluble contractile cues is a conserved characteristic of cells that prefer soft environments (Kranenburg et al., 1999).

Inhibition of ROCK with either Y27632 or H1152 (Fig. 4Aa) produced rigidity-independent motility, suggesting that ROCK plays a key role in OCC mechanosensitivity. Moreover, ROCK inhibition greatly mitigated changes in the expression of EMT-associated genes (Fig. 4Ac). When OCCs were grown on soft substrates, treatment with Y27632 reduced traction forces (Fig. 4B,Ca). Although Y27632 induced modest increases in force on hard substrates, treatment with the more-specific H1152 produced completely rigidity-independent forces and corresponding decreases in the amount of pMLC (supplementary material Fig. S4Cb). This disparity might be due to either incomplete ROCK inhibition with Y27632 (supplementary material Fig. S4Ca) or off-target effects of Y27632 (Ikenoya et al., 2002). Despite the larger traction forces on hard substrates, Y27632-treated OCCs showed no increase in motility, possibly owing to the inability of the cells to polarize forces properly (Fig. 4Cb). The near-complete force inhibition with H1152 greatly mitigated OCC motility regardless of matrix rigidity (Fig. 4Ac).

Our results are consistent with recent findings reported by Waterman and colleagues. Their results show that, below a threshold matrix stiffness, cells use a subset of focal adhesions for mechanosensing, and these focal adhesions undergo constant force fluctuations through a ‘tugging’ mechanism (Plotnikov et al., 2012). These ‘tugging’ focal adhesions exert larger forces than their stable counterparts. As observed here, ROCK inhibition in tugging cells on soft matrices decreased the amount of pMLC and the subsequent force exertion. By contrast, in non-tugging cells on hard matrices, ROCK inhibition induced tugging that could lead to the increased forces seen on hard substrates (Fig. 4C). These findings could imply that ROCK inhibition essentially decouples the mechanosensing machinery of the cell, and indeed downstream molecular and functional changes were largely mitigated by ROCK inhibition (Fig. 4A). Because cells on soft matrices can display biphasic behavior based on the density of the extracellular matrix (ECM) (Engler et al., 2004), future studies investigating the role of ECM density on Rho–ROCK-mediated mechanosensitivity might help to determine the interplay of these factors that is required for ovarian cancer metastasis.

These findings are reminiscent of Stephen Paget’s 1889 ‘seed and soil’ hypothesis, which sought to explain the observation that certain cancer cells, or ‘seeds’, seem to prefer specific metastatic sites, or ‘soil’ (Fidler, 2003). We posit that secreted soluble factors increase OCC homing to the omentum, and then mechanical cues from the matrix promote OCC engraftment, growth and migration. Interestingly, nuclear lamin levels have

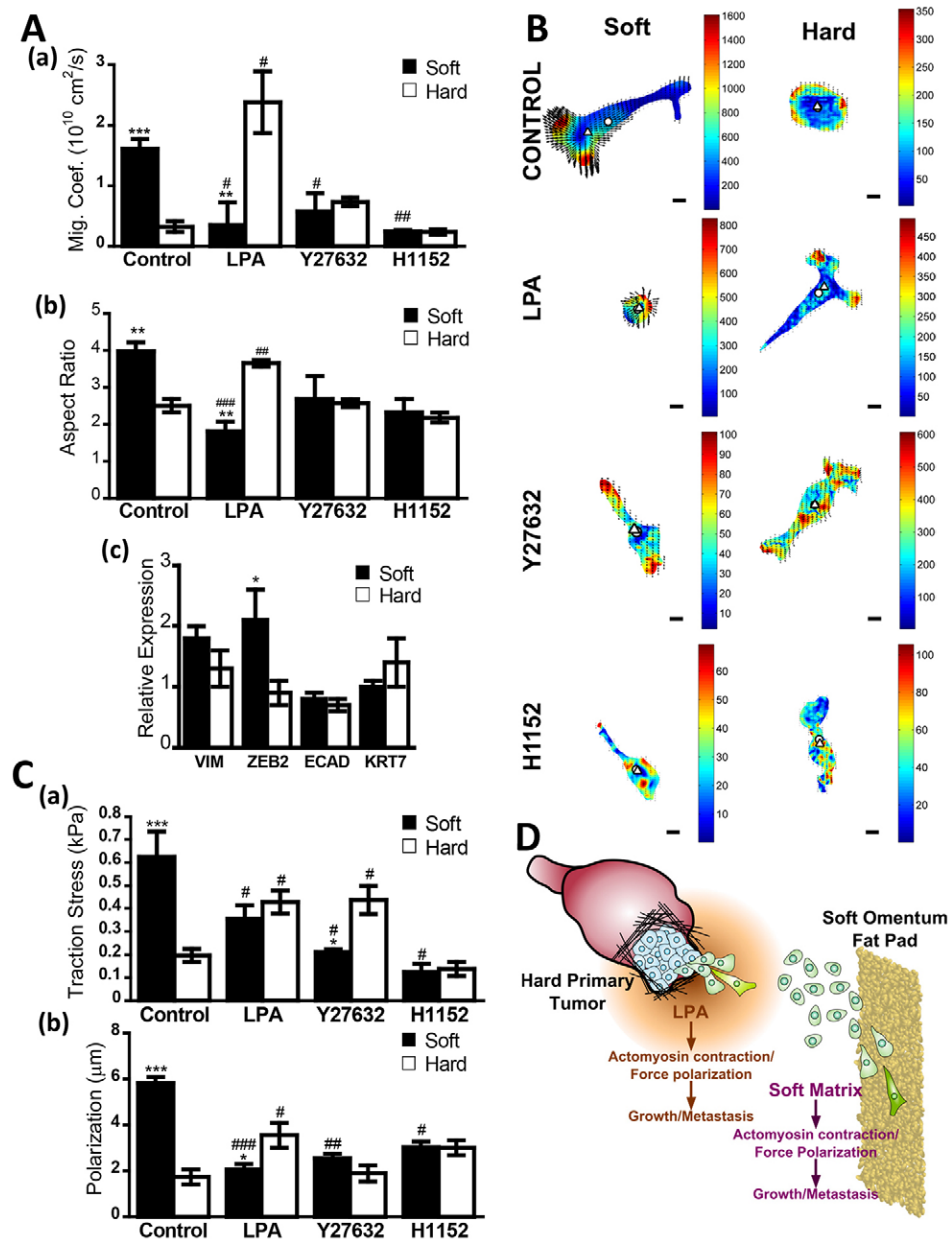


Fig. 4. Mechanical tropism is governed by a Rho–ROCK pathway, as demonstrated by Rho activation or ROCK inhibition.

(A,a) Rho activation by treatment of cells with LPA (10 μ M) induces large increases in migration on hard substrates, but inhibits migration on soft substrates. ROCK inhibition by treatment of cells with Y27632 (10 μ M) or H1152 (1 μ M) produces rigidity-independent migration with decreased migration on soft matrices and with decreased migration on soft matrices. (b) Similar trends in cell morphology were observed in response to treatment with the indicated small molecules, as quantified by calculation of the cell aspect ratio. (c) ROCK inhibition with Y27632 mitigates changes in EMT-associated gene expression. (B) Traction force maps as described in Fig. 1 after treatment with small molecules. Scale bars: 10 μ m. (C) Quantification of peak traction stresses (a) and traction force polarization (b). Data are shown as the mean \pm s.e.m.; * P <0.05, ** P <0.01, *** P <0.001 (between soft and hard substrates); # P <0.05, ## P <0.01, ### P <0.001 (between samples and their untreated rigidity-matched controls). (D) Proposed model of ovarian cancer cell metastasis, in which LPA in the hard environment of the primary tumor induces a change from a less migratory, more epithelial cell type (blue) to a more migratory mesenchymal cell type (green). After spreading into the ascites fluid, engraftment into the soft omentum likewise results in a more aggressive mesenchymal phenotype.

recently been shown to scale with tissue stiffness (Swift et al., 2013), and microarray analysis of primary and metastatic ovarian tumors have shown changes in lamin expression that are consistent with this result (Lili et al., 2013).

In conclusion, this report demonstrates a previously undocumented mechanical tropism in metastatic ovarian cancer cells that is regulated through a Rho–ROCK pathway. Based on these observations, we propose a model, as outlined in Fig. 4D, whereby high local amounts of LPA around the tumor increase the growth of OCCs and their migration from the primary tumor. Once in the peritoneal fluid, cells preferentially adhere to the omentum as they come into contact with the soft matrix. After adhering, the compliant matrix causes an increase in malignant characteristics, including growth, chemoresistance and motility. Taken together with recent studies implicating ascitic fluid flow in ovarian cancer cell EMT (Rizvi et al., 2013), this work further highlights the crucial role of mechanical cues in ovarian cancer metastasis. By furthering our understanding of the factors affecting

ovarian cancer metastatic tropism, this work might help to address the lack of effective therapies for advanced-stage disease.

MATERIALS AND METHODS

Cell culture and substrate synthesis

Ovarian carcinoma cells SKOV-3 and OVCAR-3 were cultured per the manufacturer's instructions. Human mesenchymal stem cells (hMSCs) acquired from Texas A&M University were differentiated, as described previously (McGrail et al., 2013), into adipocytes and osteoblasts (supplementary material Fig. S1A). Polyacrylamide substrates (Tse and Engler, 2010) were coated with equal densities of collagen I (supplementary material Fig. S1B).

Adhesion, proliferation, chemoresistance and cell motility

Cells labeled with carboxyfluorescein succinimidyl ester (CFSE, Biologend) were allowed to adhere for 2 hours in Hank's balanced salt solution (HBSS) with divalents before taking an initial fluorescence reading. To determine the adherent fraction, a final reading was taken after removing non-adherent cells by washing with HBSS. Cell proliferation was quantified by determining the cell number increase at

48 hours after plating. Chemoresistance was quantified by using an MTT assay on cells treated with 50 μ M carboplatin. All three parameters are reported relative to a collagen-coated glass control. For quantification of the cell-migration coefficient, cells were imaged on an environmentally controlled Nikon Eclipse Ti microscope, and traces were fitted to the persistent random-walk model (Dickinson and Tranquillo, 1993).

Traction force microscopy

Cell-induced displacements were used to determine traction forces, as described previously (Sabass et al., 2008). To capture the traction forces of OVCAR-3 cells that grow in clumps and to avoid inaccuracies arising from analyzing patches of cells, traction stress values are reported as the peak (95th percentile) of traction forces (supplementary material Fig. S1C). Polarization was defined as the difference between the centroid of the cell and the force-weighted center of mass (Fig. 1Ea).

Immunofluorescence and characterization of gene expression

Staining for cytokeratin was performed with an anti-pan-cytokeratin antibody (Biolegend), followed by incubation with Rhodamine-phalloidin and Alexa-Fluor-488-conjugated secondary antibody (Invitrogen) before sealing with Vectashield containing DAPI. Staining for pMLC was performed as described previously (Raab et al., 2012). For gene expression analysis, expression was normalized to an endogenous 18S RNA and is reported relative to that of cells cultured on collagen-coated glass (McGrail et al., 2013).

Statistical analysis

All studies were performed in triplicate or more and are reported as the mean \pm s.e.m. Statistical analysis was performed using a Student's *t*-test or ANOVA, with $P < 0.05$ considered to be statistically significant ($***P < 0.001$, $**P < 0.01$, $*P < 0.05$). For inhibitor studies, the hash symbol (#) indicates comparison between samples and their untreated rigidity-matched controls.

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Competing interests

The authors declare no competing interests.

Author contributions

The study was conceived and designed by D.J.M. and M.R.D. Both D.J.M. and Q.M.N.K. generated data. D.J.M. developed the methodologies and analyzed and interpreted data. The manuscript was written by D.J.M. and M.R.D.

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Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.144378/-/DC1>

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