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An *in vitro* correlation of mechanical forces and metastatic capacity

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Abstract

Mechanical forces have a major influence on cell migration and are predicted to significantly impact cancer metastasis, yet this idea is currently poorly defined. In this study we have asked if changes in traction stress and migratory properties correlate with the metastatic progression of tumor cells. For this purpose, four murine breast cancer cell lines derived from the same primary tumor, but possessing increasing metastatic capacity, were tested for adhesion strength, traction stress, focal adhesion organization and for differential migration rates in two-dimensional and three-dimensional environments. Using traction force microscopy (TFM), we were surprised to find an inverse relationship between traction stress and metastatic capacity, such that force production decreased as the metastatic capacity increased. Consistent with this observation, adhesion strength exhibited an identical profile to the traction data. A count of adhesions indicated a general reduction in the number as metastatic capacity increased but no difference in the maturation as determined by the ratio of nascent to mature adhesions. These changes correlated well with a reduction in active beta-1 integrin with increasing metastatic ability. Finally, in two dimensions, wound healing, migration and persistence were relatively low in the entire panel, maintaining a downward trend with increasing metastatic capacity. Why metastatic cells would migrate so poorly prompted us to ask if the loss of adhesive parameters in the most metastatic cells indicated a switch to a less adhesive mode of migration that would only be detected in a three-dimensional environment. Indeed, in three-dimensional migration assays, the most metastatic cells now showed the greatest linear speed. We conclude that traction stress, adhesion strength and rate of migration do indeed change as tumor cells progress in metastatic capacity and do so in a dimension-sensitive manner.

Introduction

The migration of mammalian cells is fundamental to normal embryonic development, tissue repair and immune function (Lauffenburger and Horwitz 1996, Ridley *et al* 2003, Chodniewicz and Klemke 2004, Vandenberg 2008). Under normal physiological conditions, most cells migrate in an adhesion-dependent manner involving the formation of adhesions at the cell-substrate interface and the subsequent generation of mechanical forces via the actin-myosin network (Fournier *et al* 2010). Adhesion formation begins with the interaction between extra-cellular matrix proteins (ECM) and integrin receptors (Riveline *et al* 2001, Calderwood 2004, Gallant *et al* 2005, Shattil *et al* 2010). What follows this initial

interaction is under intense study and involves orchestrated protein recruitment and phosphorylation events resulting in the linkage of select adhesions to the actin–myosin network. Mechanical forces produced by the contraction of this network can be measured outside the cell as traction stress (Lauffenburger and Horwitz 1996, Li *et al* 2007). The full purpose of these forces continues to be defined, but represent a variety of activities involving propulsion, probing and matrix remodeling (Thomas and DiMilla 2000, Bershadsky *et al* 2003). Nonetheless, their requirement in multiple normal cellular processes including cell division, adhesion, and migration is well documented (Fournier *et al* 2010). What remains to be established is the importance of traction stress in disease states, such as cancer and fibrosis (Mierke *et al* 2008).

Tumor cells have long been known to differ from normal cells in adhesive and contractile strength resulting in abnormal growth and migratory behavior (Thomas and DiMilla 2000, Rabinovitz *et al* 2001, Friedl and Wolf 2003). Conflicting studies of individual cell lines have found both reduced and increased traction stress produced in cells after oncogenic transformation, hinting at the importance of traction stress in cancer (Munevar *et al* 2001). However, no studies done so far show how traction stress changes as cells progress through different stages of metastasis. Several significant studies have also focused on how the compliance (stiffness) of the tumor micro-environment promotes tumor growth (Paszek *et al* 2005, Assoian and Klein 2008, Ronnov-Jessen and Bissell 2009). However, 90% of deaths result from the metastatic phase of the disease and not the primary tumor. As tumors progress to the multi-step process of metastasis their motility changes drastically often taking on adhesion-independent modes of migration after leaving the primary tumor environment. Although studies have looked at compliance and the metastatic state (Kostic *et al* 2009), how changes in cell-generated traction stress and cell–substrate adhesion strength change throughout the progression of the metastatic phase are unknown.

Inappropriate migration of a tumor cell from the primary tumor is an early step in the process of metastasis. A subsequent cascade of events include invasion of tumor cells into the stroma, intravasation into the lymphatics and blood circulation, extravasation into the secondary site and finally the formation of a secondary tumor at distant tissue (Banyard and Zetter 1998, Fidler 2003, Mierke *et al* 2008). This malignant transformation of epithelial-derived tumor cells is thought to be associated with an epithelial to mesenchymal transition in which carcinoma cells downregulate the epithelial proteins E-cadherin and cytokeratin in exchange for expression of the mesenchymal proteins N-cadherin, vimentin, and fibronectin (Ke *et al* 2008, Mani *et al* 2008, Sarrio *et al* 2008).

To our knowledge, despite evidence that migration, adhesion and traction stress are aberrant in isolated cancer cell lines, none of these parameters have been correlated with the progressive stages of cancer metastasis. In this study, we used four murine breast cancer cell lines derived from a single primary tumor, but capable of completing different stages of metastasis and ask if indeed changes in these parameters coincide with the degree of metastatic aggressiveness. Because these cells come from the same tumor, within the same mouse, they offer a significant advantage over using multiple cell lines derived from multiple different genetic backgrounds that confound interpretation. In this study, we find that traction stress, in two-dimensional cultures, and cell-substrate adhesion strength decreased as metastatic capacity increased in this panel. We also observed a general reduction of migration efficiency in two-dimensional cultures during single cell and collective migration, despite the organization of focal adhesion and actin cytoskeleton structure being grossly unaffected. However, we also found a reduction in the total number of focal adhesions and the level of active beta-1 integrin as the metastatic capacity increased. We interpreted this as a gradual shift toward a less adhesion-dependent mode of migration, one that can only be measured in three dimensions. As predicted, the highest migration

speeds in three-dimensional cultures were observed in the most metastatic cells of the panel, despite the low levels of active beta-1 integrin. Our results enhance our understanding of the significance of traction stress in metastatic progression and re-enforce the need for concurrent three-dimensional studies of these forces. Nonetheless, our study suggests that two-dimensional analysis of traction, migration and adhesion can be reflective of the metastatic capacity of a cell and could potentially be a useful prognostic tool.

Materials and methods

Cell culture and preparation of polyacrylamide substrates

Four sub-populations of murine breast cancer cell lines derived from the same primary tumor but possessing different metastatic potential (generous gift from Dr Fred Miller, Karmanos Cancer Institute) and normal murine mammary gland cell line (ATCC) were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone, UT, USA), 2 mM L-glutamine, 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin and incubated at 37 °C under 5% humidified CO₂.

Traction force microscopy (TFM) and adhesion assays were performed on polyacrylamide hydrogels that were coated with bovine plasma fibronectin (Sigma, MO, USA) at 5 µg cm⁻², as described previously (Beningo *et al* 2002). All substrates used in this study were 5% acrylamide and 0.08% *N,N'*-methylene-bis-acrylamide unless otherwise specified.

Microscopy

Images were acquired with an Olympus IX81 ZDC inverted microscope, equipped with a 10×/0.25 NA CP-Achromat lens and a 40×/0.75 NA Plan-Neofluar lens for phase-contrast images and fluorescence images. Live cells were imaged at 37 °C under a microscope equipped with a custom-stage incubator. All images were acquired with a Diagnostic Instruments (MI, USA) Boost EM-CCD-BT2000 camera driven by the IPLab software (BD Biosciences, MD, USA).

Determination of traction stress

Traction stress was measured as previously described (Beningo *et al* 2001). In brief, flexible polyacrylamide substrates (5% acrylamide and 0.08% bis-acrylamide) were coated with extracellular matrix protein fibronectin for cell adhesion and embedded with fluorescent microbeads for tracking the deformation on the substrate. The Young modulus of the substrate was estimated to be 2.4×10^4 N m⁻², determined as described previously using a bead indentation method (Lo *et al* 2000). Cells were seeded onto the substrates overnight before measuring the traction stress. The stress images were obtained in the presence of the cell and the null images were obtained by removing the cell with a microneedle. Traction stress was calculated and rendered as previously described (Dembo and Wang 1999, Marganski *et al* 2003). The magnitude of the average integrated traction stress was used directly for comparison.

Cell adhesion assay

A centrifugation assay was used to measure the strength of cell-substrate adhesion. This assay was a modified version described earlier (Guo *et al* 2006). Briefly, approximately 2.5×10^4 cells were seeded on to a fibronectin-coated polyacrylamide substrate (5% acrylamide and 0.08% bis-acrylamide) in specifically designed chambers. The cells were then allowed to adhere for 30 min at 37 °C. The chambers were then centrifuged at $1800 \times g$ for 5 min in a Beckman (CA, USA) TJ-6 centrifuge with a TH-4 rotor. The strength of adhesion was determined as the percentage of cells remaining adhered to the substrate after centrifugation.

At least three sets of experiments were done and ten fields of cells were counted for each cell type.

Cell migration assays

The migration of breast cancer and control cell lines was monitored by time-lapse video microscopy with 40× magnification. Two-dimensional migration was performed as previously described (Shiu *et al* 2004). In brief, cells were seeded at low density onto fibronectin or collagen-coated no.1 coverglass slides and incubated overnight at 37 °C. The phase contrast images of a single cell were acquired at 2 min intervals and transferred from a frame grabber to computer storage. Three-dimensional collagen gel migration assays were performed as previously described (Niggemann *et al* 2004). Briefly, 1×10^5 cells ml^{-1} were suspended in buffered liquid collagen type I at a final concentration of 1.65 mg ml^{-1} (pH 7.4; 3 mg ml^{-1} ; Pure Col, Advanced Biomatrix, CA, USA). Self-constructed glass chambers were filled with 400 μl of suspension, sealed and incubated at 37 °C. Following polymerization, sequential phase images were collected as above. The three-dimensional fibrin-fibronectin migration assay was performed as previously described (Brown *et al* 1993). In short, 1×10^6 cell were suspended in 350 μl of DME (25 mM HEPES) and mixed with 200 μl platelet poor human plasma collected in sodium citrate (Innovative Research, MI, USA). The suspension was placed into 7.25 × 55 mm glass tubes (Bio Data Corporation, PA, USA). Two hundred microliters of HEPES DMEM containing 28 mM CaCl_2 and 5 U ml^{-1} human thrombin (Innovative Research, MI, USA) were then added to the tube, mixed and incubated at 37 °C for 3 h to generate the clot. The clot was transferred to a self-contained glass chamber and phase contrast images were acquired as above.

The custom-designed dynamic image analysis system software (DIM; Y-L Wang) was used to determine the x , y coordinates of cell centroids from the phase images and to track the path of each cell during migration. The migration speed ($\mu\text{m min}^{-1}$) and persistence (minutes) were calculated by quantifying the centroid displacement every 2 min over a 120 min period. Migration speed is shown as $\pm$ S.E. Only isolated and well-spread cells were used for migration analysis. At least ten individual cells were pooled from each cell line for these assays.

Scratch wound assay

To perform the scratch wound assay, cells were cultured on fibronectin-coated glass surface ($5 \mu\text{g cm}^{-2}$), until the formation of a tight monolayer. As described earlier (Rajasekaran *et al* 2001) the confluent monolayer was then scratched with a sterile 200 μl pipette tip to create a uniform, cell-free area with a width of approximately 1 mm. Cell debris was removed by washing with fresh culture medium at 37 °C and incubated for 30 min at 37 °C. Wound closure was monitored by time lapse microscopy for a period of 24 h at 20 min intervals. At least three different wounds for each cell line were monitored. The percentage of the wound filled in micrometers was determined using the Image J software (NIH) as the average distance from the wound origin covered by cells 24 h after wounding.

Immunofluorescence

Cells adhered to no. 1 coverglass coated with fibronectin ($5 \mu\text{g cm}^{-2}$) were washed with warm cytoskeleton buffer and fixed for immunofluorescence in 4% paraformaldehyde and 0.1% Triton X-100 for 10 min, followed by blocking for 1 h with 5% BSA. Fixed cells were incubated with 1:50 Alexa Fluor 546 phalloidin (Invitrogen, CA, USA) for 1 h, 1:1000 anti-paxillin antibody (Clone 349, Chemicon International, USA) for 4 h and 1:1000 Alexa Fluor 488 anti-mouse secondary antibody (Invitrogen, CA, USA) for 1 h. All incubations with antibodies were done at room temperature unless otherwise specified. To obtain the percentage of mature focal adhesions attached to stress fibers, an arbitrary line forward of

the nucleus of a highly polarized cell was drawn separating the cell body from the leading edge. The number of paxillin plaques that co-localize with actin fibers located at the leading edge was then counted and divided by the total number of paxillin plaques. To visualize the epithelial and mesenchymal markers, cells were incubated with either 1:1000 anti-E-cadherin antibody (BD Biosciences, CA, USA) or 1:1000 anti-N-cadherin antibody (BD Biosciences, CA, USA) or 1:200 anti-fibronectin antibody (Abcam Inc., MA, USA) overnight at 4 °C followed by 1 h incubation with Alexa Fluor 488/546 anti-mouse secondary antibodies (Invitrogen, CA, USA). Collected images were pseudo-colored and overlaid to identify the areas of co-localization where necessary.

Western blot analysis

Cells cultured to 70% confluency on standard 100 mm culture plates were rinsed with ice-cold $1 \times$ PBS and lysed in triple detergent lysis buffer (2% NP40, 0.5% deoxycholic acid and 0.2% SDS) along with protease inhibitors (SIGMAFAST Protease Inhibitor, Sigma Aldrich, USA). The protein content was determined by the DC protein assay (Bio Rad, USA) according to the manufacture's instructions. Samples (50 μ g of protein, unless otherwise specified) were subjected to the SDS-PAGE on 10% mini gels and transblotted onto the PVDF membrane (Millipore, CA, USA). The membrane was blocked in 5% non-fat dry milk and 0.1% Tween-20 in PBS for 2 h at room temperature before exposure to the primary antibody (except for vimentin which was blocked in 0.1% Tween-20 in TBS). The antibody source and concentration were as follows: 1:4000 mouse monoclonal E-cadherin (Clone 36, BD Biosciences, CA, USA), 1:5000 mouse monoclonal N-cadherin (Clone 32, BD Biosciences, CA, USA), 1:300 mouse monoclonal fibronectin (Clone IST-9, Abcam Inc., MA, USA), 1:2500 mouse monoclonal vimentin (Clone RV202, BD Pharmingen, CA, USA), 1:1000 mouse monoclonal alpha-tubulin (Clone B-5-1-2, Sigma, USA) and 1:7000 mouse monoclonal GAPDH (Clone 6C5, Chemicon International, USA). Incubation with the vimentin antibody was performed for 1 h at room temperature. Incubations with all other primary antibodies were performed overnight at 4 °C. The membranes were then incubated with secondary horseradish-conjugated goat anti-mouse antibodies (BD Biosciences, CA, USA (diluted 1:1000 for E-cadherin, N-cadherin, vimentin, fibronectin and alpha-tubulin; 1:7000 for GAPDH)). Signals were developed with the ECL detection kit (GE Health Care, UK).

To determine the level of expression of active β 1 integrin, polystyrene cell culture plates were coated with either 5 μ g cm⁻², 0.05 μ g cm⁻² or no fibronectin (Sigma, USA) and incubated overnight at 4 °C. The plates were rinsed with sterile $1 \times$ PBS, blocked with 1% bovine serum albumin (BSA) and incubated overnight at 4 °C. After blocking, the plates were rinsed with sterile $1 \times$ PBS and cells were seeded, harvested and lysed as described above. Blots were probed with 1:500 rat anti-mouse monoclonal active β 1-integrin antibody (Clone 9EG7, BD Pharmingen, CA, USA), and 1:7000 GAPDH served as an indicator of loading consistency.

Results

Characterization of the metastatic panel

Four murine mammary carcinoma cell lines (67NR, 168FARN, 4TO7 and 66cl4) were used in this study. This panel of cells originated from a single parental breast tumor yet each has acquired a different capacity to complete the metastatic cascade, as described by Aslakson and Miller (1992) and summarized in table 1. Briefly, the first cell line (67NR) is non-metastatic and forms only primary tumors, the second (168FARN) can invade and enter the circulation, but can not re-colonize, the third (4TO7) completes all steps of the metastatic cascade, but only forms micro-metastases, and the fourth (66cl4) can execute all of the steps

required for the formation of a secondary tumor, although it is considered to be only moderately aggressive (Aslakson and Miller 1992).

This cell panel has been used in a number of studies and provides a powerful tool for the study of metastasis *in vitro* or *in vivo* because the cells initially derive from the same genetic background (Dexter *et al* 1978, Aslakson and Miller 1992, Tester *et al* 2000, Yang *et al* 2004, Lou *et al* 2008). Further, genetic profiling of this panel has identified new proteins involved in different stages of the metastatic process. One protein of interest is the transcription factor Twist, which activates the transdifferentiation program, known as epithelial–mesenchymal transition (EMT) (Yang *et al* 2004). This change from an epithelial-type morphology to a more migratory phenotype is thought to be a requirement for metastasis of carcinoma cells. However, the significance of this transition, and to what extent it must be completed for metastatic relevance, is not entirely clear (Christofori 2006, Lou *et al* 2008, Voulgari and Pintzas 2009).

Typically, several proteins are used as markers to determine if the EMT transdifferentiation has occurred. It is generally accepted that cells that have undergone EMT may express N-cadherin (instead of E-cadherin) and higher levels of vimentin and fibronectin (Kong *et al* 2006, Sarrio *et al* 2008). Cells that have retained the epithelial phenotype generally express E-cadherin and lower levels of the other two markers. We determined the state of EMT of this panel of breast tumor cells by immunofluorescence and western blotting for these markers. Both 67NR and normal murine mammary gland cells (NmuMg) served as controls; however 67NR is the true control as it comes from the same mouse as the other cell lines and only forms primary tumors and does not metastasize.

As expected, by immunofluorescence the epithelial marker E-cadherin was expressed only at the cell–cell junction of normal murine mammary gland cells (figure 1(A)). Surprisingly, the expression of the mesenchymal marker N-cadherin was observed only in 67NR and 66cl4 (figure 1(A)). Two intermediate cell lines, 168FARN and 4T07, did not express either of these two proteins (figure 1(A)). These observations were further confirmed by western blot (figure 1(B)).

The expressions of cellular fibronectin and the intermediate filament protein vimentin were also observed by western blot. We found that vimentin was strongly expressed by 67NR compared to the other cells (figure 1(B)). On the other hand, fibronectin was strongly expressed by 4T07 (figure 1(B)). Surprisingly, 168FARN, the cell line capable of leaving the primary tumor but unable to re-colonize, did not express any of these proteins (figure 1(B)). Our results, consistent with recent observation, show that the EM transition is incomplete in all cells of the panel and suggest that a full transition, as defined by this set of markers, was not necessary for their metastatic properties (Lou *et al* 2008). Furthermore, our observations support the growing contention that EMT may not always be a requirement for successful metastasis (Tarin *et al* 2005, Cowin and Welch 2007).

Traction stress is inversely related to metastatic capacity

The measurement of traction stress has provided valuable insight into the process of cell migration, specifically into adhesion and contraction (Balaban *et al* 2001, Munevar *et al* 2001, Geiger and Bershadsky 2002, Mierke *et al* 2008). A few studies have found differences in traction stress when comparing cancer cells with normal cells (Munevar *et al* 2001, Ghosh *et al* 2008). However, to our knowledge, this relationship has not been defined with regard to the multiple stages of metastatic ability. Using this unique panel of cancer cells, with their varying metastatic abilities, we have asked whether metastatic capacity can be linked to changes in traction stress.

For this study we have used traction force microscopy (TFM) to quantify the stress transferred from the migrating cancer cells onto the underlying substrates. We were surprised to discover that the traction stress of each of the cell panel members decreased in a highly consistent manner as we progressed from cells of the least aggressive in metastatic capacity to the cells that were most successful in establishing secondary tumors. This inverse relationship was striking, as the least metastatic line 67NR produced the highest average traction stress (0.231 Pa) and the most metastatic 66cl4 line produced the least average traction stress (0.036 Pa) (figure 2(A)). Furthermore, as a general observation, the spatial organization of the stress vectors was highly disorganized in 66cl4 and forces were not concentrated at the most leading edge as with control cells (figure 2(B)). Our results establish that a correlation does exist between traction stress and the progression of metastatic abilities, such that cells capable of completing different stages of the metastatic cascade, ultimately toward the capacity to form secondary tumors, may gradually lose their ability to produce strong traction stress and these forces appear to become less organized.

Cell–substrate adhesion strength correlates with decreasing traction stress

Cell–substrate adhesion is critical for the transmission of internal forces to the substrate resulting in cell migration in two dimensions; however how much adhesion is enough for optimal migration appears to be cell-type dependent (Palecek *et al* 1997, Schwartz and Horwitz 2006, Mierke *et al* 2008). Changes in cell–substrate adhesive characteristics have long been implicated in the progression of cancer (Cavallaro and Christofori 2001, Lu *et al* 2001, Mierke 2008). Thus we have asked if the strength of adhesion changes with metastatic capacity and how this aligns with the measurements of traction stress.

Using a centrifugation assay, we have tested the panel of metastatic breast cancer cells for adhesion strength. Briefly, cells were plated onto fibronectin-coated polyacrylamide hydrogels and allowed to adhere for 30 min before being subjected to centrifugal forces of $1800 \times g$ for 5 min (figure 2(C)). Consistent with previous observations (Cavallaro and Christofori 2001, Yeatman 2004, Mierke 2008), we found that enhanced metastatic capacity indeed reduces cell–substrate adhesion strength in two dimensions. Furthermore, this inability to bind tightly to the extracellular matrix correlates very well with the progressive reduction of traction stress as cells of this panel acquire greater metastatic capacity. It is reasonable to presume that this reduced adhesion in the most metastatic cells results in lower traction stress.

The number of focal adhesions decreases with increasing metastatic capacity

Efficient migration and traction result from a proper balance of adhesion and contraction, as specified by the function of the cell type. Our results thus far suggest a decline in function of the adhesion and contraction machinery as the cancer cell panel progresses in metastatic capacities. To test this possibility we determined the number and distribution of focal adhesions and actin in these cell lines by immunofluorescence (figure 3(A)). We were surprised to find that stress fibers were not aberrant in any of these cell lines, despite the reduction in traction stress. Furthermore, we did not observe an aberrant morphology of individual adhesions; they were of typical size and shape, as for wild-type cells. In addition, we observed the ability of adhesions to mature from the front of the cell (defined by an arbitrary line forward of the nucleus of a highly polarized cell) into the cell body and found that for all cell lines an equal ratio was maintained such that 35% of the adhesions matured into the cell body (figures 3(B) and (C)). However, we did find a reduction in the overall number of adhesions as the cells increased in metastatic capacity, which dropped from an average number of 47 adhesions/wild-type cell to 14 adhesions/cell in the most metastatic line (figure 3(B)). These results indicate that as the cells of this panel progress in metastatic

capacity they produce fewer focal adhesions for migration, perhaps progressing into a less adhesion-dependent mode of migration.

Integrin activation decreases with metastatic capacity

Previous studies have found that the amount of available ligand can affect adhesion strength and subsequent traction stress (Palecek *et al* 1997, Garcia and Boettiger 1999, Maheshwari *et al* 1999, Holub *et al* 2003, Engler *et al* 2004). We tested this response in the breast cancer panel by measuring the levels of active beta-1 integrin in each cell line grown on culture plates coated at three densities of fibronectin 0, 0.05 and 5 $\mu\text{g cm}^{-2}$, the highest being the concentration used for the TFM and adhesion studies (figure 4(A)). We chose to look at the active form of beta-1 integrin because this subunit of integrin can engage both collagen and fibronectin fibers and gave us a direct comparison of the degree of engagement with the substrate. Interestingly we discovered the same inverse relationship between the amounts of active beta-1 integrin such that the more metastatic cells had the lowest amount of integrin activated (figure 4(B)). These results provide an explanation for the weak traction, adhesion and low adhesion numbers observed in the most metastatic cells, but could support the suspicion that the more highly metastatic cells may have switched to less adhesion-dependent mode of migration.

Collective migration decreases with metastatic ability

During migration cells move not only as single entities, but also more often in groups and clusters (Friedl *et al* 2004). Cells migrating during metastasis have also been observed moving in many forms as single cells, and as small and large clusters (Bell and Waizbard 1986, Friedl *et al* 2004). To test the migratory behavior of this panel when influenced by cell–cell contacts, we used a standard scratch wound assay. In this assay, the least metastatic cells filled in the wound within a 24 h period (figure 5(A)). The normal cells and 67NR served as controls filling the gap 100% and 62%, respectively; this healing ability progressively dropped with metastatic capacity to 32% for the most metastatic cells (66cl4) (figure 5(B)). Interestingly, all of the cancer lines had cells that broke from the monolayer and migrated non-collectively to fill the wound, with the exception of the most metastatic cell line, 66cl4 (figure 5(A), see movies 1–5, available from stacks.iop.org/PhysBio/8/015015/mmedia). Individual cells from this panel did not leave the cell sheet, but maintained their cell–cell interactions to eventually fill the wound after 48 h.

Single cell migration rates decrease with metastatic capacity in 2D but increase in 3D

Given the great distances traveled by successful metastatic cells, it is generally believed that cancer cells are highly efficient in migration, indeed the majority of previous studies conclude that the migration speed of cancer cells is superior to normal cells in two dimensions (Banyard and Zetter 1998, Friedl and Wolf 2003). Furthermore, a recent study of HT1080 fibrosarcoma cells has found that the migration speed of these cancer cells is even greater in three-dimensional culture systems compared to two-dimensional systems (Fraley *et al* 2010). Finally, a summation of our data finds that in two dimensions a gradual loss in adhesion occurs as metastatic abilities progress in the panel; such a change may reflect a switch in these cells to less adhesion-dependent migration that would result in greater migration rates in three-dimensional environments. To test the relevance of this concept we have compared migration speeds and persistence in two and three dimensions in both fibrin/fibronectin and collagen for the entire breast cancer panel of cells.

To measure single cell migration in two-dimensional cultures, cells were grown on fibronectin- and collagen-coated cover slips and time-lapse imaging was used to record their migration. Consistent with the results of the scratch wound assay, the linear speed of single cells on fibronectin decreased from 51 to 23 $\mu\text{m min}^{-1}$ as the metastatic capacity increased,

a change of 45% (figure 6(A)). Furthermore, the low speeds did not appear to be dependent on the type of ECM as nearly identical measurements were observed when tested on collagen-coated slides (figure 6(B); see movies 6–9, available from stacks.iop.org/PhysBio/8/015015/mmedia). However, when the linear speed was measured in three-dimensional cultures of fibrin/fibronectin clots (Brown *et al* 1993) or collagen matrices (Niggemann *et al* 2004), the trend was clearly reversed such that speed increased with metastatic capacity (figures 6(C) and (D); see movies 10–13, available from stacks.iop.org/PhysBio/8/015015/mmedia). Interestingly the normal mammary gland NmuMg cells were unaffected by the change in dimensions maintaining a rate of 40–45 $\mu\text{m min}^{-1}$ in either environment and for both ECM proteins. Likewise, 168FARN remained unchanged under any condition. Those cells most affected by the change in dimension were 67NR, 4T07 and 66cl4. A striking ligand-dependent three-dimensional sensitivity was observed in the most metastatic cell line (66cl4) where its migration rate between 3D fibrin/fibronectin and the collagen matrices went from 20 to 50 $\mu\text{m min}^{-1}$, while the change from two-dimensional collagen to three-dimensional collagen jumped from 30 to 50 $\mu\text{m min}^{-1}$, respectively.

Migration efficiency can be defined not just by the linear speed, but by how long the cell maintains a trajectory, reflecting its persistence in a particular direction. Analysis of the persistence in two- and three-dimensional cultures of both ECM compositions defined low persistence across the entire panel when compared to normal mammary gland epithelial cells and did not show a strong trend as the metastatic capacity increased (figure 7). However, a tremendous reduction in persistence was observed in all cells in three-dimensional culture systems, producing a four- to fivefold decrease and no evidence of ECM selectivity (figure 7).

The results of both the single cell migration experiment and the scratch wound confirm that in two dimensions, the most metastatic cells lacked the migration efficiency possessed by the least metastatic cells. However, when these same measurements were taken in three-dimensional cultures the speed was greater and showed cell-specific ligand selectivity in this response. Oddly, however, the persistence parameter was low across the panel and exceedingly low in three dimensions, perhaps due to barriers created by the fibrous matrices. These results are suggestive of a lower dependence on adhesion migration, one that does not rely on adhesion strength and traction stress to pull the cell along, but could use instead ameboid migration or some hybrid variation of it.

Discussion

Each step in the metastatic cascade selects for the most competitive cancer cells. When a normal cell becomes tumorigenic and subsequently develops metastatic properties, it incorporates several biochemical and biophysical changes (Guo and Giancotti 2004, Lopez *et al* 2008, Mierke 2008, Kumar and Weaver 2009). Most of these changes are associated with higher proliferation, efficient migration, reduced adhesion and traction (Cowin and Welch 2007). In some cases, complete adhesion independent or ameboid movement is developed (Sanz-Moreno *et al* 2008). However, it is not known if these changes are acquired in a stepwise manner as cells become more and more metastatic. In this study we have asked if a relationship exists between the metastatic stages and changes in migration, adhesion, and the production of traction stress. We were able to address our question *in vitro* by using a panel of isogenic murine breast cancer cells that originated from the same tumor but possessing increasing abilities to metastasize.

Previous studies (Munevar *et al* 2001) on individual pairs of wild-type and oncogenically transformed fibroblast cells found that traction stress was reduced in the most transformed cell line. A separate study has found that metastatic sarcoma cells produce higher traction

stress as compared to parental non-metastatic cells (Rosel *et al* 2008). In our study the most metastatic cell line produced the least force compared to the isogenic control cell. The differences between the studies may be explained by the fact that our cells are epithelial-derived carcinomas versus the fibroblast-derived sarcomas. Alternatively, the degree of aggressiveness of the cells used in the different studies could also provide an explanation. In our study the cell line 66cl4 is considered as a moderately aggressive metastatic cell line (Tester *et al* 2000).

The strength of our study lies in the use of a panel of murine mammary breast cancer cells as opposed to unrelated cell lines. This panel allows us to follow the change in traction stress in progressively more metastatic cells. We were surprised at the neatly graded decline in the magnitude of traction stress as the panel of cells progressed in their ability to metastasize. One potential explanation for this stepwise decline is that a series of mutations targeting a group of genes involved in migration pathways and machinery are accumulated as the cells become more metastatic (Yang *et al* 2004). However, this would imply that the mutational events were not random but sequential and that one mutation targeting the process of migration defined the site of the next mutation. If the mutations had been random, arbitrary magnitudes of stress across the cell panel would have been observed. Another possible explanation is that mutational events involved a repeated assault on a single key regulator of the process of migration. This idea would allow for random mutational events that may not contribute to assaults on the migration system whilst providing opportunity for repeated random assaults on the same gene or gene product within the migration system thus explaining why we observed a stepwise decline in the traction.

The measurement of traction stress is essentially a readout of the health of the contractile system of the cell in a two-dimensional environment. Across the panel, adhesions and stress fibers appeared morphologically normal. At a functional level, the adhesions matured normally though the total number of adhesions per cell declined as the metastatic ability increased. This is an obvious explanation for the decline in the adhesion strength of the cell as a whole, which declined as the metastatic capacity increased. These results suggest that the formation of adhesions becomes compromised in the panel as metastatic capacity increases, yet if they are formed they will mature properly. However, the question of why the number of adhesions decreases across the panel remains to be answered. Part of the answer certainly lies in the reduced levels of engaged beta-1 integrin observed in the most metastatic cells. Taken together, the reduced level of active beta-1 engaging the substrate provides a causative explanation for the lack of adhesions, adhesion strength, as well as traction stress, in the more metastatic cells of the panel, and interestingly it changes very little in response to changes in the amount of available ligand. This might suggest a gradual switch to a less adhesion-dependent mode of migration.

Metastatic cells have often been compared to professional migratory cells, including macrophages and neutrophils. These cells are known to have few adhesions and move by less adhesion-dependent mechanisms thus producing relatively weak traction stress reflective of their stealth like migratory properties (Smith *et al* 2007). Alternatively one could argue that a tumor cell that has evolved to become aggressively metastatic is an unhealthy cell and to expect it to have remained efficient in such a highly orchestrated event as migration may be far reaching. Nonetheless, consistent with the former argument, we find that migration rates in three-dimensional cultures are much greater in the most metastatic cells than the least metastatic, a completely opposite trend from the two-dimensional migration assays. These results coupled with the two-dimensional observations of lower levels of activated beta-1, poor adhesion strength, fewer adhesions and reduced traction might suggest we have documented the gradual acquisition of less adhesion-dependent migration during the progression of metastasis. Alternatively, we may be observing a switch

in dependence upon another ECM protein; however, this is unlikely as migration rates for the most metastatic cells in both collagen- and fibronectin-based matrices were increased in three dimensions over two dimensions. Yet another possible explanation is the activity of matrix metalloproteases which are known to modify the ECM in three dimensions in front of migrating cells (Sternlicht and Werb 2001). This could explain the enhanced migration rates we observe in three dimensions. Indeed, it has previously been reported that 66cl4 secretes more MMP compared to the least metastatic cell 67NR (Tester *et al* 2000).

Many cancer biologists believe that EMT is a requirement for a tumor cell to leave the primary tumor and metastasize (Ke *et al* 2008, Mani *et al* 2008, Sarrio *et al* 2008). However, others have documented that in most cancers the complete loss of epithelial markers and the gain of mesenchymal markers were rarely observed (Christofori 2006). In our study it was intriguing to find an incomplete expression of mesenchymal markers throughout the entire panel despite the complete loss of the epithelial marker E-cadherin. The gain of the N-cadherin marker was only observed in the cells that do not metastasize (67NR) and the cells with the greatest (66cl4) capacity to metastasize. In spite of this agreement, the traction produced, and the adhesion and migration properties of these two cells were diametrically opposed. While we certainly have not exhausted the number of potential EMT markers nor can we speak to those that have yet to be discovered, based on our results we do not find a correlation between the EMT markers we have used and the degree of metastatic capacity. Alternatively one could argue that mesenchymal type migration is an adhesion-dependent mode of migration, and since the cancer panel may have progressed to less adhesion-dependent migration, a full set of these markers may be irrelevant. Our observations support the contention that a complete epithelial–mesenchymal transition is not an infallible hallmark in the gauging of metastatic capacity and that individual markers are likely to vary as well.

In summary, we have found an inverse correlation between traction stress and metastatic capacity within a panel of breast cancer cell lines (see figure 8). Furthermore, we have determined that this correlation extends to adhesive strength, migration speed and directional persistence in two-dimensional assays. In addition, we have determined that these changes correlate, not with the morphology of cell–substrate adhesions or their ability to mature, but likely their ability to engage the substrate through beta-1 integrin activation as the cells become more metastatic. Finally, based on three-dimensional migration assays we contend that as the metastatic capacity increases in our cell panel, a switch to a less adhesion-dependent mechanism occurs. Our results demonstrate for the first time a relationship between traction stress and the progression of a disease, more specifically the metastatic state of cancer cells *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The epithelial to mesenchymal transition is incomplete in the panel of breast cancer cells. (A) Immunofluorescent images of E-cadherin and N-cadherin at the cell–cell junction of Nmu Mg, 67NR and 66cl4. (B) Whole cell lysates of NmuMg (lane 1), 67NR (lane 2), 168FARN (lane 3), 4T07 (lane 4) and 66cl4 (lane 5) were prepared and analyzed by western blot for the expression of E-cadherin, N-cadherin, vimentin and fibronectin. Alpha-tubulin (50 kDa) was used as a load control for E-cadherin (120 kDa) and N-cadherin (130 kDa). GAPDH (38 kDa) was used as a load control for vimentin (57 kDa) and fibronectin (52 kDa).

Figure 2.

Two-dimensional stress production and adhesion strength are inversely related to the metastatic capacity. (A) Average integrated traction stress (Pascal) produced by the normal murine mammary gland cell line and the panel of mice breast cancer cell lines on a polyacrylamide substrate coated with $5 \mu\text{g cm}^{-2}$ fibronectin is depicted in bar graph ($n = 15$ cells). (B) Field of traction stress, shown as vectorial arrows within the boundary of the cell. In the highest metastatic cell (66cl4) stress is scattered and distributed in small pockets of short-lived protrusions, whereas in the non-metastatic cells (67NR) stress is concentrated in a single direction. (C) Cell-substrate adhesion strength decreases as metastatic potential increases in a centrifugation assay. Each bar represents mean $\pm$ s.e.m., results from three separate experiments, expressed as a percentage of control as defined by a wild-type mammary epithelial cell.

Figure 3.

The number of focal adhesion proteins at the leading edge decreases without affecting the focal adhesion maturation and cytoskeleton organization with increasing metastatic aggressiveness. (A) Immunofluorescence analysis for a focal adhesion protein paxillin (top panel) and a cytoskeleton protein actin (bottom panel) indicates that focal adhesion and stress fiber organization remain intact with metastatic progression. Scale bar 10 μm . (B) A hypothetical line is drawn forward of the nucleus of a highly polarized cell and the number of paxillin proteins forward (identified as the leading edge) and behind the line (defined as the cell body) are calculated. The analysis involves counting the total number of paxillin-containing adhesions and those associated with actin stress fibers at the leading edge and inside the cell body. (C) Each bar represents mean $\pm$ s.e.m. of the number of mature focal adhesions, expressed as a percentage calculated from the number of focal adhesions at the leading edge and inside the cell body ($n = 15$ cells).

Figure 4.

Active $\beta 1$ integrin at various concentrations of fibronectin. (A) Active $\beta 1$ integrin was detected by western blot from lysates of cells cultured on 0.05 $\mu\text{g cm}^{-2}$, 5 $\mu\text{g cm}^{-2}$ fibronectin or without fibronectin. Blots were probed with an antibody against mouse active $\beta 1$ integrin. The level of GAPDH served as a load control. (B) Line graph represents the normalized intensity of active $\beta 1$ integrin bands (Y-axis) and cell type (X-axis).

Figure 5.

Collective migration rate of the entire panel of mice breast cancer cell lines decreases with increasing metastatic capacity. (A) Motility of a confluent monolayer of mouse breast cancer cell lines in *in vitro* wound healing assay. Wound closure was monitored by phase contrast microscopy at the indicated time points. Original magnification, 10 $\times$. Scale bar = 100 μ m. (B) Percent of the wound gap filled was determined as the distance from the wound origin, in μ m, covered by cells in 24 h following initiation of the wound. Each bar represents the mean value $\pm$ s.e.m. of three independent experiments.

Figure 6.

Highly metastatic cells migrate efficiently in three dimensions but not in two dimensions. Linear migration speed of the panel of murine breast cancer cell lines on two-dimensional (A) fibronectin- and (B) collagen-coated coverslips and in three dimensions within (C) fibrin–fibronectin clot and (D) collagen gel using time-lapse video microscopy. The average rate of migration expressed in $\mu\text{m min}^{-1}$ for a period of 2 h. Each bar represents the mean value $\pm$ s.e.m. from ten sets of images.

Figure 7.

Directional persistence is reduced as metastatic capacity increases. The average persistence of migration in two-dimensional (A) fibronectin- and (B) collagen-coated plates and within three-dimensional (C) fibrin–fibrinogen clot and (D) collagen gels. Persistence is expressed in min; each bar represents the mean value $\pm$ s.e.m. from ten sets of images. Migration trajectories in two dimensions (E), (F) and three dimensions (G), (H) over a period of 2 h. Each node represents a 10 min interval.

Figure 8. Summary of murine breast cancer cell panel and their abilities to migrate and produce traction relative to metastatic capacity. Those cells that are the most metastatic produce the weakest forces in two dimensions and migrate the least efficiently, whilst in three dimensions, metastatic cells move more efficiently. These results may reflect the evolution of a less adhesion-dependent form of migration as the cells progress to greater metastatic abilities.

Table 1

Characterization of the murine breast cancer cell lines. Phase images of the normal mammary gland cell line and four mice breast cancer cell lines, along with their metastatic potential and capacity to form the primary tumor when injected into mice.

Cell Type	NmuMg	67NR	168FARN	4T07	66cl4
Phase images
Primary tumor	-	+	+	+	+
Metastatic capacity	-	Only forms primary tumor	Micrometastases to lymph node	Micrometastases to blood, lymph node, lung	Visible lung nodule, blood, lymph node

Increasing Metastatic Capacity